

Evolution of Floral Meristem Identity Genes. Analysis of *Lolium temulentum* Genes Related to *APETALA1* and *LEAFY* of *Arabidopsis*¹

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Flowering (inflorescence formation) of the grass *Lolium temulentum* is strictly regulated, occurring rapidly on exposure to a single long day (LD). During floral induction, *L. temulentum* differs significantly from dicot species such as *Arabidopsis* in the expression, at the shoot apex, of two *APETALA1* (*AP1*)-like genes, *LtMADS1* and *LtMADS2*, and of *L. temulentum* *LEAFY* (*LtLFY*). As shown by in situ hybridization, *LtMADS1* and *LtMADS2* are expressed in the vegetative shoot apical meristem, but expression increases strongly within 30 h of LD floral induction. Later in floral development, *LtMADS1* and *LtMADS2* are expressed within spikelet and floret meristems and in the glume and lemma primordia. It is interesting that *LtLFY* is detected quite late (about 12 d after LD induction) within the spikelet meristems, glumes, and lemma primordia. These patterns contrast with *Arabidopsis*, where *LFY* and *AP1* are consecutively activated early during flower formation. *LtMADS2*, when expressed in transgenic *Arabidopsis* plants under the control of the *AP1* promoter, could partially complement the organ number defect of the severe *ap1-15* mutant allele, confirming a close relationship between *LtMADS2* and *AP1*.

In *Arabidopsis* and snapdragon (*Antirrhinum majus*), two orthologous genes, *LEAFY/FLORICAULA* (*LFY/FLO*) and *APETALA1/SQUAMOSA* (*AP1/SQUA*), are important for the initiation of floral development. The *flo* mutants mostly lack flowers and produce secondary inflorescences in positions normally occupied by solitary flowers, with the secondary inflorescences repeating the pattern of the main inflorescence (Carpenter and Coen, 1990). The *squa* mutants have a similar phenotype as *flo* mutants, but flowers are produced more often than in *flo* mutants (Huijser et al., 1992). The *lfy* mutants also show flower-to-shoot conversions, but similarly to *squa* mutants produce flowers more often than *flo* mutants. These flowers lack petals and stamens and contain leaf-like organs, indicating partial shoot character (Schultz and Haughn, 1991). The weakest phenotypes are seen in *ap1* mutants, in which flower-to-shoot conversions are relatively rare. Instead, flowers have leaf-like organs in the first whorl, with secondary flowers in their axils (Irish and Sussex, 1990). The increased

proliferation of floral meristems is more pronounced when *ap1* mutations are combined with mutations in the *AP1* paralogs *CAULIFLOWER* (*CAL*) and *FRUIT-FULL* (*FUL*; Bowman et al., 1993; Ferrándiz et al., 2000). A more severe phenotype is also seen in *lfy ap1* double mutants, in which all flowers are transformed into shoot-like structures, and in combinations of weak *flo* and *squa* mutations (Huala and Sussex, 1992; Weigel et al., 1992; Carpenter et al., 1995).

LFY/FLO and *AP1/SQUA* are strongly expressed in floral meristems, consistent with a direct role in promoting floral fate. This has been further demonstrated with transgenic plants in which either gene is overexpressed from the constitutive 35S viral promoter. These plants flower early, lateral inflorescence shoots are often transformed into solitary flowers, and the shoot apex, which in wild-type *Arabidopsis* remains indeterminate, terminates in a flower (Mandel and Yanofsky, 1995a; Weigel and Nilsson, 1995). The early-flowering phenotype indicates that both genes are targets of upstream floral inductive pathways, which has been confirmed in a variety of genetic and molecular studies (Simpson et al., 1999; Blázquez and Weigel., 2000). *LFY* and *AP1* in turn bind directly to the promoters of downstream homeotic genes and activate their expression (Hill et al., 1998; Parcy et al., 1998; Tilly et al., 1998; Busch et al., 1999). There are no *LFY*-related genes in the *Arabi-*

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dopsis genome, and the DNA-binding protein encoded by *LFY* is unrelated to other classes of transcription factors (Weigel et al., 1992; Parcy et al., 1998). In contrast, *AP1* belongs to the family of MADS box genes, many of which encode transcription factors regulating different aspects of flower development (Mandel et al., 1992). The majority of plant MADS domain proteins contain three conserved regions, the MADS, I, and K domains (together the M-I-K region); however, even between orthologs, the C-terminal domain can be highly divergent (Theissen et al., 1996).

