Molecular and Genetic Mechanisms of Floral Control

Thomas Jack¹

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

INTRODUCTION

In the last 15 years, knowledge of the molecular and genetic mechanisms that underlie floral induction, floral patterning, and floral organ identity has exploded. Elucidation of basic mechanisms has derived primarily from work in three dicot species: Antirrhinum majus, Arabidopsis thaliana, and Petunia hybrida. Although Antirrhinum and petunia have contributed fundamental breakthroughs to our understanding of flower development, it is from Arabidopsis that the most detailed and comprehensive picture of the molecular mechanisms underlying flower development has been obtained. In this review, I will outline the present state of knowledge, focusing on molecular and genetic mechanisms revealed in work on Arabidopsis, specifically in three areas: the integration of floral induction signals by a small group of floral integrators, the activation of the floral organ identity genes by the floral meristem identity genes, and interactions among the floral organ identity genes, particularly the A and C class genes.

By choosing to focus on progress in Arabidopsis, I do not mean to suggest that work in other species is unimportant or uninformative. To the contrary, without studies in Antirrhinum and petunia, our knowledge and the broad impact of what has been learned would clearly be less. One of the satisfying things about the field of flower development is the applicability of the floral patterning mechanisms to a wide range of plant species; such a conclusion only comes from careful analysis in multiple distantly related species. The general pattern in the field has been that molecular and genetic mechanisms, based on work in model species, serve as the basis for work in other species, many of which are of economic importance. Ultimately, the goal is to use information discovered in the model plants to engineer economically important plants for human and ecological benefit.

UNIFYING PRINCIPLES OF FLOWER DEVELOPMENT

The first unifying principle in the flower development field is the ABC model. This model, initially proposed in the early 1990s based on genetic experiments in Antirrhinum and Arabidopsis, is striking in its simplicity and is applicable to a wide range of angiosperm species, both dicots and monocots, including economically important grass species such as rice and maize (Bowman et al., 1991; Coen and Meyerowitz, 1991; Ambrose et al., 2000; Fornara et al., 2003). The Arabidopsis flower, like most angiosperm flowers, consists of four organ types that are arranged in a series of concentric rings or whorls. From outside to inside, the flower consists of sepals in whorl 1, petals in whorl 2, stamens in whorl 3, and carpels in whorl 4. The ABC model postulates that three activities, A, B, and C, specify floral organ identity in a combinatorial manner. Specifically, A alone specifies sepals, A+B specifies petals, B+C specifies stamens, and C alone specifies carpels. A second major aspect is that A and C classes are mutually repressive. In the absence of A, C activity is present throughout the flower. Likewise, in the absence of C, A activity is present throughout the flower. Throughout the 1990s, the ABC genes were cloned from a wide range of species, and numerous molecular studies were performed. These molecular experiments largely support the major tenets of the ABC model (reviewed by Weigel and Meyerowitz, 1994; Yanofsky, 1995; Ng and Yanofsky, 2001b; Lohmann and Weigel, 2002).

The second major unifying principle involves the central role of the LEAFY (LFY) gene (Coen et al., 1990; Weigel et al., 1992). LFY orthologs are present in a wide range of flowering and nonflowering plant species (Frohlich and Parker, 2000; Gocal et al., 2001). In many developmental contexts, LFY is necessary and sufficient to specify a meristem as floral (Weigel and Nilsson, 1995). In addition, LFY serves two key roles in specifying flowers. First, LFY is a key integrator of the outputs of floral inductive pathways (Nilsson et al., 1998; Blazquez and Weigel, 2000). Second, LFY is a key activator of the floral organ identity ABC genes (Weigel and Meyerowitz, 1993; Parcy et al., 1998; Lenhard et al., 2001; Lohmann et al., 2001). Both aspects of LFY function are described in more detail below.

Broadly speaking, flower development can be divided into four steps that occur in a temporal sequence. First, in response to both environmental and endogenous signals, the plant switches from vegetative growth to reproductive growth; this process is controlled by a large group of flowering time genes. Second, signals from the various flowering time pathways are integrated and lead to the activation of a small group of meristem identity genes that specify floral identity. Third, the meristem identity genes activate the floral organ identity genes in discrete regions of the flower. Fourth, the floral organ identity genes activate downstream “organ building” genes that specify the various cell types and tissues that constitute the four floral organs.

MULTIPLE FLORAL INDUCTIVE PATHWAYS CONTROL THE TIMING OF FLOWERING

The flowering time genes function on four major promotion pathways: long-day photoperiod, gibberellin (GA), autonomous, and vernalization. Mutants in the long-day photoperiod

¹To whom correspondence should be addressed. E-mail thomas.p.jack@dartmouth.edu; fax 603-646-1347.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.017038.
promotion pathway are late flowering when grown in long-day photoperiods. Many long-day pathway genes encode proteins involved in light perception (e.g., PHYTOCHROME A and CRYPTOCHROME2) or components of the circadian clock (e.g., GIGANTEA and ELF3) (reviewed by Reeves and Coupland, 2000; Mouradov et al., 2002; Hayama and Coupland, 2003). The light and clock components ultimately lead to the activation of CONSTANS (CO). CO mutants are late flowering, particularly in long-day photoperiods (Koornneef et al., 1991). Overexpression of CO results in very early flowering (Simon et al., 1996; Onouchi et al., 2000). CO encodes a nuclear protein that contains two B-box zinc finger domains (Putterill et al., 1995). Despite the presence of zinc finger domains, there is no evidence that CO is a sequence-specific DNA binding protein. Instead, it seems likely that CO is a component of a transcriptional activation complex that is directed to specific target genes by another component of the complex that possesses sequence-specific DNA binding activity.

A second flowering time pathway involves the promotion of flowering by GA. Mutants defective in the biosynthesis of GA, such as ga1, exhibit dramatic delays in the timing of flowering when grown in short days but not long days, suggesting that GA is an important stimulator of flowering in the absence of long-day promotion (Wilson et al., 1992; Moon et al., 2003). To date, this pathway consists of only GA biosynthetic and GA response genes. In other words, no genes have been isolated that are clearly on a GA output pathway specific for flowering time control.

Genes on the third pathway, the autonomous pathway, function to control flowering in a photoperiod-independent manner. As a facultative long-day plant, Arabidopsis flowers more rapidly when grown in long days, but it does eventually flower when grown in noninductive short-day photoperiods. Autonomous pathway components play a role in this promotion. The fourth major pathway is the vernalization pathway. An extended cold treatment (vernalization) that mimics overwintering stimulates flowering in many Arabidopsis accessions.

The details of the functions and interactions among the flowering time genes have been the focus of several recent reviews (Koornneef et al., 1998; Mouradov et al., 2002; Simpson and Dean, 2002) and will not be described in detail here. Instead, I will focus on how flowering time signals are integrated and how these signals function to activate downstream meristem identity genes (Figure 1).

Ultimately, the flowering time genes function to control the activity of a much smaller group of meristem identity genes. The meristem identity genes can be divided into two subclasses: the shoot meristem identity genes and the floral meristem identity genes. Shoot meristem identity genes such as TERMINAL FLOWER1 (TFL1) specify the inflorescence shoot apical meristem as indeterminate and nonfloral (Bradley et al., 1996, 1997). In tfl1 mutants, the shoot inflorescence meristem develops as a flower, resulting in a terminal flower phenotype in Arabidopsis, a plant that normally develops indeterminate inflorescences (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Ectopic expression of TFL1 (e.g., 35S:TFL1) converts the normally floral lateral meristems that arise on the flanks of the shoot apical meristem into shoots (Ratcliffe et al., 1998).

The second subclass, the floral meristem identity genes, specify lateral meristems in Arabidopsis to develop into flowers rather than leaves or shoots. After bolting, Arabidopsis plants produce between two and five cauline leaves on the primary inflorescence before developing flowers. In the axil of each of the cauline leaves is a secondary inflorescence meristem that gives rise to a secondary shoot. In Arabidopsis, LFY and APETALA1 (API) specify the lateral primordia to develop as flowers rather than shoots. Both lfy and ap1 single mutants exhibit a partial conversion of flowers to shoots (Irish and Sussex, 1990; Schultz and Haughn, 1991; Weigel et al., 1992; Bowman et al., 1993). In lfy ap1 double mutants, lateral meristems in the plant are not specified as floral and instead strongly resemble shoots. Ify ap1 plants have a phenotype very similar to that of 35S:TFL1 (Weigel et al., 1992). Ectopic expression of LFY or API converts the inflorescence shoot apical meristem to a flower; 35S:AP1 and 35S:LFY flowers exhibit a terminal flower phenotype similar to that of tfl1 mutants (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Although API and LFY are the major floral meristem identity genes, other genes such as CAULIFLOWER (Bowman et al., 1993; Kempin et al., 1995), FRUITYFULL (Gu et al., 1998; Ferrandiz et al., 2000), and AP2 (Jofuku et al., 1994; Okamura et al., 1996, 1997b) play secondary roles in specifying floral meristem identity.

