The LEAFY target LMI1 is a meristem identity regulator and acts together with LEAFY to regulate expression of CAULIFLOWER

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The timing of the switch from vegetative to reproductive development is crucial for species survival. The plant-specific transcription factor and meristem identity regulator LEAFY (LFY) controls this switch in Arabidopsis, in part via the direct activation of two other meristem identity genes, APETALA1 (AP1) and CAULIFLOWER (CAL). We recently identified five new direct LFY targets as candidates for the missing meristem identity regulators that act downstream of LFY. Here, we demonstrate that one of these, the class I homeodomain leucine-zipper transcription factor LMI1, is a meristem identity regulator. LMI1 acts together with LFY to activate CAL expression. The interaction between LFY, LMI1 and CAL resembles a feed-forward loop transcriptional network motif. LMI1 has additional LFY-independent roles in the formation of simple serrated leaves and in the suppression of bract formation. The temporal and spatial expression of LMI1 supports a role in meristem identity and leaf/bract morphogenesis.

KEY WORDS: Meristem identity switch, LEAFY, CAULIFLOWER, Feed-forward loop, Transcription, Development, Arabidopsis

INTRODUCTION

The switch from vegetative to reproductive development is tightly regulated in plants and occurs in two consecutive steps. The first step is controlled by the flowering-time regulators. Upon perception of the proper inductive stimuli, these regulators stop vegetative growth (rosette leaf formation) and initiate bolting. During the vegetative phase and during bolting, the shoot apical meristem gives rise to leaves, which have the potential to generate branches in their axes (axillary inflorescences in the axes of rosette leaves and secondary inflorescences in the axes of the sessile leaves, or bracts). The second step in reproductive development is controlled by the meristem identity regulators. These factors are induced during bolting, and ultimately direct the primordia founder cells to cease production of bracts and secondary inflorescences, and to initiate production of the reproductive structures, the flowers.

The known meristem identity regulators comprise the transcription factors LEAFY (LFY), APETALA1 (AP1) and the closest homolog of AP1, CAULIFLOWER (CAL), as well as the factor most related to AP1 and CAL, FRUITFULL (FUL) (Alvarez-Buylla et al., 2000; Blazquez et al., 1997; Bowman et al., 1993; Ferrándiz et al., 2000; Hempel et al., 1997; Huala and Sussex, 1992; Nilsson et al., 1998; Ruiz-Garcia et al., 1997; Weigel et al., 1992). LFY is considered to be the central meristem identity regulator in Arabidopsis (Blazquez et al., 1997; Bowman et al., 1993; Hempel et al., 1997; Huala and Sussex, 1992; Weigel et al., 1992; Weigel and Nilsson, 1995). LFY is a transcription factor and directly activates AP1 during the meristem identity transition (Parcy et al., 1998; Wagner et al., 1999). In addition CAL, but not FUL, is a direct target of LFY (William et al., 2004). After flowers have been initiated, LFY has a separate role in flower patterning (Busch et al., 1999; Lamb et al., 2002; Lenhard et al., 2001; Lohman et al., 2001; Parcy et al., 1998; Weigel and Meyerowitz, 1993).

We are interested in identifying and characterizing the components of the regulatory network that leads from LFY induction to the formation of the first flower primordium. Despite the developmental importance of the changes the meristem undergoes at this point in development, events immediately downstream of LFY that culminate in the formation of the first flower primordium are poorly understood. Only two of the direct LFY targets [AP1 and CAL (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004)] are known to act as meristem identity regulators (Bowman et al., 1993; Ferrándiz et al., 2000; Nilsson et al., 1998; Ruiz-Garcia et al., 1997; Weigel et al., 1992), suggesting that additional direct LFY targets exist that are meristem identity regulators. We recently identified five additional direct LFY targets as candidate meristem identity regulators, using a genomic approach (William et al., 2004).

Here, we report our investigation of the biological role of one of these targets, the class I HD-Zip transcription factor At5g03790. Based on our phenotypic and molecular investigations, we named this regulator LATE MERISTEM IDENTITY1 (LMI1). We found that lmi1 mutants enhance the meristem identity defects of the weak lfy-10 allele, indicating that LMI1 is a meristem identity regulator. Furthermore, CAL expression is reduced in the double mutant, suggesting that LMI1 acts upstream of CAL. The observed in vivo binding of LMI1 to CAL promoter proximal regions suggests that the regulation of CAL by LMI1 is direct. LMI1 has a second, LFY-independent role in leaf and bract development.

