Review

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Tansley review

Gene networks controlling *Arabidopsis thaliana* flower development

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Summary

The formation of flowers is one of the main models for studying the regulatory mechanisms that underlie plant development and evolution. Over the past three decades, extensive genetic and molecular analyses have led to the identification of a large number of key floral regulators and to detailed insights into how they control flower morphogenesis. In recent years, genome-wide approaches have been applied to obtaining a global view of the gene regulatory networks underlying flower formation. Furthermore, mathematical models have been developed that can simulate certain aspects of this process and drive further experimentation. Here, we review some of the main findings made in the field of *Arabidopsis thaliana* flower development, with an emphasis on recent advances. In particular, we discuss the activities of the floral organ identity factors, which are pivotal for the specification of the different types of floral organs, and explore the experimental avenues that may elucidate the molecular mechanisms and gene expression programs through which these master regulators of flower development act.

I. Introduction

The emergence of flowers as reproductive units probably contributed substantially to the evolutionary success of angiosperms, or flowering plants. Darwin described the obscure origin of flowers as an 'abominable mystery' because of their sudden appearance in the fossil record (Crepet, 2000). Flowers are thought to have originated from either the male or the female cones of gymnosperms and several theories have been posited as to how this might have occurred (reviewed in Scutt *et al.*, 2006). However, there is little paleobotanical evidence to support a steady and gradual decent with modification of gymnosperm cones to angiosperm flowers (Bateman *et al.*, 2006), and thus flower evolution remains largely enigmatic. Furthermore, the lack of similarity between the

reproductive structures of flowering plants and gymnosperms precludes direct comparisons between these two phyla (Fig. 1; Frohlich & Chase, 2007). While in gymnosperms, the male and female reproductive axes are separated and the ovules are exposed (Fig. 1b), angiosperm flowers are typically bisexual and are often composed of four different organ types (Fig. 1a), which are arranged in consecutive circles or whorls (Fig. 3b). In the model plant *Arabidopsis thaliana*, the outermost whorl (the first whorl) contains four sepals, the second whorl four petals, the third whorl six stamens, which produce pollen, and the fourth whorl two fused carpels, which encompass the ovules (Fig. 1a; Smyth *et al.*, 1990).

Obtaining insights into the molecular mechanisms underlying gymnosperm and angiosperm reproductive development is at the core of solving Darwin's abominable mystery. Over the past three decades, work in *A. thaliana*, as well as in several other angiosperm species (including snapdragon (*Antirrhinum majus*), petunia *(Petunia hybrida*), rice (*Oryza sativa*) and others), has resulted in detailed insights into the genetic control of flower development. More recently, researchers have begun to delineate the gene regulatory networks (GRNs) that govern the formation of flowers on a global scale and to generate predictive mathematical models that can be used to simulate regulatory processes during flower development and to drive further experimentation. This work was made possible largely through the introduction of genomic technologies and approaches of systems biology into plant research (Wellmer & Riechmann, 2005; Sablowski, 2010). In this review, we outline some of the main concepts and findings in the field of flower development and then discuss recent progress in elucidating the architecture and composition of the flowering gene networks in *A. thaliana*.

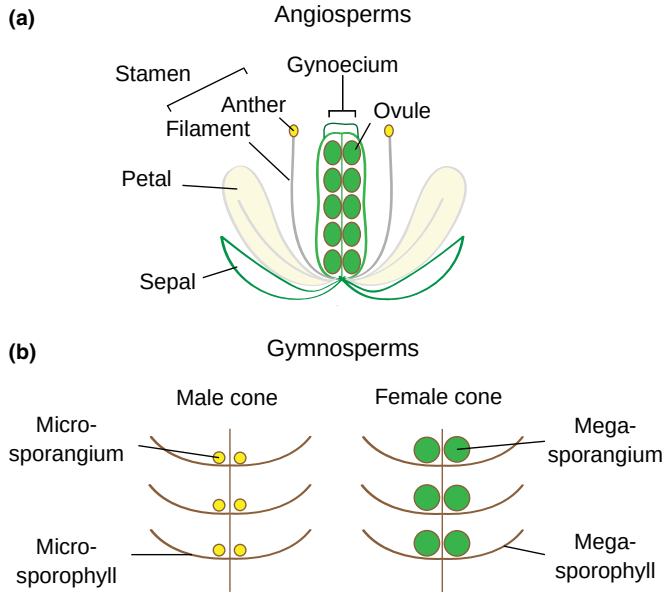

Fig. 1 Absence of homology between the organs of angiosperm flowers and gymnosperm cones. (a) An *Arabidopsis thaliana* flower, as a representative flower of the angiosperm lineage. In angiosperms, the ovules are always enclosed within a gynoecium. In *A. thaliana*, both male (stamens) and female (gynoecium) reproductive organs are present in the same flower, but in some plant species, these reproductive organs may form on separate flowers. The presence of sepals and petals also varies (Theissen & Melzer, 2007), and in some flowers both can be replaced by tepals, which are undifferentiated organs of the perianth. (b) Male and female pine (*Pinaceae*) cones representative of the gymnosperm lineage. In male cones, microsporangia, which contain the pollen grains, develop at the base of modified leaf-like organs termed microsporophylls. In female cones, uncovered ovules develop on the surface of megasporophylls instead of being enclosed in a gynoecium. Sporophylls are modified leaf-like organs, thought to be the most closely related gymnosperm structures to carpels (Melzer *et al.*, 2009).

II. Initiation of flower development

A highly complex GRN strictly regulates the transition from the vegetative to the reproductive phase of plant development, as mistimed flowering can dramatically decrease the number of offspring produced by a plant. Environmental cues such as temperature, photoperiod and nutrient availability as well as endogenous signals (Paul *et al.*, 2008; Wahl *et al.*, 2013) influence the timing of the floral transition (Srikanth & Schmid, 2011; Andres & Coupland, 2012), which leads to the conversion of the shoot apical meristem of a plant into an inflorescence meristem (IM) and subsequently to the formation of flowers. The genetic circuits that integrate these different signals ultimately converge to activate the expression of a small group of so-called floral meristem (FM) identity genes (Liu *et al.*, 2009a; Wellmer & Riechmann, 2010; Siriwardana & Lamb, 2012) in emerging FMs (or floral primordia), which arise from regions of the IM where the phytohormone auxin accumulates as a result of polar transport mechanisms (Fig. 2a; Reinhardt *et al.*, 2000, 2003). Of the FM

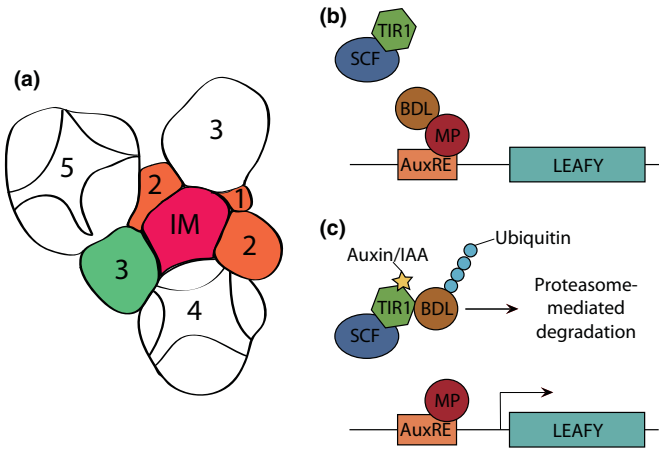

Fig. 2 Inflorescence meristem (IM) and floral meristem development in *Arabidopsis thaliana*. (a) Once the IM (shown in pink) is established, floral meristems are produced on its flanks. Orange floral meristems represent floral stages at which *LEAFY* (*LFY*) and *APETALA1* (*AP1*) are both being expressed, whereas the green floral meristem indicates the approximate stage at which the B and C class genes are activated. Numbers on the floral meristems indicate approximate floral stages (Smyth *et al.*, 1990). (b, c) Auxin signaling promotes the expression of *LFY*. (b) In the absence of auxin, MONOPTEROS (MP) is bound by BODENLOS (BDL), which prevents transcriptional activation of *LFY*. (c) In the presence of auxin, BDL is polyubiquitylated through interactions with Skp1 (S-phase kinase associated protein)-Cullin-F-box (SCF) complexes containing an auxin receptor such as TRANSPORT INHIBITOR RESPONSE 1 (TIR1). This results in the proteolytic degradation of BDL, allowing transcriptional activation of *LFY* by MP (Yamaguchi *et al.*, 2013). IAA, indole-3-acetic acid; AuxRE, auxin response element.

identity genes, *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which both encode transcription factors, are arguably the most important and have been functionally characterized in detail. These regulatory genes were identified from mutants with severe defects in flower development. A loss of *LFY* function results in the partial conversion of flowers into inflorescence shoots (Weigel *et al.*, 1992) while *ap1* flowers (in addition to exhibiting floral organ identity defects; see Section III) produce secondary flowers in the axils of first-whorl organs (Irish & Sussex, 1990). Combining *lfy* with *ap1* mutant alleles leads to a dramatically enhanced phenotype compared with each of the single mutants (Weigel *et al.*, 1992), suggesting that these regulators have some redundant functions in the control of floral development.