Both LFY and AP1 encode sequence-specific DNA binding transcription factors. AP1 is a member of the MADS family (Huijser et al., 1992; Mandel et al., 1992), whereas LFY encodes a plant-specific protein that exhibits strong similarity to other genes in Arabidopsis (Coen et al., 1990; Weigel et al., 1992). Transcription of AP1 and LFY in lateral meristems in many developmental contexts is sufficient to specify them as floral (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). However, the fact that 35S:LFY 35S:AP1 double transgenic
plants still undergo a vegetative growth phase, as indicated by the development of a small number of vegetative rosette leaves, suggests that there are other factors, independent of LFY and AP1, that determine the competence of the plant to flower (Blazquez et al., 1997).

**INTEGRATION OF FLOWERING SIGNALS BY FLC, SOC1, FT, AND LFY**

One of the key events in the development of flowers is the activation of LFY and AP1. LFY and AP1 respond, either directly or indirectly, to outputs of flowering time pathways. Some of the outputs of the flowering time pathways are integrated by LFY, whereas others are integrated upstream or in parallel to LFY by FLOWERING LOCUS C (FLC), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1), and FLOWERING LOCUS T (FT).

Repressive signals from the autonomous and vernalization pathways are integrated by the floral repressor FLC (Figure 1) (Michaels and Amasino, 1999; Sheldon et al., 2000b). FLC also integrates positive regulatory signals from the genes FRIGIDA (FRI) (Johanson et al., 2000) and PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1) (Noh and Amasino, 2003). FLC encodes a MADS transcription factor. Mutations in FLC result in early flowering, whereas overexpression of FLC causes late flowering. There is a strong correlation between the levels of FLC RNA/protein and the timing of flowering; high levels of FLC correlate with late flowering, and low levels of FLC correlate with early flowering. The autonomous pathway genes function to downregulate the levels of FLC RNA/protein. The best described molecular mechanism involves the autonomous pathway gene FLOWERING LOCUS D (FLD), which encodes a protein with similarity to a human protein that is a component of the histone deacetylase complex (He et al., 2003). Histone deacetylases function as transcriptional repressors by deacetylating histones, resulting in a transcriptionally inactive chromatin state. In fld mutants, histone H4 is hyperacetylated in the vicinity of the FLC transcription start site. The region of FLC that mediates FLD function is a 295-bp region of the first intron that, when deleted from FLC, results in both hyperacetylation of the FLC locus (resulting in high levels of FLC RNA) and a late-flowering phenotype (similar to fld mutants) (He et al., 2003). In a second autonomous pathway mutant, fve, histone H4 also is hyperacetylated at the FLC locus. At present, the molecular mechanisms for how other autonomous pathway genes control the levels of FLC are not well understood but are the focus of active investigation (Rouse et al., 2002). One intriguing possibility is that the autonomous pathway genes FY and FCA function to regulate the processing of FLC RNA (Quesada et al., 2003; Simpson et al., 2003).

Vernalization also results in a reduction in FLC RNA/protein levels. Several lines of evidence suggest that vernalization controls FLC epigenetically, either by altering the methylation state of FLC or by controlling chromatin structure (Sheldon et al., 2000a; Gendall et al., 2001). The establishment and maintenance of the downregulation of FLC by vernalization requires both 5′ promoter sequences and intragenic sequences (Sheldon et al., 2002). Deletion of the large 2.8-kb first intron of FLC, but retention of exons 1 and 2 and 5′ promoter sequences, results in a failure to maintain vernalization, suggesting that intron 1 of FLC mediates the maintenance of vernalization. This fits nicely with the putative role of VERNALIZATION2 (VRN2), a gene necessary for the maintenance of vernalization (i.e., stable downregulation of FLC levels after vernalization) (Gendall et al., 2001). VRN2 encodes a Polycomb group protein. In Drosophila and mammals, Polycomb proteins are important for stable transcriptional repression and are postulated to function by altering chromatin structure (Orlando, 2003). Interestingly, the FLC activator PIE1 encodes a protein with similarity to ATP-dependent chromatin remodeling proteins; in other systems, PIE1-like proteins function to put chromatin in a transcriptionally active state (Noh and Amasino, 2003).

In turn, FLC functions to repress the floral activator SOC1 (Figure 1) (Lee et al., 2000; Hepworth et al., 2002). SOC1, like FLC, encodes a MADS transcription factor (Lee et al., 2000). SOC1 is activated by the long-day promotion pathway via CO (Samach et al., 2000) as well as by the GA pathway (Borner et al., 2000; Moon et al., 2003). Integration of the FLC and CO signals is mediated by discrete elements in the SOC1 promoter (Hepworth et al., 2002). A consensus MADS binding sequence in the SOC1 promoter can be bound by FLC in vitro. Mutation of this binding sequence abolishes repression of SOC1 by FLC. Although a CO-responsive region of the SOC1 promoter was also defined, binding of CO to this sequence could not be demonstrated, either because the activation is indirect or because CO requires a cofactor for sequence-specific DNA binding. Future experiments will distinguish between these possibilities.

Although the GA-responsive element in the SOC1 promoter has not been defined, it is clear that removal of the FLC repression of SOC1 is not sufficient to result in high SOC1 transcript levels; upregulation of SOC1 also requires positive activation by either the GA or the long-day promotion pathway. The best evidence that the release of FLC repression is not sufficient for SOC1 upregulation comes from an analysis of ga1 mutant plants that express high levels of FLC RNA/protein because they contain functional alleles of both FRI and FLC. When short-day-grown ga1 FRI FLC plants are vernalized, levels of FLC RNA decrease in response to vernalization treatment but levels of SOC1 do not increase. Thus, the upregulation of SOC1 requires activation by the long-day pathway either via CO or via the GA pathway. In short days, the GA pathway is the only pathway that can activate SOC1 (Moon et al., 2003).

Like SOC1, LFY is a key integrator of output signals from the long-day promotion and GA pathways (Blazquez et al., 1998; Nilsson et al., 1998; Blazquez and Weigel, 2000). Separate LFY promoter elements have been shown to mediate the response to long days (photoperiod promotion) and short days (GA promotion) (Blazquez and Weigel, 2000). The GA effects on the LFY promoter require an 8-bp binding site that is a perfect match for the sequence recognized by a MYB transcription factor (Blazquez and Weigel, 2000). A MYB protein, AtMYB33, binds in vitro to a DNA probe containing the 8-bp LFY element but not to a mutant form of this element. Although AtMYB33 is upregulated by GA, it is not known if AtMYB33 is necessary for the GA activation of LFY. Analysis of atmyb33 mutants and overexpression lines should resolve this issue.
The photoperiod promotion effects on LFY may be mediated by SOC1 or by a second MADS gene, AGAMOUS-LIKE24 (AGL24). Like SOC1 loss- and gain-of-function alleles, agl24 loss-of-function mutants are late flowering, and overexpression of AGL24 results in early flowering (Yu et al., 2002; Michaels et al., 2003). At present, it is somewhat controversial whether AGL24 functions downstream of SOC1 (Yu et al., 2002) or in parallel to SOC1 (Michaels et al., 2003). However, it is clear that both SOC1 and AGL24 function upstream of LFY. What is still unclear is whether either SOC1 or AGL24 acts directly on LFY, because binding of SOC1 or AGL24 to the LFY promoter has not been demonstrated.

The third major integrator of flowering time pathways is FT (Kardailsky et al., 1999; Kobayashi et al., 1999). ft mutants are late flowering in long days (Kooymneef et al., 1991). The primary input to FT activation is long-day photoperiod promotion mediated via CO. The best evidence for this is the rapid induction of FT RNA in response to an inducible form of CO (CO fused to the rat glucocorticoid receptor) (Kobayashi et al., 1999). 35S:CO plants express increased levels of FT RNA and are very early flowering, but in 35S:CO ft, flowering time is delayed, demonstrating that FT functions downstream of CO (Onouchi et al., 2000; Samach et al., 2000). FT also receives inputs from FLC. This is best illustrated by the downregulation of FT RNA that occurs when 35S:CO is expressed together with 35S:FLC (Hepworth et al., 2002). FT is negatively regulated by EARLY BOLTING IN SHORT DAYS (EBS) (Pineiro et al., 2003). EBS encodes a protein that contains conserved motifs that suggest that EBS functions via chromatin remodeling. The molecular details of how FT integrates signals from CO, FLC, and EBS are unknown. In other words, it is not known if FT itself integrates these signals or whether the signals are integrated by a gene that functions upstream of FT.

