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A Role for Late Meristem Identity2 in the Reproductive Development of Arabidopsis

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Abstract
The switch from producing vegetative structures--branches and leaves--to producing reproductive structures--flowers--is a crucial developmental transition that significantly affects the reproductive success of flowering plants. In *Arabidopsis thaliana* , this transition is in large part controlled by the meristem identity regulator LEAFY (LFY) and the LFY direct target *APETALA1* (*AP1*). The molecular mechanisms by which LFY orchestrates a precise and robust switch to flower formation is not well understood. Here we show that the R2R3 MYB transcription factor and direct LFY target *LATE MERISTEM IDENTITY2* (*LMI2*) plays a role in the meristem identity transition. Like LFY, LMI2 directly activates *AP1* ; moreover LMI2 and LFY physically interact. LFY, LMI2 and AP1 are connected in a feed-forward and positive feedback loop network. We propose that these intricate regulatory interactions direct not only the precision of this critical developmental transition, but also contribute to its robustness and irreversibility.

Subsequent to the meristem identity transition floral primordia undergo a growth period prior to floral organogenesis. This growth phase is maintained in part by the flowering-time genes SHORT VEGETATIVE PHASE (SVP), AGAMOUS-LIKE24 (AGL24) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1). Eventually, these flowering-time genes are downregulated by AP1. This downregulation results in the termination of meristematic activity and the onset of floral differentiation. In the absence of AP1, ectopic expression of *SVP, AGL24* and *SOC1* prevents differentiation and leads to the development of floral meristems in the axils of the first whorl organs. These floral meristems give rise to "branched" flowers. Here we present a possible role for LMI2 during floral primordia growth. Similar to SVP, AGL24 and SOC1, AP1 downregulates *LMI2* in young flower primordia thus preventing the development of branched flowers. LMI2 acts in the same pathway as SVP, AGL24 and SOC1 and the similar expression patterns of LMI2 and SVP as well as the direct binding of LMI2 to *SVP* suggests a link between LMI2 and the pathways that maintain primordia growth during early flower development.

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For My Dad

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ABSTRACT

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The switch from producing vegetative structures - branches and leaves - to producing reproductive structures -flowers - is a crucial developmental transition that significantly affects the reproductive success of flowering plants. In *Arabidopsis thaliana,* this transition is in large part controlled by the meristem identity regulator LEAFY (LFY) and the LFY direct target *APETALA1* (*AP1*). The molecular mechanisms by which LFY orchestrates a precise and robust switch to flower formation is not well understood. Here we show that the R2R3 MYB transcription factor and direct LFY target *LATE MERISTEM IDENTITY2* (*LMI2*) plays a role in the meristem identity transition. Like LFY, LMI2 directly activates *AP1*; moreover LMI2 and LFY physically interact. LFY, LMI2 and AP1 are connected in a feed-forward and positive feedback loop network. We propose that these intricate regulatory interactions direct not only the precision of this critical developmental transition, but also contribute to its robustness and irreversibility.

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Chapter 1. Introduction

1.1. The reproductive transition in *Arabidopsis* and the morphological changes that occur during this time

Unlike animals, which generate all of their appendages during embryogenesis, plants develop new appendages throughout their lifecycle. Flowering plants transition through a number of distinct developmental phases and during each phase, different above ground organs are generated from a group of cells at the flanks of the shoot apical meristem (SAM) (Steeves, 1989). These cells, which form the primordia, produce new lateral meristems within the axils of developing leaves. These axillary meristems will eventually give rise to branches or flowers (Long and Barton, 2000).

In *Arabidopsis* development, during the vegetative phase the primordia cells give rise to a series of meristems located within the axils of vegetative leaves known as the basal rosette (Fig. 1). At the onset of the reproductive transition, endogenous and environmental stimuli induce the flowering-time pathways (Komeda, 2004; Baurle and Dean, 2006; Kobayashi and Weigel, 2007; Turck et al., 2008; Amasino, 2010). These pathways promote the upward growth of the SAM known as bolting. At this time, the primordia cells at the flanks of the SAM give rise to secondary inflorescence meristems in the axils of cauline leaves (Fig. 1). The last step of the reproductive transition is known as the meristem identity (MI) transition; during this phase the primordia cells generate floral meristems within the axils of developing reproductive leaves known as bracts (Long and Barton, 2000; Blazquez et al., 2006; Irish, 2010). Unlike rosette and cauline leaves, growth of the developing bract will eventually be repressed in *Arabidopsis* and the floral meristem will continue to grow and differentiate to produce the floral organs that make up the reproductive structure, the flower (Fig. 1) (Long and Barton, 2000).

1.2. Indeterminate vs. determinate growth

Similar to the lateral organs that are derived from the shoot apical meristem, floral organs are derived from a population of undifferentiated stem cells that make up the floral meristem (Blazquez et al., 2006; Sablowski, 2007). Unlike the shoot apical meristem which undergoes indeterminate growth, producing stem cells throughout the life cycle of the plant and continuously producing lateral organs, floral meristems are determinate in nature, they produce a finite number of stem cells to generate a defined number of floral organs that make up the flower (Sablowski, 2007). Although the growth patterns of the shoot and floral meristem are different, the factors involved in establishing the stem cell population for organogenesis are the same.

1.3. The first step in the reproductive transition in *Arabidopsis*-Induction of the flowering-time pathways

1.3.1. The flowering-time pathways

When to flower is an important decision that ultimately affects the reproductive success of the plant (Roux et al., 2006). Prior to flowering, plants must ensure that the energy and resources accumulated during the vegetative phase are optimally allocated to the production of offspring (Roux et al., 2006). Given the importance of this decision, plants have evolved many strategies to determine the appropriate time to flower. These strategies involve interpreting environmental stimuli including temperature, day length, light intensity and wavelength as well as endogenous cues such as developmental age and hormonal signaling (Simpson and Dean, 2002; Parcy, 2005; Amasino, 2010). These cues

are perceived by a series of regulatory pathways that ultimately transmit these signals to floral promoting factors in the SAM. The most well studied of these flowering-time pathways include the environmentally regulated photoperiod and the vernalization pathways and the endogenously controlled autonomous and GA biosynthesis pathways (Simpson and Dean, 2002; Parcy, 2005; Amasino, 2010).

1.3.2. Photoperiod pathway

The photoperiod pathway controls flowering-time by measuring the duration of day/night length (Turck et al., 2008; Amasino, 2010). This pathway relies on photoreceptors to sense and transduce the signal, as well as the circadian clock to measure day/night length (Simpson and Dean, 2002). The photoperiodic induction necessary for flowering varies amongst species (Garner, 1920). For example rice, a short-day plant, only flowers after perceiving a span of night that exceeds a critical length, whereas clover, a long-day plant, requires a shorter night span and flowers upon increases in day length (Amasino, 2010). *Arabidopsis* is considered a facultative long-day plant and as such it flowers rapidly under long-days; it will also flower under non-inductive short-day conditions but at a much slower rate (Gregory, 1953).

In plants, day length is perceived in the leaves (Knott, 1934). Upon perception in the leaf, a signal is transmitted to the SAM where flowering occurs. A component of this mobile signal termed “florigen” (Chailakhyan, 1936), was recently identified as FLOWERING LOCUS T (FT) (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007), a small protein similar to the phosphatidylethanolamine binding protein (PEBP) and the Raf kinase inhibitors in animals (Kardailsky et al., 1999; Kobayashi et

al., 1999). *FT* is activated in the leaf by a circadian regulated zinc finger protein known as CONSTANS (CO) (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Although CO is thought to be a transcription factor (Putterill et al., 1995) it has not been shown to bind to DNA, and therefore CO is hypothesized to interact with other proteins to activate its targets (Hepworth et al., 2002). Once activated, FT travels from the leaves to the shoot apex where it interacts with the bzip transcription factor FD to promote flowering. The FT/FD heterodimer upregulates the MI regulator and MADS box transcription factor *APETALA1* (*AP1*), as well as the floral promoting and MADS box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). At the moment it is not clear how FT promotes FD activity but FT is suggested to affect FD activity either through conveying transcriptional activation of FD or by postranslationally modifying FD (Abe et al., 2005; Michaels et al., 2005; Wigge et al., 2005; Yoo et al., 2005).

The photoperiod pathway promotes flowering by regulating the levels of CO RNA and protein, both of which are controlled by circadian cycles and light. Under long-day conditions, the circadian proteins known as CYCLING DOF FACTORS (CDFs) limit *CO* expression by binding to the *CO* promoter and repressing it during the early morning. During the afternoon, another protein known as the FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1), which is regulated by the blue-light receptors, is involved in the degradation of the CDF proteins. FKF1 interacts with the CDF proteins an interaction that is further mediated by the circadian protein GIGANTEA (GI). The degradation of the CDF proteins allows for the accumulation of *CO* RNA during the late afternoon (Turck et al., 2008; Amasino, 2010; Imaizumi, 2010). CO protein levels are also regulated by photoreceptors under long-day conditions. During the

early morning the red/far-red light receptor PHYTOCHROME B (PHYB) is thought to promote degradation of the CO protein, whereas in the late afternoon and evening, the CO protein is stabilized by the blue-light receptors, CRYPTOCHROME 1 and 2 (CRY1; CRY2) as well as the red/far-red light receptor PHYTOCHROME A (Valverde et al., 2004; Turck et al., 2008; Amasino, 2010). It is the accumulation of *CO* RNA and the stabilization of the CO protein that is necessary for the activation of *FT* under long-day conditions.

1.3.3. Vernalization pathway

Vernalization is the process by which an extended period of cold promotes floral competency (Chouard, 1960). Vernalization is a reproductive strategy that allows certain plant species including perennials, biannual and some *Arabidopsis* ecotypes, to remain vegetative during the cold months of winter prior to flowering during the spring and summer months (Simpson and Dean, 2002; Amasino, 2010). At the molecular level, the vernalization response causes the downregulation of the MADS box transcription factor and flowering-time repressor, FLOWERING LOCUS C (FLC). FLC is a robust floral repressor that is expressed in both the leaves and the SAM (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC functions with another floral inhibitor and MADS box transcription factor, SHORT VEGETATIVE PHASE (SVP). *SVP* is expressed throughout seedlings during vegetative growth but is repressed in the inflorescence meristem during the onset of the reproductive transition (Hartmann et al., 2000). SVP and FLC directly repress the expression of the floral promoting factors *SOC1* in the apex and *FT* in the leaves prior to the reproductive transition (Hepworth et al., 2002; Helliwell et

al., 2006; Searle et al., 2006; Lee et al., 2007; Fujiwara et al., 2008; Li et al., 2008; Amasino, 2010). Furthermore, FLC directly represses *FD* in the apex (Searle et al., 2006).

The components that make up the vernalization pathway function to reduce the level of *FLC* RNA (Amasino, 2010). Vernalization is mitotically stabilized, once cells experience a period of cold, *FLC* levels decrease and remain low throughout the remaining lifecycle of the plant (Amasino, 2010). The idea that the vernalization response maintains low levels of *FLC,* suggests it is epigenetically regulated. Indeed, many chromatin-modifying proteins have been identified that are important for conferring this vernalization response. The VRN proteins, VERNALIZATION 2 (VRN2), VERNALIZATION-INSENSITIVE 3 (VIN3) and VERNALIZATION 5 (VRN5) are a part of a complex similar to the Polycomb Repressor Complex (PRC2) that is conserved in a wide range of eukaryotes from plants to humans (De Lucia et al., 2008). The PRC2 complex functions by adding repressive marks to the histone tails of chromatin, more specifically, tri-methyl groups to H3K27 (Goodrich and Tweedie, 2002; Kohler and Villar, 2008). Therefore, during and after a long exposure to cold, low levels of *FLC* are thought to be maintained through the action of this PRC2- like complex which adds repressive H3K27 trimethylation marks onto the chromatin at the *FLC* locus (De Lucia et al., 2008).

There is evidence for a vernalization pathway that promotes flowering in an FLC-independent fashion (Michaels and Amasino, 2001). In support of this, *flc* null mutants still respond to vernalization and promote flowering faster than *flc* mutants that are not exposed to a period of cold (Michaels and Amasino, 2001). Similarly, the floral

promoting factor and MADS box transcription factor *AGAMOUS-LIKE24* (*AGL24*) is regulated by the vernalization pathway, yet this regulation does not depend on FLC as *AGL24* levels are not affected by FLC (Michaels et al., 2003).

1.3.4. Autonomous pathway

The autonomous pathway is generally defined as any non-environmental signaling pathway that influences flowering (Amasino, 2010). Most of the factors in the autonomous pathway function to downregulate the floral repressors *FLC* and *SVP* (Lee et al., 2007; Li et al., 2008). A majority of the proteins that act in this pathway, including FCA, FLOWERING LOCUS K HOMOLOGY (FLK), FPA, FY and possibly LUMINIDEPENDENS (LD), are involved in RNA metabolism (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Lim et al., 2004; Manzano et al., 2009). Other proteins including FLOWERING LOCUS D (FLD) and FVE are thought to be components of repressive chromatin modification complexes including histone deacetylases (He et al., 2003; Ausin et al., 2004; Shi et al., 2004). Furthermore, mutations in *fve* and *fld* cause changes in acetylation on the *FLC* locus (He et al., 2003; Ausin et al., 2004).

The RNA mediated gene silencing pathway is also considered to play a role in regulating *FLC* levels. For example, double mutants between *DICER-LIKE 1* and *DICER-LIKE 3* (*dcl1 dcl3*) show delays in flowering that are FLC-dependent (Schmitz and Amasino, 2007). These RNA components are thought to recruit chromatin-remodeling complexes to the *FLC* locus (Baurle and Dean, 2008). Long non-coding RNAs are involved in *FLC* regulation as well. Non-coding intronic RNAs have recently

been shown to recruit the PRC2-like complex to the *FLC* locus (Heo and Sung, 2011). Also implicated in *FLC* silencing are long non-coding RNA antisense transcripts. These transcripts, which cover the *FLC* locus, are cold induced and thought to repress *FLC* earlier than the PRC2 mediated silencing (Swiezewski et al., 2009; De Lucia and Dean, 2011). Finally, the floral repressor *SVP* is regulated by the autonomous pathway since *svp* levels are increased in both *fca* and *fve* mutants (Lee et al., 2007; Li et al., 2008).

Recently factors involved in the autonomous age-sensing pathway have been characterized. This pathway prevents flowering until plants have proceeded through a juvenile phase of development (Wu and Poethig, 2006). An important component of this pathway is the microRNA, miR156. miR156 promotes the juvenile phase, thereby preventing precocious flowering (Wu and Poethig, 2006). At the same time, a second microRNA, miR172 promotes flowering in part by downregulating factors that repress *FT* induction (Aukerman and Sakai, 2003; Jung et al., 2007; Mathieu et al., 2009). Throughout development, *miR156* and *miR172* have opposite expression patterns based on the age of the plant (Wu et al., 2009). During the juvenile phase, when *miR156* expression is high, it represses the positive regulators of *miR172*, the *SQUAMOSA PROMOTER BINDING LIKE* (SPL) transcription factor family. Later in development, when *miR156* levels are low, the SPL proteins are active and can upregulate the expression of *miR172* to promote flowering (Wu et al., 2009). Recently, the SPL transcription factors themselves have been shown to positively regulate flowering. Members of the SPL family directly upregulate *FT* expression in the leaves, as well as a number of floral promoting factors in the SAM, including, *SOC1*, and three MI regulators *LEAFY* (*LFY*), *AP1* and *FRUITFULL* (*FUL*) (Wang et al., 2009; Yamaguchi et al., 2009).

This age-dependent pathway is thought to be a conserved mechanism for flower regulation since many of the components of this pathway are found in other flowering species including maize and rice (Poethig, 2009).

1.3.5. Gibberellin (GA) pathway

Although many of the known phytohormones have been implicated in promoting flowering (Davis, 2009), the gibberellin (GA) pathway is the most widely studied to date. GA was first thought to be responsible for promoting flowering after mutants in the *GA1* gene, a gene essential for carrying out the first step in GA biosynthesis, were found to delay flowering under non-inductive short-day conditions (Wilson et al., 1992). Additional mutations in the GA signaling pathway resulted in similar delays in flowering under short-day conditions (Koornneef et al., 1991; Wilson et al., 1992; Moon et al., 2003). Further evidence for a role for GA in flowering came from increasing GA levels by endogenous or exogenous methods; this resulted in an early flower phenotype of plants grown under short-day conditions (Huang et al., 1998; Coles et al., 1999).

The molecular mechanisms governing GA regulation of flowering have recently been established. The GA pathway upregulates the expression of three floral promoting factors, *SOC1*, *AGL24* and the MI regulator, *LFY*. All three of these regulators when overexpressed, can partially rescue the delayed flowering phenotype caused by the *ga1* mutation (Blazquez and Weigel, 2000; Moon et al., 2003; Liu et al., 2008). The GA pathway also promotes flowering by downregulating *SVP* expression. Application of exogenous GA leads to reduced *SVP* levels and in *ga1* mutants, *SVP* expression is increased under short-day grown conditions (Li et al., 2008).

The regulation of *LFY* by the GA pathway has been well studied (Blazquez et al., 1998; Blazquez and Weigel, 2000; Gocal et al., 2001). Under short-day conditions, *LFY* levels increase slowly but upon application of GA, the *LFY* promoter activity dramatically increases and a rapid transition to flowering occurs (Blazquez et al., 1998). Promoter studies have lead to the identification of a GA responsive element in the *LFY* promoter that is independent of the photoperiod responsive element (Blazquez and Weigel, 2000). This element was bound in vitro by a GA-MYB like transcription factor, MYB33 (Blazquez and Weigel, 2000; Gocal et al., 2001), further confirming a role for the GA signaling pathway in *LFY* induction under short-day conditions.

1.3.6. Convergence of the flowering-time pathways on the floral integrators

Upon induction by environmental and endogenous signals the flowering-time pathways promote flowering by converging on and regulating a set of downstream genes (Fig. 2) (Simpson and Dean, 2002). These downstream genes are known as “floral pathway integrators” because their expression is regulated by more than one flowering-time pathway (Simpson and Dean, 2002). Once activated, these integrators promote the final step in reproductive development by upregulating the floral MI genes in the SAM (Fig. 2) (Simpson and Dean, 2002; Wellmer and Riechmann, 2010). The first factors to be characterized as floral pathway integrators were FT, SOC1 and LFY (Simpson and Dean, 2002) but additional factors including FD and AGL24 have since been added to this list (Fig. 2) (Wellmer and Riechmann, 2010).

The purpose of the floral pathway integrators is to optimally time the floral transition yet their regulation and specific functions in this process are not fully

redundant (Simpson and Dean, 2002). First, these integrators are not all regulated by the same environmental and endogenous signals. *SOC1*, *FT* and *LFY* are all upregulated by the photoperiod pathway, but only *SOC1* and *FT* are upregulated by the autonomous and vernalization pathway through *FLC* repression. Furthermore, only *SOC1* and *LFY* are upregulated by the GA pathway. Secondly, the floral pathway integrators can be regulated to different degrees by the same stimuli. For instance, *SOC1* is regulated through the autonomous and the photoperiod pathways, yet mutations in the autonomous pathway affect *SOC1* expression more so than mutations in the photoperiod pathway (Fig. 2) (Samach et al., 2000; Moon et al., 2005). Finally, these integrators do not all share the same functions. For instance, FT promotes the floral transition by upregulating the MI genes *AP1* and *FUL* (Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005), whereas SOC1 in conjunction with AGL24, directly upregulate *LFY* expression (Lee et al., 2008a). LFY on the other hand, is the central regulator of the MI transition and therefore it activates *AP1* as well as many additional MI genes to promote the floral transition (Fig. 2) (this study) (William et al., 2004; Saddic et al., 2006; Winter, 2011). Although these integrators can function independently, they also regulate each other. As mentioned above, SOC1 upregulates *LFY* expression prior to the floral transition and FT, in conjunction with FD, upregulates *SOC1* (Fig. 2) (Wigge et al., 2005; Yoo et al., 2005). The regulation of these integrators by different flowering- time pathways allows the plant to process a large and diverse number of signals in order to optimally determine the appropriate time to flower. Furthermore, the overlapping as well as non-redundant functions of these integrators ensures flowering will occur during the plant life cycle.

1.4. The final step in the reproductive transition in *Arabidopsis*- the meristem identity (MI) transition

1.4.1. The MI transition

Once activated by the flowering-time pathways the floral pathway integrators upregulate the expression of the MI genes in the SAM. These regulators then execute the last step in the reproductive phase known as the MI transition. Given the importance of the reproductive phase in plants, the MI transition is thought to be controlled by many redundantly acting factors within a complex regulatory network (Wellmer and Riechmann, 2010). The complexity of this pathway is thought to ensure a rapid and irreversible switch to flower formation (Tooke et al., 2005; Blazquez et al., 2006; Liu et al., 2009a; Irish, 2010; Wellmer and Riechmann, 2010). LFY is the key regulator of this process, yet not much is known about the events downstream of LFY that leads to floral initiation. So far, only a handful of MI regulators have been identified and characterized (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Ferrandiz et al., 2000; William et al., 2004; Saddic et al., 2006). Genomic approaches have aided in the identification of factors involved in the MI transition, however the function of the newly identified putative MI regulators have yet to be determined (William et al., 2004; Kaufmann et al., 2010; Winter, 2011).

1.4.2. LEAFY is the central regulator of the MI transition

In many plant species, the plant specific transcription factor LEAFY (LFY) is the central regulator of the MI transition. The importance of LFY in establishing the floral

fate of the inflorescence meristem is evident by its functional conservation throughout the angiosperms (Moyroud et al., 2010). LFY performs this role by directly regulating a set of genes that establish and maintain the development of the incipient floral primordia. *LFY* is first expressed during the vegetative phase in young leaf primordia, but at the onset of the reproductive transition, *LFY* expression increases and is localized to the primordia cells flanking the SAM (Blazquez et al., 1997; Hempel et al., 1997). *LFY* expression persists throughout stage 1 and 2 flower primordia (Blazquez et al., 1997; Hempel et al., 1997). At this time, *LFY* expression is restricted to the developing primordia of the inflorescence meristem by the FT-related protein, TERMINAL FLOWER1 (TFL1) (Ratcliffe et al., 1998). Unlike FT, which promotes flowering, TFL1 promotes inflorescence development. TFL1 represses *LFY* expression in the inflorescence meristem in order to maintain inflorescence identity (Bradley et al., 1997; Ratcliffe et al., 1998). In the absence of TFL1, *LFY* is ectopically expressed in inflorescence apices causing them to terminate in flowers (Weigel et al., 1992; Blazquez et al., 2006). LFY in turn, represses *TFL1* expression in the developing floral primordia in order to maintain floral identity. LFY represses *TFL1* expression in part by directly activating *AP1* (see below) (Weigel et al., 1992; Liljegren et al., 1999; Ratcliffe et al., 1999; Kaufmann et al., 2010). Finally, in stage 3 flowers, *LFY* expression is restricted to whorls two and three of the developing flower where it is involved in directly inducing the expression of the class B and C floral homeotic genes (Weigel et al., 1992; Blazquez et al., 1997; Lenhard et al., 2001; Lohman et al., 2001; Lamb et al., 2002; Winter, 2011).

As the central regulator of the MI transition, the precise timing of *LFY* upregulation is important for reproductive success. As mentioned above, LFY is a floral

integrator and is therefore regulated by a number of environmental and endogenous stimuli including the photoperiod and the GA signaling pathways (Blazquez et al., 1998; Gocal et al., 2001) as well as SPL3, a component of the age-sensing pathway (Yamaguchi et al., 2009). In addition, the floral pathway integrator and the flowering-time gene SOC1 directly upregulates *LFY* expression through an interaction with AGL24 (Lee et al., 2008a).

In *Arabidopsis*, LFY is necessary and sufficient for the proper timing of the MI transition as demonstrated by LFY loss and gain of function phenotypes (Weigel and Nilsson, 1995). In wild-type plants, after the first stage of the reproductive transition, primordia cells give rise to 3-4 secondary inflorescences subtended by cauline leaves (depending on ecotype), prior to the MI transition. In *lfy* mutants, the MI transition is severely delayed and secondary inflorescences develop in positions that would normally be assigned to flowers (Huala and Sussex, 1992; Weigel et al., 1992). *lfy* mutants eventually develop floral- like structures, which lack petals, stamens and have abnormal carpels (Weigel et al., 1992) (Huala and Sussex, 1992). On the other hand, if *LFY* expression is driven by the 35S cauliflower mosaic virus promoter, all of the lateral appendages that would normally give rise to secondary inflorescences and cauline leaves now give rise to flowers (Weigel and Nilsson, 1995). Indeed, increasing the *LFY* copy number by just one causes precocious floral initiation (Blazquez et al., 1997).

1.4.3. The LFY target *AP1* is a MI regulator

LFY executes its role as the central MI regulator by upregulating a number of downstream factors that play a role in establishing the floral primordia. One of the first

LFY targets identified was the MI regulator and MADS box transcription factor, AP1 (Fig. 2) (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004). LFY directly induces *AP1* expression in stage 1 floral primordia (Parcy et al., 1998; Wagner et al., 1999). Similar to LFY, *AP1* expression is restricted to the developing primordia and is absent from the inflorescence meristem by the repressive activity of TFL1 (Ratcliffe et al., 1998). In *tfl1* mutants, *AP1* is ectopically expressed in the inflorescence meristem and in the terminal flowers that form in place of inflorescence shoots (Bowman et al., 1993; Gustafson-Brown et al., 1994).

Although *AP1* is regulated by LFY, mutations in *LFY* only delay *AP1* induction implying that *AP1* is regulated by additional factors in parallel to LFY during floral initiation (Ruiz-Garcia et al., 1997; Wagner et al., 1999). Indeed, at least two LFY-independent pathways upregulate *AP1*, one involves the photoperiod flowering-time regulators, FT and FD, and the other includes components of the age-sensing flowering-time pathway, the SPL transcription factors (Abe et al., 2005; Wigge et al., 2005; Wang et al., 2009; Yamaguchi et al., 2009).

ap1 single mutants cause a subtle delay in the MI transition as floral meristems are only partially converted into inflorescences structures (Bowman et al., 1993), yet simultaneous loss-of-function mutations in both *LFY* and *AP1* results in plants that essentially lack flowers (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993). This suggests that LFY, AP1 and their respective downstream targets, comprise the essential components that regulate the floral transition. *AP1* upregulation marks the committed and irreversible step in flower formation (Bowman et al., 1993; Mandel and Yanofsky, 1995; Blazquez et al., 1997; Hempel et al.,

1997; Yu et al., 2004; Liu et al., 2007). AP1 performs this role by upregulating floral promoting pathways and repressing inflorescence promoting pathways (Kaufmann et al., 2010). During floral initiation, AP1 promotes floral fate in part by directly binding to and upregulating *LFY* (Liljegren et al., 1999; Kaufmann et al., 2010). This feedback loop further ensures the MI transition is robust. In addition, AP1 downregulates inflorescence identity pathways in part by directly binding to and downregulating *TFL1* (Liljegren et al., 1999; Kaufmann et al., 2010). The requirement for LFY and AP1 for maintaining floral identity is evident in *lfy* and *ap1* mutants, both of which develop floral primordia that have the capacity to revert back to the inflorescence program and produce inflorescences in place of flowers (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993).