Several monocot orthologs of dicot homeotic genes, which control organ identity within flowers, have recently been characterized (Mena et al., 1995; Ambrose et al., 2000). In contrast, less is known about monocot genes controlling floral meristem identity. Expression of *RFL*, a rice (*Oryza sativa*) homolog of *FLO/LEAFY*, has been reported to deviate from that of its dicot counterparts, and based on this finding a role in panicle branching has been proposed (Kyo-zuka, et al., 1998). An *AP1*-related gene from rice, *OsMADS1*, has been characterized by transgenic and mutant analysis (Jeon et al., 2000). However, *OsMADS1* is more closely related to the *SEPALLATA* (*SEP*) genes of Arabidopsis, and its mutant phenotype, with defects in all floral organs except glumes, is similar to that of the *SEP* genes as well (Jeon et al., 2000; Pelaz et al., 2000).

Here, we report the isolation and functional characterization of *LFY*- and *AP1*-related genes from the long-day (LD) grass *Lolium temulentum*. Studies of the timing and localization of expression of *LFY*- and *AP1*-related genes at the shoot apex of *L. temulentum* has revealed both similarities and differences between these genes and their dicot homologs. Transgenic studies show that at least one of the *AP1*-related genes can partially substitute for its Arabidopsis homolog.

RESULTS

Characterization of Two *AP1*-Related Genes from *L. temulentum*

cDNAs of two genes termed *L. temulentum* MADS (*LtMADS1* and *LtMADS2*; GenBank accession nos. AF035378 and AF035379) were isolated from a PCR-based cDNA library constructed from *L. temulentum* LD III shoot apex tissue. This library was screened using two 129-bp *L. temulentum* MADS probes that had been isolated from *L. temulentum* genomic DNA using primers described in "Materials and Methods." At the RNA level *LtMADS1* and *LtMADS2* were 79% identical. The encoded proteins share 88% similar amino acids and 73% identical amino acids. An alignment of *LtMADS1* and *LtMADS2* with other MADS domain proteins from the *AP1/SQUA* clade is shown in Figure 1.

Fragments specific for the 3' ends including the untranslated regions (UTR) of each of *LtMADS1* and *LtMADS2* hybridized to single bands in a genomic DNA gel blot, indicating that they are single-copy genes (data not shown).

In comparisons involving a larger number of *AP1*-related genes, the phylogenetic trees were similar for alignments of amino acids over the four sequence regions detailed in "Materials and Methods." The consensus of the most parsimonious tree for the MADS domain data set is presented in Figure 2. This tree matched results obtained by Theissen et al. (2000) but did not include sequences for several monocots. Up-to-date information on all sequences and their accession numbers are available on a Web site listed by Theissen et al. (2000).

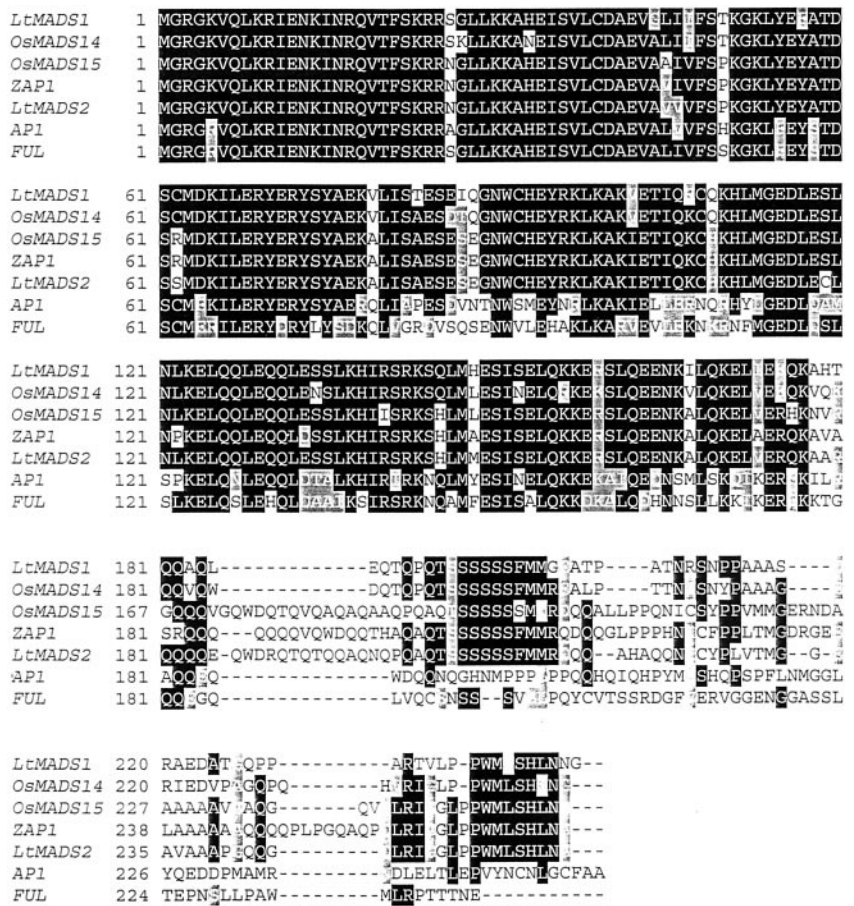
LtMADS1 and *LtMADS2* are closely related members of the monocot *AP1/SQUA* branch of the plant MADS box gene family (Fig. 2), which includes BM3, BM5, and BM8 of barley; ZAP1 and ZMM4 of maize (for the latter, only partial sequence is available); and *OsMADS14/RAP1B* and *OsMADS15/RAP1A* from rice (Fischer et al., 1995; Mena et al., 1995; Theissen et al., 1996 and EST T12733; Moon et al., 1999; Kyo-zuka et al., 2000; Schmitz et al., 2000).

