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Molecular and Genetic Mechanisms of Floral Control

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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

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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).

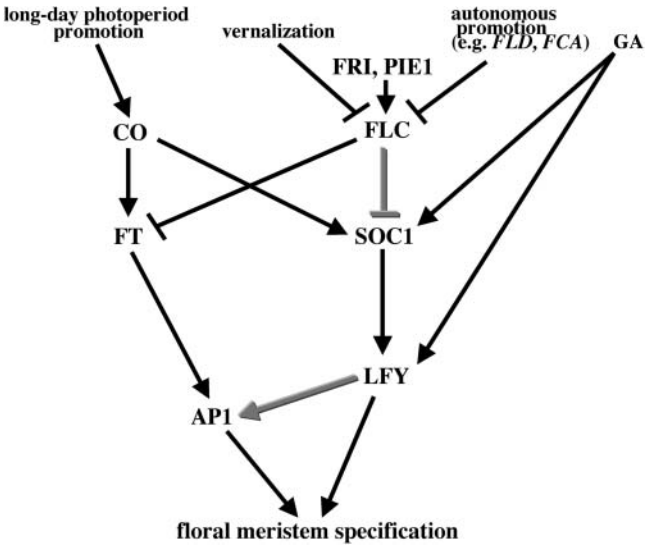

Figure 1. Major Floral Inductive Pathways.

Signals from the four major floral inductive pathways are integrated by *FLC*, *SOC1*, *FT*, and *LFY*. Interactions demonstrated to be direct are indicated in gray.

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* (*AP1*) 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. *lfy ap1* plants have a phenotype very similar to that of 35S:TFL1 (Weigel et al., 1992). Ectopic expression of *LFY* or *AP1* 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 *AP1* and *LFY* are the major floral meristem identity genes, other genes such as *CAULIFLOWER* (Bowman et al., 1993; Kempin et al., 1995), *FRUITFULL* (Gu et al., 1998; Ferrandiz et al., 2000), and *AP2* (Jofuku et al., 1994; Okamuro 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 no 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 also was 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 (Koornneef 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 (Parcy 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 (Liljegren 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 IDENTITY 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 sepals 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 *PISTILLATA* (*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 ''ABCE'' 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 *fpb11* 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.

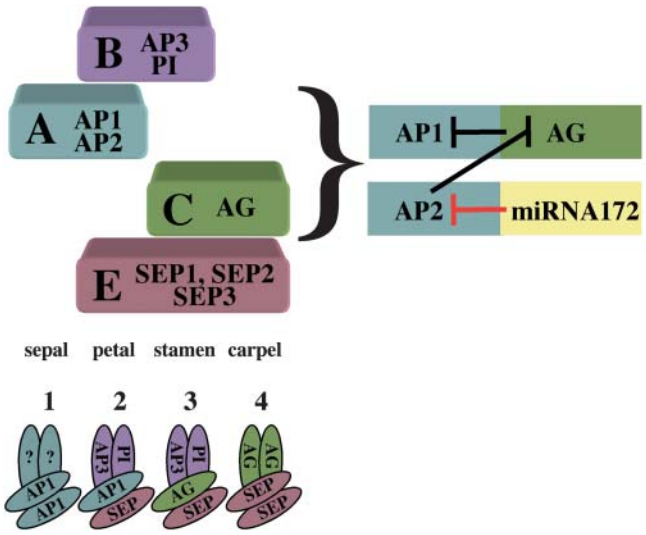

Figure 2. The Revised ABC Model of Flower Development.

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 *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 (Okamuro 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 >100 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/AP1 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 *lfy* mutants. Both the size of the domain and the level of *AP3* expression are reduced in *lfy* 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,

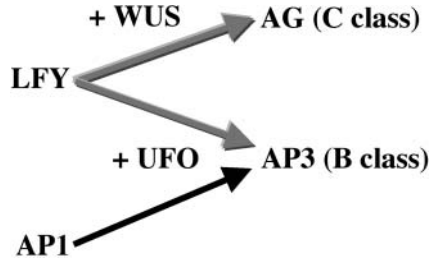

Figure 3. Initial Activation of Floral Organ Identity Genes.

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 *PI* activity (Lee et al., 1997).

All known *UFO* functions require *LFY*; *lfy* 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 proteosome (Wang et al., 2003). At present, the protein target (or targets) of SCF^{UFO} is unknown. The favored model is that *UFO*-mediated positive activation of *AP3* occurs as a result of the SCF^{UFO}-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 SCF^{UFO} degradation has not been identified. Also, there are almost certainly additional unidentified activators of *AP3*, because in strong loss-of-function *lfy* and *ufo* mutants, *AP3* RNA is still detectable. Even in *ap1 lfy* 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 *AP3* 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*), *SUESS* (*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 Gln-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 *CHORIPETALA* (*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 $(\text{sepal-petal-stamen})_n$ repeat pattern (Sieburth et al., 1995). By contrast, strong *ag* alleles such as *ag-3* exhibit a $(\text{sepal-petal-petal})_n$ 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 DOB1 (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 (35S: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:ΔmiAP2). Control 35S:AP2^{wt} 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:ΔmiAP2 plants exhibit an *ag* phenotype: stamen-to-petal transformations and loss of floral determinacy. Although the *AP2* RNA levels were not changed in 35S:ΔmiAP2 compared with 35S:AP2^{wt}, 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 in

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 in 1994 that 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* (35S:*TOE1*). These experiments suggests 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 35S 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 miRNA172a-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.

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