1.4.4. Additional MI regulators

A number of additional direct LFY targets have been identified during the MI transition (William et al., 2004; Winter, 2011). One LFY target is the MADS box transcription factor and the closest AP1 homolog, CAULIFLOWER (CAL) (Fig. 2) (William et al., 2004; Winter, 2011). *CAL* is induced in young stage 1 and 2 flower primordia (Mandel et al., 1992; Kempin et al., 1995) and *CAL* activation by LFY is thought to precede that of *AP1* induction by LFY (William et al., 2004).

cal single mutants do not have a visible MI phenotype (Kempin et al., 1995), yet the *cal* mutation combined with mutations in *ap1* enhance the MI phenotype of the *ap1* mutant (Bowman et al., 1993). In *ap1 cal* mutants, the meristems that would normally give rise to flowers are now converted to inflorescence meristems. These inflorescence

meristems then proceed to give rise to additional inflorescence meristems and this process is reiterated indefinitely producing inflorescence branches with meristems resembling a cauliflower head (Bowman et al., 1993). Similar to *lfy* null mutants, *ap1 cal* double mutants will eventually give rise to flowers (Bowman et al., 1993).

Another direct LFY target during the MI transition is the class I HD-Zip transcription factor, *LATE MERISTEM IDENTITY1* (*LMI1*) (Fig. 2) (William et al., 2004; Saddic et al., 2006). *LMI1* is first expressed in the vegetative leaves. Later during the MI transition, *LMI1* is upregulated in young floral primordia (Saddic et al., 2006). Mutations in *LMI1* enhance the MI phenotype of weak *lfy* mutants as demonstrated by the increase in secondary inflorescences and cauline leaves of *lmi1 lfy* mutants compared to *lfy* mutants (Saddic et al., 2006). LMI1 promotes floral fate by directly upregulating *CAL* expression. This is demonstrated by the reduction in *CAL* levels in *lmi1 lfy* mutants compared *lfy* mutants. LFY, LMI1 and CAL form a feed-forward loop (FFL) (Fig. 2) (Alon, 2007) regulatory network that is thought to withstand transient environmental stimuli in order to ensure the MI transition is induced at the appropriate time in development. LMI1 also plays a role in leaf development but this function is likely to be independent of LFY (Saddic et al., 2006).

In the absence of LFY, the floral transition eventually occurs as indicated by the flower-like structures that develop in *lfy* mutants (Huala and Sussex, 1992; Weigel et al., 1992). The development of flowers in *lfy* plants implies that other MI regulators act in parallel to LFY to initiate the floral transition. Indeed this is case, as mentioned above, *AP1* is activated by LFY as well as LFY-independent pathways (Abe et al., 2005; Wigge et al., 2005; Wang et al., 2009; Yamaguchi et al., 2009; Xu et al., 2010). Another MI

regulator that acts in parallel to LFY is the MADS box transcription factor FRUITFULL (FUL) (Fig. 2). During the floral transition *FUL* is upregulated by components of the photoperiod pathway as well as components of the age-sensing pathway (Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005; Yamaguchi et al., 2009).

FUL shares high sequence similarity with AP1 and CAL, yet their expression patterns during the MI transition are opposite. Whereas *AP1* and *CAL* are expressed in young stage 1 and 2 floral primordia, *FUL* is expressed in the inflorescence meristem and is excluded from stage 1 and 2 flowers (Mandel et al., 1992; Kempin et al., 1995; Mandel and Yanofsky, 1995). *FUL* is later expressed in stage 3 flowers where it plays a role in carpel development, and then again in the developing carpel, where it plays a role in fruit development (Gu et al., 1998).

Similar to *ap1* mutants, *ful* single mutants cause a subtle delay in the MI transition (Ferrandiz et al., 2000; Melzer et al., 2008) implying that FUL has a non-redundant role in promoting the floral transition. Despite their opposite expression patterns AP1, CAL and FUL are thought to act together during the MI transition to upregulate *LFY* as well as repress *TFL1* in the developing floral primordia (Ferrandiz et al., 2000). This is supported by the dramatic non-flowering phenotype as well as the low levels of *LFY* expression and the ectopic expression of *TFL1* in the floral primordia of *ap1 cal ful* mutants (Ferrandiz et al., 2000). Hence, the upregulation of *LFY* by both its downstream targets AP1, CAL as well as FUL is necessary for the establishment and maintenance of the floral transition.

1.4.5. Conservation of the MI transition in other plant species

The MI transition is the last step leading to floral development and it is controlled by a group of regulators that have homologs in other flowering species. LFY and its function in specifying floral fate are conserved throughout the angiosperm kingdom (Moyroud et al., 2010). In support of the functional conservation of LFY in floral initiation, LFY homologs from different angiosperm species are able to fully complement the *Arabidopsis lfy* mutation (Maizel et al., 2005). The efficiency of this complementation correlates with the evolutionary distance of these homologs from LFY (Maizel et al., 2005). Similarly, constitutive expression of *LFY* in woody plants including, citrus trees and poplar, can cause ectopic flowers (Weigel and Nilsson, 1995; Pena et al., 2001).

Although the functional role of LFY in specifying floral fate is conserved among angiosperms the importance of LFY in establishing this fate varies among flowering species. For instance FLORICAULA, the LFY homolog in *Antirrhinum*, is essential for flower formation (Coen et al., 1990). Unlike *lfy* mutants, which eventually develop floral-like structures, *flo* mutants only produce vegetative inflorescence-like structures and never produce flowers (Coen et al., 1990). Conversely, the LFY homologs in petunia, tomato and lotus are not sufficient to establish floral fate on their own, instead these LFY homologs interact with other factors to promote flowering(Moyroud et al., 2010). In petunia, the LFY homolog ABERRANT LEAF AND FLOWER (ALF) functions with its coactivator the UFO homolog, DOUBLE TOP (DOT). DOT is necessary to promote floral meristem identity in petunia as mutations in *DOT* alone cause almost full loss of

floral identity. Furthermore, overexpression of *DOT* causes precocious flowering (Souer et al., 2008). In petunia, as well as other *Solanaceae* species, LFY cofactors are necessary for regulating both the timing and the location of the MI transition. In agreement with this, *ALF* is already highly expressed early on during the vegetative stage whereas DOT is specifically expressed in the apical regions of the SAM but only during the floral transition (Souer et al., 2008).

In addition to specifying floral fate, certain LFY homologs have other functions. For example UNIFOLIATA (UNI), the LFY homolog in pea, is important for the development of compound leaves (Hofer et al., 1997). In pea, UNI promotes periods of temporary meristematic activity in the developing leaf. This meristematic activity is important for the development of leaflets that make up compound leaves (Moyroud et al., 2010). In monocots such as rice and maize, LFY homologs are thought to promote branching. Mutations in *ZFL*, the LFY homolog in maize, causes a reduction in the number of tassel branches and a similar reduction in branches are observed for mutations in the LFY rice homolog, RFL (Bomblies et al., 2003; Rao et al., 2008). Similar branching phenotypes have also been observed in *Arabidopsis* for mutations in *PENNYWISE* (*PNY*) and *POUNDFOOLISH* (*PNF*), two genes that are involved in patterning events that occur at the SAM (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Kanrar et al., 2006). *pny pnf* mutants produce cauline leaves devoid of secondary inflorescence branches and these plants also lack flowers (Kanrar et al., 2008). These genes have been shown to act upstream of both LFY and AP1 as the expression of *LFY* and *AP1* is dramatically reduced in these double mutants (Kanrar et al., 2008). This data combined with the meristematic activity of LFY

homologs in legumes implies that LFY may also play a general role in promoting primordia outgrowth (Moyroud et al., 2010). Finally, LFY has recently been linked to plant defense, a function that may also be conserved in other LFY homologs (Maizel et al., 2005; Winter, 2011).

The MADS proteins are classified into two groups, the eudicot AP1 (AP1-like genes) and the eudicot FUL clade (FUL-likes genes) (Litt and Irish, 2003). Functional characterization of homologs in these two clades implies that their role in floral meristem initiation is conserved in other angiosperm species (Liu et al., 2009a). For example, *SQUAMOSA* (*SQUA*), the *AP1* homolog in *Antirrhinum*, has a similar expression pattern to that of *AP1* during floral development. *SQUA* is expressed in the floral meristem and the first two whorls of the developing flower (Huijser et al., 1992). In *Antirrhinum*, SQUA may play a more pivotal role in the floral transition than that of AP1 in *Arabidopsis* since *squa* mutants rarely form flowers (Huijser et al., 1992; Blazquez et al., 2006). In pea, the function of the AP1-like gene, *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*) is also similar to AP1in that the floral meristems are partially converted to inflorescence branches in *pim* mutants and *pim* flowers have floral organ defects similar to *ap1* mutants (Taylor et al., 2002; Blazquez et al., 2006).

Unlike the euAP1 clade, which is only conserved in core eudicots, the euFUL clade is not only conserved in core eudicots but also found in certain monocotyledonous species (Benlloch et al., 2007; Preston and Kellogg, 2007; Liu et al., 2009a; Wellmer and Riechmann, 2010). Although the functions of the euFUL clade in floral meristem initiation are conserved in these species they have additional functions during the reproductive transition. For example, in wheat, the FUL-like homolog is involved in

promoting flowering through the vernalization response (Trevaskis et al., 2003; Yan et al., 2003).

Although components of the MI transition in *Arabidopsis* are found in other species, whether or not the regulatory mechanisms that control this pathway are conserved in these species is still unclear. In *Antirrhinum*, the LFY homolog FLO and the AP1 homolog SQUA are thought to be regulated independently from one another. This is demonstrated by the fact that the expression of *FLO* or *SQUA* are not changed in *squa* or *flo* mutants, respectively (Huijser et al., 1992). Furthermore, the *tendril less* (*TI*) gene, the LMI1 homolog in pea, is transcriptionally regulated by the LFY homolog, yet its role in the floral initiation is not conserved (Hofer et al., 2009).

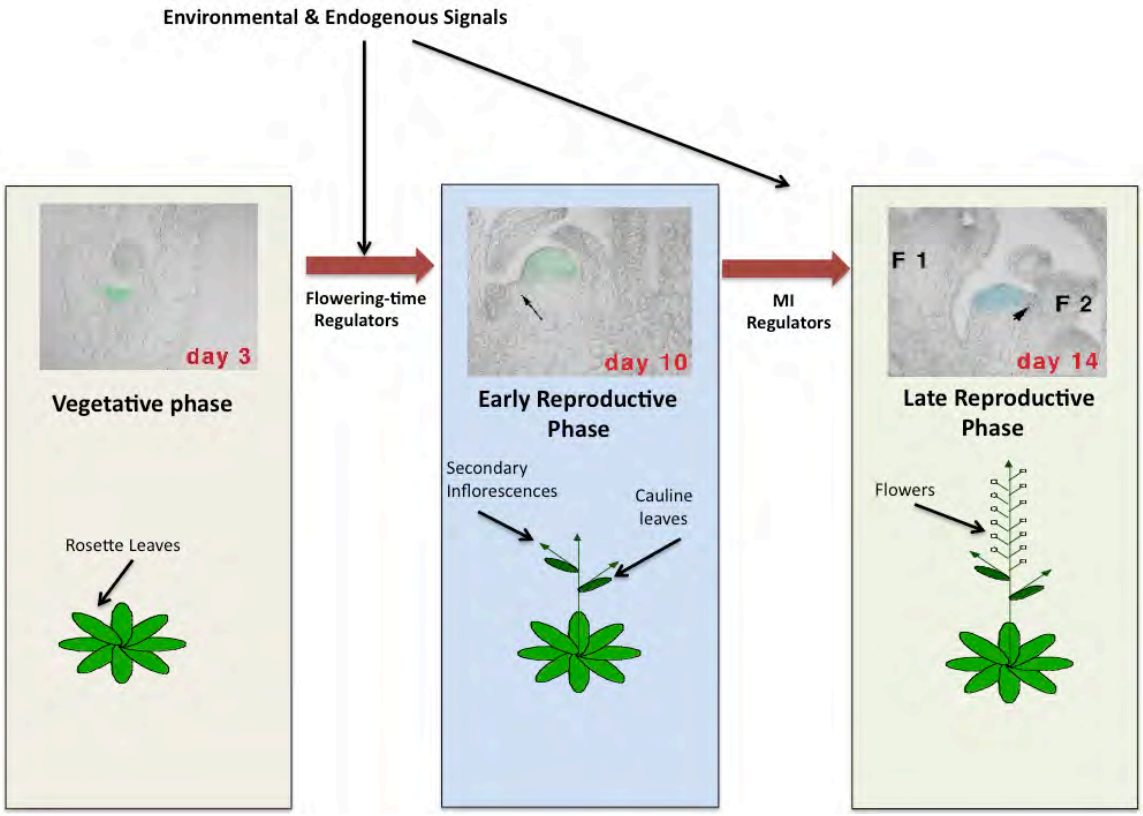

Figure 1. *Arabidopsis* reproductive development.

(**Left panel**) Vegetative phase-The SAM gives rise to axillary meristems in the axil of rosette leaves. (**Middle panel**) Early reproductive phase-The flowering-time pathways are induced upon the proper environmental and endogenous signals. During this time the SAM grows upward "bolting" and the primordia give rise to axillary meristems that will become secondary inflorescences. These inflorescences are subtended by cauline leaves. (**Right panel**) Late reproductive phase- After the MI transition the primordia cells give rise to floral meristems that will eventually produce flowers. Top images: Longitudinal sections of the SAM (L*er* ecotype) at day 3, 10 and 14. The SAM is highlighted in green prior to the MI transition and blue after the MI transition. The arrow (in image) points to a developing secondary inflorescence and the arrowhead (in image) points to a

developing floral primordia. F1 and F2 indicate two previously formed flowers. The diagrams below each image denote to the lateral organs that form as a result of each developmental phase. SAM images courtesy of Doris Wagner.

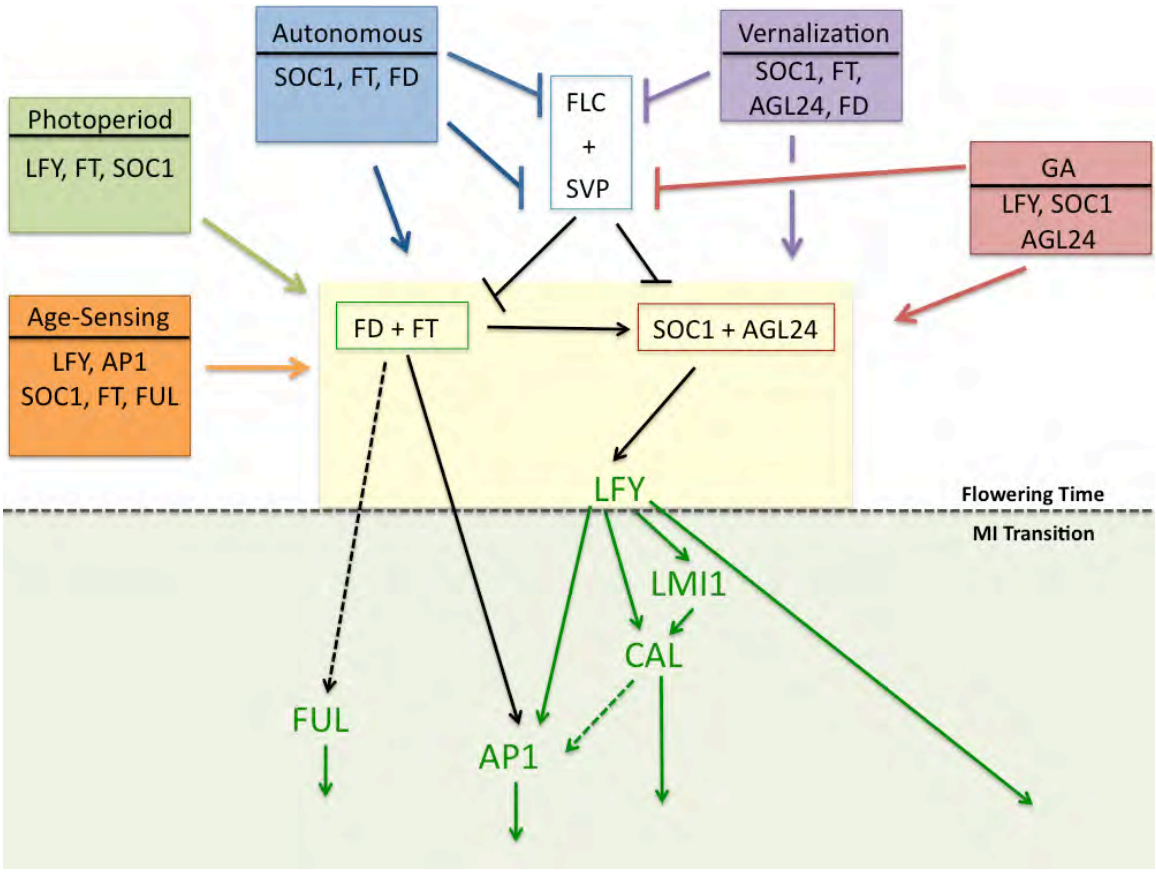

Figure 2. The pathways controlling the reproductive transition in *Arabidopsis*.

The flowering-time pathways are indicated by colored boxes. Genes that are positively regulated by components of these pathways are indicated within the box (see text for citations). Positive and negative inputs from the flowering-time pathways are indicated in color. Floral pathway integrators are located within the yellow rectangle (Simpson and Dean, 2002; Wellmer and Riechmann, 2010). Factors known to interact to regulate downstream targets are indicated by colored boxes (see text for citations). Positive and negative inputs from flowering-time genes are indicated in black. The MI regulators and their interactions are indicated in green. Dashed arrows represent indirect and/or direct interactions.

1.5. Early Floral Development- Maturation and maintenance of the floral meristem (stage 1-early stage 2 flower development)

1.5.1. Growth and proliferation of the floral meristem

After specification of floral fate by the MI regulators, floral meristems progress through a series of distinct developmental stages (Smyth et al., 1990). During stages 1 and 2, the emerging floral meristems begin a period of growth and maturation prior to entering floral differentiation at stage 3. An essential part of this growth period is the establishment and maintenance of the stem cell population. Similar to its role in the SAM, the homeodomain-containing transcription factor WUSCHEL (WUS) is necessary for establishing and maintaining the stem cell population in floral meristems (Laux et al., 1996). Proper regulation of *WUS* is important for maintaining meristem development; loss-of-function mutations in *wus* cause premature termination of floral meristems (Lenhard et al., 2001) and overexpression of *WUS* results in the over accumulation of cells with stem cell identity (Schoof et al., 2000). Analogous to what occurs in the SAM, the *WUS* expression domain is spatially restricted to the central region of the floral meristem by the CLAVATA 1-3 receptor-kinase signaling proteins (Weigel and Jurgens, 2002; Carles and Fletcher, 2003). WUS also interacts in a positive feedback loop to upregulate *CLV3* expression that in turn restricts the *WUS* expression domain (Weigel and Jurgens, 2002; Carles and Fletcher, 2003). The positive and negative feedback loops regulating the expression of WUS and the CLV proteins is important for establishing the appropriate size of the floral meristem. Accumulation of stem cells in the floral meristem

during this maturation phase is necessary to produce a sufficient number of cells for generating a complete set of floral organs that make up the flower (Wagner, 2009).

1.5.2. Active repression of floral differentiation is important for floral meristem growth

The regulatory mechanisms that lead to the formation of floral organs has been extensively studied over the past two decades (Krizek and Fletcher, 2005; Causier et al., 2010), yet not much is known about how the earlier steps of floral meristem growth and differentiation are coordinated to allow for this process to occur. Recent studies have started to reveal how growth of the floral meristem is controlled during early flower development. So far, numerous factors involved in regulating gene expression at the transcriptional as well as the chromatin level have been identified. In most cases these factors maintain the growth of the floral meristem by actively repressing genes involved in floral differentiation (Gregis et al., 2006; Gregis et al., 2009; Liu et al., 2009b).

1.5.3. Growth of the floral meristem is maintained by repressing the floral homeotic regulator and floral determinacy marker *AGAMOUS*

The class C floral homeotic gene and MADS box transcription factor AGAMOUS (AG) is crucial for specifying stamens and carpels in the center of the developing flower (Krizek and Fletcher, 2005; Irish, 2010). AG is also important for the determinate growth of the floral meristem, in *ag* loss-of-function mutants, carpels are replaced by an additional floral primordium, which will give rise to a new flower consisting of sepals and petals (Bowman et al., 1989; Bowman et al., 1991). AG promotes determinate

growth in part by downregulating the stem cell regulator *WUS* (see below-section 1.6.3.) and therefore eventually terminating stem cell proliferation in the center of the meristem (Lenhard et al., 2001; Lohman et al., 2001).

Recently, a number of factors have been identified that actively repress *AG* during early stages of floral development in order to prevent premature differentiation. Two of these factors are LEUNIG (LUG) and SEUSS (SEU). LUG and SEU are transcriptional co-regulators that interact with each other to repress *AG* in stage 1 and 2 flower primordia as well as later stages during floral differentiation (Liu and Meyerowitz, 1995; Franks et al., 2002; Sridhar et al., 2004; Gregis et al., 2006; Gregis et al., 2009). LEU is a nuclear localized protein with homology to the Tup1 corepressors in yeast as well as the Groucho protein in *Drosophila* (Conner and Liu, 2000). This group of proteins known as the GroTLE family, are recruited to DNA by transcription factors and interact with chromatin modifying proteins and the RNA polymerase II machinery to repress gene expression (Sridhar et al., 2004). SEU is a plant specific protein that shares homology with the dimerization domain of the LIM domain binding family (Ldb) (Jurata and Gill, 1997; Franks et al., 2002). The Ldb family act as adapter proteins that bridge interactions between transcriptional co-regulators and transcription factors (Agulnick et al., 1996; Bach et al., 1997; Jurata and Gill, 1997).

Several pieces of evidence suggest LUG and SEU maintain *AG* repression during early flower development. First, unlike wild-type flowers that initially express *AG* in the center of stage 3 flowers (Yanofsky et al., 1990; Drews et al., 1991), in *lug seu* double mutants *AG* expression is precociously expressed as early as stage 1 floral primordia (Franks et al., 2002). Secondly, the LUG-SEU complex associates with *AG* regulatory

regions and finally, *seu lug* double mutants exhibit a reduction in floral organ number, a characteristic phenotype of mutants that lack an appropriate stem cell population for the generation of a complete set of floral organs (Liu and Meyerowitz, 1995; Franks et al., 2002; Sridhar et al., 2004; Gregis et al., 2006).

Since LUG and SEU do not contain DNA binding domains they must be recruited to the *AG* locus by other proteins. The MADS box transcription factors and flowering-time regulators SVP and AGL24 and the MI and floral homeotic regulator *AP1*, are thought to mediate this process (Gregis et al., 2006; Gregis et al., 2009). Double mutants of *svp agl24* grown at 30°C have similar phenotypes to the *seu lug* mutants; *svp agl24* mutant flowers have a reduced number of floral organs. These phenotypes are enhanced when combined with a weak *ap1* allele (Gregis et al., 2006). Furthermore, in *svp agl24* mutants, *AG* is precociously expressed in the inflorescence meristem as well as in early stage 1 flower primordia (Gregis et al., 2006). Based on interaction studies in yeast, AP1 can form heterodimers with SVP and AGL24 and these heterodimers can then interact with the LUG-SEU protein complex. The protein interaction between the LUG-SEU complex and the AP1-SVP and AP1-AGL24 heterodimers are thought to recruit LUG and SEU to the second intron of *AG* for repression of *AG* during early stages of floral development (Fig. 3) (Gregis et al., 2006; Gregis et al., 2009).

1.5.4. Growth of the floral meristem is achieved by repressing the floral homeotic gene and floral differentiation activator *SEPALLATA3*

SEPALLATA3 (*SEP3*) is a class E floral homeotic gene that is redundantly required with the other SEP proteins (SEPALLATA1, 2 and 4) to specify petals, stamens

and carpels in the developing flower (Pelaz et al., 2000; Goto et al., 2001; Theissen, 2001; Ditta et al., 2004). SEP3 is also required in conjunction with the MI and floral patterning regulator LFY, to activate the class B and C floral homeotic genes (Castillejo et al., 2005; Liu et al., 2009b; Winter, 2011). The activation of the class B and C genes by LFY and SEP3 in stage 3 flowers is a critical time in flower development as it signifies the onset of floral differentiation (Wagner, 2009; Irish, 2010; McKim and Hay, 2010). Given its role in activating floral organ patterning, *SEP3* repression during early stages of floral development is essential for maintaining floral meristem growth and preventing premature differentiation.

In addition to actively repressing *AG* during early stages of floral development, SVP and AGL24 along with the flowering-time regulator and MADS box transcription factor SOC1 are redundantly required to repress *SEP3* prior to floral differentiation (Liu et al., 2009b). *SEP3* as well as the class B genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) and the class C gene *AG*, all of which are normally activated in stage 3 flowers, are all precociously expressed throughout stage 1 and 2 floral primordia in *agl24 soc1 svp* mutants (Liu et al., 2009b). The precocious expression of the class B and C genes explains the substantial reduction in floral organ number observed in *agl24 svp soc1* mutant flowers (Liu et al., 2009b).

Similar to *AG* regulation in early floral primordia, SVP, AGL24 and SOC1 interact with additional proteins to repress *SEP3*. SVP represses *SEP3* by interacting with the chromatin regulatory protein TERMINAL FLOWER2 (TFL2)/LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), the *Arabidopsis* homolog of HP1 of metazoans and S. pombe (Gaudin et al., 2001; Kotake et al., 2003). *TFL2* is expressed in

floral primordia and it is known to associate with repressive H3K27 trimethylation marks of histones to maintain gene repression (Turck et al., 2007; Zhang et al., 2007). It is suggested that SVP guides TFL2 to the *SEP3* locus to possibly affect trimethylation of histone H3K27 (Liu et al., 2009b). In support of this, TFL2 and SVP interact in the nucleus where both factors are known to function. TFL2 and SVP also bind to the same region of the *SEP3* promoter, and H3K27 trimethylation levels on nucleosomes are reduced in this region of the *SEP3* promoter in *svp* mutants (Liu et al., 2009b).