The long-day promotion pathway functions by activating LFY and AP1 via separate branches of the photoperiod pathway. Downstream of CO, the pathway splits; one branch functions via SOC1 and LFY, the other via FT. CO is the last identified component of the long-day promotion pathway that is upstream of both LFY and FT. The branch of the pathway that acts via FT appears to promote flowering by ultimately activating AP1 rather than LFY (Ruiz-Garcia et al., 1997; Nilsson et al., 1998).

INTERACTION BETWEEN AP1 AND LFY
Although AP1 and LFY are necessary to specify floral meristem identity, they do not function independently of one another. Instead, AP1 functions largely downstream of LFY. The best evidence for this comes from analysis of combinations of gain-of-function and loss-of-function alleles of LFY and AP1. The floral promotion effects of 35S:LFY are blocked in an ap1 mutant (Weigel and Nilsson, 1995), but the floral promotion effects of 35S:AP1 are not blocked in a lfy mutant (Mandel and Yanofsky, 1995). However, in 35S:AP1 lfy, floral organ identity is not properly specified, demonstrating that LFY is necessary for the proper expression of floral organ identity genes, and this activity of LFY is independent of AP1.

The activation of AP1 by LFY is postulated to be direct. The best evidence for this comes from experiments that use an inducible form of LFY (fusion of LFY to the rat glucocorticoid receptor [LFY-GR]). The induction of LFY-GR in the presence of a protein synthesis inhibitor results in the rapid upregulation of AP1 RNA, suggesting that LFY directly activates AP1 (Wagner et al., 1999). The AP1 promoter contains a sequence that can be bound in vitro by the LFY protein (Percy et al., 1998), but this sequence has not yet been demonstrated to be necessary in planta for LFY activation of AP1.

TFL1
The regulation of the shoot identity gene TFL1 is poorly understood. TFL1 RNA accumulates in subapical cells in the shoot apex before the vegetative-to-reproductive phase transition, at ~2 to 3 days of seedling development when plants are grown in long days (Bradley et al., 1997). TFL1 is expressed at low levels in the vegetative shoot meristem and appears to play a role in preventing premature flowering. At later stages, TFL1 is upregulated and plays a role in repressing the expression of floral meristem identity genes such as LFY and AP1 in the shoot meristem. The upstream regulators of TFL1 are unknown. TFL1 encodes a protein that likely plays a role in signaling, perhaps as an inhibitor of mitogen-activated protein kinase pathways (Corbit et al., 2003). TFL1 is closely related to FT; the two proteins are 50% identical (Kardailsky et al., 1999). This high degree of similarity is surprising because FT and TFL1 have opposite effects on flowering timing: ft mutants are late flowering, and tfl1 mutants are early flowering, TFL1 and FT are members of a six-member gene family in Arabidopsis (Mimida et al., 2001). Future work will be aimed at determining the molecular function of this enigmatic group of proteins.

There is a mutually repressive relationship between the shoot identity gene TFL1 and the meristem identity genes LFY and AP1, and the repression is mediated at the transcriptional level. In tfl1 mutants, LFY and AP1 RNAs are expressed ectopically in the shoot apex (Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Bradley et al., 1997). Similarly, in lfy mutants, TFL1 RNA is expressed in the ectopic shoots (Ratcliffe et al., 1999). In 35S:LFY and 35S:AP1, TFL1 RNA levels are reduced dramatically (Lijegren et al., 1999). Likewise, in 35S:TFL1, LFY and AP1 RNA levels are reduced dramatically (Ratcliffe et al., 1998). It is possible that LFY and/or AP1, because they are transcription factors, bind directly to the TFL1 promoter, but the TFL1 promoter has not been characterized, so the details of this regulation have not been elucidated. Because TFL1 does not encode a transcription factor, the negative regulatory effects of TFL1 on LFY and AP1 are likely to be indirect. At present, the downstream components of the TFL1 pathway have not been identified. It also is not clear where TFL1 fits with regard to the major floral inductive pathways.

FLORAL ORGAN IDENTIY GENES
One of the important functions of the floral meristem identity genes is to activate the ABC floral organ identity genes. The A class genes specify the identity of sepal and petals that develop in whorls 1 and 2, respectively. A second function of A class genes is to repress C class activity in whorls 1 and 2. In
Arabidopsis, there are two A class genes: AP1 and AP2. The B class genes AP3 and PI are required to specify the identity of petals in whorl 2 and stamens in whorl 3. The C class gene AGAMOUS (AG) is necessary to specify the identity of whorl 3 stamens and whorl 4 carpels. The second major function of C class is to repress A class activity in whorls 3 and 4.

The general rule for the floral organ identity genes is that the gene products are expressed in the region of the flower that exhibits defects in mutants. For example, AG RNA is expressed in stamen and carpel primordia and throughout these organs once they have formed (Yanofsky et al., 1990). Similarly, the B class genes AP3 and PI are expressed persistently in petals and stamens (Goto and Meyerowitz, 1994; Jack et al., 1994). AP1 functions as both a floral meristem identity gene and a floral organ identity gene, and the different aspects of AP1 function are reflected in the AP1 expression pattern. During very early floral stages, when AP1 activity is required to specify floral meristem identity, AP1 RNA is present throughout the floral primordium. At later floral stages, when AP1 activity is required to specify the identity of sepals and petals, AP1 RNA is expressed exclusively in whorls 1 and 2 (Mandel et al., 1992; Gustafson-Brown et al., 1994).

The second A class gene, AP2, is the exception to the general rule stated above. Although AP2 functions only in whorls 1 and 2, AP2 RNA is present in all four floral whorls throughout flower development. This puzzling fact was explained recently by the discovery that AP2 is translationally repressed by a microRNA (miRNA) present in whorls 3 and 4 (Chen, 2004). The experiments that led to this exciting discovery are described in more detail below.

A NEW ADDITION TO THE ABC MODEL

In the last several years, it has become clear that a fourth set of genes, the SEPALLATA (SEP) genes, are necessary for proper floral organ identity (Pelaz et al., 2000, 2001a). The first indication that SEP genes played an important role in petal, stamen, and carpel identity came from cosuppression experiments in petunia and tomato (Angenent et al., 1994; Pnueli et al., 1994). In petunia, a transgenic line designed to cosuppress the SEP3 ortholog FLORAL BINDING PROTEIN2 (FBP2) resulted in floral organ identity transformations in whorls 2, 3, and 4 as well as a loss of floral determinacy. In these FBP2 cosuppressed plants, the SEP/FBP2 subfamily member FBP5 also is downregulated, suggesting that both FBP2 and FBP5 are necessary for organ identity specification of petals, stamens, and carpels as well as for proper floral determinacy (Ferrario et al., 2003). These observations were extended with genetic analysis of the three SEP family members in Arabidopsis: SEP1, SEP2, and SEP3. Single and double sep mutant combinations fail to exhibit a dramatic phenotype in floral development. By contrast, sep1 sep2 sep3 triple mutants consist entirely of sepal-like organs, and their flowers are indeterminate (Pelaz et al., 2000). The phenotype of sep1 sep2 sep3 triple mutants is similar to that of double mutants that lack both B and C class activity, such as pi ag and ap3 ag (Bowman et al., 1989). Based on this fact, the three SEP genes are postulated to function redundantly to specify petals, stamens, and carpels as well as floral determinacy.

The discovery of the importance of the SEP genes has led to a revision of the ABC model (Goto et al., 2001; Theissen, 2001; Theissen and Saedler, 2001). The SEP genes also are referred to as E class genes. The revised “ABC’’ model postulates that sepals are specified by A activity alone, petals by A + B + E, stamens by B + C + E, and carpels by C + E (Figure 2).

D CLASS AND OVULE DEVELOPMENT

The SEP genes are referred to as E class rather than D class because a second set of genes, initially characterized in petunia, was previously named D class genes (Colombo et al., 1995). The two genes FBP7 and FBP11 function to specify placenta and ovule identity in petunia. In FBP7 and FBP11 cosuppressed plants, ovules do not develop and are replaced by carpel-like structures (Angenent et al., 1995). In the cosuppressed lines, both FBP7 and FBP11 are downregulated, and downregulation of both genes appears to be necessary for the ovule-to-carpel transformations, because single loss-of-function fbp7 and fbp11 mutants do not exhibit an ovule phenotype (Vandenbussche et al., 2003). Ectopic expression of FBP11 results in the development of ectopic ovules on whorl 1 sepals and whorl 2 petals (Colombo et al., 1995). Thus, in petunia, FBP11 is necessary and sufficient to specify ovule identity.