Specific combinations of mutants in the group of FM identity genes can lead to a dramatic delay or an abolition of flowering, again highlighting the inbuilt genetic redundancy during the floral transition. For example, *CAULIFLOWER* (*CAL*) and *FRUITFUL* (*FUL*), two genes closely related to *AP1*, are functionally redundant with *AP1* in their roles of specifying FMs (Ferrandiz *et al.*, 2000). *ap1 cal* double-mutant plants undergo a massive over-proliferation of IM-like tissue and flowering is dramatically delayed (Bowman *et al.*, 1993), while introduction of a *ful* mutation into the *ap1 cal* background abolishes flowering completely (Ferrandiz *et al.*, 2000). Notably, overexpression of *LFY* in the *ap1 cal ful* triple mutant can partially restore flowering, suggesting that a key role for the other FM identity genes is to boost *LFY* expression levels (Ferrandiz *et al.*, 2000).

The central role of LFY in the establishment of FMs is supported by the recent observation that auxin promotes *LFY* expression via the auxin response factor MONOPTEROS (MP; Yamaguchi *et al.*, 2013). In the absence of auxin, MP normally interacts with the AUX/IAA protein BODENLOS (BDL), which inhibits its activity (Fig. 2b; Hamann *et al.*, 2002). BDL-mediated repression of MP is lost once BDL is degraded in response to auxin-dependent polyubiquitylation by an Skp1 (S-phase kinase associated protein)-Cullin-F-box (SCF) ubiquitin ligase complex (Cardozo & Pagano, 2004; Mockaitis & Estelle, 2008), which contains an auxin receptor such as TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005; Fig. 2c). Using chromatin immunoprecipitation (ChIP), both BDL and MP have been shown to occupy a conserved regulatory element in the *LFY* promoter (Yamaguchi *et al.*, 2013). Furthermore, when inflorescences were treated with a chemical analog of auxin, BDL occupancy decreased dramatically while *LFY* expression was increased, and upon overexpression of *MP* or *BDL*, *LFY* transcripts were more and less abundant, respectively (Yamaguchi *et al.*, 2013). These data, together with results from a related study (Li *et al.*, 2013), provided molecular insights into how the auxin-dependent outgrowth of primordia from the IM is linked to the specification of FMs and thus the onset of flower development.

Identification of the direct targets of LFY and AP1 has been a central research focus of late, resulting in a much more complete picture of the roles of these regulators during floral initiation. One of the most important functions of LFY is to promote *AP1* transcription by direct regulation (Wagner *et al.*, 1999; Winter *et al.*, 2011). The AP1 transcription factor then binds to the regulatory region of *LFY* to form a positive feedback loop (Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010), leading to a strong and rapid up-regulation of both FM identity genes. Although *AP1* expression is controlled by LFY, it is not entirely dependent on it as *AP1* transcripts are detectable in *lfy* mutant inflorescences (Ratcliffe *et al.*, 1999). In fact, several other factors (e.g. LATE MERISTEM IDENTITY2 (LMI2), AGAMOUS-LIKE24 (AGL24) and SHORT VEGETATIVE PHASE (SVP)) are also involved in up-regulating *AP1* expression (Pastore *et al.*, 2011; Grandi *et al.*, 2012).

To specify an FM, AP1 and LFY promote the expression of other FM identity genes while concomitantly repressing the expression of negative regulators of flowering. For example, AP1 has been shown to suppress the expression of *TERMINAL FLOWER1* (*TFL1*), *TARGET OF EAT1* (*TOE1*) and *TOE3* (Kaufmann *et al.*, 2010). Furthermore, AP1 down-regulates the expression of *TEMPRANILLO1* (*TEM1*) and *TEM2* (Kaufmann *et al.*, 2010), which are known to repress the expression of the potent flowering time activator *FLOWERING LOCUS T* (*FT*; Castillejo & Pelaz, 2008). LFY also directly suppresses *TFL1*, *TOE3* and *TEM1* expression (Parcy *et al.*, 2002; Winter *et al.*, 2011), showing that AP1 and LFY can perform redundant tasks during the initiation of flowering.

AP1 and LFY also control the expression of genes involved in hormone pathways. For example, LFY has been shown to inhibit auxin biosynthesis and to promote auxin signaling in emerging FMs (Li *et al.*, 2013; Yamaguchi *et al.*, 2013), while AP1 regulates, during floral initiation, the expression of several genes involved in gibberellin metabolism and signal transduction (Kaufmann *et al.*, 2010). Together with the large number of transcription factor-coding genes that were identified to act downstream of AP1 and LFY (Kaufmann *et al.*, 2010; Winter *et al.*, 2011), it appears that these FM identity regulators constitute hubs in the flowering GRN and control FM specification by coordinating a multitude of cellular processes and developmental pathways.

III. Specification of floral organs: the ABCE model

Once the FM has been specified, AP1 and LFY activate floral organ identity genes (Lohmann & Weigel, 2002), which specify the four different types of floral organs. The floral organ identity genes were discovered through the study of mutants in *A. thaliana* and *A. majus*, which are characterized by homeotic transformations, that is, the replacement of one organ type by another (Fig. 3). Based on the phenotypes of these mutants and their genetic interactions, it was proposed that the affected genes specify either sepals and petals (termed A function), petals and stamens (B function), or stamens and carpels (C function) and thus act in a combinatorial manner to control the development of the four types of floral organs (Bowman *et al.*, 1991; Coen & Meyerowitz, 1991). This so-called ABC model (Fig. 3a) has provided, over the past two decades, the main genetic framework for research on flower development and evolution. It is now known that the underlying cause of the organ transformations in the floral homeotic mutants is the mutation of genes that code for transcription factors (Krizek & Fletcher, 2005). With the exception of the A function gene *APETALA2* (*AP2*), which is a