AGL24 and SOC1 also bind to the *SEP3* promoter and repress its expression, but AGL24 and SOC1 associate with SAP18, a structural protein that is known to stabilize the repressive Sin3/Histone deacetylase (HDAC) complex and its interacting components (Silverstein and Ekwall, 2005). Histone deacetylase complexes are known to remove active acetylation marks from histones of actively transcribed genes, therefore leaving these genes in a repressed state (Marks et al., 2001). A role for AGL24, SOC1 and SAP18 in *SEP3* repression is supported by interaction studies showing AGL24 and SOC1 can physically interact with SAP18 and *soc1 svp* and *soc1 svp agl24* mutants have an increase in histone H3 acetylation on nucleosomes located on the *SEP3* promoter (Liu et al., 2009b). The interaction of AGL24 and SOC1 with SAP18 is thought to repress *SEP3* by recruiting a HDAC complex to the nucleosomes on the *SEP3* promoter therefore preventing the accumulation of acetylation marks on these histones. Out of these three proteins, SVP is thought to be the prominent repressor of *SEP3* since *SEP3* expression is already upregulated in *svp* single mutants, and AGL24 and SOC1 only bind to the *SEP3* promoter in the absence of SVP (Fig. 3) (Liu et al., 2009b).

1.6. Onset of Floral differentiation (late stage 2-early stage 3 flower development)

1.6.1. The AP1 branched flower phenotype

The MADS box transcription factor AP1 has many different functions during reproductive development. *AP1* is initially upregulated in the emerging floral primordia (Mandel et al., 1992; Gustafson-Brown et al., 1994) where it acts as a MI regulator (Bowman et al., 1993). As the floral primordia grow and flower development proceeds, *AP1* levels increase throughout the developing primordia (Mandel et al., 1992; Gustafson-Brown et al., 1994). AP1 has two main repressive functions during this time that are separated by a temporal delay. First, in stage 1 and 2 primordia as discussed above, AP1 supports the growth of the floral meristem by repressing the genes involved in floral differentiation through its interaction with SVP, AGL24 and the LEU-SEU complex. Subsequently, in late stage 2 and early stage 3 primordia, AP1 in fact has an opposite role, it downregulates genes involved in promoting meristematic growth to begin floral differentiation. After floral differentiation is initiated, *AP1* is restricted to the first and second whorls of the flower where it functions along with the other class A homeotic regulator APETALA2 (AP2), to specify sepals and petals (Mandel et al., 1992; Gustafson-Brown et al., 1994).

The functions of AP1 during flower development are evident by the distinct defects observed in *ap1* mutant flowers. *ap1* flowers are characterized by the conversion of sepals into bract-like structures and the absence of petals. Often *ap1* flowers have a reduced number of these bract-like sepals and stamens (Irish and Sussex, 1990; Bowman et al., 1993). These floral defects primarily effect whorls one and two and are a

consequence of the role AP1 has as a class A floral homeotic regulator in these whorls (Irish and Sussex, 1990; Bowman et al., 1993). The second floral defect of *ap1* mutants is the presence of "branched flowers"; flowers that develop from within the axils of the first whorl organs of the developing flower (Irish and Sussex, 1990; Bowman et al., 1993; Blazquez et al., 2006). The second order flowers also have the capacity to develop branched flowers. The branched flower phenotype is not considered a homeotic transformation of the petal primordia because these flowers do not develop from within the second whorl, but instead arise from the axil of the first whorl organs, the sepals (Irish and Sussex, 1990). Furthermore, branched flowers have been described for other organisms, for example, *Nasturtium officinale* (Arber, 1931; Irish and Sussex, 1990). In the case of *Nasturtium* flowers, they produce branched flowers as well as petals, suggesting the loss of petals and the formation of branched flowers are independent defects in *ap1* mutants (Arber, 1931). This branched flower phenotype is caused by the retention of meristematic potential in the axils of the first whorl organs of an existing flower (Irish and Sussex, 1990; Blazquez et al., 2006). This region can form new floral meristems which give rise to these secondary flowers or can acquire inflorescence meristem characteristics and can develop inflorescence-like structures from the axils of the first whorl organs (Irish and Sussex, 1990; Bowman et al., 1993; Blazquez et al., 2006). The development of branched flowers in *ap1* mutants is therefore a consequence of the role AP1 plays in reducing the meristematic potential of the developing flower and is linked to its role in promoting floral differentiation (see below).

1.6.2. Downregulation of *SVP, SOC1* and *AGL24* by AP1 leads to floral differentiation and reduces the ectopic meristematic growth of *ap1* mutants

During the initial stages of flower development the floral meristem undergoes a growth phase characterized by the accumulation of a finite number of stem cells that will give rise to the floral organs. The meristematic potential of the flower is gradually reduced within specific spatial domains of the developing flower from the outside (whorl 1) to the inside (whorl 4) and it is eventually completely terminated in the center region of the floral meristem by stage 6 when carpel development is initiated (Krizek and Fletcher, 2005; Sun and Ito, 2010). The decline in meristematic potential is linked to the onset of floral differentiation in stage 3 flowers. Patterning of the floral organs starts from the outer region of the developing flower in whorl one, and proceeds inward to whorl two, three and four (Steeves, 1989; Krizek and Fletcher, 2005).

As described above, the flowering-time genes SVP, SOC1 and AGL24 interact with chromatin regulatory proteins to repress *SEP3* expression in stage 1 and 2 floral primordia. The repression of *SEP3* by these flowering-time genes allows growth and development of the floral meristem prior to the onset of differentiation. In late 2 and early stage 3 flowers, AP1 directly downregulates *SVP*, *SOC1* and *AGL24* in the floral primordia (Yu et al., 2004; Liu et al., 2007). The downregulation of these flowering-time genes by AP1 terminates the meristematic activity in the region between the developing first whorl organs and the floral meristem and allows differentiation to occur (Fig. 3). In support of this, all three of these genes are ectopically expressed in stage 2 and 3 flowers in the absence of AP1 (Yu et al., 2004; Liu et al., 2007). This ectopic expression of *SVP*, *AGL24* and *SOC1* is in part responsible for the production of branched flowers in *ap1*

mutants; mutations in these flowering-time genes alleviate the branched flower phenotype of *ap1* mutants (Liu et al., 2007). By contrast, overexpression of these genes in combinations, using the 35 cauliflower mosaic virus promoter, causes production of branched flowers (Yu et al., 2004; Liu et al., 2007). *SVP*, the major factor involved in preventing premature floral differentiation is expressed in stage 1 and 2 flowers and is undetectable by stage 3 (Hartmann et al., 2000; Liu et al., 2007). However, in *ap1* mutants *SVP* is ectopically expressed in stage 3 and 4 flowers. In stage 3, *SVP* expression is localized in the axil of the first whorl organs, the same region where new floral meristems develop and where branched flowers form (Liu et al., 2007).

1.6.3. Upregulation of the floral homeotic genes leads to floral organ patterning and promotes further termination of meristematic growth

SEP3 is a key regulator of floral differentiation and as such it is upregulated by many factors. At the onset of floral differentiation in stage 3 flowers *SEP3* is indirectly and directly activated by *AP1* (Yu et al., 2004; Liu et al., 2007; Liu et al., 2009b; Kaufmann et al., 2010). *SEP3* is also activated by LFY during this time and subsequently, SEP3 and LFY interact to activate the class B and C genes (Liu et al., 2009b; Winter, 2011). Once activated SEP3 takes part in many feedback loops to reinforce the floral organ program. SEP3 directly binds to and upregulates its own expression as well as the expression of *AP1* (Kaufmann et al., 2009). Finally, SEP3 directly downregulates *SVP*, *AGL24* and *SOC1* and may do this in combination with other SEP proteins, to promote floral differentiation and also to repress meristematic activity (Kaufmann et al., 2009).

This latter function of SEP3 is supported by the fact that double and triple mutant combinations of the *sep* genes produce branched flowers (Fig. 3) (Ditta et al., 2004).

In stage 3 flowers the Class B floral homeotic genes *AP3* and *PI* are upregulated by SEP3 and LFY (Liu et al., 2009b; Winter, 2011). LFY interacts with UNUSUAL FLORAL ORGANS (UFO), an F-box protein and a component of the SCF ubiquitin ligase complex to induce *AP3* (Chae et al., 2008) and SEP3 to activate *AP3* as well as *PI* (Liu et al., 2009b; Winter, 2011). The class B and C proteins specify the petals in whorl two and stamens in whorl three (Krizek and Fletcher, 2005). Finally, activation of the class C gene *AG* promotes differentiation of stamens in whorl three and carpels in whorl four and eventually leads to the termination of the meristematic activity in the center of the floral meristem (Krizek and Fletcher, 2005). In addition to acting with SEP3 to initiate *AG* expression, LFY acts with the stem cell regulator WUS to upregulate *AG* (Lohman et al., 2001). WUS and LFY bind to adjacent regions of the *AG* second intron to upregulate its expression (Lohman et al., 2001). AG in turn causes the downregulation of *WUS* in the center of the floral meristem (Lenhard et al., 2001).

WUS activity is necessary to maintain the stem cell population for carpel development in stage 6 flowers. Hence, upon *AG* activation in stage 3 flowers, *WUS* is not immediately terminated(Sun and Ito, 2010). Recent studies have identified how AG regulates *WUS* in the developing flower. AG is thought to indirectly downregulate *WUS* by activating factors both transcriptionally as well as using epigenetic mechanisms (Sun and Ito, 2010). These regulatory mechanisms are thought to provide the necessary delay between *AG* activation in stage 3 flowers and *WUS* termination later in stage 6 flowers (Sun and Ito, 2010).

ag mutants are characterized by floral homeotic defects for example, petals replace stamens in the third whorl and carpels are absent in the fourth whorl. In place of carpels a new floral primordium develops initially producing sepals in the first whorl and both petal and stamen primordia. As this flower continues to develop only sepals, sepal – petaloid structures as well as petals are generated (Bowman et al., 1989; Bowman et al., 1991). This phenotype is repeated continuously. This flower within a flower phenotype of *ag* mutants is in part a consequence of the remaining WUS activity in the central region of the floral meristem (Lenhard et al., 2001).

1.6.4. Similarities between AP1 and AG

The *ag* indeterminate flower phenotype is analogous to the branched flowers in *ap1* mutants, yet it occurs in a different region and at a later time point in the developing flower meristem. Similar to *ap1* mutants, *SVP* and *AGL24* are also ectopically expressed in *ag* mutants suggesting that AG may also function to promote determinacy by downregulating these flowering-time genes in the center of the floral meristem (Gregis et al., 2008). Furthermore, overexpression of *AG* by the cauliflower mosaic virus 35 S promoter reduces the inflorescence branching structures of *ap1* mutants (Mizukami and Ma, 1997) further supporting a role for AG in repression of these flowering-time genes.

1.6.5. Functional conservation of the components involved in early flower development in other species

The early stages of flower development are essential for generating the complete set of floral organs that make up the flower (Liu et al., 2009a; Wagner, 2009; Irish, 2010;

McKim and Hay, 2010). The importance of this coordinated growth and differentiation suggests the components that make up these pathways and their functions may be conserved in other flowering species. Indeed, this seems to be the case in many angiosperm species (Liu et al., 2009a).

Homologs for the flowering-time genes AGL24 and SVP have been found in other species. Overexpression studies of these homologs suggest their regulation is important for maintaining the determinacy of the floral meristem. For instance, overexpression of *AGL24* and *SVP* homologs from *Antirrhinum* and *Eucalyptus* in *Arabidopsis* causes indeterminate flowers, and overexpression of rice homologs in *Arabidopsis* causes both indeterminate and branched flowers (Brill, 2004; Masiero et al., 2004; Fornara et al., 2008; Lee et al., 2008b). In addition, the SVP homolog in orange PtSVP, is thought to play a role in maintaining floral meristem development (Li et al., 2010). In agreement with this, when *PtSVP* is expressed in *Arabidopsis* under the 35S cauliflower mosaic promoter, carpels are replaced by flower-like structures. Furthermore, PtSVP interacts with the orange AP1-like gene PtAP1, suggesting a possible conservation of the regulatory mechanisms found in early flower development in *Arabidopsis* (Li et al., 2010).

The role of SEP3 in promoting floral differentiation and inhibiting meristematic activity is conserved in other flowering species. Unlike *Arabidopsis* where loss of the SEP proteins causes development of additional floral meristems, loss of SEP3 function in other species is more extreme and these flowers develop inflorescence meristems. In tomato the SEP-LIKE gene, *TM29* is important for maintaining floral determinacy. Downregulation of *TM29* by cosuppression or antisense RNA causes flowers to be

replaced by inflorescence-like structures (Ampomah-Dwamena et al., 2002). Furthermore, a SEP3 homolog in petunia FBP2 (Ferrario et al., 2003) is also involved in inhibiting meristematic activity of the developing flower. Downregulation of *FBP2* by cosuppression causes the development of inflorescences in the axils of carpels (Angenent et al., 1994).

AG homologs have been found in both monocots and dicot species and their function in determinate growth is mainly conserved yet it varies among species. In *Antirrhinum*, the AG homolog PLENA (PLE) is important for floral meristem determinacy. In the absence of PLE, new floral meristem develop inside the forth whorl of a developing flower (Davies et al., 1999). AG homologs in monocots such as rice and maize also have a conserved role in floral determinacy (Schmidt et al., 1993; Yamaguchi et al., 2006). RNAi knockout lines of the AG rice homolog OsMADS58 causes flowers to reiterate floral organ whorls containing lodicules, stamens and carpels. Similar to the continued WUS activity in *ag* mutants, in OsMADS58 knockout lines the marker gene for meristematic activity, OSHI is expressed continuously even after carpel development (Yamaguchi et al., 2006). Interesting to note, in some species where AG homologs do not function in floral determinacy, for instance in impatiens, these homologs when ectopically expressed in *Arabidopsis* show determinate characteristics (Ordidge et al., 2005). Similar to SEP3 homologs, certain AG homologs are important for maintaining the overall identity of the floral meristem. For example, silencing the AG homolog in petunia *pMADS3* lead to the development of inflorescence like structures in the third whorl of the developing flower (Kapoor et al., 2002).

Whether or not the function of AP1 in floral determinacy is conserved in other flowering species is less clear. For example, mutations in *PIM*, the AP1 homolog in pea, do not form obvious branched flowers or inflorescence like structure within developing flowers (Taylor et al., 2002). Furthermore in *Antirrhinum*, the occasional flowers that are produced in *squa* mutants do not develop branched flowers from the bract like-sepals (Huijser et al., 1992). The floral phenotypes of the AP1 homologs in *Antirrhinum* and pea suggest other genes in these species may convey the floral determinate function of AP1.

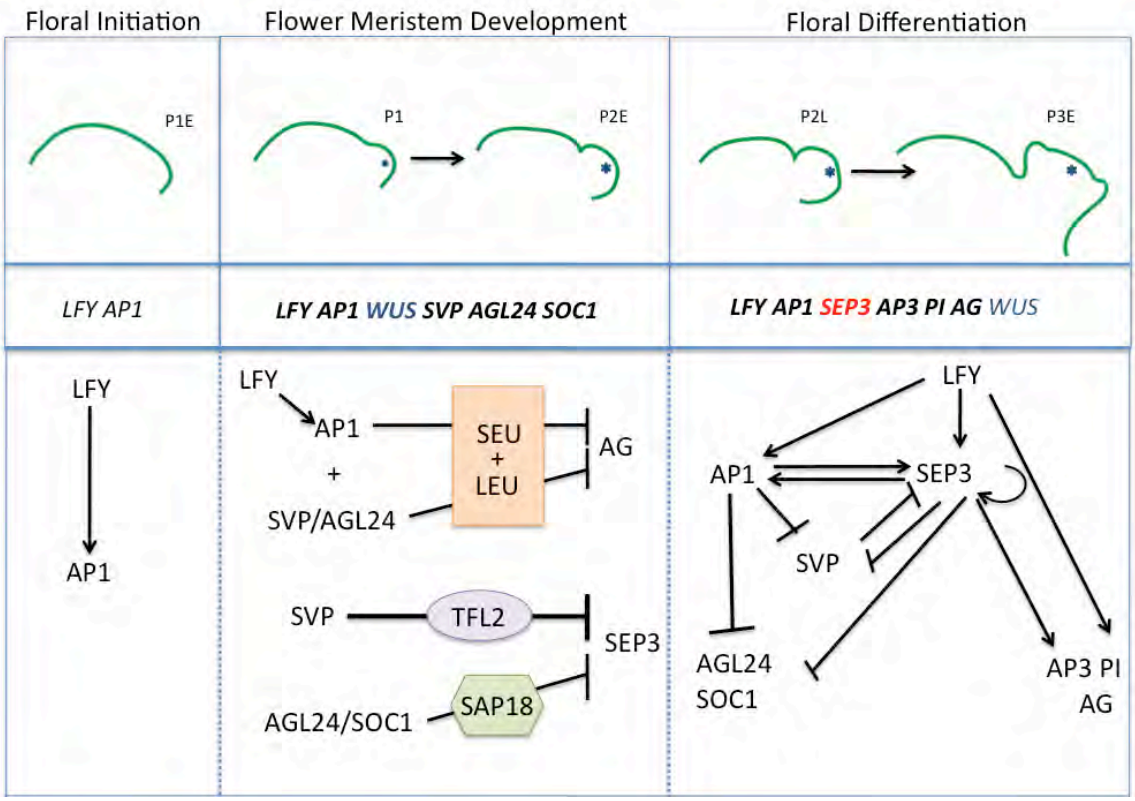

Figure 3. Early stages of flower development.

(Top panel)- Floral primordia stages represented at each phase in early flower development (Smyth et al., 1990). The specific floral stages are indicated above the developing primordia. "E" indicates "early" and "L" indicates "late" in terms of development. Asterisks denote location and intensity of *WUS* expression. (**Middle panel**)- The genes expressed in the floral primordia at each stage. Standard print denotes low expression, bold print denotes high expression. WUS and SEP3 are highlighted in blue and red respectively to denote their importance in the particular developmental phase indicated. (**Bottom panel**)- The regulatory pathways active during each stage of early floral development. Chromatin regulators are indicated by colored shapes (see text

for citations). Factors that physically interact are indicated by "+". Bold lines indicate a key regulatory step in the pathway.

In the following two chapters I will present the data for my two thesis projects. In Chapter 2, I will discuss my main project, the role of LMI2 in the MI transition. This chapter has been adapted from "LATE MERISTEM IDENTITY2 acts together with LEAFY to activate *APETALA1*." Pastore et al., Development, In press. I will begin Chapter 2 by briefly describing LMI2 and the rationale behind this project. In Chapter 3, I will present the data for my second and smaller project, the role of LMI2 in early flower development. I will again begin by discussing the rationale behind this project prior to presenting the results. This second project is incomplete and critical experiments are still needed to make final conclusions. I have highlighted the data that are missing within Chapter 3. In Chapter 4, I have discussed the experiments that are in progress to address these unanswered questions.

Chapter 2. The role of LMI2 in the Meristem Identity Transition

2.1. Background

Although the MI transition is a key developmental switch, our understanding of the events that lead from *LFY* upregulation to flower formation is still incomplete. The MI transition is essential for establishing floral fate and is therefore likely regulated by many redundant factors. In agreement with this, *lfy* null mutants have a more dramatic MI phenotype compared to *ap1 cal*, indicating that there are additional factors downstream of LFY that are required for the MI transition (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993). We previously used a microarray approach followed by chromatin immunoprecipitation to identify direct downstream targets of LFY during the MI transition (William et al., 2004).

This genomic approach identified the MI regulator and direct LFY target *CAL* as well as five new direct LFY targets. Mutations in these new LFY targets enhanced the MI phenotype of a weak *lfy* mutant and were therefore later renamed the *LATE MERISTEM IDENTITY* (*LMI*) genes. Of these LMI genes, only LMI1 has so far been characterized as a bona fide MI regulator (William et al., 2004; Saddic et al., 2006).

Another direct target of LFY identified was LATE MERISTEM IDENTITY2 (LMI2) (William et al., 2004). LMI2, also known as AtMYB17 is a member of the R2R3 class of MYB transcription factors, which play important roles in many processes in plants including cell fate specification, metabolism, and biotic and abiotic stress responses (Martin and Paz-Ares, 1997; Kranz et al., 1998; Stracke et al., 2001; Dubos et al., 2010). The *Arabidopsis* homologs of AtMYB17, AtMYB16/MIXTA and ATMYB106/NOECK, have been reported to function in the determination of cell shape

in the petal epidermis and in the repression of trichome branching (Baumann et al., 2007; Jakoby et al., 2008). The biological function of AtMYB17 is not understood. In my thesis work I have taken a genetic and molecular approach to characterize the role of LMI2 in the MI transition.

2.2. Results

2.2.1. Characterization of *LMI2* T-DNA alleles

To elucidate the role of LMI2 in the MI transition, we analyzed three T-DNA insertion alleles (Alonso and Stepanova, 2003), *lmi2-1*, *lmi2-2* and *lmi2-3* (Fig. 4A). In *lmi2-1*, the T-DNA insertion was located in the promoter region (116 bp from the transcription start site), while the insertions in *lmi2-2* and *lmi2-3* were located in the conserved MYB DNA binding domain (Fig. 4A). All three T-DNA insertions caused deletions in the *LMI2* locus ranging from 4 to 41 bp (Fig. 4A).

Both *lmi2-2* and *lmi2-3* expressed RNA upstream of the T-DNA insertion, suggesting that they are not RNA null alleles (Fig. 4B). However, I did not detect *LMI2* expression in either the *lmi2-2* or the *lmi2-3* mutant using primers flanking the T-DNA insertions (Fig. 4B). Hence, these insertions likely disrupt the conserved MYB DNA binding domain and give rise to a non-functional LMI2 protein. The *lmi2-1* mutant, on the other hand, expressed elevated levels of *LMI2* RNA (Fig. 4B). Since the T-DNA insertion in *lmi2-1* is located in the promoter region, it is likely that this insertion generates a full length *LMI2* transcript. Nonetheless, this combined data (see below)

suggests that *lmi2-1* is a loss-of-function allele. Because *lmi2-2* and *lmi2-3* have similar T-DNA insertion sites, I chose to focus on the *lmi2-1* and *lmi2-2* alleles.

2.2.2. MI phenotypes of *lmi2* mutants

The timing of the MI transition was assessed in *lmi2* mutants compared to the wildtype by counting the number of secondary inflorescences and cauline leaves formed prior to the formation of the first flower (Saddic et al., 2006; Yamaguchi et al., 2009). Flowering-time was measured by counting the number of rosette leaves (Yamaguchi et al., 2009). *lmi2-2* displayed a statistically significant increase in the number of cauline leaves and secondary inflorescences formed compared to the wildtype in five independent experiments (Table 1; Fig. 5A) suggesting that LMI2 plays a non-redundant role in the MI transition. *lmi2-1* exhibited a more subtle delay in the MI transition that differed significantly from the wildtype only in some of the experiments performed (Table 1; Fig. 5A).

All three *lmi2* alleles significantly enhanced the MI phenotype of the weak *lfy-10* mutant in at least six independent experiments (Table 1; Fig. 5B,C). *lmi2-2 lfy-10* double mutants showed the strongest MI delay, essentially phenocopying the *lfy-1* null mutant (Fig. 5B). In addition, in the *lfy-10* background, *lmi2-2* and to a lesser extent *lmi2-1* caused a delay in the MI transition as heterozygotes (Fig. 6). Hence, *LMI2* is a dosage sensitive gene at least under conditions when LFY activity is impaired. *LFY* itself is also dosage dependent (Okamuro et al., 1996; Blazquez et al., 1997), highlighting the sensitivity of this pathway to the level of both regulators. Finally, *lmi2-2* and *lmi2-1*

displayed a subtle delay in flowering-time (Table 2) both as single mutants and in the *lfy-10* genetic background.

I next tested whether the mutations in *LMI2* caused the delay in the MI transition by performing phenotypic rescue. Transformation of *lmi2-2 lfy-10* with a genomic copy of *LMI2* (pLMI2:LMI2) restored *LMI2* expression to a level similar to that observed in *lfy-10* (Fig. 5E). In addition, pLMI2:LMI2 fully rescued the enhanced MI defects of *lmi2-2 lfy-10* and *lmi2-1 lfy-10* relative to *lfy-10* (Fig. 5D and Fig. 10A).

2.2.3. LMI2 acts downstream and in parallel to LFY

To test whether LMI2 has additional LFY-independent roles in the MI transition we crossed the *lmi2-2* allele to the *lfy-1* null mutant. *lmi2-2* significantly enhanced the MI transition defect of *lfy-1* (Table 1), indicating that LMI2 acts both downstream and in parallel to LFY in this pathway. This is similar to AP1, which also acts downstream and in parallel to LFY (Bowman et al., 1993).

2.2.4. *LMI2* is expressed in the inflorescences meristem, in young flower primordia, and in flowers

We first examined the expression of *LMI2* during the MI transition using a bacterial beta-glucuronidase (GUS) transcriptional reporter. In nine-day-old wild-type seedlings, *LMI2:GUS* was expressed in the center of the rosette close to the shoot apex (Fig. 7A) in a pattern roughly similar to that of *pLFY:GUS* (Fig. 7E). In the inflorescence, the *LMI2:GUS* reporter was expressed in the meristem proper, in young floral primordia, as well as the carpels of older flowers (Fig. 7B-D; Fig. 8A). By contrast, as previously

reported (Blazquez et al., 1997), *pLFY:GUS* expression was absent from the meristem proper but observed in young floral primordia as well as older flower primordia (Fig. 7F,G; Fig. 8B). In addition, both *LMI2:GUS* and *pLFY:GUS* were strongly expressed in secondary inflorescences (Fig. 7D,H). Thus, *LMI2:GUS* and *pLFY:GUS* have overlapping, but not identical, expression patterns during reproductive development.

LMI2:GUS expression was reduced in the shoot apex of intermediate *lfy-9* mutants compared to wild-type seedlings (Fig. 8C,D). Conversely, steroid treatment of an inducible version of LFY, LFY-GR (William et al., 2004) resulted in elevated *LMI2:GUS* expression in seedlings, this was not observed in steroid treated wild-type seedlings expressing *LMI2:GUS* (Fig. 8E,F). Hence, LFY acts on *LMI2* cis regulatory elements present in this reporter construct, this is consistent with in vivo LFY binding to this locus (Winter, 2011).

I next examined endogenous *LMI2* expression by in situ hybridization. *LMI2* was expressed throughout the shoot apical meristem of primary inflorescences, with the highest expression observed in the young flower primordia (Fig. 9A). *LMI2* expression was reduced, but not absent, in the young flower primordia of *lfy-1* null mutant apices (Fig. 9B). No signal was observed using a sense probe (Fig. 9C). The residual *LMI2* expression in *lfy-1* is consistent with the genetic data that revealed a LFY independent role for LMI2 in addition to its function downstream of LFY.