Figure 2. The Revised ABC Model of Flower Development. A, B, C, and E are four activities that are present in adjacent whorls of the flower. These four activities are postulated to function combinatorially to specify the identity of the four organs in the flower: sepals, petals, stamens, and carpels. A second major tenet of the ABC model is that A and C activities are mutually repressive. The specific molecular interactions between A and C class genes as well as their regulators are shown at right. The majority of ABC genes encode MADS domain transcription factors. Recent evidence suggests that MADS proteins function together in complexes larger than a dimer. The “quartet’’ model postulates that tetramers of MADS proteins specify floral organ identity (shown as colored ovals). Interactions demonstrated to be direct are indicated in red.
The Arabidopsis ortholog of FBP11 is AGL11, recently renamed SEEDSTICK (STK) (Pinyopich et al., 2003). STK functions redundantly with the closely related genes AG, SHATTERPROOF1 (SHP1), and SHP2 to specify ovule identity. Although carpels and ovules fail to develop in ap2 ag single mutants, carpelloid organs and ovules do develop in ap2 ag double mutants, demonstrating that genes independent of AG can specify carpel and ovule identity. This residual carpelloid identity in ap2 ag double mutants can be removed by eliminating either SPATULA or SHP1/2 activity (Alvarez and Smyth, 1999; Pinyopich et al., 2003). shp1 shp2 double mutants exhibit defects in valve margin development and seed dehiscence, but ovule development is normal (Liljegren et al., 2000). stk single mutants have defects in the development of the funiculus, the stalk that attaches the ovule to the placenta, as well as defects in release of the mature seed from the seed pod, but ovule identity is normal. By contrast, in stk shp1 shp2 triple mutants, most ovules arrest, suggesting that these three genes function redundantly to specify ovule identity. Consistent with this, ectopic expression of STK, SHP1, or SHP2 results in ectopic ovule development (Favaro et al., 2003), a phenotype similar to that observed when FBP11 is overexpressed in petunia. In the end, the Arabidopsis genes STK, SHP1, and SHP2 could be considered as D class genes because they function similarly to the petunia genes FBP7 and FBP11 in specifying ovule identity.

THE MAJORITY OF ABCE GENES ENCODE MADS TRANSCRIPTION FACTORS

Most ABCDE genes are members of the MADS transcription factor family, including the A class gene AP1, the B class genes AP3 and PI, the C class gene AG, the D class genes STK, SHP1, and SHP2, and the E class genes SEP1, SEP2, and SEP3. The A class gene AP2 is the exception; AP2 encodes a putative transcription factor that is a member of a small plant-specific gene family (Okamura et al., 1997a; Riechmann and Meyerowitz, 1998). In addition, several flowering time proteins (FLC, SOC1, AGL24, and SHORT VEGETATIVE PHASE [SVP] [Hartmann et al., 2000]) and meristem identity proteins CAULIFLOWER (CAL and FUL) also are MADS proteins. In Arabidopsis, there are >60 MADS genes (Alvarez-Buylla et al., 2000; de Bodt et al., 2003; Parenicova et al., 2003). The MADS family can be divided into two groups based on molecular evolutionary criteria. The vast majority of plant MADS genes of known function are type II MADS genes. The majority of type II MADS proteins have a characteristic domain structure. The MADS domain is located at the N-terminal end and encodes DNA binding, nuclear localization, and dimerization functions (McGonigle et al., 1996; Riechmann and Meyerowitz, 1997; Immink et al., 2002). A second conserved domain, the K domain, mediates protein–protein interaction and dimerization functions (Fan et al., 1997; Yang et al., 2003a). In a subset of plant MADS proteins, the C domain encodes a transcriptional activation domain (Moon et al., 1999; Honma and Goto, 2001). The C domain also has been reported to play a role in the formation of higher order MADS complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001). Recently, the extreme C-terminal end of AP3 was demonstrated to play a role in functional specificity (Lamb and Irish, 2003).

Type I MADS genes do not encode a K domain. Even though there are ~60 type I MADS genes in Arabidopsis, it was not until recently that a function was determined for the first member of this class. The type I MADS gene PHERES functions in early seed development (Kohler et al., 2003).

All MADS proteins studied to date bind to DNA as dimers, either homodimers or heterodimers. In Arabidopsis, AG has been demonstrated to bind to DNA either as a homodimer or as a heterodimer with SEP1 (Huang et al., 1996). By contrast, AP3 and PI do not form DNA binding homodimers but instead bind to DNA only as a heterodimer (Riechmann et al., 1996a, 1996b). The fact that neither A (AP1) nor C (AG) class proteins form DNA binding heterodimers in vitro with AP3 or PI makes heterodimerization an unlikely molecular explanation for why petal development requires A+B activities and stamen development requires B+C activities. At present, the in vivo significance of different MADS dimer combinations is not well understood.

COMBINATIONS OF ABCE GENES ARE SUFFICIENT TO DIRECT FLORAL ORGAN IDENTITY

The failure of floral organs to develop with the correct identity in A, B, C, and E class mutants demonstrates that the ABCE genes are necessary to specify floral organ identity. When expressed ectopically, the ABCE genes are sufficient to direct organ identity in flowers but not in vegetative leaves. For example, ectopic expression of both B class genes (35S:AP3 and 35S:PI) results in flowers that consist of two outer whorls of petals and two inner whorls of stamens, but leaves remain largely vegetative (Krizek and Meyerowitz, 1996). Based on this finding, it was concluded that AP3 and PI are sufficient, within the flower, to direct petal and stamen identity. Similarly, 35S:AG, 35S:SEP1, 35S:SEP2, and 35S:SEP3 do not alter the identity of the vegetative leaves. However, when the E class gene SEP3 is expressed ectopically together with AP3 and PI, both rosette and cauline leaves are converted to organs that resemble petals (Honma and Goto, 2001; Pelaz et al., 2001b). Furthermore, when AG is expressed ectopically together with AP3, PI, and SEP3, the cauline leaves are converted to organs that resemble stamens (Honma and Goto, 2001). These studies demonstrate that the E class genes, together with the ABC genes, are sufficient to direct floral organ identity in vegetative organs.

HIGHER ORDER MADS COMPLEXES?

Plant MADS proteins have been demonstrated to associate in complexes larger than dimers (Egea-Cortines et al., 1999; Honma and Goto, 2001; Ferrario et al., 2003; Yang et al., 2003b). This has led to a molecular model, the “quartet” model, which has received broad publicity but in fact is supported by only a limited amount of experimental evidence (Jack, 2001; Theissen, 2001; Theissen and Saedler, 2001; Eckardt, 2003). The quartet model (Theissen, 2001; Theissen and Saedler, 2001) postulates that tetramers of MADS proteins determine floral organ identity (Figure 2). Each tetramer is proposed to consist of two MADS dimers, each of which binds to a single
MADS binding site. The tetramers are formed by protein–protein interaction between MADS dimers, resulting in a tetramer that is simultaneously bound to two MADS binding sites. There are at least two molecular mechanisms that explain how these MADS tetramers result in an active transcription complex. One mechanism is that binding to two MADS binding sites is required, but the binding of MADS dimers is cooperative; specifically, binding of one dimer in the tetramer results in increased affinity for local binding of the second dimer in the tetramer. Some target genes have multiple consensus MADS binding sites in their promoters (e.g., GLOBOSA [GLO] in Antirrhinum [Tröbner et al., 1992] and AP3 in Arabidopsis [Hill et al., 1998; Tilly et al., 1998]). A second mechanism is that one or more subunits provides an activation domain to the tetramer to allow for efficient transcriptional activation. For example, both AP3 and PI lack activation domains, but SEP3 and AP1 possess activation domains (Honma and Goto, 2001). Thus, the inclusion of SEP3 or AP1 together with AP3/PI might result in a transcriptionally active complex.

The quartet model makes predictions about the composition of the tetramers in the four whorls of the flower (Figure 2). Specifically, in whorl 2, a combination of AP3/PI-SEP/AP1 is postulated to specify petals; in whorl 3, AP3/PI-SEP/AG is postulated to specify stamens; and in whorl 4, AG/AG-SEP/SEP is postulated to specify carpels.

One line of evidence that MADS proteins form higher order complexes comes from yeast two-hybrid and three-hybrid experiments. A two-hybrid screen using AG as bait identified SEP1, SEP2, and SEP3 as interacting proteins (Fan et al., 1997). A yeast three-hybrid screen, designed to identify proteins that interact with the AP3/PI heterodimer, but not with AP3 or PI alone, led to the isolation of SEP3 and AP1 (Honma and Goto, 2001). Interactions among AP3/PI/SEP3 and AP3/PI/SEP3 were confirmed by coimmunoprecipitation experiments (Honma and Goto, 2001), lending support to the hypothesis that MADS proteins form complexes that consist of more than two monomers. Similarly, in petunia, yeast three- and four-hybrid experiments demonstrated the existence of complexes that consist of $B+E$ and $B+E+C$ MADS proteins (Ferrario et al., 2003; Immink et al., 2003).