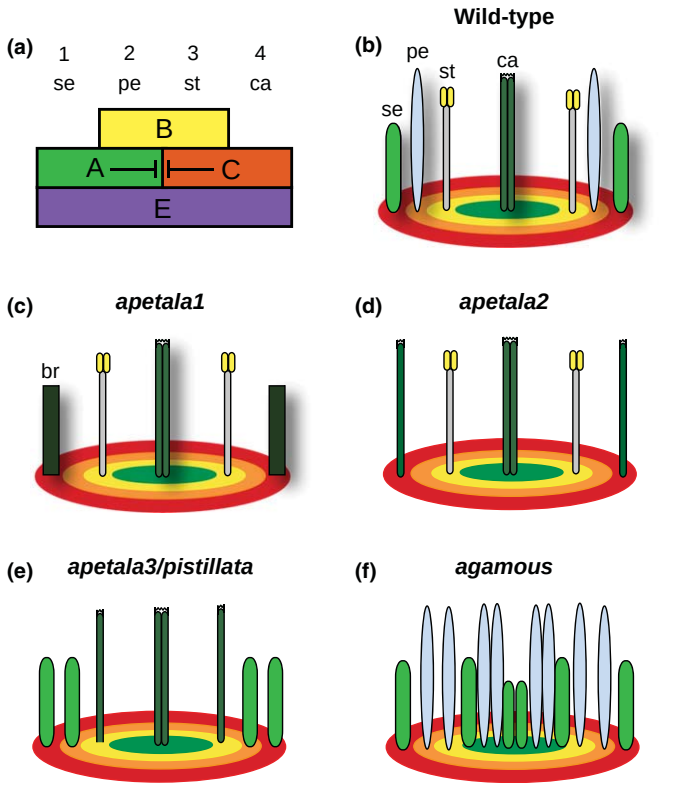

Fig. 3 Schematic representation of the ABCE model of flower development in *Arabidopsis thaliana* and of the homeotic transformations in the corresponding mutants. (a) A graphical representation of the ABCE model. (b–f) Schematic representations of the floral organ types present in a wild-type *A. thaliana* flower and in flowers of the homeotic mutants. (b) A wild-type flower contains sepals, petals, stamens and carpels, from the outer to the inner whorl. (c) Strong *apetala1* (*ap1*) mutants form bracts in the first whorl instead of sepals and do not contain second-whorl organs. (d) Strong *ap2* mutants have carpelloid organs in the first whorl and do not contain organs in the second whorl. (e) Strong *ap3* and *pistillata* (*pi*) mutants have sepals in the second whorl instead of petals, and stamens are replaced by carpels (or filaments; not shown) in the third whorl. (f) Strong *agamous* (*ag*) mutants do not form reproductive organs. Their flowers show reiterations of consecutive whorls containing sepals, petals, and then again petals. se, sepal; pe, petal; st, stamen; ca, carpel; br, bract.

founding member of the AP2/ETHYLENE RESPONSE FACTOR (ERF) transcription factor family (Okamuro *et al.*, 1997), all of the *A. thaliana* 'ABC' genes code for MIKC^c-type MADS-domain transcription factors (Yanofsky *et al.*, 1990; Jack *et al.*, 1992; Mandel *et al.*, 1992; Goto & Meyerowitz, 1994; Jofuku *et al.*, 1994; Parenicova *et al.*, 2003). These genes are typically expressed in the organ primordia that they help to specify (Yanofsky *et al.*, 1990; Jack *et al.*, 1992; Mandel *et al.*, 1992; Goto & Meyerowitz, 1994; Wollmann *et al.*, 2010).

von Goethe (1790) proposed that floral organs are modified leaves and, indeed, leaf-like organs arise instead of floral organs when all three classes of floral organ identity genes are mutated (Bowman *et al.*, 1991). However, ectopic expression of the floral organ identity factors does not result in the transformation of leaves into floral organs (Honma & Goto, 2001), suggesting that flower-specific co-factors must exist that are required for their activity. Through reverse genetic analyses, it was found that four closely related and partially redundant *SEPALLATA* genes (*SEP1* to *SEP4*), which also encode MADS-domain transcription factors and exhibit flower-specific expression, function together with the floral organ identity genes. In fact, removing the activities of all *SEP* genes simultaneously leads to a conversion of floral organs into leaf-like structures (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). Also, when *SEP* genes are ectopically expressed in conjunction with certain combinations of floral organ identity genes, leaves can be converted into floral organs (Honma & Goto, 2001; Pelaz *et al.*, 2001). These genetic interactions formed the basis of the expanded ABCE model, which states that the *SEP* genes (conferring E function) encode flower-specific co-factors that are required for organ specification in all floral whorls (Theissen & Saedler, 2001; Lohmann & Weigel, 2002; Krizek & Fletcher, 2005).

IV. Floral patterning: activating the floral organ identity genes in specific domains

As mentioned in Section III, the expression of the floral organ identity genes is directly promoted by LFY, which is expressed throughout the emerging FM (Parcy *et al.*, 1998; Siriwardana & Lamb, 2012). At the same time, LFY suppresses the expression of negative regulators of the floral organ identity genes, further contributing to their activation. For example, the protein EMBRYONIC FLOWER1 (EMF1) is involved in the polycomb group-mediated suppression of B and C class genes outside of FMs (Calonje *et al.*, 2008), and LFY down-regulates *EMF1* expression (Winter *et al.*, 2011). For the direct activation of the floral organ identity genes, LFY appears to require co-factors. Recently, SEP3 and LFY have been shown to interact *in vitro* and it appears that a SEP3/LFY-containing complex activates B and C class gene expression (Liu *et al.*, 2009b). Although SEP3 behaves as an LFY co-factor, it is expressed in the inner three floral whorls during early flower development (Mandel & Yanofsky, 1998) and therefore cannot significantly contribute to the domain-specific activation of the floral organ identity genes.

As described in Section II, *AP1* expression is directly promoted by LFY, and *AP1* transcripts initially accumulate throughout the FM (Wagner *et al.*, 1999). During early flower development, *AP1* expression becomes restricted to the outer two whorls where it functions as an A class gene in the specification of sepals and petals (Mandel *et al.*, 1992). The suppression of *AP1* in the center of the flower is (directly or indirectly) mediated by the C class gene *AGAMOUS* (*AG*), as in strong *ag* mutants, *AP1* transcripts are present in the center of the flower (Gustafson-Brown *et al.*, 1994).

Regulation of B function, which activity is mediated in *A. thaliana* by the MADS-domain transcription factors APETALA3 (AP3) and PISTILLATA (PI) that forms as obligate heterodimers (Riechmann *et al.*, 1996a; Yang *et al.*, 2003), is more complex. Although *AP3*/*PI* expression is reduced in *lfy* mutants, it is not abolished (Weigel & Meyerowitz, 1993), indicating that other factors regulate their expression. Moreover, overexpression of *LFY* alone is not sufficient to activate the expression of *AP3* in seedlings (Parcy *et al.*, 1998). The F-box protein UNUSUAL FLORAL ORGANS (UFO) proved to be an excellent candidate for a co-regulator of B class gene expression, as plants without functional

copies of *UFO* have homeotic transformations similar to those of B class mutants (Wilkinson & Haughn, 1995; Lee *et al.*, 1997). The first indication that UFO behaves as an LFY co-factor came from experiments in which a fusion between a dominant transcriptional repressor domain and UFO was overexpressed in plants (Chae *et al.*, 2008). This resulted in transgenic lines that strongly resembled *lfy* mutants, suggesting that UFO is present at the same regulatory sites as LFY (Chae *et al.*, 2008). As mentioned in Section II, F-box proteins like UFO are part of SCF-type ubiquitin ligase complexes that polyubiquitylate their targets, marking them for degradation via the 26S proteasome. UFO has been shown to physically interact with ASK1 (Arabidopsis Skp1-like) (Samach *et al.*, 1999; Wang *et al.*, 2003), another component of SCF complexes; it has also been demonstrated that *ASK1* genetically interacts with *LFY*, *PI* and *AP3* (Zhao *et al.*, 2001; Ni *et al.*, 2004). Therefore, it was proposed that UFO mediates the degradation of a factor that inhibits LFY-driven gene expression (Zhao *et al.*, 2001). Subsequently, it was found that LFY and UFO proteins interact *in vitro* and *in vivo*. Furthermore, it was shown that LFY is required to direct UFO to the *AP3* promoter (Chae *et al.*, 2008). These data led to an alternative proposal where UFO mediates the polyubiquitylation of LFY after a number of rounds of transcription, thereby promoting the turnover of LFY. This proteolysis of LFY is thought to increase transcriptional activity by allowing 'unspent' LFY transcription factors to bind to the promoters of target genes. While this mode of activity has not yet been demonstrated for LFY, several examples of transcription factor degradation being necessary for transcriptional activation have indeed been found in yeast, mammals and plants (Muratani & Tansey, 2003; Spoel *et al.*, 2009).