Subsequent to the MI transition, *LMI2* was expressed in stage 2 to stage 4 flowers (Fig. 9D) (Smyth et al., 1990) and in the developing stamens and carpels of older flowers from stage 6 to stage 8 (Fig. 9E,F). Eventually, in stage 8 flowers, *LMI2* expression decreased in the developing stamens but continued to persist in the carpels (Fig. 9F).

2.2.5. *lmi2-1* acts as a loss-of-function allele

lmi2-1 displayed elevated *LMI2* expression in seedlings based on semi-quantitative RT-PCR (Fig. 4B), yet behaved as a loss-of-function allele (Table 1; Fig. 5A; Fig. 6). Moreover, the defect in *lmi2-1 lfy-10* was rescued by pLMI2:LMI2 (Fig. 10A). In contrast to the wildtype, *LMI2* expression was undetectable in *lmi2-1* shoot apices and young flower primordia (Fig. 10B,C), similar to the sense control (Fig. 10D). Thus in *lmi2-1* mutants, *LMI2* is absent from the initiating floral primordia where it is required for the MI transition. This suggests that the increased *LMI2* levels observed by RT-PCR could be due to ectopic *LMI2* expression. Indeed, while *LMI2* expression was very low in roots and leaves of nine-day-old wild-type seedlings, it was strongly expressed in these tissues in *lmi2-1* (a 40-fold and 400-fold increase, respectively; Fig. 10E). Based on these combined findings, we conclude that the T-DNA insertion in *lmi2-1* apparently disrupts the *LMI2* promoter, causing loss of *LMI2* expression in the SAM and in the young flower primordia. At the same time, the insertion causes ectopic and elevated *LMI2* expression, perhaps from a promoter located in the T-DNA insertion.

2.2.6. LMI2 is required for proper *AP1* upregulation

To place LMI2 in the MI pathway, I examined the expression of the direct LFY targets *AP1*, *CAULIFLOWER* (*CAL*), *LMI1* to *LMI5,* and that of another MI regulator, *FRUITFULL* (*FUL*) (Wagner et al., 1999; Ferrandiz et al., 2000; William et al., 2004) in *lfy-10* single mutants compared to *lmi2-2 lfy-10* double mutants during the MI transition (Fig. 11A,B). I conducted a time-course experiment spanning time-points prior to, during,

and immediately subsequent to the MI transition for all genotypes tested (Fig. 11A,B) (William et al., 2004; Yamaguchi et al., 2009). While I did not observe a reduction in the expression of *LMI1*, *LMI3*, or *LMI5*; a subtle reduction in the expression of *LMI4* was observed, and a pronounced (ca. four-fold) reduction in the expression of *AP1* in *lmi2-2 lfy-10* compared to *lfy-10* at day 13 (Fig. 11A,B). Indeed, *AP1* expression was induced more slowly in the double mutant compared to *lfy-10* (Fig. 11A). By contrast, induction of *CAL* and *FUL* expression was very similar in *lfy-10* and *lmi2-2 lfy-10* plants suggesting that the observed defect in *AP1* upregulation is specific. *AP1* expression was also reduced in *lmi2-2/+ lfy-10* plants relative to *lfy-10* mutants (Fig. 11C) consistent with the observed dosage sensitivity of *LMI2*, as well as in *lmi2-2* single mutant seedlings compared to the wildtype (Fig. 13B). These combined data suggest that LMI2 acts upstream of *AP1*.

lfy null mutants cause a delay, but not a loss in *AP1* expression; *AP1* is expressed in the flowers that eventually form in these mutants (Ruiz-Garcia et al., 1997; Wagner et al., 1999). Likewise, based on qRT-PCR, *AP1* is upregulated in *lmi2-2 lfy-10*, reaching expression levels similar to those in *lfy-10* at day 15 (Fig. 11A), when flower patterning is initiated(Fig. 11D).

We next examined *AP1* upregulation in wild-type, *lfy-10* and *lmi2-2 lfy-10* seedlings using in situ hybridization. By day 13, all three genotypes had initiated the first flowers. *AP1* expression was much reduced in stage 1 or 2 flower primordia in thirteen-day-old *lmi2-2 lfy-10* and the *lfy-10* mutants relative to the wildtype (Fig. 12A-D and data not shown). In addition, *AP1* expression levels were slightly more reduced in developing flower primordia of *lmi2-2 lfy-10* compared to *lfy-10* (Fig. 12B-D), and in the double

mutants especially in the shoot meristem proximal region of stage 2 flower primordia (Fig. 12C,D).

To test whether LMI2 can directly regulate *AP1* expression I scanned the *AP1* locus for presence of plant MYB binding sites using AthaMap (http://www.athamap.de/) (Steffens et al., 2004). Eight predicted MYB binding sites were found in the 5' upstream region, two in the introns, and one in the first exon of *AP1* (Fig. 13A). We next examined whether LMI2 binds to *AP1* regulatory regions in vivo by anti-HA chromatin immunoprecipitation (ChIP) followed by qPCR using plants expressing a HA-tagged genomic version of *LMI2* driven from its own promoter (pLMI2:LMI2-HA). The LMI2-HA fusion protein is biologically active, as pLMI2:LMI2-HA *lmi2-2* rescued the reduced *AP1* expression observed in *lmi2-2* mutants (Fig. 13B). LMI2-HA was recruited to the *AP1* promoter and bound to region six of *AP1,* which is very close to the known or predicted binding sites of other regulators of *AP1*, including LFY (Fig. 13A,C) (Parcy et al., 1998; William et al., 2004; Abe et al., 2005; Wigge et al., 2005; Wang et al., 2009; Yamaguchi et al., 2009; Xu et al., 2010; Winter, 2011). By contrast, we did not see enrichment of LMI2-HA relative to control *lmi2-2* plants in the remaining regions of the *AP1* locus, suggesting the binding of LMI2 at region six is specific (Fig. 13C). Taken together, these data suggest that LMI2 directly activates *AP1* expression during the MI transition.

2.2.7. LMI2 acts upstream and in parallel to AP1 during the MI transition

To test whether LMI2 acts solely to induce *AP1* or whether it regulates other factors during the MI transition, I crossed *lmi2-2* to the strong *ap1-10* mutant and

examined the timing of the MI transition. I did not observe an increase in the number of secondary inflorescences in *lmi2-2 ap1-10* compared to *ap1-10.* There was, however, a significant increase in the number of cauline leaves produced in *lmi2-2 ap1-10* compared to *ap1-10* (Table 3). AP1 does not play a significant role in cauline leaf suppression during the floral transition (Bowman et al., 1993; Schultz and Haughn, 1993). Thus like LFY (Liljegren et al., 1999), LMI2 functions through an AP1-independent pathway to suppress cauline leaf formation. We conclude that LMI2 acts both upstream of and in parallel to AP1 during the MI transition.

2.2.8. LMI2 and LFY interact

LMI2 binds very close to the known LFY binding site in the *AP1* locus (one putative LMI2 binding site in region six is six base pairs downstream of the LFY binding site (data not shown) (Winter, 2011). Hence, LMI2 and LFY may physically interact. Indeed, based on pulldown assays, LMI2 interacted with GST-LFY (Fig. 14A). Full length LFY protein homodimerized, as previously proposed (Hames et al., 2008), serving as a positive control. A negative control protein (see materials and methods for details) did not interact with GST-LFY, confirming the specificity of the observed interactions (Fig. 14A).

Based on yeast two-hybrid assays, the N-terminal half of LMI2 (LMI2N) showed a weak, but reproducible interaction with LFY (Fig. 14B). The C-terminal domain of LMI2 also interacted with LFY in yeast (data not shown). This interaction was more difficult to observe because -as previously reported (Zhang et al., 2009)- this domain of LMI2 displays transcriptional activation activity. Finally, bimolecular fluorescence

complementation (BiFC) was used to test for an in vivo interaction between LMI2 and LFY. Both the N-terminal half of LMI2 (LMI2N) and -to a lesser extent- the C-terminal half of LMI2 (LMI2C) interacted with LFY (Fig. 14C). Again, LFY interacted with itself (positive control). By contrast, a negative control protein did not interact with LFY, suggesting the observed interactions were specific. The combined data suggest that LFY and LMI2 can form heterodimers.

2.2.9. LMI2 is required for proper *LFY* upregulation

During the floral transition LFY and AP1 act in a positive feedback regulatory loop (Liljegren et al., 1999; Ferrandiz et al., 2000; Kaufmann et al., 2010). In light of this, I examined whether LMI2 can also feedback regulate *LFY*. Indeed, *LFY* levels were reduced in *lmi2-2 lfy-10* compared to *lfy-10* mutants throughout the MI transition (Fig. 15A). Furthermore, *LFY* levels were reduced in eleven-day-old *lmi2-2* seedlings compared to the wildtype (Col) (Fig. 15B). To determine whether the reduction in *LFY* in *lmi2* mutants was an indirect consequence of reduced *AP1* expression in these mutants or whether LMI2 directly regulated *LFY* levels, we used ChIP to examine LMI2 binding to *LFY* regulatory regions. We tested binding of LMI2 to three predicted MYB binding sites in the 5' upstream regulatory region, two sites in exon one, and two sites in the second intron of *LFY* (Fig. 15C). We did not see binding of LMI2-HA to the promoter or intron regions of *LFY*, but we did observe a subtle enrichment at region four in exon one (Fig. 15D). Although one other LFY regulator has previously been shown to bind this region (Yamaguchi et al., 2009), further experiments are needed to determine whether the feedback from LMI2 to *LFY* is direct.

2.2.10. SOC1 and AGL24 are possible regulators of *LMI2*

The genetic and expression data suggests that in addition to LFY, at least one other factor regulates *LMI2* during the MI transition. Since *lmi2* mutants have a subtle flowering-time delay, *LMI2* expression could be induced by a flowering-time regulator. We noted that the expression domains of the MADS box transcription factor and flowering-time genes *SOC1* and *AGL24* overlap temporally and spatially with that of *LMI2* in the shoot apical meristem (Fig. 8A; 9A) (Lee et al., 2000; Samach et al., 2000; Yu et al., 2002; Yu et al., 2004; Lee et al., 2008a). In addition, it is known that SOC1 and AGL24 act together by forming a heterodimer to activate *LFY* expression prior to the MI transition (Lee et al., 2008a). Furthermore, the 5' upstream region of *LMI2* contains four CArG boxes, which are the consensus binding motif for MADS box transcription factors (Fig. 16C). Therefore, it is possible that SOC1 and/or AGL24 could upregulate *LMI2* expression.

To examine whether *LMI2* expression is regulated by SOC1 we generated 35S:SOC1-GFP lines, which displayed early flowering phenotypes as previously reported (Fig. 16A) (Moon et al., 2005; Lee et al., 2008a), indicating the chimeric protein was biologically active. 35S:SOC1-GFP plants undergo the floral transition significantly earlier than the wildtype (Moon et al., 2005; Lee et al., 2008a), therefore I examined LMI2 expression at day 3, 5 and 7. Indeed, I saw an increase in *LMI2* expression over the entire timecourse in the 35S:SOC1-GFP line compared to the wildtype (Fig. 16B). I next tested *LMI2* levels in the *soc1-2* null mutant at day 7, 9 and 11. Although subtle, *LMI2* expression was reduced in day 9 and day 11 *soc1-2* mutants compared to the wildtype

control (Fig. 16B). Since SOC1 is a known regulator of *LFY* it is possible that the differential expression of *LMI2* in these SOC1 lines is a result of changes in *LFY* levels. Additional experiments are needed to differentiate between a direct and/or an indirect effect of SOC1 on *LMI2* expression.

One way to examine a direct vs. indirect effect of SOC1 and/or AGL24 on *LMI2* expression is to test for potential SOC1 and AGL24 binding to the CArG boxes found on the *LMI2* regulatory regions. To test for binding of SOC1 and AGL24 on *LMI2* we used a representative 35S:SOC1-GFP line (see above) and a previously characterized pAGL24:AGL24-RFP transgenic line (Gregis et al., 2009). We performed ChIP-qPCR using anti-GFP antibodies in seedlings. We tested binding to all four regions containing CArG boxes upstream of *LMI2* as well as to one region downstream of *LMI2* (Fig. 16D). We observed enrichment of both SOC1-GFP and AGL24-RFP at regions two and three of the *LMI2* locus. By contrast, no enrichment was observed for both SOC1-GFP and AGL24-RFP at regions one or four, suggesting that the observed binding was specific (Fig. 16D). Hence, both SOC1 and AGL24 bind to regulatory regions of the *LMI2* locus and occupy the same regions in this locus.

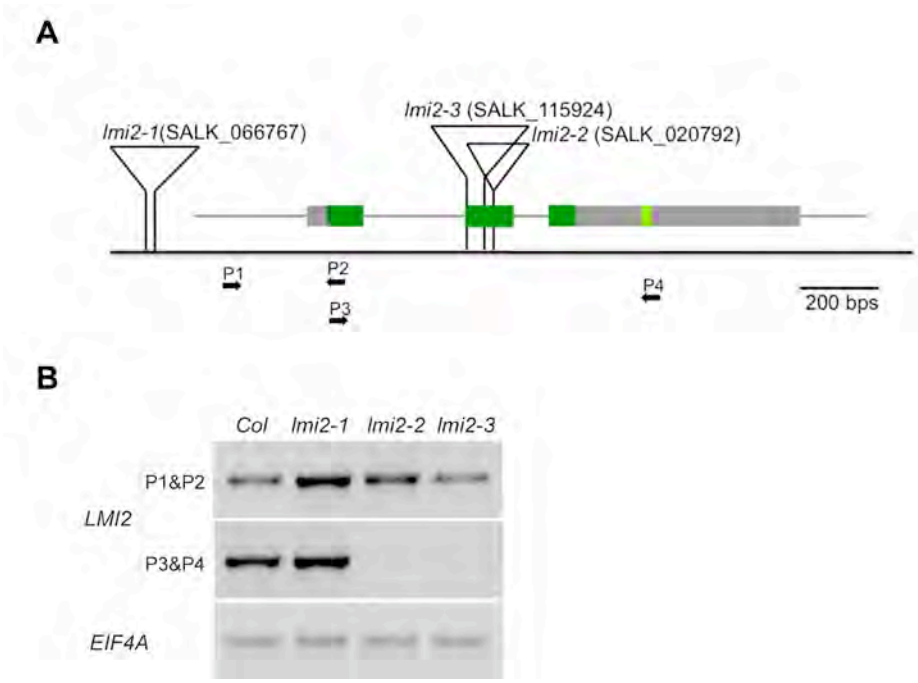

Figure 4. Characterization of LMI2 T-DNA insertion alleles.

(**A**) Map of the *LMI2* locus. Dark grey boxes: exons, grey line: 5' and 3' UTR and introns. Dark green boxes; MYB DNA-binding domain; light green box: conserved amino acid motif found in LMI2 and its homologs (Kranz et al., 1998; Stracke et al., 2001). Triangles: T-DNA insertions. The lines connecting each T-DNA to the sequence denote the size of the deletion caused by each insertion. (**B**) Semi-quantitative RT-PCR of *LMI2* expression performed on nine-day-old seedlings for each T-DNA insertion line and Col (wildtype). Primers used (see **A** for location) are indicated at the left. The *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (*EIF4A*) gene was used as an internal control.

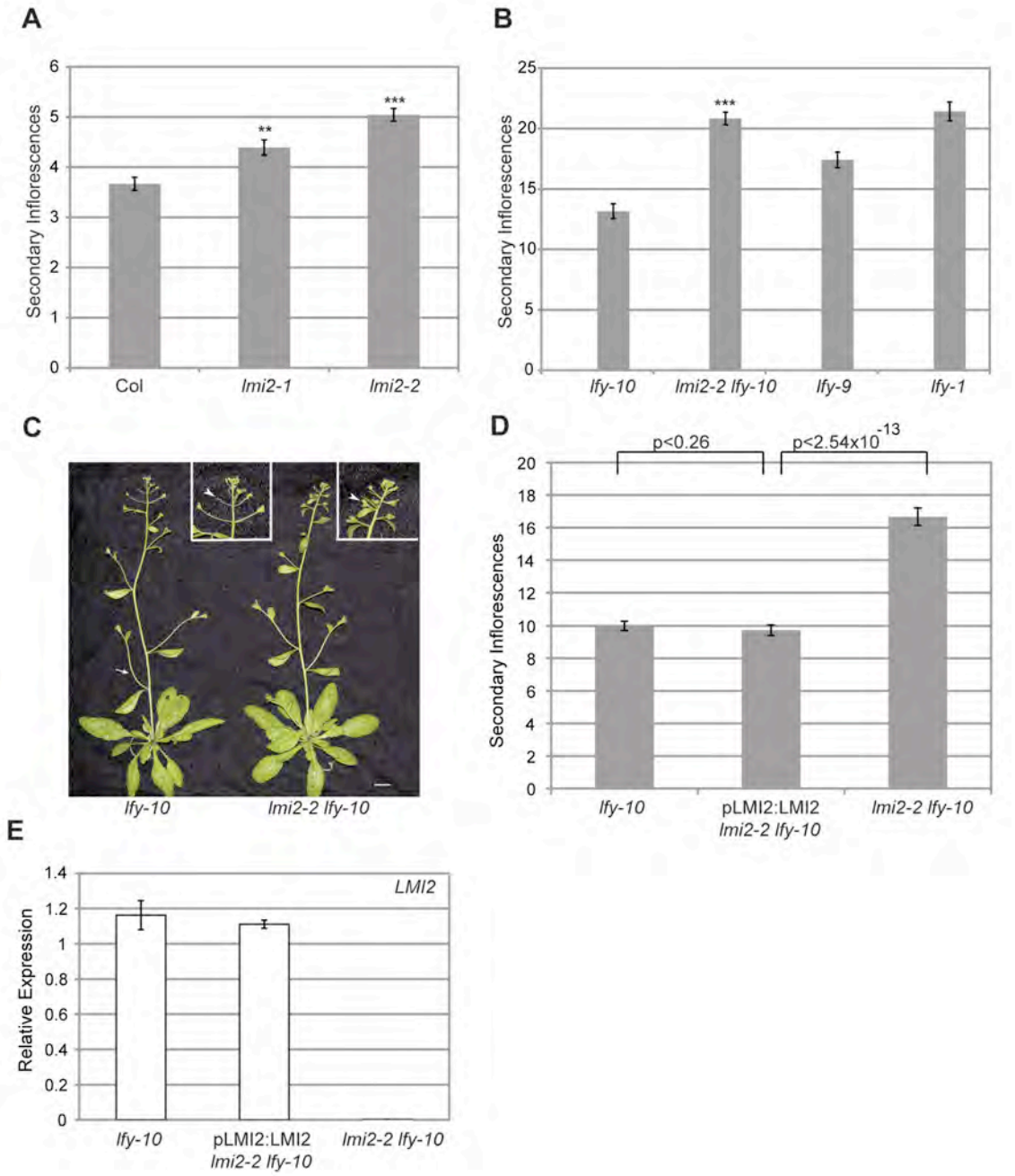

Figure 5. *lmi2* mutants cause a MI phenotype.

(**A**) Number of secondary inflorescences formed in *lmi2* single mutants compared to Col (wildtype). (**B**) Number of secondary inflorescences of *lmi2-2 lfy-10* compared to *lfy-10* (weak), *lfy-9* (intermediate) and *lfy-1* (strong) alleles. (**C**) *lfy-10* and *lmi2-2 lfy-10* mutant phenotypes, with close-ups of the inflorescence apices (insets). Arrow: a secondary

inflorescence subtended by a cauline leaf. Arrowheads: lateral organs formed in *lmi2-2 lfy-10* and *lfy-10* at a comparable stage. Scale bar: 1 cm. (**D**) Number of secondary inflorescences formed in pLMI2:LMI2 *lmi2-2 lfy-10* compared to *lfy-10* and *lmi2-2 lfy-10*. P-values for one-tailed Student's t-test are indicated. (**E**) qRT-PCR of *LMI2* expression in thirteen-day-old *lfy-10*, pLMI2:LMI2 *lmi2-2 lfy-10*, and *lmi2-2 lfy-10* seedlings. Asterisks: a statistical significant difference based on one-tailed Student's t-test: **$p< 10^{-3}$ (*lmi2-1* compared to Col) and ***$p<10^{-9}$ (*lmi2-2* and *lmi2-2 lfy-10* compared to Col and *lfy-10*, respectively). All values represent mean ± s.e.m.

Table 1. MI phenotypes of *lmi2* mutants

Genotype	Cauline leaves	Student's t-test	Secondary inflorescences	Student's t-test
WT (Col)	3.1±0.1 (33)		3.1±0.1 (33)	
lmi2-1	3.5±0.1 (32)	3/5	3.5±0.1 (32)	2/5
lmi2-2	4.1±0.1 (33)	5/5	4.1±0.1 (33)	5/5
lfy-10	6.0±0.1 (28)		11.4±0.4 (28)	
lmi2-1 lfy-10	11.5±0.5 (24)	6/6	15.7±0.5 (24)	6/6
lmi2-2 lfy-10	13.6±0.3 (28)	9/9	14.9±0.3 (28)	9/9
lfy-10	7.0±0.2 (37)		12.4±0.4 (37)	
lmi2-3 lfy-10	11.8±0.4 (14)	6/6	17.1±0.7 (14)	6/6
lfy-1	10.7±0.3 (21)		21.3±0.6 (21)	
lmi2-2 lfy-1	13.1±0.3 (17)	3/3	37.5±1.9 (17)	3/3

Average number of cauline leaves and secondary inflorescences ± s.e.m. for one representative experiment are shown. The number of plants counted is indicated in the parentheses. All phenotypic experiments were performed multiple times and one-sided Student's t-tests were performed for each experiment. The alternative hypothesis (H_1) is *lmi2* mutants have more lateral organs compared to the control genotype. Listed under Student's t-test are the number of experiments with a p-value less than 0.05 out of the total number of experiments performed.

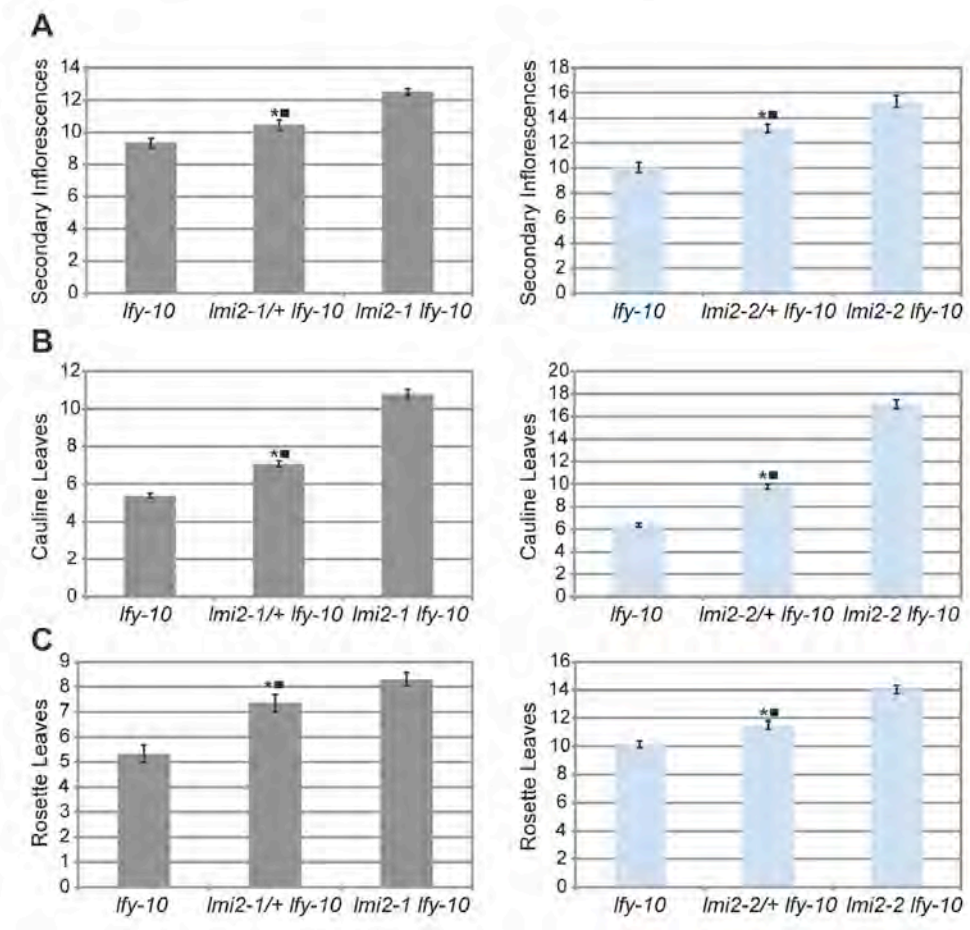

Figure 6. *lmi2-1/+* and *lmi2-2/+* cause an enhanced MI phenotype in the *lfy-10* mutant background.

(**A-C**) The number of secondary inflorescences (A), cauline leaves (B) and rosette leaves (C) formed in *lmi2-1/+ lfy-10*, *lfy-10* and *lmi2-1 lfy-10* (grey bars) or *lmi2-2/+ lfy-10*, *lfy-10* and *lmi2-2 lfy-10* (light blue bars). Asterisks denote a statistically significant difference (one-tailed Student's t test ($p<0.05$)) between *lfy-10* and the *lmi2/+ lfy-10* mutants, rectangles denote a statistically significant difference ($p<0.05$) between the *lmi2/+ lfy-10* and the *lmi2 lfy-10* mutants.

Table 2. Flowering-time phenotypes of *lmi2* mutants

Genotype	Rosette Leaves	Student's t-test
WT (Col)	9.9±0.2 (33)	
lmi2-1	10.0±0.2 (32)	1/5
lmi2-2	10.5±0.2 (33)	5/5
lfy-10	9.4±0.2 (28)	
lmi2-1 lfy-10	9.3±0.2 (24)	2/5
lmi2-2 lfy-10	11.8±0.3 (28)	8/8
lfy-10	8.5± 0.3 (37)	
lmi2-3 lfy-10	8.1±0.3 (14)	0/6
lfy-1	12.5±0.2 (21)	
lmi2-2 lfy-1	12.7±0.2 (17)	0/3
ap1-10	5.9±0.2 (12)	
lmi2-2 ap1-10	6.5±0.3 (13)	2/5

Average number of rosette leaves ± s.e.m. for one representative experiment are shown. The number of plants counted is indicated in the parentheses. All phenotypic experiments were performed multiple times and one-sided Student's t-tests were performed for each experiment. The alternative hypothesis (H_1) is *lmi2* mutants have more lateral organs compared to the control genotype. Listed under Student's t-test are the numbers of experiments with a p-value less than 0.05 out of the total number of experiments performed.