MATERIALS AND METHODS

Plant growth, transgenic plants and phenotypes

T-DNA insertion lines for LMI1 were obtained from the SALK collection (Alonso et al., 2003) and twice backcrossed to the wild type (Col). LFY alleles were obtained from the Arabidopsis Biological Resource Center
RT-PCR and real-time PCR

Total RNA was isolated from above ground plant tissues grown in continuous light at 55 μmol/m²s on half-strength Murashige and Skoog medium. RNA isolation and RT-PCR was performed as described (William et al., 2004). Quantitative real-time PCR was performed on RNA treated with DNase Set (Qiagen, Valencia, CA) in a 20-μM PCR reaction using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on a DNA Engine Opticon Thermal cycler (MJ Research). Thermal cycling conditions were as follows: 15 minutes at 95°C, 44 cycles of 15 seconds at 94°C, 30 seconds at 54°C and 30 seconds at 72°C, followed by a melting curve analysis. Relative amounts of all mRNA were calculated from threshold cycle values and normalized with the level of eukaryotic translation initiation factor 4A-1 (EIF4A) for mRNA and input for ChIP. For details of the primers used for amplification see Table S1 in the supplementary material.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed essentially as previously described (Kwon et al., 2005; William et al., 2004), except that 100 mg of tissue was employed per sample. The MYC antiseraum was used from the Myc1-9E10 cell line. After binding of the immunocomplexes to protein G magnetic beads (Dynal, Brown Deer, WI), washes were performed according to Ricci et al. (Ricci et al., 2002). For details of the primers employed for ChIP, see Table S1 in the supplementary material.

RESULTS

We previously identified the putative class I HD-ZIP transcription factor At5g03790 as a direct target of LFY (William et al., 2004). The biological role of this regulator was not characterized. Based on our morphological and molecular analyses of loss-of-function alleles of this gene, we named it LATE MERISTEM IDENTITY1 (LMI1).

To investigate the role of LMI1 in development, we characterized four insertion alleles from the SALK T-DNA collection (Alonso et al., 2003). Lmi-1 to lmi-4 (Fig. 1A). We found that, in addition to the T-DNA insertion, each lmi allele also had a small deletion ranging from 10 to 56 bp (Fig. 1A).

To determine whether any of the insertions destabilized the LMI1 message and resulted in a RNA null allele, we performed semi-quantitative RT-PCR using primers flanking the T-DNA. LMI1 message was present in all mutant lines, suggesting that none of the four insertion lines are RNA null alleles (LMI1up; Fig. 1B). Three of the insertions (in lmi-1, lmi-2 and lmi-4) had LMI1 message levels similar to those of the wild type, whereas increased LMI1 levels were observed in lmi-3. The reason for this increase is not understood, although it is possible that the large deletion observed in this line stabilizes the truncated message. As expected, when we examined LMI1 mRNA by semi-quantitative RT-PCR analysis using primers flanking the T-DNA insertion sites, we could not detect any LMI1 message in the four mutants (Fig. 1B). Thus, all alleles are likely to result in 3' truncated LMI1 mRNAs. lmi-1 is therefore predicted to give rise to a polypeptide lacking both the homeodomain and the leucine zipper motif. The remaining lines are predicted to give rise to versions of LMI1 containing a functional homeodomain and a truncated leucine zipper domain, lacking one or two of the conserved leucines (lmi-1, lmi-4 or lmi-2, respectively). Because of the absence of the putative DNA-binding domain, LMI1-1 should encode a non-functional protein.

Because LMI1 has not been identified as a meristem identity regulator in any forward genetic screen to date, we hypothesized that loss-of-function alleles in this gene may have subtle or no meristem identity phenotypes. We therefore first crossed all four T-DNA
alleles to the weak lfy-10 mutant (http://www.weigelworld.org/resources/mutants/lfy) and assayed for enhanced meristem identity defects and/or flowering time defects in this sensitized genetic background. The timing of the meristem identity switch is commonly measured by counting the number of secondary inflorescence branches formed prior to flower formation (e.g. Ratcliffe et al., 1998). In addition, the number of bracts formed is a measure of the formation of true flowers. Flowers with residual inflorescence identity, such as those observed early during the reproductive phase in many meristem identity mutants, are unable to suppress bract formation (Bowman et al., 1993; Dinneny et al., 2004; Huala and Sussex, 1992; Long and Barton, 2000; Ohno et al., 2004; Weigel et al., 1992). We measured flowering time by counting the number of rosette leaves produced prior to bolting.