The expression of *AP3/PI* is also promoted by AP1, as indicated by the absence of their transcripts in *lfy ap1* double-mutant flowers (Weigel & Meyerowitz, 1993). By fusing AP1 to a strong transcriptional activation domain, it was shown that B class gene expression can be induced by AP1; however, as with LFY, this also appears to require the activity of UFO (Ng & Yanofsky, 2001; Lamb *et al.*, 2002), as well as that of SEP3 (Sridhar *et al.*, 2006; Kaufmann *et al.*, 2010; Wu *et al.*, 2012).

Numerous regulators of the C class gene *AG* have been identified. These include repressors, such as the polycomb group protein CURLY LEAF, that prevent *AG* expression outside of the flower through epigenetic mechanisms (Goodrich *et al.*, 1997; Calonje *et al.*, 2008), as well as activators that promote the transcription of *AG* in the center of the flower (Liu & Mara, 2010). For example, overexpression of *WUSCHEL* (*WUS*), encoding a homeodomain transcription factor, in flowers resulted in the ectopic formation of stamens and carpels (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001), an effect that also required *LFY* activity (Lenhard *et al.*, 2001). When the regulatory regions of *AG* were fused to the *uidA* reporter gene, β-glucuronidase activity was stronger in the presence of both WUS and LFY than in the presence of either WUS or LFY alone (Lohmann *et al.*, 2001). Gel-shift assays further showed that WUS can bind to sequences in the second intron of *AG* that are in close proximity to known LFY binding sites, although WUS and LFY do not appear to interact directly with each other (Lohmann *et al.*, 2001). *WUS* is required for stem cell maintenance and is expressed in a small number of cells in the center of the FM (Laux *et al.*, 1996; Mayer *et al.*, 1998). Given that *WUS* is only expressed in a subset of the cells expressing *AG* (Yanofsky *et al.*, 1990; Drews *et al.*, 1991; Mayer *et al.*, 1998), it is not clear how *AG* expression is promoted by WUS throughout its full domain. However, it has been shown recently that WUS protein can move within meristems from the region where it is being synthesized into neighboring cells (Yadav *et al.*, 2011). Thus, the activation of *AG* may depend, at least in part, on a non-cell-autonomous function of WUS.

Several other transcriptional regulators boost the expression of *AG* in the center of FMs, including the basic leucine zipper (bZIP) transcription factor PERIANTHIA (PAN). PAN binds directly to the second intron of *AG* (Das *et al.*, 2009; Maier *et al.*, 2009) and appears to activate *AG* expression, as flowers overexpressing a fusion between PAN and a transcriptional repressor domain are indeterminate (Das *et al.*, 2009), a hallmark of *ag* mutant flowers (Bowman *et al.*, 1991). PAN seems to act redundantly with other factors to maintain high levels of *AG* expression, which are required to maintain floral determinacy (Prunet *et al.*, 2009).

The original ABC model predicted that A and C function genes act in a mutually antagonistic manner (Bowman *et al.*, 1991). In fact, AP2 suppresses *AG* in the outer two floral whorls; however, AG does not appear to control *AP2* expression levels, as *AP2* transcripts do not accumulate in the center of *ag* mutant flowers (Wollmann *et al.*, 2010). Rather, it has been shown that this function is mediated by the microRNA miR172, which prevents the accumulation of *AP2* mRNA and protein in the third and fourth whorls (Chen, 2004; Wollmann *et al.*, 2010). Perturbation of AP2 activity in the third whorl resulted in the conversion of stamens into petals (Wollmann *et al.*, 2010), implying that AP2 is sufficient to confer petal identity to floral organs, although it is not strictly necessary for the specification of petals (Krogan *et al.*, 2012). Recent genetic evidence implies that the transcriptional co-repressor family TOPLESS/TOPLESS RELATED (TPL/TPR) and HISTONE DEACTYLASE 19 (HDA19) are also required to suppress *AG* expression, and *in situ* hybridizations confirmed that the *AG* expression domain expands in these mutant backgrounds (Krogan *et al.*, 2012). Remarkably, expression of a fusion of the AP2 DNA-binding domain to the transcriptional co-repressor TPL under the control of the *TPL* regulatory sequence was sufficient to restore AP2 function in a strong *ap2* mutant background. Yeast two-hybrid and pull-down assays demonstrated that AP2 and TPL physically interact, while bimolecular fluorescence complementation (BiFC) and yeast two-hybrid assays showed that TPL interacts with the histone deacetylase HDA19 (Krogan *et al.*, 2012), whose activity leads to gene repression. Furthermore, ChIP experiments confirmed that AP2, TPL and HDA19 bind to the second intron of *AG*, which contains important regulatory elements. All of these data together indicate that an AP2-TPL-HDA19-containing complex is required to prevent *AG* expression in the outer two whorls. Using ChIP, the AP2-TPL-HDA19 complex members were also shown to bind to the regulatory regions of *AP3* and *SEP3*. Furthermore, expression of *AP3*, *PI* and *SEP3* was detected in the outer whorls of plants lacking functional copies of *AP2*, *TPL* or *HDA19* (Krogan *et al.*, 2012). Together, these data show that

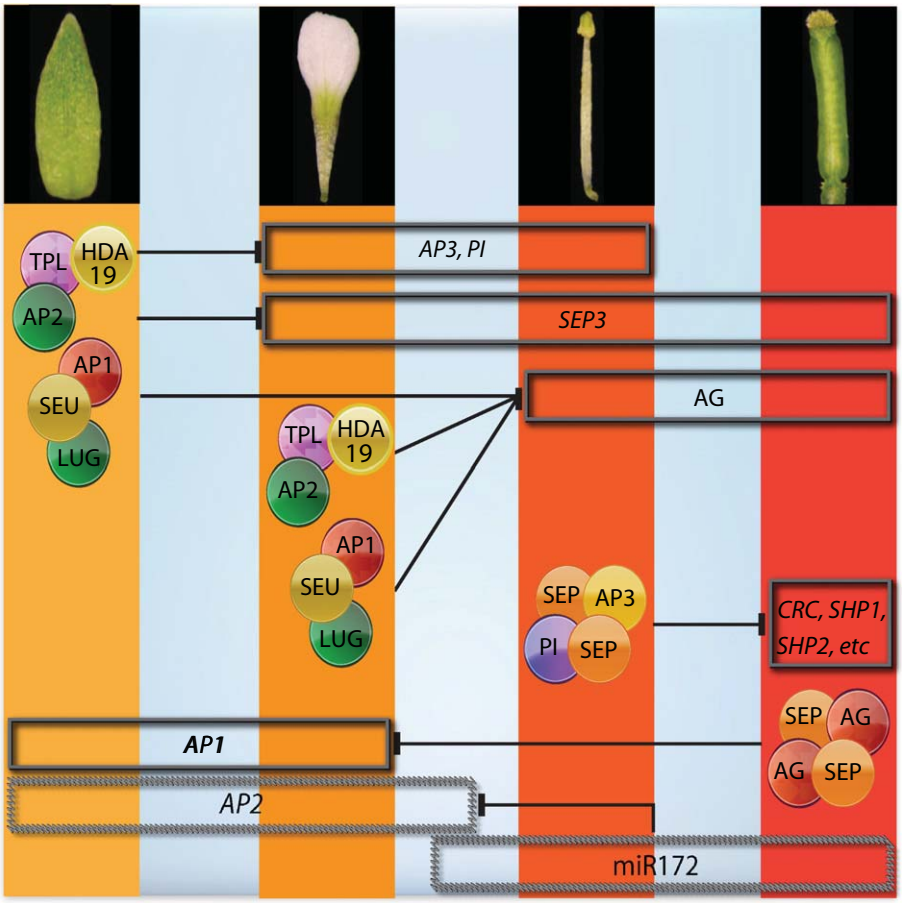

Fig. 4 Floral organ boundary maintenance by the ABCE proteins in *A. thaliana* flowers. First-whorl and second-whorl floral organ fate is promoted by the A-function proteins APETALA1 (AP1) and AP2. AP1 also interacts with SEUSS (SEU) and LEUNIG (LUG), while AP2 forms a complex with TOPLESS (TPL) and HISTONE DEACTYLASE 19 (HDA19). AP1 and AP2 repress C function in the first two whorls. These same protein complexes have been shown to suppress the expression of the B class homeotic regulators *AP3* and *PISTILLATA* (*PI*), as well as the E class gene *SEPALLATA 3* (*SEP3*) in sepals. AP3 and PI have been shown to transcriptionally suppress the expression of carpel-specification genes, such as *CRABS CLAW* (*CRC*), *SHATTERPROOF1* (*SHP1*) and *SHP2* in the third whorl. AGAMOUS (AG) suppresses, directly or indirect, *AP1* expression in the third and fourth whorls while miR172 prevents *AP2* mRNA accumulation in the inner two whorls. Whorls are represented by colored rectangles, from light orange (first whorl) to red (fourth whorl). Blue shading represents organ boundaries. Boxes with smooth outlines represent promoter activities, while boxes with jagged outlines represent RNA accumulation. Circles represent proteins and blunt-end lines indicate repression.