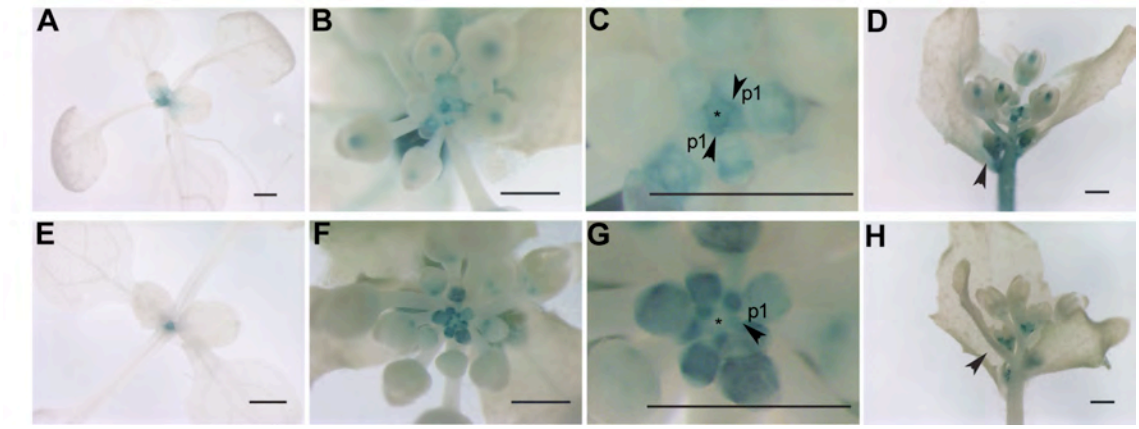

Figure 7. *LMI2:GUS* and *pLFY:GUS* expression is largely overlapping in seedlings and inflorescences.

Expression of *LMI2:GUS* (**A-D**) and *pLFY:GUS* (**E-H**); Scale bars: 1 mm. (**A,E**) Nine day-old seedlings. (**B, F**) Young (1 cm bolt) primary inflorescences. (**C, G**) Higher magnification of the shoot apices shown in (**B, F).** The black arrowheads point to stage 1 floral primordia (p1) and the asterisks indicate the shoot apical meristem. (**D, H**) *GUS* reporter expression in flowers and secondary inflorescences formed on 1 cm bolt primary inflorescences. The black arrowheads point to secondary inflorescences.

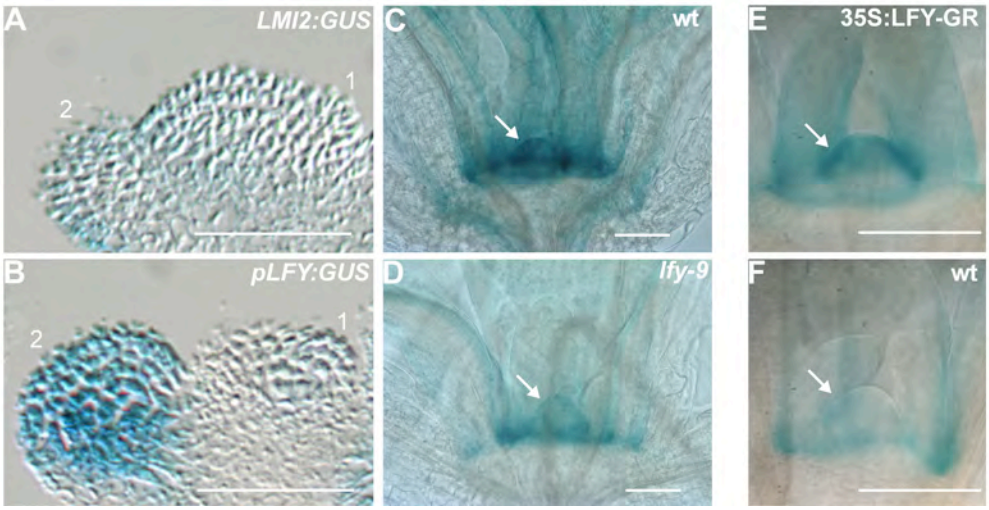

Figure 8. *LMI2:GUS* and *pLFY:GUS* expression overlaps in young floral primordia and *LMI2:GUS* expression in seedlings is dependent on LFY.

(A-B) Histological sections of 1 cm bolt primary inflorescence apices expressing *LMI2:GUS* (A) or *pLFY:GUS* (B). Numbers indicate the developmental stages of the young floral primordia (Smyth et al., 1990). Scale bars: 50 μm. **(C-D**) Whole-mount *LMI2:GUS* expression in wild-type (C) and *lfy-9* (D) seven-day-old seedlings. White arrows indicate shoot apex. (**E-F**) Whole-mount *LMI2:GUS* expression in eight-day-old 35S:LFY-GR (E) and wild-type (F) seedlings treated with dexamethasone. *LMI2:GUS* is induced at the flanks of the shoot apex. It is unclear why *LMI2:GUS* is induced in this spatially restricted domain. It is possible that the LFY protein is not uniformly expressed in 35S:LFY-GR seedlings. Alternatively, a spatially restricted co-factor may be required for *LMI2* induction. White arrows indicate the flanking regions of the shoot apex. Scale bars: 100 μm.

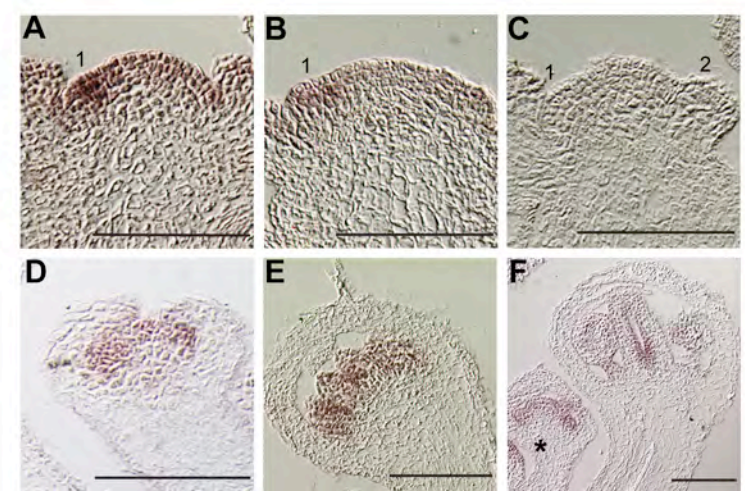

Figure 9. *LMI2* expression in the initiating floral primordia and developing flowers and dependence of the expression in the floral primordia on LFY.

LMI2 expression based on in situ hybridization; scale bars: 100 μm. Numbers indicate the developmental stage of young floral primordia (Smyth et al., 1990). Expression in wild-type (**A,C,D-F**) and *lfy-1* (**B**). Tissues assayed were: primary inflorescence apices (1 cm bolt; **A-C**), developing flowers; stage 4 (**D**), stage 7 (**E**) and stage 6 (asterisk), as well as stage 8 (**F**). Sense probe control (**C**).

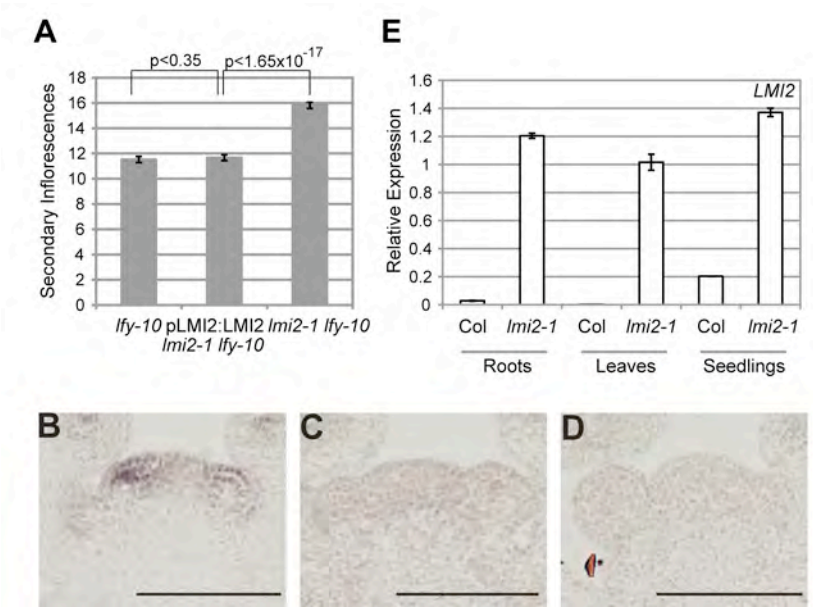

Figure 10. The T-DNA insertion in *lmi2-1* causes misregulation of *LMI2*.

(A) The number of secondary inflorescences in pLMI2:LMI2 *lmi2-1 lfy-10* compared to *lfy-10* and *lmi2-1 lfy-10*. P-values for one-tailed Student's t-test are indicated. (**B-C**) *LMI2* expression based on in situ hybridization in wild-type (**B**) and *lmi2-1* (**C**) 1 cm bolt primary inflorescences. (**D**) *LMI2* sense probe control. Scale bars: 100 μm. (**E**) qRT-PCR analysis of *LMI2* expression in roots (including hypocotyls), leaves (including cotyledons) and whole seedlings from nine-day-old wild-type (Col) and *lmi2-1* mutants. Values are mean ± s.e.m.

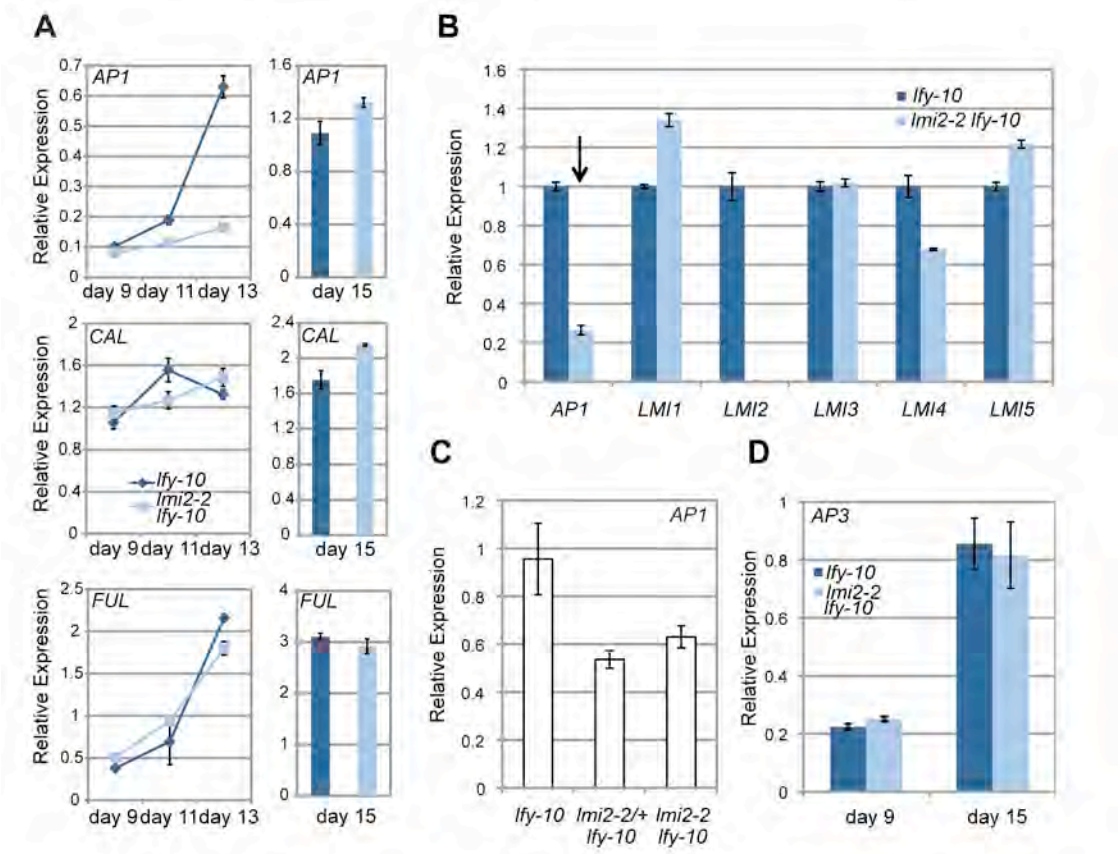

Figure 11. LMI2 is required for proper activation of *AP1* expression.

(**A**) *AP1*, *CAL* and *FUL* expression in *lfy-10* and *lmi2-2 lfy-10* seedlings based on qRT-PCR at days 9,11,13 and 15. Values represent mean ± s.e.m. (**B**) *AP1* and *LMI1-5* gene expression in thirteen-day-old *lfy-10* and *lmi2-2 lfy-10* seedlings. To facilitate comparison, expression of each gene in the *lfy-10* sample was set to one. (**C**) *AP1* expression in thirteen-day-old *lfy-10*, *lmi2-2/+ lfy-10* and *lmi2-2 lfy-10* seedlings. **(D)** Flower patterning was initiated in *lfy-10* and *lmi2-2 lfy-10* by day 15. Expression of the floral homeotic regulator *APETALA3* (*AP3*) in nine and fifteen-day-old *lfy-10* and *lmi2-2 lfy-10* seedlings. Values are mean ± s.e.m.

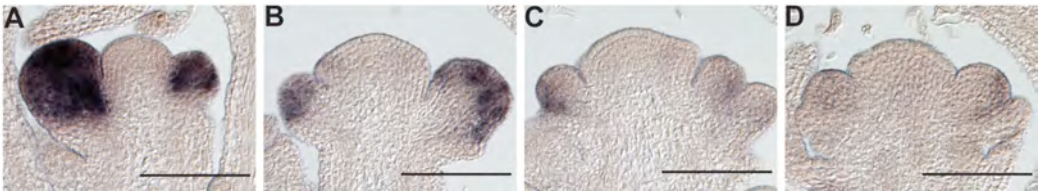

Figure 12. *AP1* expression is reduced in *lfy-10* and *lmi2-2 lfy-10* floral primordia.

(**A**-**D**) *AP1* expression based on in situ hybridization of eleven-day-old wild-type (Col) (**A**), and thirteen-day-old *lfy-10* (**B**), and *lmi2-2 lfy-10* (**C, D**) seedlings. Scale bars: 100 μm.

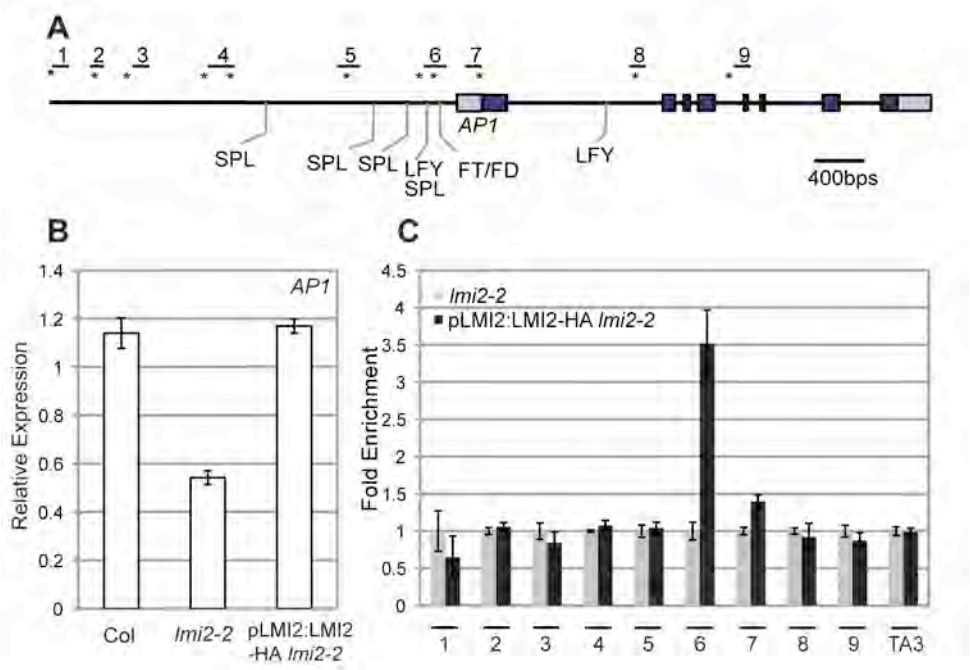

Figure 13. LMI2 binds to *AP1* regulatory regions.

(**A**) Map of the *AP1* locus. Light purple boxes: 5' and 3' UTRs; dark purple boxes: exons; black lines: introns and intergenic regions. Asterisks: predicted plant MYB binding sites with a score exceeding the threshold score (Steffens et al., 2004). Binding sites of known regulators of *AP1* are shown below the locus (see text for details). Horizontal bars: regions amplified in ChIP q-PCR. (**B**) Rescue of *AP1* expression in eleven-day-old pLMI2:LMI2-HA *lmi2-2* seedlings. (**C**) ChIP-qPCR in eleven-day-old *lmi2-2* and pLMI2:LMI2-HA *lmi2-2* seedlings to assess LMI2 binding to *AP1* regulatory regions. Immunoprecipitated DNA is represented as fold enrichment relative to the *lmi2-2* control. Shown are mean ± s.e.m. The heterochromatic *TA3* retrotransposon (Konieczny et al., 1991) served as a negative ChIP control.

Table 3. MI phenotypes of *lmi2 ap1* mutants

Genotype	Cauline Leaves	Student's t-test	Secondary inflorescences	Student's t-test
ap1-10	3.6±0.2 (12)		3.8±0.3 (12)	
lmi2-2 ap1-10	4.2±0.2 (13)	4/5	4.0±0.2 (13)	0/5

Average number of cauline leaves and secondary inflorescences ± s.e.m. for one representative experiment are shown. The number of plants counted is indicated in the parentheses. All phenotypic experiments were performed multiple times and one-sided Student's t-tests were performed for each experiment. The alternative hypothesis (H_1) is *lmi2* mutants have more lateral organs compared to the control genotype. Listed under Student's t-test are the number of experiments with a p-value less than 0.05 out of the total number of experiments performed.

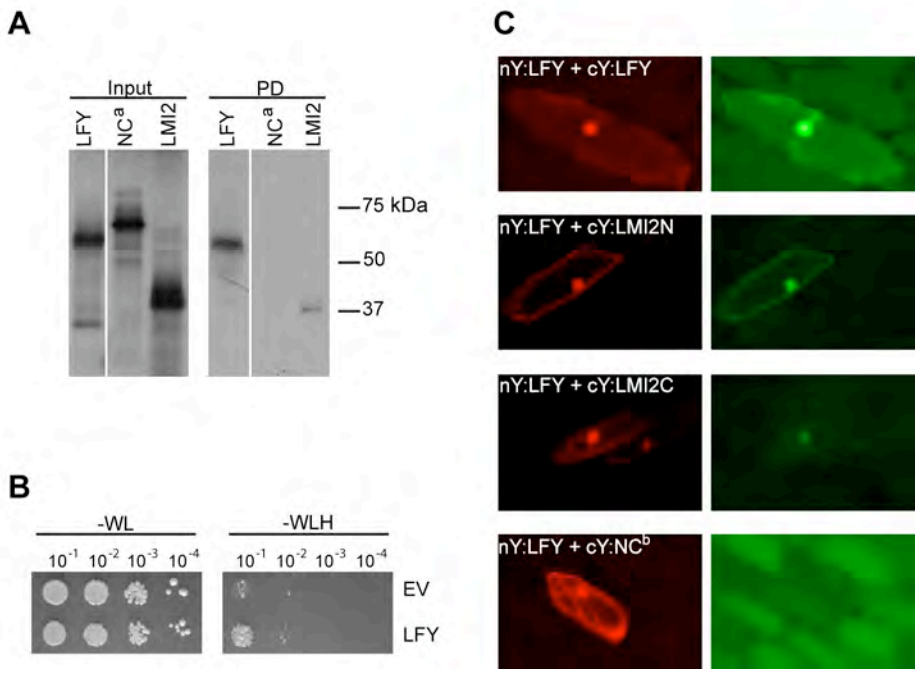

Figure 14. LMI2 physically interacts with LFY.

(**A**) In vitro GST-Pull-down assay. GST-tagged LFY protein incubated with in vitro translated LFY, LMI2, and a negative control peptide (NC^a). 5% input is shown. Input and pull-down (PD) were run on the same gel, spaces between lanes denote irrelevant samples removed from the gel image. Right: molecular weight markers (kDa). (**B**) Yeast two-hybrid assay. Growth of yeast transformed with pDBLeu-LMI2N bait construct and pDEST22-LFY or pDEST22 alone (EV) on -Trp-Leu/SD plates (-WL) or -Trp-Leu-His/SD plates (-WLH). (**C**) Interaction of LMI2N and LMI2C with LFY based on bimolecular fluorescence complementation (BiFC). Left: 35S:2XMCherry transformation control, right: protein interactions. Positive control: nY:LFY and cY:LFY, Negative control: nY:LFY and $cY:NC^b$.

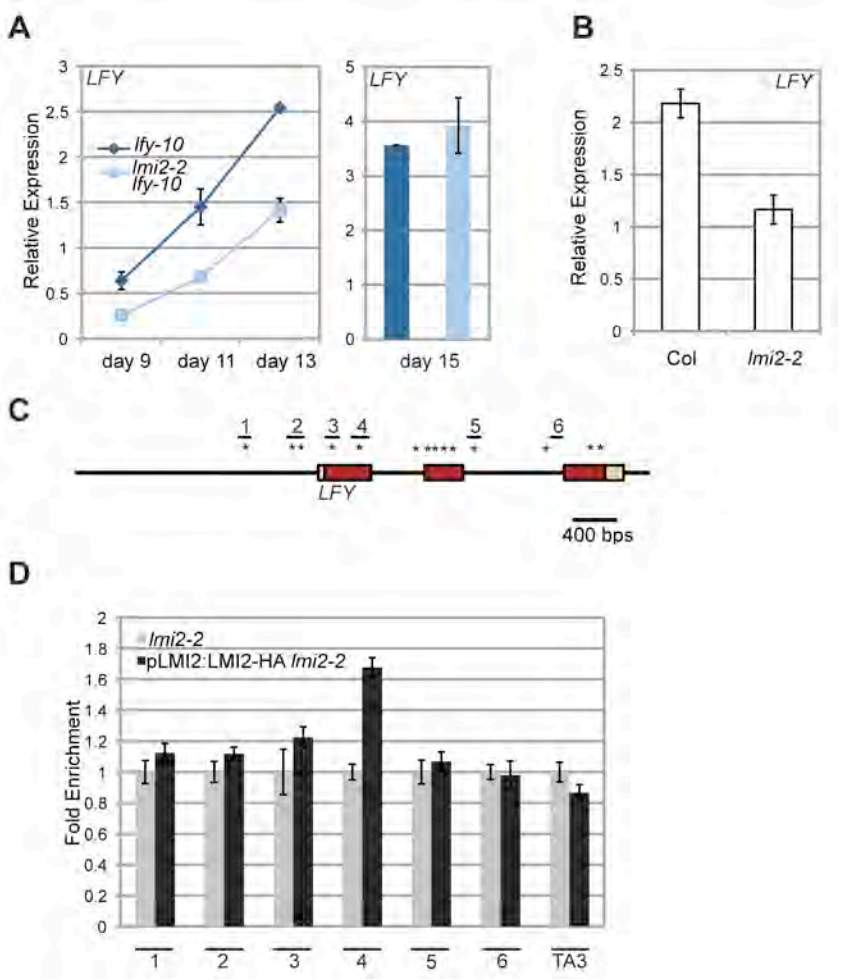

Figure 15. LMI2 positively feedback regulates *LFY* expression during the floral transition.

(**A**) *LFY* expression based on qRT-PCR in *lfy-10* and *lmi2-2 lfy-10* seedlings at days 9,11,13 and 15. (**B**) *LFY* expression in eleven-day-old wild-type (Col) and *lmi2-2* seedlings. (**C**) *LFY* Locus. Light red boxes: 5' and 3' UTRs; dark red boxes: exons; black lines: introns and intergenic regions. Asterisks: predicted plant MYB binding sites (see Fig. 13A). (**D**) ChIP q-PCR to test for LMI2-HA binding to *LFY* regulatory loci. See Fig. 13C for details on the ChIP analysis. Shown are the mean ± s.e.m.

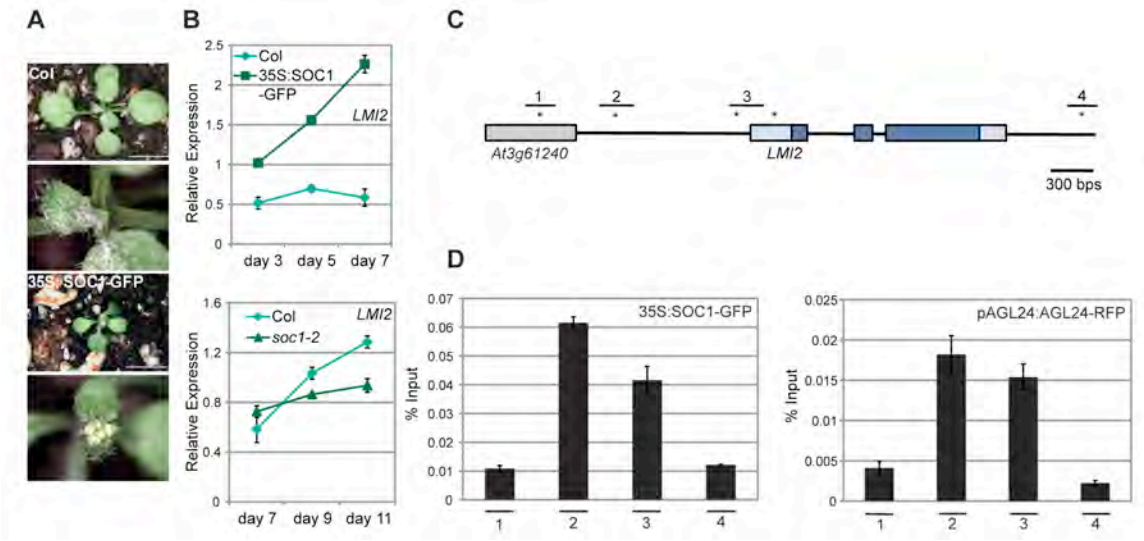

Figure 16. SOC1 and AGL24 are possible regulators of *LMI2*.