Evidence suggesting that these higher order MADS complexes are functional comes from DNA binding assays performed with Antirrhinum MADS proteins. In one key experiment, a probe containing two MADS binding sites exhibited enhanced DNA binding in the presence of both SQUAMOSA (SQUA) (the AP1 ortholog) and DEFICIENS (DEF)/GLO (the AP3/PI orthologs) compared with DEF/GLO or SQUA alone (Egea-Cortines et al., 1999). Based on this finding, the authors concluded that the B class proteins DEF/GLO and the A class protein SQUA formed a multimeric DNA binding complex.

At present, the nature of MADS protein complexes in planta is completely uncharacterized. For example, even though there is abundant evidence that AP3 and PI form a heterodimer in vitro and in yeast, an AP3/PI heterodimer has not been isolated from plant cells. Even less is known about other proteins that might be components of plant MADS protein complexes. Future work will focus on the biochemical characterization of MADS protein complexes from plant cells.

**ACTIVATION OF FLORAL ORGAN IDENTITY GENES BY FLORAL MERISTEM IDENTITY GENES**

Not only are AP1 and LFY necessary to specify floral meristem identity, they also are crucial to activate the floral organ identity genes. During early stages of flower development, both LFY and AP1 are expressed throughout the floral meristem (Mandel et al., 1992; Weigel et al., 1992; Gustafson-Brown et al., 1994; Blazquez et al., 1997). Despite the broad expression of LFY and AP1, the B class genes AP3 and PI and the C class gene AG are activated in only a subset of cells in the floral meristem. The B class genes are activated in the precursor cells for petals and stamens in whorls 2 and 3, and the C class gene AG is activated in the precursor cells for the stamens and carpels in whorls 3 and 4. Clearly, other factors must act in concert with LFY and AP1 to properly activate B and C class genes in spatially restricted patterns.

**INITIAL ACTIVATION OF THE B CLASS GENE AP3**

Evidence that LFY is important for the initial activation of AP3 comes from analysis of the AP3 expression pattern in ify mutants. Both the size of the domain and the level of AP3 expression are reduced in ify mutants (Weigel and Meyerowitz, 1993). Positive regulation of AP3 by LFY may be direct, because LFY binds in vitro to a LFY binding site located in an AP3 promoter element that directs the establishment of AP3 expression during early floral stages (Figure 3) (Hill et al., 1998). Surprisingly, mutation of this LFY binding site does not disrupt LFY activation of AP3 (Lamb et al., 2002). It appears that either this element is not the bona fide LFY binding site in vivo or there are redundant LFY activation elements in the AP3 promoter. Some of these LFY activation elements might function indirectly, thus explaining why LFY fails to bind to other regions of the AP3 promoter.

Proper activation of the B class gene AP3 is also dependent on UNUSUAL FLORAL ORGANS (UFO). ufo mutants resemble B class mutants in that petal and stamen numbers are reduced, which correlates with a reduction in the level of AP3 RNA during early floral stages (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Ectopic expression of UFO (35S:UFO) results in the partial conversion of first whorl sepals to petals and fourth whorl carpels to stamens; these organ identity conversions resemble those in 35S:AP3 and 35S:PI. Not surprisingly,

**Figure 3. Initial Activation of Floral Organ Identity Genes.**

LFY activates floral organ identity genes by functioning together with the coactivator WUS to activate the C class gene AG and together with UFO to activate the B class gene AP3. Interactions demonstrated to be direct are indicated in gray.
the sepal-to-petal and carpel-to-stamen transformations in 35S:UFO require AP3 and IPl activity (Lee et al., 1997).

All known UFO functions require LFY; Ify is epistatic to both ufo and 35S:UFO (Lee et al., 1997). The present model is that UFO functions together with LFY to activate AP3 (Figure 3). Although simultaneous ectopic expression of both LFY and UFO causes a seedling-lethal phenotype, when an AP3;β-glucuronidase reporter is placed together with 35S:UFO and 35S:LFY, 

β-glucuronidase is activated in the leaves of seedlings, demonstrating that LFY and UFO together are sufficient to activate AP3 (Parcy et al., 1998).

UFO RNA is expressed in three discrete patterns during early floral development. First, although UFO RNA is not detected in very young floral buttresses that have only recently differentiated from the inflorescence shoot meristem (stage 1), UFO RNA is detectable in slightly older floral meristems, before the morphological differentiation of any of the floral organ primordia (stage 2), in the precursor cells for whorls 3 and 4 (Ingram et al., 1995; Lee et al., 1997). Second, during floral stages 3 and 4, when the sepal primordia emerge, UFO RNA is detectable in the precursor cells for the petals and stamens (whorls 2 and 3) but not in whorl 4 carpel primordia. Third, beginning at stage 5, UFO RNA is detectable exclusively at the base of whorl 2 petals. Recent evidence suggests that each of these temporal expression patterns of UFO is associated with a discrete function (Ng and Yanofsky, 2001a; Durfee et al., 2003; Laufs et al., 2003).

UFO encodes an F-box protein (Simon et al., 1994; Ingram et al., 1995; Samach et al., 1999). In yeast, mammals, and plants, F-box proteins have been shown to be components of a complex, named the SKP1-cullin-F-box (SCF) complex, that selects substrates for ubiquitin-mediated protein degradation. UFO functions as a component of an SCF complex (Wang et al., 2003). Evidence that protein degradation is involved in UFO function comes from the suppression of UFO-overexpression phenotypes by mutants in the COP9 signalosome, a multisubunit complex that is postulated to constitute the lid of the 26S proteasome (Wang et al., 2003). At present, the protein target (or targets) of SFCF is unknown. The favored model is that UFO-mediated positive activation of AP3 occurs as a result of the SFCF-mediated degradation of a repressor of AP3. The expression of UFO in second and third whorl primordia during floral stages 3 and 4 is postulated to be associated with the initial transcriptional activation of AP3.

In addition to its role in activating B class genes, UFO has at least two other important functions. ufo mutants exhibit dramatic defects in floral organ positioning; the arrangement of organs, particularly in the second and third whorls, varies dramatically from flower to flower (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Early expression of UFO in whorls 3 and 4 during stage 2 is thought to be important for patterning the arrangement of floral organs.

A role for UFO in whorl 2 petal development is suggested by analysis of a class of weak ufo alleles, such as ufo-11, that fail to develop petals but have normal floral organ positioning. The fact that these ufo mutants develop normal stamens suggests that the ability of UFO to activate B class genes has not been compromised (Durfee et al., 2003). A function for UFO in whorl 2 organ development also is supported by the petal-specific phenotype observed in flowers that express UFO transiently for a restricted developmental period, only between stages 2 and 4 (Laufs et al., 2003). Based on these studies, it appears that the expression of UFO at the base of the petals beginning at stage 5 is necessary for whorl 2 organ development (Durfee et al., 2003; Laufs et al., 2003). Surprisingly, the petal-less phenotype of ufo alleles such as ufo-11 is dependent on AG, because ufo-11 ag double mutants develop a normal number of petals (Durfee et al., 2003). More surprisingly, AG RNA is not detected in whorl 2 in ufo-11 mutant flowers, suggesting that AG functions non-cell autonomously (i.e., AG-expressing cells in whorls 3 and 4 signal whorl 2 cells, leading to the suppression of organ development). One possible explanation is that the AG protein itself moves from the inner whorls to the outer whorls, perhaps via plasmodesmata. A second explanation is that AG could control a second gene whose RNA or protein could move from whorl 3 cells to whorl 2 cells. A third possibility is that either AG or an AG target could control signaling from the inner whorls to the outer whorls.

AP1 also plays a role in activating UFO. Specifically, AP1 is necessary for the accumulation of UFO RNA that occurs at the base of the petals during later floral stages (i.e., stage 5). In ap1 mutants, UFO RNA is not detectable at the base of the petals and whorl 2 organ development is largely suppressed (Ng and Yanofsky, 2001a). This is consistent with the role of UFO as a promoter of whorl 2 organ development, because whorl 2 organs rarely develop in ap1 mutants.

Like UFO and LFY, AP1 plays a role in activating B class genes (Figure 3). Although B class expression in whorls 2 and 3 is normal in ap1 mutants during early floral stages (Weigel and Meyerowitz, 1993), ap1 mutants rarely develop petals, suggesting that B class function is compromised, at least in whorl 2. One interpretation is that in the absence of AP1, UFO fails to be activated in the petals, and the failure to activate UFO results in a failure of petal development in whorl 2. This model offers a good explanation for why, despite the fact that B class gene expression is normal in ap1 mutants during early floral stages, petals most often fail to form. The failure of whorl 2 organ development in ap1 mutants is dependent on AG, as shown by the fact that whorl 2 organs develop in ap1 ag flowers (Irish and Sussex, 1990; Bowman et al., 1993).