AP2-containing complexes act as spatial regulators of gene expression during floral patterning (Fig. 4).

V. Mechanistic insights into floral organ identity factor function

Through the use of genomic technologies, such as microarray analysis and ChIP coupled to next-generation DNA sequencing (ChIP-Seq), it has been shown that the floral organ identity factors influence the expression of thousands of downstream genes, through either direct or indirect regulation (Ito *et al.*, 2004; Wellmer *et al.*, 2004, 2006; Gomez-Mena *et al.*, 2005; Mara & Irish, 2008; Kaufmann *et al.*, 2009, 2010; Jiao & Meyerowitz, 2010; Wuest *et al.*, 2012). To directly control transcription, the MADS-domain transcription factors bind to DNA motifs with a similarity to the sequence 5′-CC(A/T)$_6$GG-3′, known as a CArG-box (Riechmann *et al.*, 1996a). Given that CArG-box motifs are extremely common in the *A. thaliana* genome (over 15 000 unique coding sequences are associated with a CArG-box in the *A. thaliana* genome) and that imperfect CArG-boxes (i.e. with mismatches to the consensus sequence) have also been shown to be functional (de Folter & Angenent, 2006), it is unlikely that the presence of this sequence in the promoter of a given gene is the solitary factor that dictates a transcriptional response (de Folter & Angenent, 2006). Indeed, genome-wide studies revealed that only a subset of genes that are associated with MADS-domain protein binding exhibit altered expression when their activities are perturbed (Table 1; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012). Similar observations have been made for several non-MADS-domain transcription factors from plants (Busch *et al.*, 2010; Yant *et al.*, 2010; Winter *et al.*, 2011) as well as from other eukaryotic model organisms (Farnham, 2009). Whether or not these 'transcriptionally silent' binding events are functional is currently unclear. However, in mammals, transcription factors are thought to be able to scan the entire genome for binding availability in a matter of minutes (Hager *et al.*, 2009). Given that ChIP merely identifies a snapshot of transcription factor binding without any kinetic information, the association of a transcription factor with sites it displays affinity for is perhaps unsurprising. Also, transcriptional control by the floral organ identity factors may require the presence of additional regulators at target gene promoters to elicit a transcriptional response, and it is from this perspective that the so-called floral quartet model provides a functional framework.

1. The floral quartet model

Aside from the highly conserved MADS DNA-binding domain, MIKC^c-type MADS-domain proteins contain at least three other functional domains, termed the I, K and C domains (Parenicova *et al.*, 2003). The K domain has been shown to be important for protein–protein interactions and is linked to the C domain, which

Table 1 Percentage of high-confidence target genes among genome-wide transcription factor-DNA localization studies

Protein of interest	Antibody	Genotype	Tissue	% DEG and bound	No. genes bound	References
AP1	αAP1	$35S_{pro}$:AP1-GR *ap1-1 cal-1*	Inflorescence meristem	10.8	2298	Kaufmann *et al.* (2010)
WUS	αWUS	Wild type	Seedlings	6.8	159	Busch *et al.* (2010)
AP2	αAP2	Wild type	Leaf	7.7	2135	Yant *et al.* (2010)
LFY	αLFY	$35S_{pro}$:LFY-GR	Seedlings	22	980	Winter *et al.* (2011)
LFY	αLFY	Wild type	Whole inflorescences	24.6	662	Winter *et al.* (2011)
AP3/PI	αGFP	$35S_{pro}$:AP1-GR $AP3_{pro}$: AP3-GFP *ap1-1 cal-1 ap3-3/pi-1*	Stage *c.* 5 flowers	22.3	1558	Wuest *et al.* (2012)

cal, *cauliflower*; DEG, differentially expressed gene; GR, glucocorticoid; WUS, WUSCHEL.
The experimental design (i.e. protein of interest, antibody used for immunoprecipitation, tissue and genotype) is indicated. The 'percentage of DEG and bound' genes was calculated by determining the overlap between genes that responded to a change in the transcription factor's activity and the genes associated with transcription factor binding, and dividing that number by the number of genes associated with transcription factor binding. The 'No. genes bound' refers to the number of genes that were within the vicinity of a binding peak, as defined in each individual study. In Kaufmann *et al.* (2010), *c.* 44% of genes that were bound by APETALA1 (AP1) showed some transcriptional response; however, only 10.8% of genes were defined as being 'robustly' differentially expressed. In Wuest *et al.* (2012), given that AP3/PISTILLATA (PI) are known to act as obligate heterodimers, the chromatin immunoprecipitation coupled to next-generation DNA sequencing (ChIP-Seq) and transcriptome studies performed on AP3 and PI were combined to define a set of high-confidence targets (22.3% of all bound genes). In Winter *et al.* (2011), *c.* 41% of genes bound by LEAFY (LFY) were rapidly differentially expressed upon LFY-GR activation. Some putative direct targets in Busch *et al.* (2010) and Winter *et al.* (2011) were omitted from the calculations, as they were not represented on the microarray platforms used.

has been suggested to act as a trans-activation domain, by the weakly conserved I domain (Riechmann & Meyerowitz, 1997; Gramzow & Theissen, 2010). Riechmann *et al.* (1996a,b) showed that AP1 and AG homodimers as well as AP3/PI heterodimers can form, in addition to higher order complexes composed of AP1/AP3/PI or AG/AP3/PI (Riechmann *et al.*, 1996a). Thus, it was proposed that the floral organ identity factors might target diverse sets of genes by exhibiting partner specificity. As mentioned above, the *SEP* genes were identified as being required for floral organ identity gene function (Pelaz *et al.*, 2000; Ditta *et al.*, 2004), but the mechanism underlying their activities was not initially clear. Through yeast two-hybrid and co-immunoprecipitation experiments, the SEP proteins were shown to interact with the MADS-domain floral organ identity factors (Honma & Goto, 2001). These findings led to the proposal that tetrameric complexes, consisting of SEP proteins with specific combinations of the floral organ identity factors, could selectively influence the transcription of bound target genes (Fig. 5; Theissen & Saedler, 2001). Several lines of evidence have been produced that are consistent with this scenario, including results obtained from *in vitro* DNA-binding assays, fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) experiments, affinity purification and mass spectrometry, BiFC of *in vivo* samples and genome-wide DNA–protein interaction studies (Riechmann *et al.*, 1996a; Immink *et al.*, 2009; Kaufmann *et al.*, 2009, 2010; Smaczniak *et al.*, 2012; Wuest *et al.*, 2012).

The floral quartet model predicts that quaternary complexes composed of MADS-domain proteins recognize two different CArG-boxes at the same time to regulate the expression of downstream targets (Melzer *et al.*, 2010). However, it has been demonstrated that multimeric complexes can bind single CArG-boxes or two CArG-boxes at the same time (Fig. 5a–d; Smaczniak *et al.*, 2012). Moreover, it has been shown that SEP3 homotetramers can mediate DNA looping, which suggests that regulation of gene expression may require distal enhancers (Fig. 5d; Melzer *et al.*, 2009).