(**A**) Flowering of fifteen-day-old wild-type (Col) (top two panels) and 35S:SOC1-GFP (bottom two panels) seedlings grown under long-day conditions. Scale bars: 0.5 cm. (**B**) *LMI2* expression in 35S:SOC1-GFP and wild-type (Col) seedlings (top graph). Expression was monitored at days 3, 5, and 7. *LMI2* expression in *soc1-2* and wild-type (Col) seedlings (bottom graph). Expression was monitored at days 7, 9 and 11. Values represent mean ± s.e.m. (**C**) Map of the *LMI2* locus. The 5' and 3'UTR of *LMI2* are shown as light blue boxes and the 3'UTR of the upstream gene is shown as a grey box. *LMI2* exons are represented by dark blue boxes, while introns, upstream and downstream intergenic regions are represented by black lines. The consensus binding sites for the MADS box transcription factors ($CC(A/T)_6GG$ or one mismatch) are indicated by asterisks. Regions of the *LMI2* locus amplified by qPCR after anti-GFP ChIP are indicated by horizontal lines. (**D**) ChIP-qPCR of nine-day-old 35S:SOC1-GFP seedlings and ChIP-qPCR of twelve-day-old pAGL24:AGL24-GFP seedlings. Values represent mean ± s.e.m.

Chapter 3. The role of LMI2 in Early Flower Development

3.1. Background

It is clear from the GUS and in situ hybridization studies described in Chapter 2 that *LMI2* is not only expressed in the incipient floral primordia during the MI transition but *LMI2* is also expressed in young developing flower primordia and throughout later stages in flower development. This suggests LMI2 may have a role after the MI transition during flower development. Furthermore, during my characterization of the MI phenotype of LMI2, I noticed a significant reduction in the *ap1* branched flower phenotype in the *ap1 lmi2* compared to *ap1* mutants (see below). These data along with the already well defined role of AP1 in maintaining determinate growth of the floral meristem (Yu et al., 2004; Liu et al., 2007) lead us to focus our attention on a possible role for LMI2 in early flower development.

3.2. Results

3.2.1. Branched flower phenotypes of *ap1* and *lmi2 ap1* mutants

We previously identified LMI2 as a MI regulator acting upstream of AP1 during the floral transition (Chapter 2). After the MI transition, *LMI2* is expressed in young flower primordia and older developing flowers (Fig. 9A,D-F) suggesting LMI2 may play a later role in floral development. *lmi2* single mutants do not present any apparent floral defects on their own but when combined with a strong or an intermediate allele of *ap1* (*ap1-10*, *ap1-11* respectively), the branched flower phenotype of *ap1* mutants is significantly reduced (Fig. 17A, B). Similar to the reduction in branched flowers previously observed in double mutants between *ap1* and *svp*, *agl24* and *soc1* (Yu et al.,

2004; Liu et al., 2007), our phenotypic analyses suggest that mutations in *lmi2* also suppress the branched flower phenotype of *ap1* mutants.

3.2.2. AP1 downregulates *LMI2* in young flower primordia

AP1 downregulates meristematic activity in late stage 2 and early stage 3 floral primordia by directly repressing *SVP*, *AGL24*, and *SOC1* (Fig. 3) (Yu et al., 2004; Liu et al., 2007). The downregulation of these flowering-time genes by AP1 promotes the onset of floral differentiation in stage 3 flowers (Liu et al., 2009b; Wagner, 2009). To test whether *LMI2* is also repressed by AP1 during these early stages of floral development we examined *LMI2* expression in primary inflorescences of wild-type (Col) and *ap1-10* mutants. *LMI2* expression was increased in *ap1-10* mutants compared to wild-type inflorescences and this increase was similar to the increase in *AGL24* expression in *ap1-10* mutants (Fig. 18A). To address whether the change in *LMI2* expression is regulated by AP1 we examined *LMI2* expression in primary inflorescences of 35S:AP1-GR *ap1-1* plants in the presence and absence of dexamethasone. *LMI2* expression was markedly reduced eight hours after AP1 activation by dexamethasone treatment (Fig. 18B). The combined expression data suggests that AP1 likely downregulates *LMI2* during early flower development. Furthermore, a recent genome-wide AP1 binding study has shown AP1 binds to the upstream region of *LMI2* in inflorescences (Fig. 18C) (Kaufmann et al., 2010) suggesting that AP1 may directly downregulate *LMI2* in a manner similar to that described for *SVP*, *AGL24*, and *SOC1* during flower development (Yu et al., 2004; Liu et al., 2007).

In order to determine the exact timing and location of *LMI2* downregulation by AP1 we used in situ hybridization to examine the spatial and temporal pattern of *LMI2* in young wild-type and *ap1-10* mutant flowers. In the wildtype, *LMI2* is expressed in late stage 2 (Fig. 8A) and early stage 3 flowers, this *LMI2* expression pattern overlaps with that of *AP1* (Mandel et al., 1992; Gustafson-Brown et al., 1994). In early stage 3 flowers, *LMI2* was expressed throughout the floral meristem (Fig. 18D). In contrast to the wildtype, in early stage 3 flowers of *ap1-10* mutants, *LMI2* was predominantly observed in a region between the floral meristem and the developing sepal primordia (Fig. 18E). Branched flowers arise within the axils of the first whorls organs (Irish and Sussex, 1990; Bowman et al., 1993), the same location where *LMI2* is misexpressed in *ap1* mutants. Hence, this region is likely where LMI2 contributes to the *ap1* branched flower phenotype.

3.2.3. LMI2, AGL24, SOC1 and SVP act in the same pathway

The three flowering-time regulators SVP, AGL24 and SOC1 together prevent premature differentiation of the floral meristem (Liu et al., 2009a; Wagner, 2009; Irish, 2010; McKim and Hay, 2010). More recently it was shown that all three proteins repress a common direct target gene, *SEP3* (Fig. 3), which encodes for an activator of flower differentiation (Liu et al., 2009b). Consistent with this, the reduction of the number of branched flowers formed relative to *ap1* in *agl24 ap1* was comparable to that in triple and quadruple mutants (i.e. *svp soc1 agl24 ap1*) (Liu et al., 2007), suggesting they act in a common pathway. We therefore next crossed *lmi2-2 ap1-10* to *agl24-3* and examined the branched flower phenotype of the triple mutant compared to *ap1-10*, *ap1-10 lmi2-2* and

ap1-10 agl24-3. We did not see a significant reduction in the number of branched flowers in the *lmi2-2 agl24-3 ap1-10* triple mutants compared to *lmi2-2 ap1-10* and *agl24-3 ap1-10* (Fig. 19). These phenotypic observations suggest that LMI2 likely acts in the same pathway as AGL24, SVP and SOC1 downstream of AP1. Furthermore, the *LMI2* expression pattern in young stage 3 *ap1* mutant flowers (Fig. 18E) was very similar to the expression pattern observed for *SVP* in *ap1* mutants at the same stage (Liu et al., 2007) suggesting a possible link between LMI2 and SVP in this pathway.

3.2.4. LMI2 binds to the upstream region of *SVP*

SVP functions along with AGL24 and SOC1, to maintain proper floral development (Liu et al., 2009b). *SVP* is expressed in stage 1 and 2 floral primordia where it is known to repress the class B and C floral homeotic genes as well the class E gene *SEP3*, to prevent premature differentiation (Gregis et al., 2006; Gregis et al., 2009; Liu et al., 2009b). This allows the floral meristem to develop and generate the proper number of stem cells for later stages of floral organ differentiation (Liu et al., 2009a; Wagner, 2009; Irish, 2010; McKim and Hay, 2010). During late stage 2 and into early stage 3 of floral development, *SVP* is repressed by AP1 to initiate floral differentiation (Liu et al., 2007; Liu et al., 2009b).

One possible role for LMI2 in this pathway could be to upregulate *SVP* in young stage 1 and 2 flowers. To test this hypothesis we examined whether LMI2 directly binds to *SVP* during early stages of flower development by ChIP. First, I scanned the *SVP* genomic region for putative MYB binding sites using AthaMap and AGRIS (http://arabidopsis.med.ohio-state.edu/, http://www.athamap.de/) (Fig. 20A)(Davuluri et

al., 2003; Steffens et al., 2004). We next performed ChIP-qPCR using anti-HA antibodies in young pLMI2:LMI2-HA *lmi2-2* and *lmi2-2* inflorescences and tested for binding of LMI2 to four regions on the *SVP* locus. Strong binding of LMI2-HA compared to *lmi2-2* was observed for regions two and three upstream of the *SVP* transcriptional start site (Fig. 20B). No enrichment was observed for regions one and four on *SVP* as well as the *EIF4A* control locus, suggesting the binding of LMI2 to *SVP* at regions two and three are specific (Fig. 20B). Although LMI2 binds to *SVP*, critical expression analyses are still needed to address whether LMI2 regulates *SVP* during early flower development. These experiments are underway and are discussed in detail in Chapter 4 of this dissertation.

3.2.5. *AG* is precociously expressed in *lmi2* mutants

Although the expression studies for this project are ongoing, while performing expression analyses for the role of LMI2 in the MI transition I obtained very interesting data for the class C homeotic regulator *AG*. During this time, I examined the expression of MI regulators during the floral transition in *lmi2-2 lfy-10* and *lfy-10* seedlings (Fig. 21). *AG* expression was tested in these seedlings as a control for floral initiation. Surprisingly, I found *AG* levels were increased (2-fold) in day 9 *lmi2-2 lfy-10* seedlings relative to *lfy-10* seedlings (Fig. 21). This data suggests LMI2 may have a possible role in repressing *AG* prior to floral organogenesis. At the moment it is not known whether this repression is a direct or an indirect consequence resulting from LMI2 regulating other factors in this pathway that control *AG* expression. The additional experiments needed to address this question are discussed in detail in Chapter 4 of this dissertation.

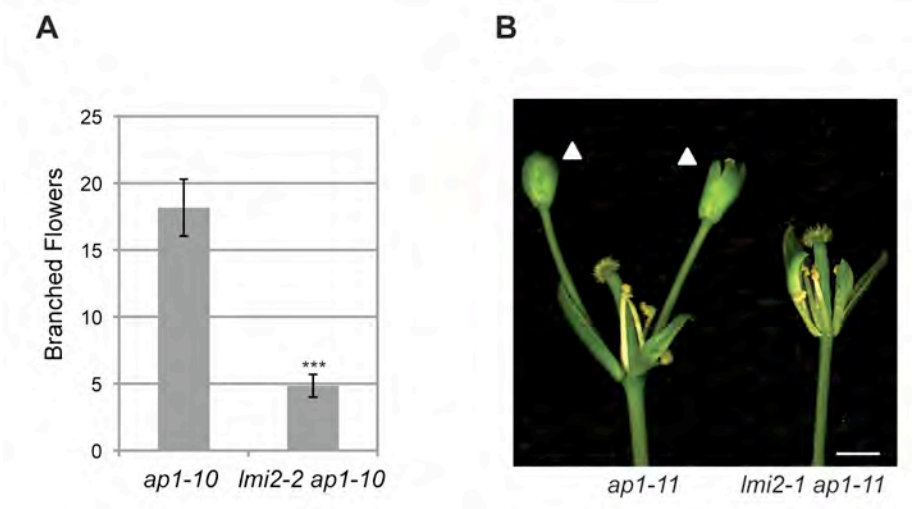

Figure 17. *lmi2* mutants suppress the branched flower phenotype of *ap1*.

(**A**) A representative experiment depicting the number of branched flowers formed in strong *ap1-10* mutants compared to *lmi2-2 ap1-10*. The mean ± s.e.m. is shown. Asterisks: statistical significance based on one-tailed Student's t-test *** $p<10^{-05}$ (**B**) Representative picture of *ap1-11* and *lmi2-1 ap1-11* mutant flowers. White arrowheads indicate branched flowers. Scale bar represents 1 mm.

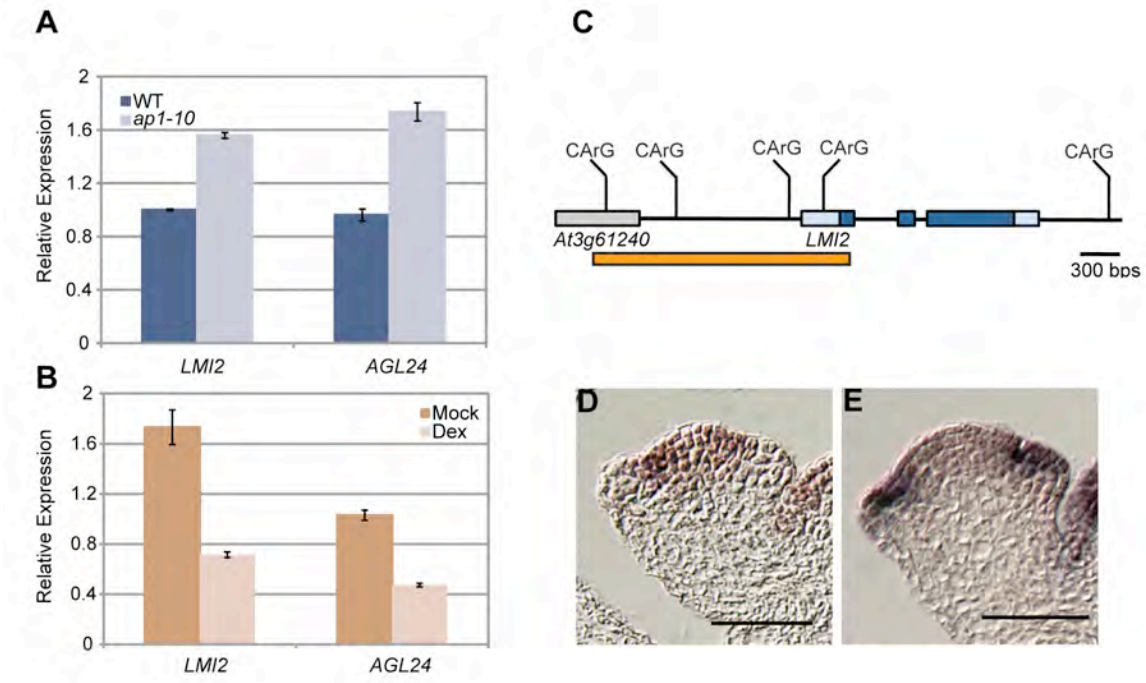

Figure 18. AP1 downregulates *LMI2* during early flower development.

(**A**) qRT-PCR analysis of *LMI2* and *AGL24* expression in Col (wild-type) and *ap1-10* 6-10 cm bolt primary inflorescence apices. (**B**) 1-2 cm bolt primary inflorescence apices of 35S:AP1-GR *ap1-1* plants treated with either 10μM dexamethasone or mock solution for eight hours. Mean values ± s.e.m. are shown. (**C**) Schematic diagram depicting the *LMI2* genomic region. The light blue rectangles represent the 5' and 3'untranslated (UTR) regions of *LMI2*. The 3' UTR of the upstream gene is represented by a grey rectangle. Dark blue rectangles represent exons and black lines represent introns and the upstream and downstream intergenic regions of *LMI2*. The consensus binding sites for MADS box transcription factors (CArG boxes) are indicated. These motifs contain either a perfect consensus or 1 base pair mismatch. Orange bar: region significantly bound by AP1 reported by Kaufmann et al. 2010 (http://published.genomics.upenn.edu/2010/LEAFY).

(**D,E**) *LMI2* expression based on in situ hybridization of wild-type (D) and *ap1-10* (E) in early stage 3 flowers. Scale bars represent 100 μm.

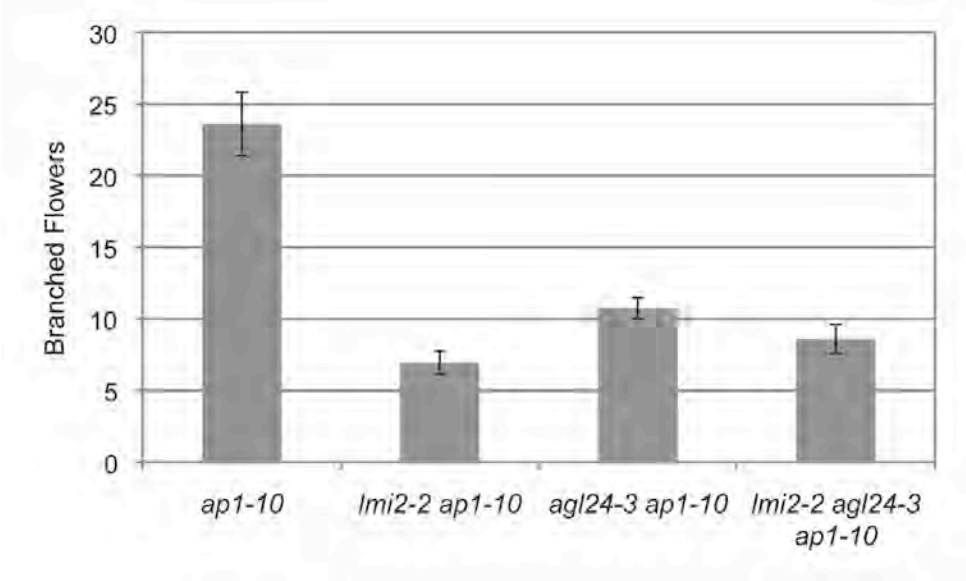

Figure 19. LMI2 acts in the same pathway as AGL24, SVP and SOC1 during early floral development.

Number of branched flowers formed in *ap1-10*, *lmi2-2 ap1-10*, *agl24-3 ap1-10* and *lmi2-2 agl24-3 ap1-10* mutants. The mean ± s.e.m. are shown.

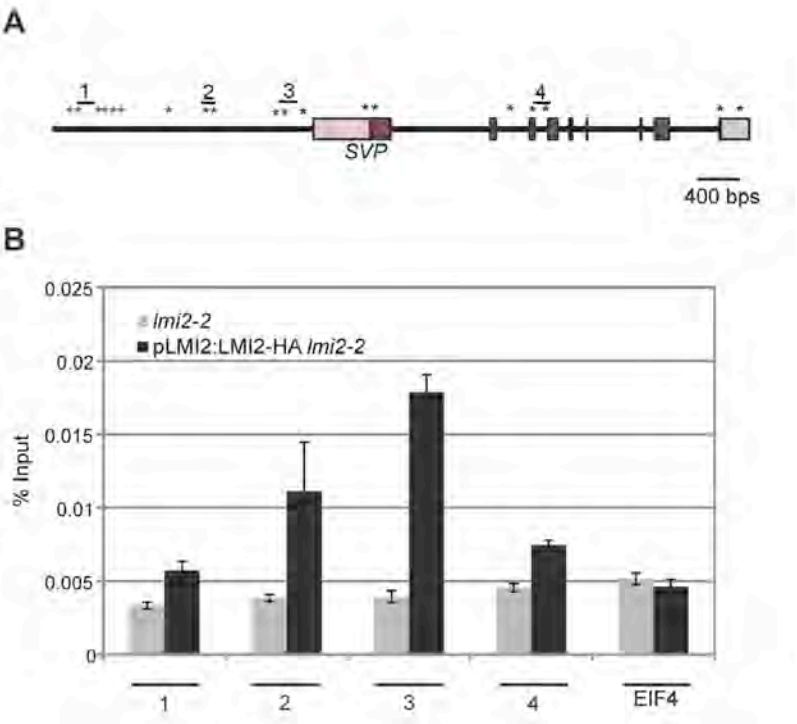

Figure 20. LMI2 binds to *SVP* regulatory regions.

(**A**) Map of the *SVP* locus. The 5' and 3'UTR are indicated by light pink boxes. The exons are represented by dark pink boxes and the 5'upstream region and introns are represented by black lines. The asterisks above the locus represent predicted MYB binding sites identified by AthaMap and AGRIS (Davuluri et al., 2003; Steffens et al., 2004). The numbers and horizontal lines represent regions amplified by ChIP-qPCR. (**B**) ChIP-qPCR from *lmi2-2* and pLMI2:LMI2-HA *lmi2-2* 1-2 cm bolted primary inflorescences. EIF4 served as a negative ChIP control. Immunoprecipitated DNA is represented by percent input. Shown are the mean ± s.e.m.

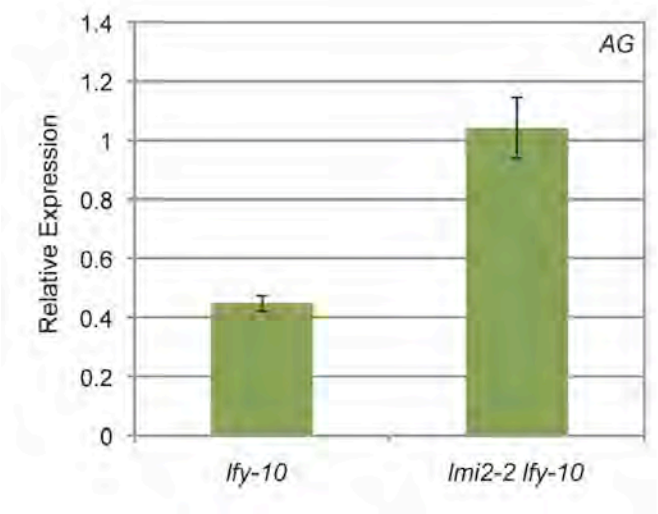

Figure 21. *AG* levels are elevated in *lmi2-2 lfy-10* seedlings compared to *lfy-10* seedlings.

qRT-PCR analysis of *AG* expression from nine-day-old *lmi2-2 lfy-10* and *lfy-10* seedlings. Mean values ± s.e.m. are shown.

Chapter 4. Discussion/Future Directions

4.1. LMI2 is a MI regulator

In Chapter 2 of my dissertation I examined a role for LMI2 in the MI transition. The genetic analyses suggest LMI2 is a key MI regulator acting downstream of LFY as well as in parallel to LFY during the MI transition. The molecular and genetic analyses revealed LMI2 acts primarily upstream of AP1 in this pathway and LMI2, possibly through an interaction with LFY, directly regulates *AP1* expression. Finally, my work has revealed a key regulatory module consisting of LFY, LMI2 and AP1 that controls the precise timing of the MI transition. This section of Chapter 4 has been adapted from Pastore et al., Development, In press.

4.1.2. LMI2 is a MI regulator downstream of LFY

We show here that the direct LFY target and MYB transcription factor LMI2 is required for correct timing of the MI transition in *Arabidopsis*. *LMI2* was identified by two independent genomic approaches as a direct LFY -regulated and -bound target during the MI transition (William et al., 2004; Winter, 2011). Notably, unlike two other known MI regulator mutants (*cal* and *lmi1*) (Bowman et al., 1993; Saddic et al., 2006), *lmi2* single mutants displayed a statistically significant delay in the MI transition, suggesting a central role for this transcription factor in the timing of flower formation. Thus far, only one other direct LFY target has a non-redundant role in this vital developmental transition: *AP1* (Weigel et al., 1992; Bowman et al., 1993).

4.1.3. Additional roles for LMI2 at other stages of reproductive development

The observed *LMI2* expression pattern suggests that LMI2 may have a broad role in reproductive development. Like many flowering-time regulators (Hempel et al., 1997; Lee et al., 2000; Abe et al., 2005; Wigge et al., 2005), *LMI2* was expressed in the shoot apex, and LMI2 controls the timing of bolting. In addition, both *LFY* and *LMI2* were expressed in older flower primordia. Unlike *lfy* mutants (Huala and Sussex, 1992; Weigel et al., 1992), *lmi2* mutants did not display noticeable floral homeotic defects nor did they enhance the floral homeotic defects of weak *lfy* mutants (data not shown), suggesting that LMI2 may have a different role in flower development.

4.1.4. LMI2 directly activates *AP1* to promote floral fate

AP1 upregulation signals commitment to flower formation, and therefore must be tightly controlled for proper timing of the MI transition (Mandel et al., 1992; Bowman et al., 1993; Kaufmann et al., 2010; Wellmer and Riechmann, 2010). Here we provide evidence that LMI2 directly upregulates *AP1* expression during the MI transition. The effect of LMI2 on *AP1* expression is specific and not due to a general delay in phase transitions, because accumulation of other MI regulators, such as *FUL* or *CAL* (Bowman et al., 1993; Ferrandiz et al., 2000) are not altered in *lmi2-2* mutants. *LMI2* induction precedes that of *AP1* and both are expressed in stage 1 floral primordia, where AP1 directs flower development (this study) (Mandel et al., 1992; Liljegren et al., 1999; Schmid et al., 2005). LMI2 binds to a region of the *AP1* locus also occupied by many other transcription factors in vivo, including LFY (William et al., 2004; Wang et al.,

2009; Yamaguchi et al., 2009; Xu et al., 2010), thus defining a critical *AP1* cis regulatory module (Jeziorska et al., 2009; Wilczynski and Furlong, 2010).

4.1.5. LMI2 and LFY physically interact

The LMI2 and LFY binding sites on the *AP1* promoter are very close to each other and based on three independent assays, the LMI2 and LFY proteins physically interact. MYB proteins are known to interact with other transcription factors to regulate gene expression (Zimmermann et al., 2004; Shin et al., 2007; Li et al., 2009). LFY also interacts with cofactors, including at least one other downstream target, to regulate gene expression (Lenhard et al., 2001; Lohman et al., 2001; Chae et al., 2008; Liu et al., 2009b; Winter, 2011). For example, LFY directly upregulates the floral homeotic regulator *SEPALLATA 3* (*SEP3*) and in turn, these two factors physically interact to activate the class B and C floral homeotic genes (Liu et al., 2009b; Winter, 2011).

Based on the recent finding that LFY acts as both a direct transcriptional activator and repressor (Parcy et al., 2002; William et al., 2004; Winter, 2011), it seems likely that cofactors modulate the effect of LFY on gene expression. Consistent with this idea, LFY alone is unable to activate gene expression from the *AP1* promoter in yeast, it can only act as a transcriptional activator in this system when fused to a strong activation domain (Parcy et al., 1998; Winter, 2011). It is likely that LFY also needs a co-activator for *AP1* induction in vivo. LMI2 is a good candidate for this LFY co-activator; it has strong transactivation activity based on yeast assays, is induced by LFY prior to *AP1* upregulation, and can form heterodimers with LFY (this study) (Blazquez et al., 1997; Hempel et al., 1997; Schmid et al., 2003; Schmid et al., 2005; Zhang et al., 2009).

Moreover, the temporal delay in the formation of the first flower is very similar in *ap1* and *lmi2* single mutants (this study) (Xu et al., 2010), thus LMI2 may be sufficient for LFY-dependent activation of *AP1* expression. However, we cannot rule out that other LFY co-factors contribute to this process.

4.1.6. The LFY, LMI2 and AP1 regulatory network may contribute to an abrupt and robust MI transition

The observed interactions between LFY, LMI2 and AP1 represent a coherent feed-forward loop (Fig. 7) (Alon, 2007), a regulatory circuit with crucial roles in control of developmental processes in many organisms (Shen-Orr et al., 2002; Mangan et al., 2003; Alon, 2007). The type of coherent feed-forward loop observed here serves as a persistence detector for inductive signal(s) and as a temporal delay element (Alon, 2007). Thus, transient inductive cues that cause a temporary increase in *LFY,* but not in *LMI2,* will delay LFY-dependent upregulation of *AP1*.