Additional evidence that the positive regulatory effects of AP1 on B class activation are dependent on UFO comes from analysis of an activated form of AP1 that contains the strong viral VP16 activation domain. ap1 mutant flowers expressing an AP1:VP16 fusion exhibit a conversion of whorl 1 bracts to petal-like organs. The effects of AP1:VP16 are dependent on UFO because petals fail to develop in AP1:VP16 ufo (Ng and Yanofsky, 2001a).

Although our understanding has been clarified considerably in recent years, it remains unclear precisely how B class genes are activated during the early stages of flower development. The long-postulated repressor of AP3 that is the putative target of SFCF degradation has not been identified. Also, there are almost certainly additional unidentified activators of AP3, because in strong loss-of-function Ify and ufo mutants, AP3 RNA is still detectable. Even in ap1 Ify double mutants, a low level of AP3 RNA is detectable in a small group of cells (Weigel and Meyerowitz, 1993), suggesting the existence of an AP3-activation pathway that is independent of LFY and AP1.
**INITIAL ACTIVATION OF THE C CLASS GENE AG**

LFY is a positive activator not only of AP2 but also of AG. An activated form of LFY, fused to the strong VP16 activation domain, results in flowers with carpels and stamens in whorls 1 and 2, respectively, similar to AG overexpression lines, suggesting that one function of LFY is to activate AG (Parcy et al., 1998). Two redundant control regions that mediate the activation of AG by LFY map to the large second intron of AG (Busch et al., 1999). AG is unusual in that its second intron contains regulatory signals that are sufficient to mimic the wild-type AG temporal and spatial expression patterns (Sieburth and Meyerowitz, 1997; Bomblies et al., 1999; Busch et al., 1999; Deyholos and Sieburth, 2000). LFY protein binds in vitro to two elements in the AG second intron. Mutation of the LFY binding sites in these elements severely compromises the ability of LFY to activate AG (Busch et al., 1999).

As is the case with AP3, LFY functions together with a region-specific coactivator to activate AG in whorls 3 and 4. LFY activates AG in a subset of AG-expressing cells, together with the stem cell–promoting gene WUSCHEL (WUS) (Figure 3) (Mayer et al., 1998). ag mutants exhibit a loss of floral determinacy in addition to the floral organ identity defects in stamens and carpels. WUS has the opposite effect on meristems; WUS is necessary for meristems to retain their proliferative state. In wus mutants, the shoot meristem arrests and fails to develop past the embryonic stage (Laux et al., 1996). The notion that WUS might have a role in specifying organ identity comes from the demonstration that plants with reduced WUS activity lack the organs normally specified by C class (i.e., stamens and carpels) (Lenhard et al., 2001; Lohmann et al., 2001). wus is epistatic to ag with regard to floral meristem determinacy, suggesting that WUS is necessary for the indeterminacy observed in ag mutants (Laux et al., 1996). WUS is expressed during very early stages of flower development (stages 1 and 2) in a subset of the precursor cells for whorls 3 and 4, before the initial activation of AG. WUS, which encodes a homeodomain transcription factor, functions to activate AG in whorls 3 and 4 by binding directly to control sequences located in the second intron of AG. Mutation of these WUS binding sites eliminates WUS activation of AG (Lohmann et al., 2001). In summary, the initial activation of AG is dependent on the activities of both LFY and WUS, which function by binding to sequences in the second intron of AG.

After AG is activated in whorls 3 and 4 during early floral stages, AG, in turn, downregulates WUS in whorls 3 and 4. Failure to downregulate WUS in an ag mutant results in a loss of floral determinacy attributable to the meristem proliferation activity of WUS (Lenhard et al., 2001; Lohmann et al., 2001). It is not known if the AG downregulation of WUS is direct or indirect.

**INTERACTIONS AMONG ABC GENES**

The ABC genes are activated initially by floral meristem identity genes such as LFY, AP1, and UFO, but later patterns of expression are refined by interactions among the ABC genes themselves. The mutually repressive interaction between A and C class genes is one of the basic tenets of the ABC model (Figure 2). We have a partial understanding of how this repression is mediated. The two A class genes AP1 and AP2 are the first ABC genes to be expressed in the flower. As mentioned above, AP1 is not only an A class gene but also functions to specify floral meristem identity. To perform its role in floral meristem specification, AP1 is expressed in all four whorls of very young flower primordia (stage 1 flowers). Similarly, AP2 RNA accumulates in all four whorls of the flower throughout flower development; the AP2 RNA expression pattern is not spatially restricted in the flower at any stage (Jofuku et al., 1994). At the time when floral organs begin to morphologically differentiate from the floral meristem (stage 3), AG is activated in whorls 3 and 4 by LFY and WUS (Lenhard et al., 2001; Lohmann et al., 2001). AG, in turn, represses AP1 in whorls 3 and 4 (Gustafson-Brown et al., 1994). At present, it is not known if the AG protein binds directly to the AP1 promoter to mediate this repression.

By stage 5, AP1 RNA accumulates in whorls 1 and 2 and AG RNA accumulates in whorls 3 and 4. However, AP2 RNA remains detectable in all four floral whorls (Jofuku et al., 1994). AP2 activity is necessary for the repression of AG because in ap2 mutants, AG is expressed ectopically in whorls 1 and 2, resulting in organ identity transformations: sepals develop as carpels and petals develop as stamens (Drews et al., 1991). AP1, however, plays no role in AG repression, because AG is not expressed ectopically in whorls 1 and 2 in ap1 mutants (Weigel and Meyerowitz, 1993).

To complicate the picture further, the repression of AG does not depend solely on AP2. Several other genes, including LEUNIG (LUG), SUCESS (SEU), STERILE APETALA (SAP), and AINTEGUMENTA (ANT), also contribute to AG repression in whorls 1 and 2 (Liu and Meyerowitz, 1995; Elliott et al., 1996; Klucher et al., 1996; Byzova et al., 1999; Conner and Liu, 2000; Franks et al., 2002). In lug, seu, and sap single mutants, AG is expressed ectopically in whorls 1 and 2 (Liu and Meyerowitz, 1995; Byzova et al., 1999; Franks et al., 2002). In ant single mutants, AG is not expressed ectopically, but ant does enhance the weak ap2-1 allele, such that ant ap2-1 double mutants exhibit increased AG misexpression in whorls 1 and 2 (Krizek et al., 2000). A fifth repressor of AG is CURLY LEAF (CLF) (Goodrich et al., 1997). Although CLF represses AG in whorls 1 and 2 late in flower development, the more important function of CLF is to negatively regulate AG in vegetative tissues; in clf mutants, vegetative leaves are curled as a result of ectopic AG expression in leaves.

Several of these putative AG repressors encode proteins that function as repressors in yeast and animals. CLF encodes a homolog of the Drosophila Polycomb group protein Enhancer of Zeste (Goodrich et al., 1997). LUG encodes a protein that contains several WD repeats and is similar in overall structure to transcriptional corepressors such as Tup1 of yeast and Groucho of Drosophila (Conner and Liu, 2000). SEU encodes a protein with two Gin-rich domains and shares overall similarity with animal LIM domain binding transcriptional coregulators (Franks et al., 2002). ANT encodes a DNA binding protein related to AP2 (Elliott et al., 1996; Klucher et al., 1996).

In whorls 1 and 2, LUG, SEU, ANT, and AP2 function to repress AG. One model suggests that LUG and SEU form a corepressor
complex that is targeted to DNA by sequence-specific DNA binding proteins such as AP2 and ANT. It has been shown that LUG and SEU interact in a yeast two-hybrid assay (Franks et al., 2002). Both LUG and AP2 repression of AG have been demonstrated to be mediated by the second intron of AG (Sieburth and Meyerowitz, 1997; Bomblies et al., 1999; Deyholos and Sieburth, 2000); this is the same control region that mediates the activation of AG by LFY and WUS (Busch et al., 1999; Lohmann et al., 2001). Future experiments will define the precise nature of the repression complex and the specific sequences in the AG second intron by which this putative repressor complex functions.

For several years, it has been unclear how AG repression is confined to whorls 1 and 2, because RNA for AP2, SEU, LUG, and ANT is detectable in all four whorls of the flower. Recent work suggests that AP2, one of the proteins of the putative AG repression complex, is localized to whorls 1 and 2. Interestingly, this post-transcriptional regulation of AP2 is mediated by translational repression mediated by a miRNA (Chen, 2004).