Two lmi1 mutants, lmi1-1 and lmi1-4, significantly enhanced the meristem identity defect of lfy-10 with respect to the number of secondary inflorescences formed (Fig. 2A,D). The secondary inflorescence number in these double mutants was comparable to that of the intermediate lfy-9 allele, but lower than that of the lfy-1 null mutant (Fig. 2A). This enhanced meristem identity defect is readily detected in adult lmi1-1 lfy-10 plants; the first flower forms much higher on the primary inflorescence (after the SAM has given rise to more secondary inflorescences) than in lfy-10 plants (Fig. 2D). In addition, all lmi1 lfy-10 double mutants had significantly increased numbers of bracts when compared with the lfy-10 single mutants (Fig. 2B). The bract numbers of the weak lmi1-3 and lmi1-2 alleles in the lfy-10 background are similar to those of lfy-9, whereas lmi1-1 lfy-10 and lmi1-4 lfy-10 give rise to more bracts than lfy-9, but still fewer than lfy-1 null plants (Fig. 2B). Thus, although defects in LMI1 increase both the number of secondary inflorescences and the number of bracts formed in lfy-10, the effect on bract formation is more pronounced, suggesting that LMI1 plays a central role in this process. No significant alterations in rosette leaf number were observed in lmi1 lfy-10 double mutants when compared with lfy-10 (Fig. 2C). The lmi1-1 mutant did not enhance the floral homeotic defects of lfy-10 (Fig. 2E), suggesting that LMI1 does not play a role in the activation of floral homeotic gene expression. These combined results indicate that LMI1 is a meristem identity regulator. As we have identified LMI1 as a direct target of LFY, LMI1 is likely to regulate meristem identity downstream of LFY.

As noted, LMI1 has not been identified as a meristem identity regulator in forward genetic screens performed to date. Indeed, none of the lmi1 alleles exhibited significant meristem identity defects or flowering time defects as single mutants [in the wild-type (Col) background; Table 1]. We next tested whether lmi1 was able to enhance the meristem identity defects in an even more mildly sensitized genetic background than lfy-10, such as in lfy-10/+.

Table 1. Meristem identity and flowering time phenotypes of lmi1 mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Secondary inflorescences</th>
<th>Bracts</th>
<th>Rosette leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>lmi1-1</td>
<td>4.1±0.10 (20)</td>
<td>4.1±0.10 (20)</td>
<td>14.3±0.36 (20)</td>
</tr>
<tr>
<td>lmi1-3</td>
<td>4.1±0.13 (20)</td>
<td>4.1±0.13 (20)</td>
<td>13.7±0.30 (20)</td>
</tr>
<tr>
<td>Col</td>
<td>3.8±0.12 (18)</td>
<td>3.8±0.12 (18)</td>
<td>13.6±0.41 (18)</td>
</tr>
<tr>
<td>lfy-10</td>
<td>10.0*±0.27 (8)</td>
<td>6.38*±0.32 (8)</td>
<td>11.4±0.38 (8)</td>
</tr>
<tr>
<td>lfy-10/+</td>
<td>3.8±0.14 (20)</td>
<td>3.8±0.14 (20)</td>
<td>12.8±0.26 (20)</td>
</tr>
<tr>
<td>lmi1-1 lfy-10/+</td>
<td>5.1*±0.13 (24)</td>
<td>5.1*±0.13 (24)</td>
<td>12.3±0.29 (24)</td>
</tr>
</tbody>
</table>

Shown is the mean number of lateral appendages formed ±s.e.m. The number of plants counted is indicated in parentheses.

*Significantly different from value of lfy-10/+ (Student’s t-test, P<0.0001).
that LMI1 acts redundantly with other meristem identity regulators under conditions of full LFY activity, but is required for the meristem identity switch when LFY activity is only slightly reduced.