Although the floral quartet model posits an explanation for how the floral organ identity factors bind DNA, it does not explain how target genes are selectively regulated. In this context, it is important to note that when the DNA-binding domains between AP1/AP3/PI/AG were swapped, their DNA-binding properties were not altered (Riechmann *et al.*, 1996a,b). Furthermore, when the amino-terminal halves of the MADS-domains of AP1, AP3, PI and AG were replaced with the corresponding regions of mammalian MADS-domain proteins, which have DNA-binding specificities distinct from those of the floral organ identity factors, their overexpression could (partially) rescue the defects of the floral homeotic mutants (Riechmann & Meyerowitz, 1997). These data strongly suggest that the DNA-binding specificity conferred by the MADS-domain is only one input for transcriptional regulation by the floral organ identity factors.

Data from genome-wide localization studies of floral MADS-domain transcription factors provided clues as to how the floral organ identity factors might specifically regulate downstream targets (Kaufmann *et al.*, 2009; Wuest *et al.*, 2012). Aside from CArG-box motifs, putative binding sites for several other transcription factor families were identified in close proximity to the genomic regions bound by the MADS-domain transcription factors. These include binding motifs for basic helix-loop-helix; bZIP; TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP); and zinc-finger transcription factors (Kaufmann *et al.*, 2009; Wuest *et al.*, 2012). Therefore, the combined activities of the floral organ identity factors and additional transcriptional regulators are probably necessary to determine when and where the expression of downstream genes is promoted or suppressed (Fig. 5a–d).

2. Co-factors and co-factor candidates

While interactions between MADS-domain proteins are well established in *A. thaliana* and other angiosperms (Immink *et al.*, 2010), candidates for proteins that can associate with them, and

may be required for their function, have been identified only sporadically until recently. For example, yeast-two hybrid screens and genetic analyses indicated that LEUNIG (LUG) and SEUSS (SEU), which are components of a transcriptional co-repressor complex, can form higher order regulatory complexes with AP1 or SEP3 to suppress *AG* expression in the outer two floral whorls (Fig. 4; Conner & Liu, 2000; Franks *et al.*, 2002; Sridhar *et al.*, 2006). A recent proteomic analysis of floral MADS-domain protein complexes confirmed the interactions between AP1 and SEU while also revealing several other candidate co-factors (Smaczniak *et al.*, 2012). For instance, using affinity purification and mass spectrometry, BELLRINGER (BLR), a homeodomain transcription factor, was shown to interact with AP1. Yeast three-hybrid assays further indicated that AP1/SEP3 or AP1/SEP4 heterodimers interact with BLR (Smaczniak *et al.*, 2012). Furthermore, using ChIP assays, it was shown that BLR binds to regulatory regions of *LFY*, *AP2* and *TOE1* (Smaczniak *et al.*, 2012) that are also targeted by AP1 during floral transition (Kaufmann *et al.*, 2010). Therefore, BLR/AP1 complexes probably cooperate to mediate the initiation of flowering.

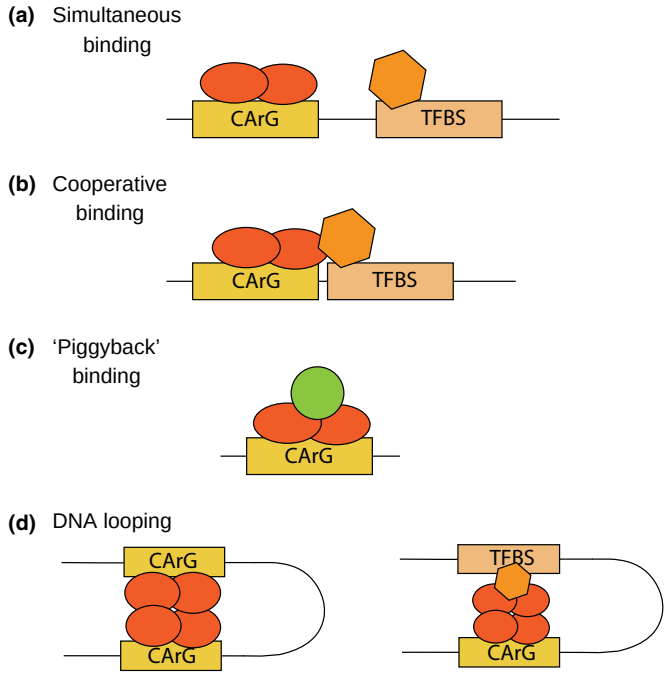

Fig. 5 Models for higher order MADS-domain protein complexes and their binding to DNA. Please note that the different scenarios shown are not mutually exclusive. (a) Dimers of MADS-domain proteins (represented by two ovals) may require the DNA binding of an additional transcription factor (s) (shown as a hexagon) to activate gene expression. In this model, a physical interaction between these different transcriptional regulators is not a prerequisite for activation of the target genes. (b) Dimers of MADS-domain proteins can directly interact with other transcription factors that bind to neighboring *cis* regulatory elements. (c) The transcriptional activity of MADS-domain transcription factor dimers may require interaction with an additional regulator (green circle) that does not bind DNA. (d) Floral quartets may mediate DNA looping to influence transcription *in vivo* (left). In some cases, DNA looping is thought to require interaction with additional regulators that bind to distinct regulatory elements in the promoter of target genes (right). TFBS, transcription factor binding site.

A number of proteins involved in chromatin modification also appear to interact with the floral organ identity factors. These include CHROMATIN REMODELING 4 (CHR4), CHR11 and CHR17, which associate with AP1, AP3, PI, AG and SEP3 (Smaczniak *et al.*, 2012). *In vitro* studies indicated that interactions between PI and CHR4, CHR11 and CHR17 are stabilized in the presence of double-stranded DNA, while *chr11 chr17* double mutants displayed several floral defects consistent with a role in organ specification (Smaczniak *et al.*, 2012). The ATPase chromatin remodelers BRAHMA (BRM) and SPLAYED (SYD) associate with AP1 and SEP3 (Smaczniak *et al.*, 2012). BRM and SYD are partially functionally redundant and appear to play a role in activating B and C class gene expression (Wagner & Meyerowitz, 2002; Hurtado *et al.*, 2006). In fact, LFY and SEP3 have been shown to interact with BRM and SYD to promote the expression of the floral organ identity genes (Wu *et al.*, 2012). The B and C function regulators also associate with SYD and BRM, although the functional significance of this interaction is currently unclear (Smaczniak *et al.*, 2012). The histone H3 lysine 27 (H3K27) demethylase RELATIVE OF EARLY FLOWERING 6 (REF6) appears to form a complex with AP1 (Smaczniak *et al.*, 2012). Overexpression of *REF6* produces pleiotropic defects including strong floral phenotypes such as carpelloid sepals (Lu *et al.*, 2011). Although AP1 has not yet been shown to influence H3K27 methylation levels, AG activity reduces the degree of H3K27 trimethylation at the promoter of *KNUCKLES* (*KNU*; Sun *et al.*, 2009), a key regulator of FM development, suggesting that the floral organ identity factors are indeed involved in the control of epigenetic modifications.

The results outlined above imply that the floral organ identity factors interact with many other proteins to control the transcription of target genes. However, the protein interactions described thus far do not indicate how transcriptional regulation of different sets of genes is achieved. A recent study of cardiac cell fate in *Drosophila* implied that DNA sequence specificity of a given transcription factor is only required to a certain degree to control gene expression during development (Junion *et al.*, 2012). Instead, it was proposed that a subset of transcription factors associate with *cis*-regulatory elements with extensive motif flexibility and that these transcription factors then recruit the remainder of the transcriptional complex. Although the general applicability of this model has yet to be tested, it provides an attractive scenario for the activity of the floral organ identity factors and for other regulators involved in the control of flower development.

VI. Gene expression programs controlled by the floral organ identity factors

As described above, there is now ample evidence that the activity of a small number of regulatory proteins is sufficient to superimpose floral organ identity upon an underlying developmental program that specifies leaves. However, the mechanistic basis of this organ transformation remains largely unknown. A combination of transcriptomics and genome-wide localization studies can be used to identify direct and indirect targets of transcription factors

(Malone & Oliver, 2011; Furey, 2012), and these types of studies have been performed for a number of floral organ identity factors. They showed that genes that are controlled by these master regulators have diverse functions (Gomez-Mena *et al.*, 2005; Mara & Irish, 2008; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012), suggesting that the regulation of a multitude of developmental pathways is involved in floral organ specification. In this section, we will review recent progress in the understanding of the gene expression programs controlled by the floral organ identity factors. We will start by discussing some of the technical approaches and innovations that have made these advances possible.