This finding is consistent with prior observations. For example, *LFY* upregulation is directed by environmental cues, such as changes in day length or ambient temperature (Kobayashi and Weigel, 2007; Liu et al., 2009a; Amasino, 2010); these stimuli are inherently noisy inputs, yet the transition to flower formation is abrupt in *Arabidopsis*, without formation of intermediate structures (Parcy, 2005). In addition, as discussed above, *AP1* induction is delayed with respect to that of *LFY* and *LMI2* and reduced in both single mutants. Finally, as predicted by the feed-forward loop model, *LMI2* was a haplo-insufficient, rate-limiting factor for *AP1* induction downstream of LFY, at least under conditions when LFY activity was compromised.

In addition to the feed-forward loop uncovered here, LFY directs at least two additional coherent feed-forward loops, one of which is also linked to the MI transition and involves the direct LFY targets *LMI1* and *CAL* (Fig. 7) (William et al., 2004; Saddic et al., 2006; Kaufmann et al., 2009; Liu et al., 2009b; Winter, 2011). Amongst these feed-forward loops involving LFY, the LFY/LMI2/AP1 feed-forward loop stands out since it alone is comprised of three regulators that have non-redundant roles in the process they regulate, hence it may represent a critical regulatory module in the MI transition.

In *Arabidopsis*, the MI transition is not only precise (occurs after formation of a defined number of secondary inflorescences subtended by cauline leaves), but also robust (no reversion from flower to inflorescence fate is observed) (Tooke et al., 2005; Blazquez et al., 2006; Liu et al., 2009b; Amasino, 2010). As outlined above, the LFY/LMI2/AP1 feed-forward loop likely contributes to the precision of this developmental transition; its robustness on the other hand, may be due to positive feedback (Alon, 2007). Indeed, a positive direct feedback from AP1 to *LFY* has recently been described (Liljegren et al., 1999; Kaufmann et al., 2010). We show here that LMI2 also positively regulates *LFY*: *LFY* expression was reduced in *lmi2-2* single and double mutants. This reduction of *LFY* expression could be an indirect effect, triggered by the reduced *AP1* expression levels observed in *lmi2-2* mutants. However, the positive feedback may in part be direct as LMI2 was weakly recruited to the *LFY* locus (Fig. 22). The observed enhancement of the *ap1* mutant MI defect by *lmi2* is consistent with this hypothesis. It is likely that the AP1 and possible LMI2 feedback loops keep the LFY/LMI2/AP1 feed-forward loop active after full *AP1* upregulation has been achieved. Indeed, AP1 directly downregulates upstream activators of itself and of LFY (Kaufmann et al., 2010), providing further

support for the idea that the combined feed-forward and feedback loop is self-maintained (Fig. 22).

It will be of interest to examine these regulatory interactions in other flowering plant species. In light of this question, *LMI2* separated from its closest homologs, the *MIXTA*/*MYB16* and *MYB106* genes, before the split of the monocots from the eudicots, ca. 100 million years ago (Baumann et al., 2007). This raises the possibility that the function of LMI2 in reproductive development evolved early in the flowering plant lineage and may be conserved in other angiosperm species.

4.1.7. The flowering-time genes, SOC1 and AGL24, are possible regulators of *LMI2*

Our combined data suggest that during the MI transition, *LMI2* is regulated not only by LFY but also by a LFY-independent pathway. *lmi2* mutants enhanced the MI defect of the *lfy* null mutant and *LMI2* expression was reduced but not lost in the initiating primordia of the *lfy* null mutant (this study) (William et al., 2004; Schmid et al., 2005). A previous report suggested that *LMI2* expression may be regulated by the MADS box transcription factor AGL15 (Zhang et al., 2009). *AGL15* is expressed in the developing embryo and in the vegetative shoot apex, but is not expressed in the shoot meristem at the onset of the reproductive transition (Fernandez et al., 2000; Schmid et al., 2005). Therefore, AGL15 is most likely not involved in induction of *LMI2* expression during the MI transition.

We propose here that the MADS box transcription factors SOC1 and AGL24 may be possible upstream activators of *LMI2*. Both SOC1 and AGL24 were bound to and occupied the same regions on the *LMI2* promoter, suggesting they may jointly regulate

LMI2. Indeed, SOC1 and AGL24 are known to heterodimerize in the shoot apex (Lee et al., 2008a; Liu et al., 2008). While future experiments are necessary to determine whether *LMI2* expression is indeed regulated by SOC1 and AGL24 (see Chapter 4), several pieces of evidence support this hypothesis. Consistent with the idea that the flowering-time regulators SOC1 and AGL24 may activate *LMI2*, *lmi2* mutants displayed a subtle but significant flowering-time delay and an increase in *LMI2* expression by 35S:SOC1-GFP and a reduction in *LMI2* expression in *soc1-2* mutants was observed. In further agreement with this, *LMI2* expression is upregulated by inductive photoperiod even in the absence of LFY (Schmid et al., 2003). Finally, the three proteins share overlapping temporal and spatial expression patterns in the shoot apical meristem (this study) (Lee et al., 2000; Yu et al., 2004; Lee et al., 2008a).

SOC1 and AGL24 also directly activate *LFY* (Lee et al., 2008a; Liu et al., 2008). Intriguingly, mutations in *SOC1* or *AGL24* enhance the MI defects of *lfy* null mutants (Moon et al., 2005; Xu et al., 2010). Thus, these two MADS-box transcription factors act both upstream of and in parallel to LFY. This latter activity may be mediated at least in part by LMI2, mutations in which also enhance *lfy* null mutants.

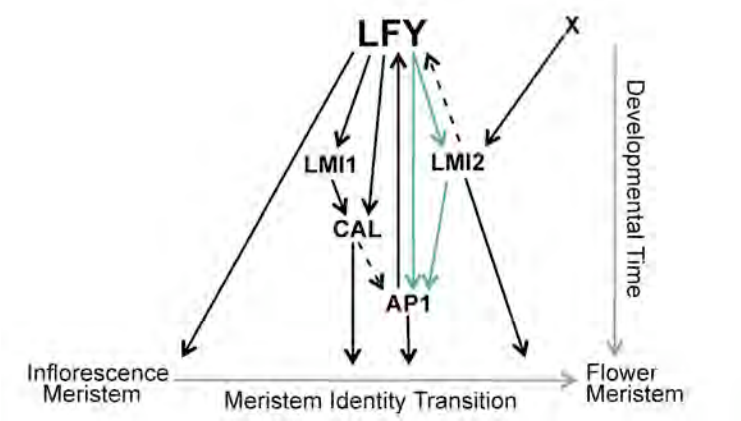

Figure 22. MI pathway downstream of LFY.

The LFY transcription factor directly activates multiple downstream factors during the MI transition, including *CAL*, *LMI1*, *AP1*, and *LMI2* (William et al., 2004; Saddic et al., 2006). *LMI2* is also upregulated by another factor X (perhaps AGL24/SOC1) in a pathway parallel to LFY. LFY, LMI2 and AP1 act in a feed-forward loop (blue arrows) to initiate the MI transition and LMI2 and AP1 positively feedback to *LFY* (this study) (Kaufmann et al., 2010). Interactions, which could be indirect or direct, are indicated by dashed arrows.

4.2. A possible role for LMI2 in early flower development

In Chapter 3 of my dissertation I explored a possible role for LMI2 in early flower development. Mutations in *LMI2* reduce the branched flower phenotype of *ap1* mutants. Furthermore, expression studies suggest AP1 downregulates *LMI2* in young stage 3 flowers in a similar fashion to the downregulation of the flower time genes *SVP*, *AGL24* and *SOC1* by AP1 (Yu et al., 2004; Liu et al., 2007). The repression of *LMI2* likely reduces the meristematic activity in the axils of the first whorl organs of *ap1* flowers and therefore reduces the development of branched flowers. Based on the similarities between *SVP* and *LMI2* expression in *ap1* mutants (this study) (Liu et al., 2007) and the binding of LMI2 to the upstream region of *SVP*, we propose that LMI2 promotes the growth of the floral meristem during early flower development in part by directly upregulating *SVP* expression in young stage 1 and 2 flower primordia.

4.2.1. Increased expression of *LMI2* in the axils of the first whorl floral organs contributes to the branched flower phenotype of *ap1* mutants

LMI2 is expressed after the MI transition suggesting it may play a later role in flower formation. Although mutations in *lmi2* alone show no obvious floral defects, when combined with mutations in *ap1*, *lmi2* showed a significant reduction in the development of branched flowers. Mutations in the flowering-time genes *SVP*, *AGL24* and *SOC1* also reduce the branch flower phenotype of *ap1* mutants raising the question of whether there is a link between LMI2 and the regulatory pathways involved in early flower development (Yu et al., 2004; Gregis et al., 2006; Liu et al., 2007; Gregis et al., 2009; Liu et al., 2009b).

The increase and ectopic expression of *SVP*, *AGL24* and *SOC1* in late stage 2 to stage 4 *ap1* mutant flowers in part causes the branched flower phenotype (Yu et al., 2004; Liu et al., 2007). In a similar manner to this, *LMI2* expression was more prominent in young stage 3 flowers of *ap1* mutants than that of the wildtype. The increase in *LMI2* levels was restricted to the axil regions of the developing sepal primordia, the same region where branched flowers arise (Irish and Sussex, 1990; Bowman et al., 1993). Hence, the increase in *LMI2* expression between the developing sepals and the floral meristem is likely to contribute to the branched flower phenotype. Interestingly, out of the three flowering-time genes that play a role in the formation of branched flowers, *SVP* is the only one that is ectopically expressed in the axils of the first whorl organs of *ap1* flowers (Liu et al., 2007). The *ap1 lmi2* phenotype and the overlap between *LMI2* and *SVP* expression in this region suggest two things: 1. LMI2 likely plays a prominent role in branched flower formation. 2. The function of LMI2 in early stages of flower development may be linked to SVP.

4.2.2. AP1 downregulates *LMI2* in the axils of the developing sepals to terminate meristematic activity and repress the development of additional floral meristems

The similarities between *lmi2*, *svp*, *agl24* and *soc1* mutants in terms of the reduction in branched flowers when combined with *ap1*, and the increase and/or the ectopic expression of these genes in *ap1* mutants suggests that *LMI2*, like these flowering- time genes, may be downregulated by AP1 during flower development (Yu et al., 2004; Liu et al., 2007). Indeed, we have shown that *LMI2* levels are affected by mutations in *ap1* and that *LMI2* is downregulated by AP1. First, *LMI2* levels are

increased in young *ap1* mutant inflorescences. This data is also consistent with publically available expression data (Schmid et al., 2005). The increase in *LMI2* that we observed in *ap1* inflorescences is subtle (less than 2-fold), yet this subtlety is not so unexpected. For one, inflorescences consist of flowers at different stages in development and *LMI2* is expressed in both early and late floral stages. Therefore, the differential changes in *LMI2* expression in early stage 3 flowers may be masked by its expression in other floral tissue, making it difficult to observe by qRT-PCR. Furthermore, unlike *SVP* and the other flowering-time genes, which are ectopically expressed in these young stage 2 and early stage 3 *ap1* flowers (Liu et al., 2007), *LMI2* is normally expressed throughout the floral meristem in stage 3 wild-type flowers, including the region where branched flowers develop. In *ap1* mutants, the level of *LMI2* is only increased in the axils of these first whorl organs. Hence, rather than an absolute increase in *LMI2* levels we see a spatial change in the *LMI2* expression pattern.

In addition to observing increased levels of *LMI2* in *ap1* mutants, *LMI2* was reduced upon AP1 induction in 35S:AP1-GR *ap1-1* inflorescences, confirming that AP1 downregulates *LMI2* expression. The repression of *LMI2* by AP1 is also supported by previous floral induction assays using 35S:AP1-GR in *ap1 cal* mutants (Wellmer et al., 2006). Finally, AP1 binds to the 5' upstream region of *LMI2* (Kaufmann et al., 2010), implying that like *SVP*, *AGL24* and *SOC1* (Yu et al., 2004; Liu et al., 2007), AP1 directly downregulates *LMI2* during flower development.

Flower development is controlled both spatially and temporally with the outside organs, the sepals, developing first and the most inner organs, the carpels, developing last (Steeves, 1989; Krizek and Fletcher, 2005). During this time meristematic activity and

floral differentiation are coordinated to reflect this growth pattern. Meristematic activity declines in the outside whorl first and the most inner whorl last, whereas differentiation begins in the outside whorl and ends with the inner most whorl (Steeves, 1989; Krizek and Fletcher, 2005; Sun and Ito, 2010). AP1 is essential for properly coordinating meristematic activity and floral differentiation between whorl one and the floral meristem (Irish and Sussex, 1990; Bowman et al., 1993; Yu et al., 2004; Liu et al., 2007). In young stage 2 and early stage 3 flowers, AP1 downregulates the expression of *SVP*, *AGL24* and *SOC1* in this region (Yu et al., 2004; Liu et al., 2007). This downregulation terminates the meristematic activity that these genes promote and leads to the upregulation of *SEP3* and the class B and C floral homeotic genes, and therefore differentiation (Liu et al., 2009a; Liu et al., 2009b; Wagner, 2009; Irish, 2010; McKim and Hay, 2010).

In this study, we have shown that *LMI2* levels must be downregulated by AP1 in the region between the first whorl organs and the floral meristem for proper flower formation. In the absence of AP1, the retention of *LMI2* expression in these cells likely promotes the meristematic activity in this region. Given its role as a MI regulator, LMI2 may have the potential to promote the development of additional floral meristems in the axil of the developing sepal thus leading to the eventual production of branched flower.

In a similar fashion to *LMI2*, AP1 represses *SVP* in the axil of the first whorl organs (Liu et al., 2007). Although, it is known that SVP prevents premature differentiation it is unclear how SVP promotes the production of branched flowers (Liu et al., 2007; Liu et al., 2009b). In light of our results here, one possibility is the SVP activity in *ap1* mutants prolongs the meristem growth phase by preventing differentiation in the

axils of the first whorl organs. This prolonged growth phase allows MI genes that are expressed in this region, like LMI2, to give rise to another floral meristem.

4.2.3. A possible role for LMI2 in *AG* repression prior to the floral transition

Prior to floral organ patterning *AG* repression is maintained by both transcriptional as well epigenetic mechanisms (Gregis et al., 2006; Gregis et al., 2009; Liu et al., 2009b). Here we show *AG* is precociously expressed in *lmi2-2 lfy-10* compared to *lfy-10* seedlings. This expression occurs at day 9, when *lfy-10* mutants have not yet undergone the floral transition. This early upregulation of *AG* may suggest LMI2 is important for its repression prior to floral initiation. At the moment we do not know if this repression by LMI2 is direct or whether it is an indirect effect of LMI2 regulating other factors that control *AG* expression, for instance SVP. So far, LMI2 has only been transcriptionally characterized as an activator (this study) (Zhang et al., 2009)and there is no a priori indication that LMI2 has a repressive function. Therefore, if LMI2 directly regulates *AG*, this may perhaps link LMI2 with an epigenetic phenomenon. For instance, LMI2 may recruit a repressive chromatin complex to the *AG* locus in similar way SVP and AGL24 have been shown to do during early flower development (Gregis et al., 2006; Gregis et al., 2009).

4.2.4. Deciphering the role of LMI2 in early flower development

So far, we have shown that AP1 downregulates *LMI2* to terminate the meristematic activity in the axils of the first whorl organs, but a function for LMI2 in early flower development is yet to be determined. Based on our data thus far, one

possible role for LMI2 during this time may be to upregulate the expression of *SVP* in young flower primordia (Fig. 23). First, the branched flower phenotypic analyses between LMI2, AGL24 and AP1 suggest LMI2 acts in the same pathway as SVP, AGL24, and SOC1. Second, out of the three flowering-time genes, *SVP* has the most similar expression pattern to that of *LMI2* in stage 1 and 2 wild-type flowers and in early stage 3 *ap1* flowers (Fig. 18E) (Hartmann et al., 2000; Liu et al., 2007). The temporal expression of *LMI2* and *SVP* is also consistent with this hypothesis, with *LMI2* being upregulated prior to *SVP*, during the MI transition (Hartmann et al., 2000; Liu et al., 2007). Finally, LMI2 binds to the promoter region of *SVP* in inflorescences.

There are a few critical experiments that are needed to fully characterize the role of LMI2 in early flower development and the particular hypothesis proposed above. In the subsequent paragraphs in this section I will present three remaining questions and the experiments that will be performed to address these questions.

The first and most critical question is whether LMI2 regulates *SVP*, as well as any of the factors that control early flower development, including *AGL24*, *SOC1* and the downstream genes, *SEP3* and the class B and C floral homeotic genes. At the moment, I have only tested the expression of these genes in young inflorescence apices by qRT-PCR. So far, we have only observed very subtle changes in gene expression in *lmi2* single mutants compared to the wildtype as well as *lmi2 ap1* mutants compared to *ap1*. Expression studies using inflorescences is challenging because of the many cell types found in this tissue as previously discussed. There are three experiments that we will perform to avoid this problem. First, we will test the expression of these genes in seedlings that have just undergone the floral transition and therefore are mostly composed

of young stage 1-3 floral primordia. These experiments should be straightforward since the tissue as well as the RNA from several biological replicates has already been generated for the MI transition studies.

In a second approach, we will test whether any of these factors are regulated by LMI2 during early flower development by examining their spatial expression by in situ hybridization using *lmi2* and wild-type young inflorescences as well as young inflorescences of *ap1 lmi2* and *ap1* mutants. We have probes for a majority of the prominent genes involved in this pathway including: *SVP*, *AGL24*, *SEP3* and *AG*. We also have the appropriate tissue embedded for this experiment. Given that in situ hybridization is more time consuming than qRT-PCR we will first test the expression of theses genes by qRT-PCR (above) to narrow down the most important factors, then confirm these results by in situ. Finally, we are also crossing *ap1*, *lmi2* and *lmi2 ap1* into pSVP:SVP-GFP, pSEP3:SEP3-GFP, pAGL24:AGL24-RFP and pAG:AG-GFP reporter lines. As a follow up to the expression studies, we will use the pLMI2:LMI2-HA line to perform ChIP in young inflorescences or seedlings after the MI transition, to determine whether any of these regulatory interactions are direct.

The second question to address is whether SOC1 and/or AGL24 regulate *LMI2* during early flower development. As mentioned previously in the MI section, SOC1 and AGL24 bind to and perhaps regulate *LMI2* during the floral transition. It is also possible that SOC1 and/or AGL24 may upregulate *LMI2* during early stages of flower development. To address this question additional *LMI2* expression analyses in various genetic backgrounds including, *soc1*, *agl24*, *lfy*, *soc1 agl24*, and *soc1 lfy* are needed. All

of these various mutants I have started to generate or have kindly received from other investigators.

The last question to address is whether SEP3 downregulates *LMI2* in stage 3 flowers. SEP3 has already been shown to directly repress the expression of *SVP*, *AGL24*, and *SOC1* during floral differentiation (Kaufmann et al., 2009). SEP3 also binds to the upstream regulatory regions of *LMI2* (Kaufmann et al., 2009). Interesting to note, the SEP3 binding site overlaps with the AP1 binding site on *LMI2* (Kaufmann et al., 2009; Kaufmann et al., 2010) and both of these proteins have been shown to interact and have been proposed to regulate genes together during floral organogenesis (Pelaz et al., 2001; Jack, 2004; Gregis et al., 2008; Gregis et al., 2009; Immink et al., 2009; Kaufmann et al., 2009; Liu et al., 2009b; Kaufmann et al., 2010). In addition to the 35S:AP1-GR lines, we have 35S:SEP3-GR and can therefore test whether *LMI2* is downregulated by SEP3 during the onset of floral differentiation.

4.2.5. Similarities and differences between AP1 and AG floral determinacy

AP1 and AG establish determinate growth of the floral meristem by patterning the floral primordia and by terminating meristematic activity of the developing flower (Krizek and Fletcher, 2005; Liu et al., 2009a; Wagner, 2009; Irish, 2010; McKim and Hay, 2010; Sun and Ito, 2010). The mechanisms by which AG and AP1 repress meristematic activity are similar, yet they occur at a different time and place in the developing flower. For instance, to promote floral differentiation of the meristem, both AP1 and AG downregulate factors that initially upregulate their expression (Fig. 24). In addition, the timing of this repression is essential for proper flower development. In the

case of AG and WUS, WUS activity is initially maintained after *AG* is activated. This delay in *WUS* repression is necessary to establish and maintain stem cells for the last floral organ formed, the carpel (Sun and Ito, 2010). AG eventually terminates *WUS* expression in stage 6 flowers allowing for carpel development (Fig. 24) (Lenhard et al., 2001). The function of LMI2 in early flower development is not known, but based on the expression pattern of *LMI2* in stage 1 and 2 flowers, and its possible link to *SVP* as well as *AG* regulation, LMI2 activity may be important for the maintenance and growth of the floral meristem early on in stage 1 and 2 flowers (Fig. 24). Finally, *LMI2* must be downregulated by AP1 in late stage 2 and early stage 3 to allow for proper differentiation of whorls two and three of the developing flower (Fig. 24).

Although similar, AG and AP1 also maintain determinate growth of the floral meristem in different ways. During flower development, AG indirectly regulates *WUS* expression through both transcriptional as well as epigenetic mechanisms and it is this indirect regulation of *WUS* that allows for the delay in *WUS* repression (Fig. 24) (Sun and Ito, 2010). Conversely, AP1 promotes determinate growth by directly downregulating *LMI2* (this study) as well as *SVP, AGL24* and *SOC1* (Yu et al., 2004; Liu et al., 2007). It is still unclear how this downregulation is properly timed in late stage 2 flowers. It has been suggested that the different protein complexes AP1 forms with other MADS box proteins throughout flower development helps regulate its various functions (Liu et al., 2009a; Irish, 2010; Kaufmann et al., 2010). Finally, unlike AG which only promotes determinate growth, AP1 also promotes indeterminate growth of the floral meristem in early stage 1 and 2 flowers by repressing *AG* and the class B homeotic genes in part

through its interactions with SVP, AGL24 and the SEU-LEU repressive chromatin complex (Gregis et al., 2006; Gregis et al., 2009).

4.3. The role of LMI2 in the reproductive development of *Arabidopsis*

In my thesis work I have taken a reverse genetic approach to characterize the role of the MYB transcription factor LMI2, during the reproductive development of *Arabidopsis*. In my main thesis project, I have elucidated the role of LMI2 in the final step of the reproductive phase known as the MI transition. This work has been submitted for publication. In my second thesis project, I have begun to uncover a second role for LMI2 in early flower development. Although this work is incomplete, a number of experiments are in progress to finalize this project.

During the MI transition, upregulation of *LMI2* by LFY and a LFY-independent pathway is essential for establishing floral fate. This important role of LMI2 is confirmed by its single mutant molecular and morphological MI phenotype. LMI2 promotes floral fate by directly upregulating *AP1* expression along with LFY, and thereby promoting the last and irreversible step for flower formation (Bowman et al., 1993; Mandel and Yanofsky, 1995; Blazquez et al., 1997; Hempel et al., 1997; Yu et al., 2004; Liu et al., 2007).

The reproductive success of plants depends on the regulatory networks that control the timing of flowering (Simpson and Dean, 2002; Parcy, 2005; Roux et al., 2006). In *Arabidopsis*, this is in part controlled by the robust regulatory module that was identified in this thesis work. This module includes LFY, LMI2, and AP1, three MI regulators with non-redundant roles in this pathway, thus making their interactions a key

regulatory component of the floral transition. The feed-forward and feedback loops that LFY, LMI2, and AP1 participate in ensure the proper timing of floral initiation as well as guarantees flowering.

The function of LMI2 after the MI transition is less clear, but the preliminary data we have generated suggests LMI2 may promote the growth and maintenance of the floral meristem. This might be a common function among important MI regulators as both LFY and AP1 promote primordia growth; AP1 through its indirect repression of the class B and C genes prior to differentiation and LFY, through its proposed role in stimulating primordia growth in *Arabidopsis* as well as in other legume and monocot species (Hofer et al., 1997; Bomblies et al., 2003; Gregis et al., 2006; Kanrar et al., 2008; Rao et al., 2008; Gregis et al., 2009; Moyroud et al., 2010). Meristematic activity is gradually terminated in the flower upon differentiation (Krizek and Fletcher, 2005; Wagner, 2009; Irish, 2010; McKim and Hay, 2010; Sun and Ito, 2010). During this time AP1 and LFY, likely transition from MI regulators to floral homeotic regulators by interacting with other proteins and cofactors that are now expressed. LMI2 on the other hand, may continue its role in maintaining floral meristem growth throughout other stages of flower development, a function that is thus far, regulated by AP1.

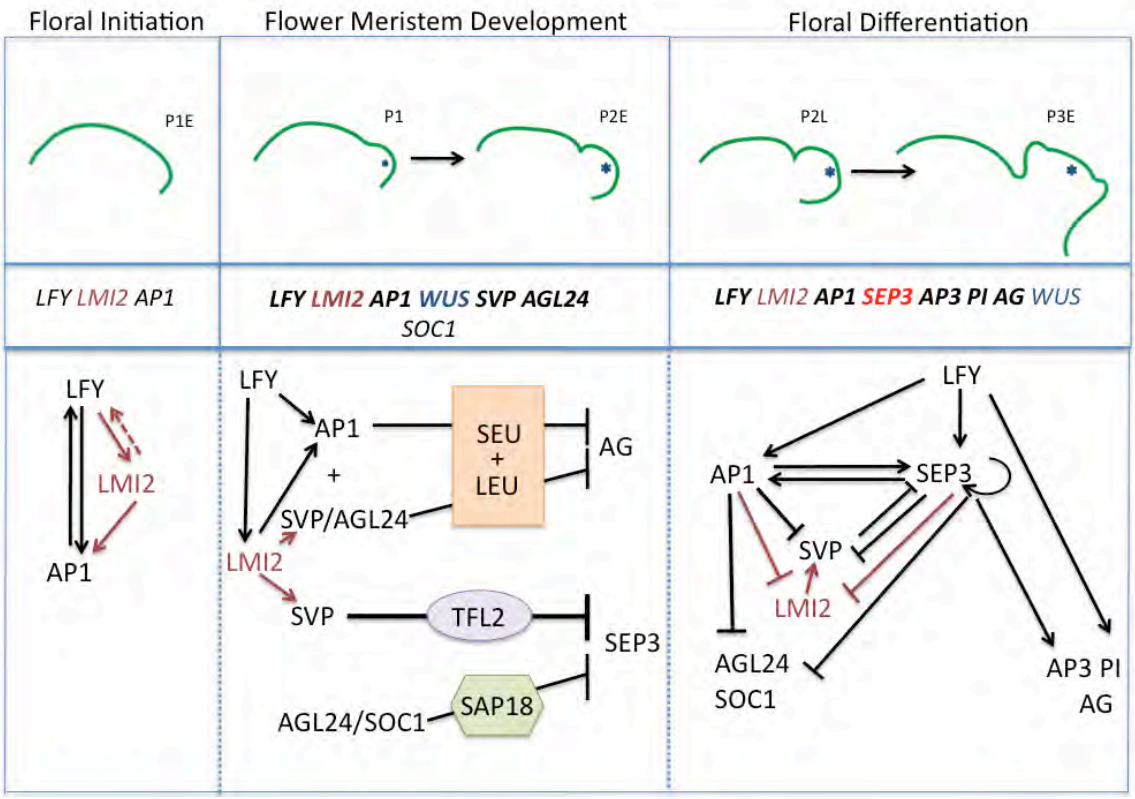

Figure 23. A role for LMI2 in early flower development.