To examine whether LMI1 acts only downstream of LFY or whether it has additional roles in meristem identity regulation, we compared the meristem identity defect of the lfy-1 null mutant to that of lmi1-1 lfy-1 (Fig. 3A). We did not observe an increase in the number of secondary inflorescences or rosette leaves formed in lfy-1 lmi1-1 when compared with lfy-1 (Fig. 3A, C). This suggests that LMI1 acts primarily downstream of LFY in meristem identity regulation. However, we did observe a marked increase in the number of bracts formed in lfy-1 lmi1-1 compared with lfy-1 (Fig. 3B). One characteristic of lfy-1 mutants is that flowers formed directly after the meristem identity transition are subtended by bracts, whereas most later-arising flowers lack bracts. By contrast, all flowers in lmi1-1 lfy-1 plants were subtended by bracts (Fig. 3D). The finding that lmi1 single mutants do not have increased numbers of bracts (Table 1) indicates that LMI1 acts together with LFY to regulate bract formation. The additional bracts formed in lmi1-1 lfy-1 double mutants compared with the lfy-1 null mutant suggest that LMI1 also has a LFY-independent role in this process.

In our combined microarray-based and candidate gene approaches for isolation of LFY targets during the meristem identity switch, we identified a total of seven genes that are directly upregulated by LFY (Wagner et al., 1999; William et al., 2004). In order to investigate the relationship between LMI1 and the other six LFY targets, we assayed the expression levels of all seven genes in lmi1-1 lfy-10 double mutants compared with lfy-10 during the meristem identity switch using quantitative real-time PCR (Fig. 4). The expression of most LFY target genes was not dependent on LMI1 (Fig. 4A) in eleven-day-old seedlings. However, CAL expression was reduced in lmi1-1 lfy-10 double mutants under these conditions (Fig. 4A). The severity of the defect in CAL expression observed correlated with the allelic strength of the lmi1 mutants (data not shown). AP1 expression was also strongly reduced in the lmi1-1 lfy-10 double mutant.

We next investigated CAL and AP1 expression in lmi1 lfy-10 versus lfy-10 mutants at several time points before, during and after the meristem identity transition (Fig. 4B). CAL expression was low in all lines at day 7. In lfy-10, there was a rapid, strong increase in CAL expression at day 9 followed by a slow decline at days 11 and 13. The slow decline of CAL levels might be due to an increase in non-CAL-expressing cells during later stages of seedling development (as entire seedlings were assayed). Alternatively, the...
decline might be caused by a downregulation of CAL expression in the CAL-expressing cells after the transition to flower formation (after AP1 induction). By contrast, in the lmi1-1 lfy-10 and lmi1-4 lfy-10 double mutants, CAL expression was not induced at day 9, leading to a fourfold reduction in CAL levels at this stage. Furthermore, CAL expression remained low at all subsequent time points. We conclude that LMI1 is required together with LFY to induce CAL expression during the meristem identity transition.

AP1 upregulation occurred by day 11 in lfy-10, and AP1 levels continued to increase until day 13 (Fig. 4B). By contrast, AP1 levels remained low in lmi1-1 lfy-10 until day 13, when we observed an upregulation of expression. Thus, CAL expression is reduced and AP1 expression is delayed in the double mutants. CAL expression precedes that of AP1 (Schmid et al., 2003; William et al., 2004). In addition, CAL is known to be required for the proper induction of AP1 expression (Bowman et al., 1993). Hence, the delay in AP1 expression in lmi1-1 lfy-10 compared with lfy-10 may be due to the reduction in CAL levels in the double mutant.

Because AP1 upregulation marks the formation of the first flower primordium (Bowman et al., 1993; Hempel et al., 1997; Mandel et al., 1992), formation of the first flower primordium is likely delayed by approximately one to two days in lmi1-1 lfy-10 compared with lfy-10. Consistent with this notion, transverse sections of fifteen-day-old inflorescences revealed the formation of two flowers in lfy-10, whereas lmi1-1 lfy-10 meristems had not formed any flowers at this stage (Fig. 4C). This difference indeed corresponds to a meristem identity transition delay of one to two days in lmi1-1 lfy-10 compared with lfy-10 (Smyth et al., 1990).

When we examined the effect of the lmi1-1 single mutant on CAL or AP1 expression by quantitative real-time PCR, we did not observe a significant decrease in expression (data not shown). It is possible that under strong inductive conditions (continuous light), CAL expression is not dependent on LMI1. Alternatively, LMI1 may act redundantly in its role as an activator of CAL expression.