1. Experimental approaches for dissecting the flowering GRN

Cell specification is driven by differential gene expression, which is largely determined by the interaction between *cis*-regulatory elements and transcription factors (Wellmer & Riechmann, 2005). Complex combinations of transcription factor binding sites can exist within the regulatory regions of genes, and spatio-temporal expression changes of transcription factors further complicate the dissection of the GRNs that control cellular differentiation. Advanced techniques such as RNA-Seq or ChIP-Seq are now available that allow, on a genome-wide scale, the assessment of gene expression changes and transcription factor occupancy during development or in response to a perturbation of a regulatory gene. These novel approaches have led to detailed insights into the structure and composition of GRNs that would probably have remained elusive using traditional genetic and molecular techniques.

In the field of flower development, the application of these methods has been hampered by the fact that flowers of the main model *A. thaliana* are small, and young floral primordia are minute and difficult to dissect (Smyth *et al.*, 1990). Furthermore, *A. thaliana* flowers are initiated sequentially by the IM so that all flowers of an inflorescence are at different developmental stages (Fig. 2a; Smyth *et al.*, 1990), making the collection of sufficient amounts of tissue for further analysis prohibitively difficult. The so-called floral induction system offers easy access to relatively homogenous populations of young flower buds, allowing the assessment of the GRNs underlying *A. thaliana* flower development through the use of genomic and proteomic technologies (Wellmer *et al.*, 2006). The system is based on the overexpression of a fusion between AP1 and the hormone-binding domain of the rat glucocorticoid receptor in an *ap1 cal* double-mutant background (Wellmer *et al.*, 2006). As mentioned in Section II, *ap1 cal* plants overproduce IM-like tissue in place of flowers. Upon treatment with a steroid hormone, the AP1-GR (glucocorticoid) fusion translocates from the cytoplasm to the nucleus where it can influence transcription (Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010). Once the AP1-GR protein is activated, synchronous flowering is initiated throughout the inflorescence-like meristems. This system has been extensively used to investigate the molecular mechanisms underlying early flower development through a wide range of methods including genomic as well as proteomic approaches (Wellmer *et al.*, 2006; Das *et al.*, 2009; Sun *et al.*, 2009; Jiao & Meyerowitz, 2010; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012).

From genome-wide analyses of gene expression in young and more mature flowers, it has been concluded that the topology of the GRN underlying flower development changes considerably over time (Wellmer *et al.*, 2004, 2006; Gomez-Mena *et al.*, 2005; Wuest *et al.*, 2012). However, the stage-specific activities of the floral organ identity factors and of other floral regulators are currently not well defined. Also, given that the floral organ identity genes are expressed in more than one type of floral organ and that their expression patterns vary over time (Yanofsky *et al.*, 1990; Drews *et al.*, 1991; Jack *et al.*, 1992; Goto & Meyerowitz, 1994; Gustafson-Brown *et al.*, 1994), the current lack of organ- or tissue-specific information on gene expression during flower development greatly hampers the understanding of the gene networks mediating organ morphogenesis. Although fluorescence-activated cell sorting (FACS) and laser capture microdissection (LCM) have been successfully used to isolate specific cell types in plants (Birnbaum *et al.*, 2003; Wuest *et al.*, 2010; Liu *et al.*, 2011), these methods are extremely time-consuming and offer suboptimal yields and purity of specific cell types (Wang *et al.*, 2012). Translating ribosome affinity purification (TRAP; Heiman *et al.*, 2008) has also been used to produce organ- or cell-specific expression data in plants. TRAP entails epitope-tagging of ribosomal subunits so that polysomes can be immunoprecipitated and the associated mRNAs can be analyzed. Jiao & Meyerowitz (2010) used this technique in an elegant study to isolate transcripts present in the *AP1*, *AP3* and *AG* expression domains at two stages of early flower development using the floral induction system. By overlapping the over-represented transcripts from each expression domain, organ- and domain-specific transcripts were inferred.

An alternative method to TRAP utilizes affinity-based isolation of tagged nuclei from which either chromatin or RNA can be purified (Deal & Henikoff, 2010). Isolation of nuclei tagged in specific cell types (INTACT) has been used to identify differences in chromatin features and expression profiles of hair and non-hair cells in the *A. thaliana* root (Deal & Henikoff, 2010). In contrast to TRAP, which only allows analysis of polysome-bound RNA, INTACT also facilitates the analysis of chromatin through the isolation of nuclei. The yields of the tagged cell type(s) are extremely high and the technical set-up required to perform INTACT is relatively simple and cost-effective. By using the INTACT method in combination with the floral induction system described above, it should be possible to obtain data on gene expression and transcription factor occupancy with high spatial and temporal resolution.

2. Global insights into floral organ identity factor function

Genome-wide studies have revealed that many of the genes that are regulated by the floral organ identity factors encode transcriptional regulators (Table 2; Gomez-Mena *et al.*, 2005; Mara & Irish, 2008; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012), which are involved in controlling a multitude of diverse processes, from hormone signaling to boundary formation. There is now mounting evidence

that, in some cases, these regulatory proteins perform subsets of the functions initially attributed to the floral organ identity factors. Arguably the best example of such a mechanism is the transcription factor-coding gene *NOZZLE/SPOROCYTELESS* (*NZZ/SPL*), which is a regulator of sporogenesis (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). Both AG and AP3/PI have been shown to promote *NZZ/SPL* transcription through direct interactions with sites in the 3′ (Ito *et al.*, 2004) and 5′ (Wuest *et al.*, 2012) regions of the gene, respectively, and ectopic expression of *NZZ/SPL* can induce microsporogenesis in petals of *ag-1* mutant flowers (Ito *et al.*, 2004). Thus, it appears that the B and C function regulators control the most central aspect of reproductive floral organ formation through the activation of a single master regulatory gene.

The floral organ identity factors also directly modulate the expression of genes with general roles in leaf and shoot development. One example of this is *JAGGED* (*JAG*), which encodes a zinc-finger transcription factor that regulates the proliferative phase of lateral organ growth. Plants without a functional copy of *JAG* have serrated leaves and floral organ margins (Dinneny *et al.*, 2004, 2006; Ohno *et al.*, 2004) as a consequence of impaired anisotropic growth (Schiessl *et al.*, 2012). ChIP-Seq performed on whole inflorescences and young floral buds identified *JAG* regulatory sequences as being bound by SEP3 and AP3/PI, respectively (Kaufmann *et al.*, 2009; Wuest *et al.*, 2012). Moreover, *JAG* mRNA levels responded to an induction of AG activity (Gomez-Mena *et al.*, 2005) and the removal of AP3/PI activity (Wuest *et al.*, 2012), respectively, suggesting that B and C functions control *JAG* expression. Therefore, it appears that B and C function regulators have assimilated *JAG*, which performs a more general role during development, into the floral GRN.

Sharp gene expression boundaries exist during floral organ specification in eudicots; however, the same appears not to be not true for at least some basal angiosperms (Theissen & Melzer, 2007), where, for example, the expression of the B class genes is found outside of the petal and stamen domains (Kim *et al.*, 2005). This spatially expanded B class gene expression is reflected in the altered morphology of flowers of basal angiosperms which show gradual transitions from outer tepals to inner tepals, inner tepals to stamens, and stamens to carpels (Theissen & Melzer, 2007). These observations are summarized by the 'fading borders' model, which proposes that the floral organ identity genes are expressed strongly at the center, and weakly at the edges, of their expression domains (Soltis *et al.*, 2007). This then leads to a scenario where weak and partially overlapping expression domains of different classes of floral organ identity genes result in the production of chimeric or intermediate organs (Soltis *et al.*, 2007). Although there is currently only limited experimental support for this model (Kim *et al.*, 2005; Chanderbali *et al.*, 2010), it provides an attractive hypothesis for the molecular mechanism underlying the differences in floral morphology between basal angiosperms and other flowering plants.