One of the ongoing debates in the field concerns whether AP2 orthologs function as A class genes throughout the angiosperms. A class function was initially postulated based on the phenotype of mutants such as ap2 in Arabidopsis and ovulata in Antirrhinum, both of which result in homeotic conversions of sepals to carpels and petals to stamens (Bowman et al., 1989, 1991; Carpenter and Coen, 1990). However, the semidominant ovulata mutations are gain-of-function mutations in the C class gene PLENA, an Antirrhinum AG family member (Bradley et al., 1993). Despite extensive mutant screens in Antirrhinum, recessive single mutants with an ap2 phenotype have not been isolated, suggesting either that A function does not exist or that simultaneous mutation of more than one gene is required to eliminate A function. Support for the hypothesis that A function is not specified by AP2-like genes comes from experiments in petunia demonstrating that mutations in the AP2 ortholog in petunia (Phap2A) do not exhibit an ap2 phenotype (Maes et al., 2001).

Recently, a reverse-genetics approach was used in Antirrhinum to isolate mutations in two genes, LIP1 and LIP2, with high sequence similarity to AP2 (Keck et al., 2003). lip1 and lip2 single mutants exhibit a wild-type phenotype. By contrast, lip1 lip2 double mutants exhibit organ identity defects in whorls 1 and 2 and more subtle defects in whorls 3 and 4. Specifically, whorl 1 sepals develop as bract-like organs but do not exhibit carpelloid features. Whorl 2 petals are missing the lip and palate regions, but the petals do not exhibit staminoid features. Thus, LIP1 and LIP2 function redundantly to specify proper organ identity of whorl 1 sepals and whorl 2 petals, but they do not appear to play a role in the repression of C class in whorls 1 and 2. In Antirrhinum, C class repression is mediated by genes such as FISTULATA (FIS), STYLOSA (STY), and CHORIPETALAA (CHO) that do not play a major role in organ identity specification in whorls 1 and 2. In this regard, FIS, STY, and CHO function analogously to LUG, SEU, ANT, and CLF in Arabidopsis. In the end, it appears that the organ identity specification function of AP2-like genes is conserved in angiosperm species as divergent as Antirrhinum and Arabidopsis. By contrast, the C class repression function of AP2 may be restricted to Arabidopsis and its close relatives in the Brassicaceae.

NEW INSIGHTS INTO AP2 REGULATION

The solution to the AG repression dilemma came out of experiments aimed at characterization of the C class pathway. To isolate additional components of the C class pathway, enhancers of a weak ag allele, ag-4, were characterized. ag-4 flowers are indeterminate and exhibit a (sepal-petal-stamen), repeat pattern (Sieburth et al., 1995). By contrast, strong ag alleles such as ag-3 exhibit a (sepal-petal-petal), repeat pattern. One strong extragenic ag-4 enhancer was identified that produced flowers that resembled ag-3. The enhancer phenotype was attributable to mutations in two unlinked genes named HUA1 and HUA2 (Chen and Meyerowitz, 1999). As single mutants, both hua1 and hua2 exhibit weak enhancement of ag-4. Similarly, hua1 hua2 double mutants (in an AG− background) exhibit a very weak ag-like phenotype. Surprisingly, both hua1 and hua2 single mutants exhibit a wild-type floral phenotype. In retrospect, it is quite fortunate that both hua1 and hua2 were mutated simultaneously in the original ag-4 enhancer screen, because the hua1 hua2 double mutant served as the basis for the isolation of the next set of very important genes.

The second genetic screen was designed to isolate enhancers of the hua1 hua2 double mutant. A number of enhancers were isolated that exhibited an enhanced ag phenotype. These enhancers were categorized into several loci called the HUA enhancers, HEN1, HEN2, and HEN4 (Chen et al., 2002; Western et al., 2002; Cheng et al., 2003). The hua1 hua2 hen mutants exhibit a partial organ identity conversion of stamens to petals and partial loss of floral determinacy, but these mutants do not have a phenotype as strong as that of putative ag null mutants such as ag-3. In an otherwise wild-type background, single hen2 and hen4 mutants do not exhibit floral organ identity phenotypes. However, several of the hen single mutants exhibit a phenotype in vegetative organs, suggesting that HEN gene function is not restricted to the AG/C class pathway (Chen et al., 2002; Western et al., 2002; Cheng et al., 2003). For example, hen2 mutants exhibit phyllotaxy defects in the inflorescence and defects in the number and position of sepals and petals (Western et al., 2002). In addition, the hua1 hua2 double mutant and the hen2 and hen4 single mutants exhibit a small plant size phenotype (Western et al., 2002; Cheng et al., 2003).

HUA1, HUA2, HEN2, AND HEN4 FUNCTION IN THE PROCESSING OF AG mRNA

The first clue that the HUA and HEN genes might be involved in RNA metabolism came from the cloning of HUA1. HUA1 encodes a nuclear RNA binding protein with six CCCH zinc fingers (Li et al., 2001). Similarly, HEN2 encodes a DExH-box RNA helicase, similar to yeast DDB1 (Western et al., 2002). HEN4 encodes a KH domain protein (Cheng et al., 2003); the KH domain has been demonstrated to possess single-stranded RNA binding activity. HUA2 encodes a protein with less obvious similarity to proteins involved in RNA metabolism (Chen and Meyerowitz, 1999).

HUA1, HUA2, HEN2, and HEN4 play roles in the proper processing of AG mRNA. As described above, the large second intron of AG contains critical regulatory signals that mediate
activation by LFY and WUS and repression by A class activity. In the hen and hua mutants, partially processed AG RNAs accumulate at the expense of the fully processed AG mRNA. Characterization of these partially processed RNAs reveals that they contain exons 1 and 2 and the majority of the large intron 2, but not intron 1 or exons 3 to 7. These partially processed AG RNAs contain poly(A) sequences adjacent to intron 2 sequences, suggesting that premature transcriptional termination and polyadenylation have occurred within intron 2. Although processing defects were observed in hua1, hua2, and hen4 single mutants, increasingly severe defects were observed in double and triple mutants. Evidence that the ag-like phenotype that is observed in the hen and hua mutants is attributable to a reduction in fully processed AG mRNA comes from experiments showing that transgenic plants that ectopically express an AG cDNA (3SS:AGcDNA), which is not dependent on RNA processing for function, is able to rescue the stamen and carpel defects of hua1 hua2 hen4 triple mutants (Cheng et al., 2003).

Evidence suggesting that HUA1 and HEN4 function together in a complex comes from the demonstration that the nuclear localization of HEN4 is dependent on HUA1; specifically, in a hua1 mutant, HEN4 is not properly localized to the nucleus. In addition, both fluorescence energy resonance transfer and yeast two-hybrid analyses support the hypothesis that HUA1 and HEN4 exhibit a direct protein–protein interaction (Cheng et al., 2003).

The pleiotropic phenotypes of hen and hua mutants suggest that the HUA/HEN genes are not specific for the AG pathway. However, it is not the case that the HEN/HUA genes are general factors that function in RNA processing of all genes, because large aberrantly processed RNAs were not detected for the MADS genes AP3, PI, AP1, and FLC (Cheng et al., 2003). At present, the source of the specificity of the HEN/HUA proteins for particular transcripts such as AG is not understood.

The present model is that HUA1 and HEN4 form a complex that may or may not include HUA2 and that this complex either (1) suppresses cryptic polyadenylation sequences in the AG second intron or (2) promotes the removal of intron 2 before the cryptic polyadenylation sites in intron 2 are activated. Future work on the role of the HEN and HUA genes in AG mRNA processing will distinguish between these two models.

**HEN1 FUNCTIONS TO PROCESS A miRNA THAT TRANSLATIONALLY REpresses AP2**

HEN1 encodes a protein that functions similarly to DICERLIKE1 (DCL1), a protein important for the production of miRNAs and small interfering RNAs (reviewed by Bartel and Bartel, 2003). Mutations in DCL1 were isolated in a variety of screens for mutants in embryo development (named abnormal suspensor [Golden et al., 2002]), ovule development (named short integuments [Ray et al., 1996]), and flower development (named carpel factory [Jacobsen et al., 1999]). miRNAs are postulated to function to control the expression of specific genes by complementary base pairing with mRNA, resulting in either degradation of the mRNA or translational repression.