Class I HD-Zip proteins act as homo- or heterodimers and bind the palindromic cis-regulatory sequence CAATNATTG (Frank et al., 1998; Johannesson et al., 2001; Meijer et al., 2000; Meijer et al., 1997; Sessa et al., 1993; Tron et al., 2004; Wang et al., 2005). Our data indicate that LMI1 regulates CAL expression together with LFY. We have previously demonstrated that LFY binds CAL promoter proximal sequences (Fig. 5A) (William et al., 2004). When we checked the region bound by LFY for potential LMI1-binding sites, we located a CAATNATTG motif in the CAL promoter proximal region, less than 100 bp upstream of the LFY-binding site (CCANTG; Fig. 5A). There is precedent for LFY acting in concert with another transcription factor to induce expression of a target gene. LFY and WUSCHEL (WUS) together activate AGAMOUS (AG), and bind to cis elements adjacent to each other in the AG regulatory region (Lenhard et al., 2001; Lohman et al., 2001). A genome-wide survey of binding elements (see Table S2 in the supplementary material) revealed that candidate LFY target genes showed a fivefold enrichment in regulatory regions that contain a LFY- and a LMI1-binding site in close proximity when compared with non-LFY targets, suggesting that LMI1 and LFY could act together to regulate the expression of several LFY targets. Of the seven verified direct LFY targets, AP1, CAL, and LMI1 through LMI5, CAL was the only gene that contained proximal LFY- and LMI1-binding sites in the region 1 kb upstream of the translation start site (see Table S2 in the supplementary material).

We next investigated whether LMI1 binds to the CAL promoter proximal CAATNATTG element by using chromatin immunoprecipitation (ChIP) analysis. Towards this end, we generated transgenic plants expressing a MYC epitope-tagged version of LMI1 (see Materials and methods). When we assayed for ChIP of CAL promoter proximal DNA regions using anti-MYC antibodies in plant nuclear extracts either containing (+) or not containing (−) MYC-LMI1, we saw an enrichment of the CAL upstream regulatory region in plants containing MYC-LMI1 (Fig. 5B). By contrast, we did not observe a similar enrichment when we assayed for MYC-LMI1 occupancy at AP1 regulatory regions, consistent with the absence of a predicted LMI1-binding site at this locus. As a control for the ChIP procedure, we included an anti-LFY reaction, which resulted in strong signal for both CAL and AP1 ChIP (data not shown). These data suggest that LMI1 is a direct upstream activator of CAL. Taken together with the previous observation that LFY binds to CAL (William et al., 2004), and with the observed reduction of CAL expression in lmi1 lfy-10 double mutants, the data suggest that LMI1 acts together with LFY to induce CAL during the meristem identity switch.

LFY expression is upregulated at the onset of reproductive development first in young leaves and later in the incipient flower primordia (Blazquez et al., 1997; Hempel et al., 1997). CAL is expressed strongly in young flower primordia; its expression domain is entirely contained within the LFY expression domain (Kempin et al., 1995). To test whether LMI1 expression overlaps with that of LFY and CAL, we performed in situ hybridization experiments to determine the temporal and spatial expression of LMI1. We were unable to obtain a signal above background with our antisense probe. This is likely to be due to low overall message abundance. A similar lack of detection for LMI1 using northern hybridization was recently reported in a study of class I HD-Zip regulators (Henriksson et al., 2005). We generated a reporter construct using bacterial β-
glucuronidase (GUS) flanked by 5’ and 3’ intergenic regions to maximize duplication of the regulatory constraints acting on LMI1 expression. The LMI1 reporter assay showed staining in the leaves of young seedlings during the vegetative stage (Fig. 6A). The staining is mostly limited to the leaf tip (Fig. 6A). During bolting, we observed LMI1:GUS expression in the bract primordia (Fig. 6B,C). After bolting, LMI1:GUS is strongly expressed in the incipient flower primordia (Fig. 6D-G,I). We infer from these data that the expression domain of LMI1 overlaps with those of CAL and LFY; all three genes are strongly expressed in young flower primordia. Like LFY expression, LMI1:GUS expression precedes formation of the flower primordium. The observed expression of LMI1:GUS is consistent with a role of LMI1 as a meristem identity regulator downstream of LFY and upstream of CAL. No LMI1 reporter expression was observed in the shoot apical meristem (Fig. 6A-G). LMI1:GUS is expressed throughout stage 1 and 2 flowers [Fig. 6G,F, respectively; flower stages as described by Smyth et al. (Smyth et al., 1990)]. It is also expressed in sepals and stamens in stage 7 flowers (Fig. 6F), and in petals in stage 9 flowers (Fig. 6G). In older flowers, staining is restricted to the tips of these organs (Fig. 6H). We also noticed staining in the L1 (and perhaps the L2) layer on the abaxial side of flowers and flower pedicels from stage 2 onwards (arrows in Fig. 6F). When we compared LMI1:GUS expression in 17-day-old wild-type and lfy-1 null mutant inflorescence meristems, we observed a marked reduction of LMI1:GUS expression in the flower primordia (compare Fig. 6I with 6J). By contrast, LMI1:GUS was expressed in the sepals of stage 4 flowers of lfy-1 (Fig. 6I). LMI1:GUS expression was as strong as that of the wild type in lfy-1 bracts (compare Fig. 6K with 6L).