(Top panel)- Floral primordia stages represented at each phase in early flower development (Smyth et al., 1990). Specific floral stages are indicated above developing primordia. "E" indicates "early" and "L" indicates "late" in terms of development. Asterisks denote location and intensity of *WUS* expression. (**Middle panel**)- The genes expressed in the floral primordia at each particular stage. Standard print denotes low expression, bold print denotes high expression. WUS and SEP3 are highlighted in blue and red respectively, to denote their importance in the particular developmental phase indicated. (**Bottom panel**)- The regulatory pathways active during each stage of early floral development. The LMI2 regulatory interactions highlighted in this dissertation are indicated in maroon. The positive input from LMI2 to *SVP* and the negative input from

SEP3 to *LMI2* are only “possible” interactions. Additional data is needed to confirm these interactions. Chromatin regulators are indicated by colored shapes (see text for citations). Factors that physically interact are indicated by “+”. Bold lines indicate a key regulatory step in the pathway.

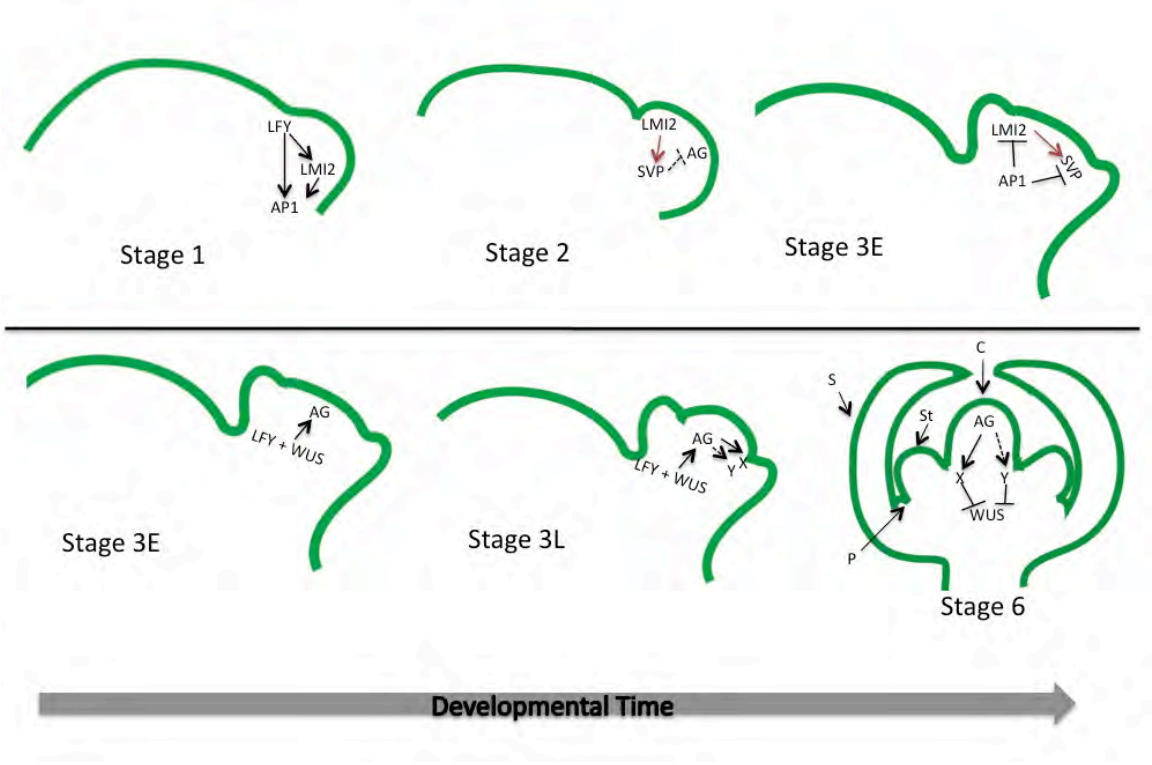

Figure 24. AP1 and AG regulatory pathways controlling floral determinacy. (**Top panel**)- LMI2 and AP1 regulatory interactions: In stage 1 floral primordia *AP1* is upregulated by LFY and LMI2 for floral initiation. In stage 1-2 flower primordia LMI2 likely maintains the growth of the floral meristem by upregulating *SVP*, which in turn indirectly (via *SEP3*) and possibly directly (via LEU/SEU complex) represses *AG* (Gregis et al., 2006; Gregis et al., 2009; Liu et al., 2009b). In early stage 3 flowers *LMI2* and *SVP* are directly downregulated by AP1 (this study) (Liu et al., 2007; Kaufmann et al., 2010) in the axil of the developing sepal in order to terminate meristematic activity in this region and to allow floral differentiation to commence (Liu et al., 2009b). (**Bottom panel**)- AG and WUS regulatory interactions: In early stage 3 flowers LFY and WUS together upregulate *AG* in the center of the floral meristem (Lohman et al., 2001). In stage 3 and later floral stages (not depicted above) AG upregulates the expression of other transcription factors (*X* and *Y*, known factors (Gomez-Mena et al., 2005; Lee et al.,

2005; Sun et al., 2009) not specifically discussed in this dissertation) by transcriptional as well as epigenetic mechanisms (Sun and Ito, 2010). In stage 6 flowers “X” and “Y” directly repress *WUS* in the center of the meristem during carpel development (Sun and Ito, 2010). “S”- sepals, “P”- developing petals, “St”- developing stamens, “C” developing carpel, “E”- early, “L”- late. Maroon arrows denote possible interactions. Dashed arrows indicate “epigenetic” mechanisms for activation. Negative inputs represented by dashed lines indicate both indirect and possibly direct interactions. Solid lines indicate direct interactions.

Chapter 5. Materials and Methods

5.1. Plant lines, growth conditions, phenotyping, and *LMI2* rescue construct

lmi2 T-DNA insertion lines were obtained from the SALK collection (Alonso and Stepanova, 2003) and twice backcrossed to Columbia (wildtype). The *agl24-3* T-DNA insertion line (SALK_095007) was provided by Richard Amasino. *lfy* and *ap1* alleles used were previously described (Saddic et al., 2006; Yamaguchi et al., 2009). *lfy-2* and *lfy-10* carry the same lesion (Weigel et al., 1992; Schultz and Haughn, 1993) and were used interchangeably. *soc1-2* was provided by Ilha Lee (Lee et al., 2000). The 35S:AP1-GR *ap1-1* line was provided by H. Yu (Yu et al., 2004) and the pLFY:GUS line was originally described in (Blazquez et al., 1997). For all genotyping primers see Table 4.

All plant growth was in inductive photoperiod. Seeds were stratified for seven days at 4°C and either grown in white fluorescent lights at 22°C in soil in long-day conditions (16hrs light, 8hrs dark; 110μmol/m^2s) for experiments involving phenotyping and inflorescences, or on plates (0.5 x MS media) in long-day conditions for three days followed by growth in continuous light (90μmol/m^2s) for seedling experiments.

The MI and flowering-time phenotypes were examined as previously described (Saddic et al., 2006; Yamaguchi et al., 2009). To examine the branched flower phenotype all flowers originating from the axils of the first whorl organ within an existing flower were counted from the first node through to the 15th node. Node one was considered the first node after the last secondary inflorescence (cauline leaf bearing branch). Solitary flowers were only considered branch flowers and inflorescence like branches were not considered in the counting.

For genomic rescue, the *LMI2* locus including 2150 bps upstream of the translational start site was PCR amplified, sequenced and Gateway® cloned into pGWB1

(Nakagawa et al., 2007). The resulting construct was transformed into *lmi2-2 lfy-10* plants. A representative pLMI2:LMI2 *lmi2-2 lfy-10* transgenic line was further characterized. For all cloning primers see Table 5.

5.2. Semi-quantitative and quantitative PCR

For seedling assays, developmental age was determined based on days of growth and adjusted by developmental stage (emergence and size of true leaves (Saddic et al., 2006)). RNA was extracted from entire seedlings except for the study of *LMI2* misexpression in *lmi2-1* mutants.

For Col and *ap1-10* primary inflorescence assays, 6-10 cm bolt apices were harvested. Any open flowers and flowers with obvious petals were discarded from these apices. For 35S:AP1-GR inducible experiments, 1-2 cm bolt primary inflorescences of L*er* and 35S:AP1-GR (*ap1-1*) were harvested. Any open flowers or flowers with obvious petals were removed from inflorescence apices. Prior to harvesting, apices were treated with 10uM dexamethasone or mock solution (Wagner et al., 1999) at time 0, 4hrs and then harvested at 8 hrs.

RNA purification, reverse transcription and qRT-PCR were previously described (Yamaguchi et al., 2009). All real-time RT-PCR experiments were normalized over the ubiquitously expressed *EIF4A* gene (At3g13920). The mean and s.e.m. were calculated for each biological replicate using three technical replicates. One representative experiment is shown. See Table 6 for qRT-PCR primers used.

5.3. Bacterial ß-Glucuronidase (GUS) assays

Upstream and downstream intergenic regions (2150 bps upstream of the translation start site and 2699 bps downstream of the translation termination site) were PCR amplified, sequenced and cloned into pBI101 (Clontech, Mountain View, CA, USA). Wild-type plants (Col) were transformed and a representative transgenic line was characterized. To test the role of LFY on *LMI2:GUS* expression, LMI2:GUS was crossed to *lfy-9*, 35S:LFY-GR in L*er* (Wagner et al., 1999), and L*er* (wildtype). GUS assays were performed as in (Yamaguchi et al., 2005; Saddic et al., 2006) using seven-day-old seedlings or 1-2 cm bolted primary inflorescences. For transient induction assays, seven-day-old F1 seedlings (LMI2:GUS x L*er* or LMI2:GUS x 35S:LFY-GR) were incubated overnight with 10µM dexamethasone at room temperature as previously described (Wagner et al., 1999) prior to GUS staining. Whole-mount samples and histological sections were visualized using an Olympus SZX12 dissecting or an Olympus BX51 compound microscope.

The *LMI2:GUS* reporter showed ectopic expression in the L1 layer of stems, petioles and leaves not detected by *LMI2* in situ hybridization analyses. This may be due to missing cis regulatory elements located in *LMI2* introns (Sieburth and Meyerowitz, 1997; Liu et al., 2007; Oh et al., 2009).

5.4. In situ Hybridization

For the *LMI2* antisense and sense probes the genic region downstream of the MYB DNA binding domain was used. The *AP1* in situ probe (construct made by Dr.

Miin-Feng Wu) contained the genic region downstream of the MADS box. The constructs were PCR amplified, cloned into pGEM T-easy (LMI2) and pGEM-T (AP1;Promega, Madison, WI, USA), and sequenced. Sense and antisense *LMI2* probes were digested with Sal I and transcribed with the T7 polymerase, while the antisense *AP1* probe was transcribed using the T7 polymerase following digestion with EcoRI. The Riboprobe® Combination System (Promega) and DIG RNA labeling mix (Roche, Branchburg, NJ, USA) were used for probe synthesis. In situ hybridization was performed as described in (Long and Barton, 1998).

5.5. pLMI2:LMI2-HA Construct and LMI2-HA ChIP

The pLMI2:LMI2 rescue construct excluding the translation termination codon was Gateway® cloned into pGWB13 (Nakagawa et al., 2007). pLMI2:LMI2-HA was transformed into *lmi2-2* plants followed by test for phenotypic rescue. For chromatin immunoprecipitation (ChIP) seedling assays, 300 mgs of tissue from eleven-day-old seedlings of a representative line were used with 3 μg/sample or 4 μg/sample of anti-HA antibody (sc-805 (Santa Cruz, Santa Cruz, CA, USA) or 12CA5 (Roche) respectively). For inflorescence ChIP assays, 1-2 cm bolt primary inflorescences were harvested from LMI2-HA *lmi2-2* and *lmi2-2* plants. 600 mgs of tissue were used for inflorescence ChIP with 10 μL/sample of anti-HA antibodies (Roche). ChIP was performed using published procedures (William et al., 2004; Kwon et al., 2005). LMI2 occupancy on genomic DNA was calculated by computing the enrichment over the respective input and normalized over *lmi2-2* for MI transition ChIP experiments and LMI2-HA occupancy on *SVP* was calculated as previously described (Yamaguchi et al., 2009). The mean and s.e.m. were

calculated using at least three technical replicates, one representative biological replicate is shown. For all ChIP-qPCR primers see Table 7.

5.6. 35S:SOC1-GFP and pAGL24:AGL24-RFP ChIP

The pAGL24:AGL24-RFP line was a gift from Martin Kater. For 35S:SOC1-GFP (construct and lines characterized by Dr. Ayako Yamaguchi), the coding region of *SOC1* was amplified and transferred into pGWB5 (Nakagawa et al., 2007). The resulting construct was transformed into wild-type plants. Functional chimeric proteins were identified from among the transformants by gain-of-function phenotypes. For chromatin immunoprecipitation (ChIP) 300 mgs of tissue were used. Fixation and ChIP were performed as previously described (William et al., 2004; Kwon et al., 2005) with 5 µl/sample of anti-GFP antibodies (Invitrogen, Carlsbad, CA, USA; A6455; 35S:SOC1-GFP and pAGL24:AGL24-RFP). SOC1 and AGL24 occupancy on genomic DNA were calculated as previously described (Yamaguchi et al., 2009). The mean and s.e.m. were calculated using at least three technical replicates for each biological replicate shown.

5.7. Glutathione S-transferase (GST) Pull-down

The LFY coding region was amplified and inserted between the EcoRI/NotI sites into pGEX-5X-1 (construct made by Dr. Yi Sang) (GE Healthcare, Piscataway, NJ, USA). The fusion protein was expressed in E.coli (AD494). After induction with 0.1mM IPTG at 37°C for one hour, cells were harvested by centrifugation and resuspended in ice-cold PBS containing 1mM EDTA, 1mM PMSF, 1mg/ml lysozyme and 1% Triton X-100. Following a 20-minute incubation at room temperature, the cell lysate was cleared

by centrifugation. Protein extracts were incubated with Sepharose 4B slurry (GE Healthcare) at 4°C for one hour. The beads were washed five times with PBS containing 1mM EDTA and 1mM PMSF. The protein-bound beads were directly used for pull-down assays. In vitro transcription and translation of LFY, LMI2 and NC[a] (1-464 amino acid fragment of the chromatin remodeling ATPase SYD; (Wagner and Meyerowitz, 2002)) and the pull-down assay were performed as previously described (Sang et al., 2005).

5.8. Yeast 2-Hybrid

LMI2N consisted of the N-terminal protein coding region of LMI2, including the MYB domain and the subgroup 9 motif, while LMI2C contained the remainder protein coding region of LMI2. The LMI2 fragments were amplified and inserted between the SalI and NotI sites of pDBLeu (construct made by Dr. Yi Sang) (Invitrogen, Carlsbad, CA, USA). The coding region of LFY was amplified and Gateway® cloned into pDEST22 (construct made by Dr. Jennifer Pfluger) (Invitrogen).

pDBLeu-LMI2N or LMI2C bait constructs were cotransformed into yeast (PJ69-4A) with either pDEST22-LFY or pDEST alone. After transformation, cells were plated on -Trp -Leu/SD media. Double transformants were grown in -Trp-Leu/SD liquid media overnight, adjusted for equal cell density, serially diluted (10^{-1}~10^{-4}) and spotted on -Trp -Leu- His/SD plates.

5.9. Bimolecular Fluorescence Complementation

LMI2N and LMI2C fragments were inserted into pENTR3C (Invitrogen) and Gateway® cloned into pCL113 (pBATL) (constructs made by Dr. Yi Sang). The coding

region of LFY was cloned into pCL112 (pBATL) (construct made by Dr. Jennifer Pfluger) to create the nYFP. p35S:2xmCherry was cloned into pEarley102 (Earley et al., 2006) (construct made by Dr. Miin-Feng Wu). The control protein (NC^b: TDY1-NLS in pCL113) was previously described (Ma et al., 2009). Constructs were transformed into onion epidermal cells using the PDS-1000/He Biolistic Particle Delivery System (BioRad, Hercules, CA, USA) as described by (Ma et al., 2009). Protein interactions were observed using an Olympus MVX10 fluorescent microscope.

Table 4. Genotyping primers

Primer Name	Sequence (5' to 3') Forward	Reverse
LMI2[1]	TCTGAAGGAGACCTGTAGTTGCTG	GAGCCTGTGAGTAGCTGTCAATGT
lmi2-1	(LB1.3) ATTTTGCCGATTTCGGAAC	GAGCCTGTGAGTAGCTGTCAATGT
lmi2-2	TCTGAAGGAGACCTGTAGTTGCTG	(LB1.3) ATTTTGCCGATTTCGGAAC
LMI2[2]	CTGAAAGCTTCACCAATCTCG	AAATTCATTGCTTCCTTTGGC
lmi2-3	CTGAAAGCTTCACCAATCTCG	(LB1.3) ATTTTGCCGATTTCGGAAC
lfy-1[3]	AAGCAGCCGTCTGCGGTGTCAGCAG CTGTT	CTGTCAATTTCCCAGCAAGACAC
lfy-2 & *lfy-10*[4]	AGAGAGACAGAGGGAGGATC	TGTCGCATTTTAGGCTTGTTT
AGL24[4]	GAATGAGAGACATATTGGGAAGGT A	AAGTGTCGGAGTCATCCTCAAG
agl24-3	(Lb1.3) ATTTTGCCGATTTCGGAAC	GAGCCTGTGAGTAGCTGTCAATGT
ap1-10[5]	CACATTTCTATCTAGGAAATCGATC G	GTATGGCCTTCTCCTGTCATTTCC
ap1-11[6]	TGAGCTTTTGGAGAGAAACCA	AACGAAATAGCAGAAGGCAGTA

[1] Gene specific primers for *lmi2-1* and *lmi2-2*. [2] Gene specific primers for *lmi2-3*. [3]Cut wild-type band with BstAP1. [4]Cut wild-type band with BamH1.[4] Gene specific primers for *agl24-3*. [5]Cut with BS II. [6] Cut with Sca I.

Table 5. Cloning primers

	Sequence (5' to 3')	
Construct	Forward	Reverse
pLMI2:LMI2	aaaaagcaggctGAAACGTGTCTCCACCCAAT	agaaagctgggttCTAGAATTTGGAAACCATGGA
LMI2:GUS [1]	GTCGACACTTGTAACTGTGCATGAAAC	CCCGGGGATTGTTCCTCACCCCACTAACA
LMI2:GUS[2]	GAGCTCGTCTTATGAGAGCCTAATATC	CAATTGAATTTTCTCAAGCATTGTCAC
LMI2 in situ	CTTTTATCTATGGGTCTTGATCCC	GAATGGTTAATTGTTTAATGTTCTGCAA
AP1 in situ	CGGAATTCCTTACGCCGAAAGACAGCTT	CGGGATCCCGTTCATTCTCTCTGACCTTCA
pLMI2:LMI2-HA	aaaaagcaggctGAAACGTGTCTCCACCCAAT	agaaagctgggttGAATTTGGAAACCATGGAAAC
GST-LFY	TAGAATTCATGGATCCTGAAGGTTTCAC	ATGCGGCCGCCTAGAAACGCAAGTCGTCGC
LMI2N	GAGGTCGACCATGGGAAGAACACCTTGTTG	TTAGCGGCCGCCTATGATTCTCTAGAAAGCCTTGC
LMI2C	GAGGTCGACCAGAGAATCAATGCTCTTTAGC	ATAGTTTAGCGGCCGCCTAGAATTTGGAAACCATGGA
LFY[3]	aaaaagcaggctacATGGATCCTGAAGGTTTC	agaaagctgggttCTAGAAACGCAAGTCGTC
LFY[4]	CACCATGGATCCTGAAGGTTTCACGAG	GAAACGCAAGTCGTCGCC
2xmCherry[4]	CACCATGGTGAGCAAGGGCGAGGAG	CTTGTACAGCTCGTCCATGCCG
35S:SOC1-GFP	aaaaagcaggctacATGGTGAGGGGCAAAACTC	agaaagctgggttCTTTCTTGAAGAACAAGGTAAC

Lower case sequences are *att*B1 and *att*B2 sequence specific. [1] Primers used to amplify *LMI2* upstream intergenic region. [2] Primers used to amplify *LMI2* downstream intergenic region. [3] Primers used for yeast two hybrid constructs. [4] Primers used for BiFC constructs.

Table 6. Semi-quantitative and quantitative PCR (qRT-PCR) primers

	Sequence (5' to 3')		
Gene	Forward	Reverse	PCR cycle
LMI2[1]	(P1)TCATTGCTTCCTTTGGCTTT	(P2) CTCATCTTCTTCAGGCGTCC	32
LMI2[1]	(P3) GACGCCTGAAGAAGATGAGG	(P4) TGTGGCGAGTTGTTGGTGAAGAT	32
EIF4A[2]	AAACTCAATGAAGTACTTGAGGGACA	TCTCAAAACCATAAGCATAAATACCC	24
EIF4A 1[2]	GCCATGGGTCTTCAAGAGAA	CCCTTACAGAAGGGGACGAT	NA
AP1[2]	GAAGGCCATACAGGAGCAAA	ACTGCTCCTGTTGAGCCCTA	NA
AP1[2]	AGGGAAAAAATTCTTAGGGCTCAACAG	GCGGCGAAGCAGCCAAGGTTGCAGTTG	NA
AP3	GCCCTAACACCACAACGAAGG	CTCACCTAGCCTCTGCTTGATC	NA
CAL	CATTTCAACACCCCCATCTT	GCCGTTTGGTCTTCTTCTTG	NA
FUL	TTGCAAGATCACAACAATTCGCTTCT	GAGAGTTTGGTTCCGTCAACGACGAT	NA
LFY	ACGCCGTCATTTGCTACTCT	CTTTCTCCGTCTCTGCTGCT	NA
LMI1	ATGGCCGGAGTCTAGTTCCT	GTTGTTCGGAAATCGGTACG	NA
LMI2[2]	GACGCCTGAAGAAGATGAGG	GAATGGCATGAAGCTGGATAA	NA
LMI2[2]	CGACCTCATCTGCACTTCTG	CGCCACAGTAACCTCTTTCC	NA
LMI2[2]	GGGAAGAACACCTTGTTGTGA	CCAGCTTCCATGTCCATTTT	NA
LMI3	GAACGAATGGGACACGTTAT	CAGACAATTCAGGATTGCCAG	NA
LMI4	AATGGTGTCCGGTGAGATTT	CATAACCACCGAATCCAACC	NA
LMI5	AACTGGTGGTCGAACAGCTC	AAGTGCATCTTCCCACATCC	NA
AGL24	GAGGCTTTGGAGACAGAGTCGGTG	AGATGGAAGCCCAAGCTTCAGGGA	NA
AG	CAAAACTCCAACAGGCAATTG	CATTTTCAGCTATCTTTGCAC	NA

[1] Primers used for T-DNA allele analysis. [2] Primer sets used interchangeably for quantitative PCR. Both sets give similar results. NA: not applicable-primers used for quantitative PCR.

Table 7. ChIP q-PCR primers

	Sequence (5’ to 3’)	
Locus (Region)	Forward	Reverse
AP1 (1)	CAAAGCTTAATGGGCCTTGA	GTCCGTGAGCTTTGTTTTGG
AP1 (2)	TCGAACGTGGTGGTTAGAAG	CGCAGCAGCTAGCATCTATTT
AP1 (3)	AGAATGGTGGGGCTAAAAGC	CAATCCAGCCACATCAAATG
AP1 (4)	CAAACCTTCCTGCCTTCTTTT	AATATCTCGATCCACTAAGATACGG
AP1 (5)	GCAAATGCCGAATCTGTTTT	AAAAACCTTTGCTCAATTTGC
AP1 (6)	ACACTTGGGGAAGGACCAGT	ATGTCGGGTCCATGATTTTT
AP1 (7)	GGGGGTCTTTGTTTTGTTTG	CCCTTCCCATTTTTGATCCT
AP1 (8)	AATGTGTCGCATCTAAGAAGATTT	TCGAGTTCTAACTGCGGTTTC
AP1 (9)	TGGGTTGTTAATGTTGATGTGTG	TGGACTCGTACATAAGTTGGTTC
LFY (1)	GCCAGTATTGCCAACTTTCC	GGCCAACCTACGTCTTTTTC
LFY (2)	TCACCACAGTGAAAACCCTAA	TGTGTCTTGCTGGGAAATTG
LFY (3)	GGAACCCAACGAGAGCATT	TCTAAACCACCAAGTCGCATC
LFY (4)	CTTTCGTTGGGAGCTTCTTG	AGCGTGATGAGTACCGGAAT
LFY (5)	ATGGGGTATGGTAGGGGAAC	TGAAAACCCTGAGAAATCGTG
LFY (6)	TTGATGTTGGGGAAAATGTG	TCCTGATTTCTTCGCGTACC
LMI2 (1)	TGAGACTCCCTTTGACTTGG	AATTCCGTGGAAGCAAAAAT
LMI2 (2)	ATGGACCCCACTGAGTGTCT	TGCTGAGCTATTTACTTCAATTTCA
LMI2 (3)	AGCTCGCTGATCGTCCTTT	AAGCCAAAGGAAGCAATGAA
LMI2 (4)	TTTCCGATAAGCGATGATGA	CAGAAGACCCAAAAAGAAGCA
SVP (1)	ACCTCACCAGTTGTGTCACG	ACATCCATCAATTGGCGTTT
SVP (2)	ATGATGATTGTGGCGATTGA	TTCACCAACGTCAACAACAGA
SVP (3)	GCCCTTGATGTTCTTCAGGT	TGGGGAATTTCTTTTTATAGGG
SVP (4)	GAGCCACCGACTAAGGTACG	TGATCATGTAAACACACAGTTAGAAA
EIF4	TGTTTTGCTTCGTTTCAAGGA	GCATTTTCCCGATTACAAC
TA3	CGAAGACAGTTCCGCTTACC	GCTTGTTCCGATTGTTCGAT

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