The discovery that animal genomes encoded hundreds of miRNAs (Carrington and Ambros, 2003) stimulated two groups to identify and characterize miRNAs in Arabidopsis (Park et al., 2002; Reinhart et al., 2002). As with animals, multiple candidate miRNAs were identified in Arabidopsis. Bioinformatics was used to predict potential target genes for these miRNAs (Park et al., 2002; Rhoades et al., 2002). One miRNA, miRNA172, was found to be complementary to a region of the A class gene AP2. This finding led to the hypothesis that perhaps HEN1 was involved in the production of a miRNA that regulated AP2. An elegant experiment that demonstrated that this might be the case involved the construction ap2-2 hua1 hua2 hen quadruple mutants. The majority of the HEN genes function directly on AG, likely by controlling the processing of AG mRNA, and thus function independently of AP2. As a result, most hen ap2 mutant combinations would be predicted to exhibit an additive phenotype. By contrast, if the phenotypic effects of HEN1 on AG are mediated via AP2, then hen ap2 mutant combinations would resemble ap2 mutants. The demonstration that the ap2-2 hua1 hua2 hen4 mutant resembles an ap2 ag double mutant and the ap2-2 hua1 hua2 hen1 mutant resembles an ap2 mutant supports the hypothesis that HEN1 acts via AP2 (Chen, 2004).

Additional evidence that miRNA172 regulates AP2 comes from ectopic expression experiments. Ectopic expression of miRNA172 (35S:miRNA172) results in flowers that exhibit an ap2 phenotype, suggesting that miRNA172 downregulates AP2 activity. Surprisingly, AP2 RNA levels are unaffected in 35S:miRNA172 but AP2 protein levels are reduced. This finding suggests that miRNA172, unlike other known plant miRNAs, functions by regulating the translation of AP2, not by promoting the degradation of the AP2 RNA. In situ hybridization experiments indicate that miRNA172 is expressed at the highest levels in whorls 3 and 4 of the flower, the region of the flower where A class activity does not function to repress C activity (Chen, 2004).

There is a single putative miRNA172 binding site in AP2 located near the 3’ end of the protein coding region. To test whether this putative binding site is functional, mutations were introduced and the mutated AP2 gene was expressed ectopically (35S:△miAP2). Control 35S:AP2wt flowers exhibit a wild-type phenotype; this is not surprising because AP2 RNA is expressed throughout the flower in wild-type plants (Jofuku et al., 1994). By contrast, 35S:△miAP2 plants exhibit an ag phenotype: stamen-to-petal transformations and loss of floral determinacy. Although the AP2 RNA levels were not changed in 35S:△miAP2 compared with 35S:AP2wt, the levels of AP2 protein were increased. Concomitantly, the levels of AG protein were decreased (Chen, 2004).

These experiments support the following model (Figure 2). AP2 RNA is expressed in all four floral whorls. In whorls 3 and 4, AP2 RNA is repressed translationally by miRNA172, which is expressed at the highest levels in whorls 3 and 4 (Chen, 2004). Although this is the first example of translational repression by a miRNA in plants, there are well-studied examples of translational repression mediated by miRNAs described in animals (Olsen and Ambros, 1999). In summary, miRNA172 appears to function as a negative regulator of AP2 in whorls 3 and 4. It is not clear at present what restricts miRNA172 to whorls 3 and 4; clearly, this will be an important area for future research. In addition, although it seems likely that AP2 accumulates only
whorls 1 and 2, it still has not been formally demonstrated, using AP2 antisera, that AP2 accumulates preferentially in whorls 1 and 2.

The finding that in Arabidopsis the AP2 mRNA was not restricted spatially in the flower (Jofuku et al., 1994) did not agree with the major tenets of the ABC model. In retrospect, the failure to determine the spatial expression pattern of the AP2 protein prevented the translational control of AP2 from being revealed. The flower development field has been quite complacent in its willingness to equate RNA expression pattern with domain of function. This dogma clearly holds true for the B class genes AP3 and PI and the C class gene AG, but not for the A class gene AP2.

The role of miRNA172 in AP2 repression does help explain another old observation that was puzzling and lacked a satisfactory explanation. In ag mutants, AG mRNA remains expressed in whorls 3 and 4 throughout flower development (Gustafson-Brown et al., 1994). This was a surprising result because the ABC model postulates that A and C classes are mutually repressive, so the prediction would be that in the absence of C class activity, A class activity would be present in all four floral whorls and would lead to the transcriptional repression of AG in whorls 3 and 4. Perhaps miRNA172 is the key; in an ag mutant, miRNA172 still can translationally repress AP2 in whorls 3 and 4. Without AP2 in whorls 3 and 4, the transcriptional repression of AG does not occur. If this is true, one prediction is that in an ag miRNA172 double mutant, AG would be repressed transcriptionally in whorls 3 and 4 at later floral stages.

**miRNA172 ALSO FUNCTIONS TO CONTROL AP2-LIKE GENES INVOLVED IN FLOWERING TIME CONTROL**

AP2 is not the only target of miRNA172; recent work also demonstrates that miRNA172 also controls an AP2 family gene involved in flowering time control (Aukerman and Sakai, 2003). This AP2 family gene, named TARGET OF EAT1 (TOE1), was identified because it exhibited a late-flowering phenotype when it was overexpressed in an activation-tagged line. The late-flowering phenotype could be mimicked by ectopically expressing TOE1 (3SS:TOE1). These experiments suggest that TOE1 normally functions as a repressor of the vegetative-to-reproductive floral transition. Consistent with this hypothesis, expression analysis in wild-type flowers revealed that TOE1 RNA levels were downregulated at the floral transition.

A second activation-tagged line, named early activation tagged, dominant (eat-D), exhibited an early-flowering phenotype and produced flowers that resembled ap2 mutant flowers (Aukerman and Sakai, 2003). Initial efforts to identify the gene responsible for the early-flowering phenotype focused on the two genes adjacent to the activation tag T-DNA insertion, as predicted by various gene prediction algorithms. Surprisingly, neither flanking gene, when expressed ectopically under 3SS control, reproduced the early-flowering phenotype. The 1.4-kb region between the two annotated genes then became an area of focus. Careful examination of this sequence revealed the presence of a noncoding RNA, specifically miRNA172a-2. Overexpression of the 1.4-kb region containing miRNA172a-2 phenocopied the eat-D early-flowering phenotype. However, a construct that overexpressed a version that contained a 21-bp deletion of the predicted mature miRNA172a-2 sequence failed to result in an early-flowering phenotype. This finding strongly suggested that the early-flowering phenotype was attributable to the overexpression of miRNA172-2. Examination of the TOE1 sequence revealed the presence of a putative miRNA172 binding site. Evidence that this binding site is functional comes from an analysis of plants that contain both the eat-D and toe1 activation tags; these plants exhibit an early-flowering phenotype, suggesting that the overexpression of miRNA172 leads to the inactivation of TOE1. Future work will analyze loss-of-function toe1 mutants to determine where in the flowering time hierarchy TOE1 functions. It also will be critical to examine the levels of TOE1 RNA and protein in response to changes in the levels of miRNA172. At present, it appears likely that miRNA172 controls TOE1 function via a translational repression mechanism that reduces the level of TOE1, similar to the mechanism described above for the miRNA172 regulation of AP2.

**FUTURE PROSPECTS**

The progress in the flower development field in the last 15 years has been impressive, but there are still many unanswered questions. Although the idea that the floral control genes function in a temporal hierarchy has been around for more than a decade, there are very few examples in which a regulatory relationship can be backed up with molecular and biochemical data that demonstrate a direct interaction (the gray arrows in Figures 1 and 3 and the red arrow in Figure 2 indicate direct interactions). Part of the problem is attributable to the fact that it has been difficult, until recently, to determine the target genes for transcription factors. Microarray technology should allow the targets of the floral control genes to be determined. Efforts to characterize targets for floral transcription factors are beginning (Zik and Irish, 2003) and should accelerate in the next several years. In these efforts, it will be important to distinguish direct from indirect targets. For the direct targets, bioinformatics will play a key role in identifying cis-acting sequences in the promoters for the target genes that are upregulated and downregulated in the presence or absence of a given transcription factor.

Another key area of future research is the biochemical characterization of biological complexes. Much better tools are available at present for isolating and purifying proteins from plant extracts. Proteomics approaches such as mass spectroscopy have the potential to define components of biochemical complexes. For a given floral transcription factor, an important future goal is to characterize the types of complexes involved in transcriptional activation and repression in various organs at different stages of development. The long-term goal is to try to correlate cis-acting promoter sequences in target genes (identified by microarray/bioinformatics) with specific protein complexes (identified by protein purification and mass spectrometry).

The efforts of many scientists, working with Arabidopsis and many other species, promise to lead to a better understanding of the molecular and genetic mechanisms of floral control in the years to come.
ACKNOWLEDGMENTS

I thank Xuemei Chen for communicating results before publication. I am grateful to laboratory members for comments on the manuscript. T.J. is supported by funds from the National Science Foundation (MCB-0090742) and the U.S. Department of Agriculture (2003-02586).

Received September 12, 2003; accepted January 9, 2004.

REFERENCES