In whole-mount images, LMI1 reporter expression is readily observed in young leaves (Fig. 7A-D) and is limited to their margins (Fig. 7B-D). In slightly older, still expanding, leaves the expression is more pronounced in the proximal leaf margins and is very strong in the serrations (Fig. 7D). Similarly, the margins,
serrations and stipules in the bracts are stained (Fig. 7E). LMI1::GUS is not expressed in the hypocotyl (Fig. 7A) or in the roots (Fig. 7C). In maturing flowers, LMI1::GUS expression is observed in the distal margins of the sepals, petals and anthers (Fig. 7F,G), suggesting that LMI1 may play a role in the maturation of floral organs. Fully expanded above-ground tissues (cotyledons, leaves or flower organs; Fig. 7A,C,D,F,G) do not show any staining for LMI1::GUS, with the exception of weak expression at the hydathode (Fig. 7A) and in serrations (Fig. 7D). Staining in the carpels appears to be limited to the ovules (Fig. 7G). We also observed staining in pollen grains (not shown). In heart-stage embryos, we observed staining in the cotyledons (Fig. 7H). In slightly older (torpedo stage) embryos, the staining was restricted to the cotyledon margins, similar to the leaf margin staining in young seedlings (Fig. 7H, inset).

The LMI1::GUS expression in expanding leaves (Fig. 7D, Fig. 8A) correlates with a second phenotype we observed in the loss-of-function mutants: lmi1 mutant rosette leaves were divided at the base and formed one or two leaflets (Fig. 8C). This is in contrast to the simple, undivided, leaves typical of wild type (Fig. 8A). This phenotype was readily observed in all four lmi1 single mutants. Although the leaf division was apparent in long-day growth conditions, it was enhanced when lmi1 mutant plants were grown in a non-inductive photoperiod (not shown). In addition, although leaf division was increased in lmi1 compared with wild type, leaf serration was decreased (Fig. 8C), suggesting that the leaf division was not a consequence of elevated leaf serration. The loss of serration was correlated with allelic strength, it was more severe in lmi1-1 than in lmi1-3 (Fig. 8C). When we examined whether the leaf margin defect was dependent on the presence of LFY activity, we found that lmi1-1 ify-1 plants exhibited leaf division and leaflet formation at a rate comparable with that observed in lmi1-1 (Fig. 8D, data not shown). Furthermore, ify-1 single mutants do not exhibit leaflet formation on the rosette leaves when grown in long- or short-day conditions (Huala and Sussex, 1992; Schultz and Haughn, 1991). Thus, LMI1 regulates leaf morphogenesis independently of LFY. Consistent with this, LMI1::GUS staining in the leaf margins was indistinguishable from that of wild type in ify null mutants (compare Fig. 8A and 8B).

The type of leaflet formation observed in lmi1 mutants has been correlated with an increased expression of the KNOX class of homeodomain transcription factors, particularly with mis-expression of BREVIPEDICELLUS (BP), also known as KNAT1 (Ha et al., 2003). We could readily detect increased BP expression in expanding lmi1-1 leaves compared with those of the wild type (Fig. 8E) using quantitative real-time PCR, suggesting that LMI1 is required for the proper regulation of BP expression.