In eudicots, such as *A. thaliana*, several genetic pathways promote the formation of sharp organ boundaries and of spatially well-defined expression domains of key floral regulators. Interestingly, the floral organ identity factors seem to play a decisive role in this process. For example, ChIP-Seq and transcriptome analysis showed that AP3 and PI directly suppress the expression of genes with known functions in carpel and ovule development (Wuest *et al.*, 2012). These data were further supported by the finding that an important regulator of carpel development, *CRABS CLAW* (*CRC*), is precociously expressed in the third whorl of B function mutants (Bowman & Smyth, 1999). Furthermore, artificial microRNAs (amiRNAs) that specifically target the transcripts of *AP3* or *PI* were used to perturb the function of these genes at various stages of flower development, and this resulted in abrupt transformations of stamens into carpels, without any chimeric

Table 2 Percentage of transcription factor-coding genes identified in microarray experiments on floral tissues

Genotypes	Comparison	Tissue	% DEGs encoding TFs	References
WT/*ap1-1/ap2-2/ap3-3/pi-1/ag-3*	RNA from wild-type samples was compared with RNA from mutant samples	Whole inflorescences	5.5	Wellmer *et al.* (2004)
$35S_{pro}$:AG-GR *ap1-1 cal-1*	RNA from nontreated samples was compared with RNA from DEX-treated samples	*ap1-1 cal-1* inflorescences	26	Gomez-Mena *et al.* (2005)
$35S_{pro}$:AP1-GR *ap1-1 cal-1*	RNA from mock-treated samples was compared with RNA from DEX-treated samples	*ap1-1 cal-1* inflorescences	13.4	Wellmer *et al.* (2006)
$35S_{pro}$:AP3-GR $35S_{pro}$:PI *ap3-3*	RNA from nontreated samples was compared with RNA from DEX-treated samples	Whole inflorescences	10.9	Mara & Irish (2008)
$35S_{pro}$:AP1-GR *ap1-1 cal-1*/$35S_{pro}$:AP1-GR *ap1-1 cal-1 ap3-3*/$35S_{pro}$:AP1-GR *ap1-1 cal-1 pi-1*	RNA from B-function mutant samples was compared with RNA from non-B-function mutant samples after DEX treatment	*ap1-1 cal-1* inflorescences	13	Wuest *et al.* (2012)

ag, *agamous*; *ap*, *apetala*; *cal*, *cauliflower*; DEG, differentially expressed gene; GR, glucocorticoid; *pi*, *pistillata*; TF, transcription factor; DEX, dexamethasone; WT, wild type.
The experimental design (i.e. genotype, samples being compared and tissue type) is indicated. Transcription factors represent *c.* 6% of the coding genes in the *Arabidopsis thaliana* genome (Riechmann *et al.*, 2000).

intermediates (Wuest *et al.*, 2012). Together these data indicate that B function behaves as a molecular switch that determines whether female or male reproductive organs are specified, and this mechanism may underlie the formation of bisexual axes in angiosperm flowers (Theissen *et al.*, 2002).

Another factor contributing to the formation of sharp organ boundaries in *A. thaliana* might be the formation of feedback loops upon floral organ identity gene activation (Gomez-Mena *et al.*, 2005; Theissen & Melzer, 2007). This type of regulation would lead to amplification of small differences, resulting in a switch-like 'on–off' behavior of gene expression. In support of this idea, the B and C function regulators in *A. thaliana* have been shown to boost their own expression (Goto & Meyerowitz, 1994; Gomez-Mena *et al.*, 2005; Wuest *et al.*, 2012). Also, they directly regulate several other genes that are required to maintain floral organ boundaries, such as *RABBIT EARS* (*RBE*) and *SUPERMAN* (*SUP*), which themselves control floral organ identity gene expression (Sakai *et al.*, 1995, 2000; Takeda *et al.*, 2004; Krizek *et al.*, 2006; Wuest *et al.*, 2012). These mechanisms, together or separately, could have been central to the transition between the putative fading borders situation found in flowers of some basal angiosperms and the sharp borders observed in eudicot flowers (Theissen & Melzer, 2007).

VII. Network modeling

Mathematical models can accurately describe how genetic interactions translate into phenotypic traits (Hammer *et al.*, 2006). Once these models have been generated, discrepancies between experimental data and the proposed model can be identified and investigated. Furthermore, modeling can be used to analyze causality, as parameters can be explicitly defined and explored by simulation (Prusinkiewicz & Runions, 2012). Initially, mathematical models for flower development were based on experimental data derived from a small number of genes (Espinosa-Soto *et al.*, 2004). More recently, production of comprehensive floral GRNs has become possible as a result of the availability of functional genomics tools. However, the ability to translate these large-scale data sets into GRNs and to mathematically model their dynamic behavior is currently limited (Moreno-Risueno *et al.*, 2010).

A discrete model of the ABCE model of floral organ identity specification was generated based on logical rules that were derived from existing experimental data (Espinosa-Soto *et al.*, 2004). The model showed that all possible starting conditions of the network converge to one of five steady states (i.e. IM, sepals, petals, stamens or carpels). Recovery of steady states that correspond to described expression patterns was also possible in mutant backgrounds, supporting the robustness of the model (Espinosa-Soto *et al.*, 2004). It was noted that the steady states of reproductive organs appeared to be more stable than those of sepals or petals. This observation can be correlated with the fact that the presence and phenotypes of perianth organs are more variable than those of reproductive organs in angiosperms (Theissen & Melzer, 2007). The model also correctly predicted that AG is involved in a positive feedback loop to maintain its own expression (Gomez-Mena *et al.*, 2005).

Although gene network models are useful to predict genetic interactions, they typically do not include information on cell division patterns during morphogenesis. Time-lapse imaging of biological samples, commonly referred to as live imaging, allows spatio-temporal assessments of development to be made (Roeder *et al.*, 2011). The expression patterns of selected regulators can be studied and, once the acquired images have been processed and analyzed, mathematical models can be generated. Live imaging was used, for example, to investigate the appearance of different cell sizes in the *A. thaliana* sepal, which depend on whether or not a cell undergoes endoreduplication (Roeder *et al.*, 2010). A computational model predicted that the decision to divide and to arrest division was made largely at random. By incorporating the probabilities of each decision, which were calculated from the *in vivo* imaging data, the authors' model could recapitulate the dividing pattern of a wild-type sepal. Furthermore, when these probabilities were altered in accordance with *in vivo* imaging data from mutant lines, the model accurately reflected cell division patterns produced in plants mutant for cell cycle regulators (Roeder *et al.*, 2010).

These examples show that mathematical modeling can be useful in predicting developmental outcomes. In fact, several developmental systems have already been extensively modeled using network analysis and live imaging approaches to facilitate hypothesis-driven research (Roeder *et al.*, 2011; Ryu *et al.*, 2013). Although impressive models have been generated that help to explain how the shape and growth of lateral floral organs are achieved (Cui *et al.*, 2010; Green *et al.*, 2010), predictive models that can accurately describe the dynamic behaviors of large-scale networks required for tissue differentiation and organ specification remain elusive.

VIII. Conclusions

Over the past 25 yr, considerable progress has been made in deciphering the GRNs and molecular mechanisms underlying the formation of flowers. Despite these advancements, several important aspects of flower development remain poorly understood. These include processes such as boundary formation as well as the differentiation and maturation of floral organs. Several lines of investigation must be followed to address these knowledge gaps and to further unravel the structure and composition of the flowering gene network. First, the activities of the known floral regulators must be analyzed with stage- and tissue-specific resolution. The availability of new experimental approaches, such as the TRAP or INTACT method, should dramatically facilitate this work. Secondly, the regulatory complexes that control gene expression during flower development must be characterized. To this end, advanced proteomics techniques are now available. A combination of these different approaches and the development of more sophisticated computational models with predictive functions should lead in the coming years to major advances in our understanding of the GRNs underlying the formation of flowers in *A. thaliana* as well as in other angiosperm species.

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