**DISCUSSION**

**LMI1 is a meristem identity regulator**

LMI1 is a member of the class I HD-Zip transcription factor family. Members of this family are known to play a role in mediating developmental responses to environmental signals (Aoyama et al., 1995; Olsson et al., 2004; Wang et al., 2003). Several class I HD-Zip regulators control leaf shape and size (Aoyama et al., 1995; Hanson et al., 2001; Wang et al., 2003). We show here that the direct LFY target LMI1 is a meristem identity regulator. Reduction in LMI1 activity delays the meristem identity transition of a weak ify mutant with respect to both initiation of flower formation and suppression of bract formation. Based on the delay observed in induction of AP1 expression, and on the observed increase in secondary inflorescences formed, the meristem identity switch occurs one to two days later in lmi1-1 ify-10 than in ify-10. As LMI1 was one of five genes identified as candidate meristem identity regulators and direct LFY targets using a microarray-based approach, the finding that LMI1 is a bona fide meristem identity regulator validates our genomic approach aimed at identifying the missing meristem identity regulators that act directly downstream of LFY.

The enhanced meristem identity of lmi1 is observed in mildly sensitized genetic backgrounds such as ify-10 heterozygotes, but not in wild type. Hence, under conditions of full LFY activity, another factor acts redundantly with LMI1 in the regulation of meristem identity. The additional factor could be one of the other direct LFY targets we identified (William et al., 2004) or another class I HD-Zip regulator. The well-studied (distantly related) class III HD-Zip regulators act redundantly in development, such that single loss-of-function mutants generally have no discernible phenotypes (Baima et al., 2001; Emery et al., 2003; Prigge et al., 2005). Although we did not observe significant upregulation of any other class I HD-Zip regulators in response to LFY activation in tissue culture (Wagner et al., 2004), the class I HD-Zip gene At4g36740 was significantly upregulated in response to LFY activation in tissue culture (Wagner et al., 2004). In addition, the class I HD-Zip transcription factor HAT7/ATHB3 (At5g15150) was shown to be activated in a LFY-dependent manner (Schmid et al., 2003). Future investigations will reveal whether any of the other class I HD-Zip genes are direct LFY targets, and whether they or the remaining LMIs act together with LMI1 in meristem identity regulation.
On the basis of our reporter studies, LMI1 is expressed in flower primordia, in young flowers, at the tips of floral organs, and in the margins of cotyledons, rosette leaves and bracts. LMI1 expression increases over time, reaching high levels around bolting. Expression remains elevated until young flowers are formed. Only the LMI1 expression in the flower primordia and in very young flowers is dependent on LFY. Our data is in good agreement with publicly available ‘virtual’ expression datasets. Based on Genevestigator, there is an eight- to tenfold increase in LMI1 expression after bolting (Zimmermann et al., 2004). This is confirmed using AVT on AtGenExpress data (Schmid et al., 2005). High LMI1 expression was observed prior to, during, and after the meristem identity switch (day 7 to day 21) (Schmid et al., 2005), corresponding to the stages when we observe expression in bracts, flower primordia and young flowers. LMI1 expression was also high in lfy null mutant inflorescence apices and flowers (Schmid et al., 2005). This is not surprising given the strong LMI1 expression we observe in bracts of lfy null mutants and the increased number of bracts formed in lfy compared with wild type. The LMI1 expression we observe in young flowers is consistent with a reduction of LMI1 expression in inflorescence apices of floral homeotic mutants [AtGenExpress (Schmid et al., 2005)]. Thus, our reporter studies agree well with publicly available, microarray-based expression studies and indicate that LMI1 expression in the floral primordia is controlled by LFY during the meristem identity transition.

**LMI1 activates CAL together with LFY**

We show here that LMI1 is a direct upstream activator of a second meristem identity regulator, the MADS-box transcription factor CAL (Kempin et al., 1995). LMI1 acts together with LFY to induce CAL expression. The observed temporal and spatial expression of LFY, LMI1 and CAL fits well with their proposed interaction. This interaction between LFY, LMI1 and CAL resembles a coherent feed-forward loop (FFL), a common transcriptional network motif found in *E. coli* and *S. cerevisiae* (Lee et al., 2002; Milo et al., 2002; Shen-Orr et al., 2002; Yeger-Lotem et al., 2004), and in multicellular organisms (Milo et al., 2004; Penn et al., 2004). This FFL consists of two transcription factors, A and B, with A directly activating B, and both A and B directly activating their common target C (Mangan and Alon, 2003). This network motif was proposed to act as a persistence detector for noisy inputs (Dekel et al., 2005; Mangan and Alon, 2003; Shen-Orr et al., 2002; Yeger-Lotem et al., 2004). This prediction is supported by the LFY (A), LMI1 (B) and CAL (C) interactions we describe here (Fig. 9). Even when LFY levels are just slightly reduced, LMI1 is required for a proper meristem identity transition; the meristem identity transition was significantly delayed in lmi1-lfy-10/+.

The onset of reproductive development is tightly controlled in flowering plants. This is to ensure that the switch to the formation of flowers (and ultimately allocation of valuable resources to the seeds) occurs only when the appropriate conditions are met. As such, correct timing of the onset of reproductive development is important for the survival of the species. As sessile organisms, plants are dependent on environmental input into the timing of this switch. The physiological relevance of this FFL would be to ensure that a key step in this transition, the meristem identity switch, is not triggered by a transient alteration in an environmental stimulus. Upregulation of LFY expression and activity is known to be controlled by several environmental inputs, the foremost among which is photoperiod (Blazquez et al., 2003; Blazquez et al., 1997; Hempel et al., 1997; Nilsson et al., 1998; Parcy, 2005). Indeed, when plants are moved from non-inductive, short-day conditions to weak-inductive conditions, a threshold for the upregulation of LFY expression and for the initiation of flower formation was identified (Blazquez et al., 1997; Hempel et al., 1997). We predict that more transient inductive conditions than those described for the wild type should trigger the meristem identity switch if LMI1 were constitutively supplied (Penn et al., 2004), leading to precious flower formation. Furthermore, a delayed meristem identity transition may be observed in lmi1-1 mutants under weak inductive conditions that are sufficient for flower formation in the wild type. Future experiments will be aimed at testing the role of LMI1 in photoperiod-induced flower formation.

**Roles of LMI1 in bracts and leaves**

The LMI1 expression in the bracts of wild-type and lfy null mutant plants suggests that LMI1 expression is controlled by another factor besides LFY in this tissue (Fig. 9). LMI1 has both LFY-dependent and LFY-independent roles in bract formation. By contrast, LMI1 acts independently of LFY in leaf development. LMI1 is strongly expressed in the leaf margins in wild type and in lfy null mutants. In addition, LMI1 is required for the production of simple, serrated leaves. In lmi1 mutants, leaflets form at the base of the leaves. Two general hypotheses have been proposed to explain the formation of compound leaves. One model postulates increasing subdivision of a simple leaf by sequentially increased serration, lobing and, finally, division. A second model instead suggests that compound leaves result from increased indeterminacy and branch formation; each leaflet is considered a small, simple leaf (Champagne and Sinha, 2004). The lmi1 phenotype supports the latter hypothesis; we see increased lobing and division at the base of the leaf, yet a reduction in overall serration. Although LMI1 is expressed throughout the leaf margin in young leaves, it is more strongly expressed at the base of expanding leaf margins, consistent with the observed phenotypic defects. Other factors may control simple leaf formation at the lateral and distal margins.

Loss of LMI1 activity enhances bract formation in the lfy null mutant, and LMI1 is expressed in the region of the cryptic bract (Long and Barton, 2000), below the incipient flower primordium and
on the abaxial side of early flowers. A gene regulating bract formation, \textit{JAGGED}, is excluded from this region, except in meristem identity mutants (Dimmey et al., 2004; Ohno et al., 2004). Future experiments will reveal whether LMI1 contributes to the repression of \textit{JAG} expression in the cryptic bract domain. The \textit{BLADE-ON-PETIOLE (BOP)} genes are upstream, negative regulators of both \textit{JAG} and \textit{BP} expression (Ha et al., 2004; Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). In addition, \textit{bop1} mutants have similar leaf and bract phenotypes to those we describe here (Ha et al., 2004; Ha et al., 2003; Norberg et al., 2005). It is thus possible that the roles of LMI1 in the suppression of leaflet and bract formation could be due to regulation of \textit{BOP1} or the \textit{BOP1} pathway. Future experiments will reveal whether this is indeed the case.

We thank J. Wagner, T. Cashmore and J. Pastore for critical comments and suggestions regarding this manuscript. This research was supported by the National Science Foundation grant IBN 0516622 and a seed grant from the University of Pennsylvania Research Foundation.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/9/1673/DC1

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