LtMADS2 is 88% identical to ZAP1 (Figs. 1 and 2) and has only six amino acid changes within the M-I-K region (the first 170 amino acid residues). In this same region, *LtMADS1* has 16 differences compared with ZAP1. The high degree of sequence similarity between barley BM8, maize ZAP1, *L. temulentum* *LtMADS2*, and *OsMADS15* of rice suggests that they are orthologs (*RAP1A* appears to be the same gene as *OsMADS15*). Likely *LtMADS1* orthologs are BM5, *OsMADS14/RAP1B*, and ZMM4. Some of the *AP1/SQUA*-related proteins in dicots have a complete or partial prenylation motif at the carboxy terminus (Yalovsky et al., 2000), but this is absent from all known monocot *AP1/SQUA*-like proteins, including *LtMADS1* and *LtMADS2*.

An alternatively spliced version of *LtMADS2*, designated *LtMADS2'*, was also isolated. This cDNA had a 20-bp insertion after the sequence coding for the K box as shown in Figure 3. Comparison with genomic DNA sequence in this region showed that the insertion resulted from differential processing of intron 4. This intron has two splice donor sites, separated by 20 bases, and a single acceptor site. The *LtMADS2* transcript is produced when the entire intron of 268 bp is removed and contains an ORF with 261 codons. The *LtMADS2'* transcript results when the downstream donor site is used, adding a 20-bp insertion, which changes the reading frame (Fig. 3).

These splice sites are conserved in *AP1* and *SQUA* (Huijser et al., 1992; Mandel et al., 1992) and, although alternative splicing occurs in a *Eucalyptus globulus* *AP1* homolog (*EAP2*), it occurs in intron 7 (Kyo-zuka et al., 1997).

Figure 1. Alignment of the predicted amino acid sequences for *LtMADS1* and *LtMADS2* with some other members of the *API/SQUA* branch: Arabidopsis, *API* (accession no. Z16421) and *FUL* (accession no. U33473); *Oryza sativa*, *OsMADS14* (accession no. AF058697) and *OsMADS15* (accession no. AF058698); and *Zea mays*, *ZAP1* (accession no. L46400). Dashes indicate gaps to maximize alignment. Amino acids that are identical in four or more of the proteins are blocked. The MADS box is amino acids 1 through 60, the I region is amino acids 61 through 93, and the K box is amino acids 94 through 170.



***L. temulentum* LFY**

LtLFY (GenBank accession no. AF321273) is almost identical at the nucleotide level to an *LFY* ortholog from *Lolium perenne* (C. Anderson, unpublished data). The encoded protein is highly similar to RFL from rice, with 84% amino acid identity (Kyojuka et al., 1998). Amino acid identity to Arabidopsis *LFY* is 54% (Weigel et al., 1992). As noticed before (Frohlich and Parker, 2000), the *LFY* homolog from common rush (*Juncus effusus*) is divergent from grass *LFY* homologs, it shares only 56% identical amino acid identity with *LtLFY*. As with other *LFY* orthologs, the carboxy-terminal region, which binds DNA (M.A. Busch and D.W., unpublished data) is more highly conserved than the amino terminal region. DNA gel-blot analysis showed *LtLFY* was present as a single-copy gene in *L. temulentum*.

Expression Patterns of *LtMADS1*, *LtMADS2*, and *LtLFY*

When blots of total RNA were probed with gene-specific probes, *LtMADS1* but not *LtMADS2* RNA was detected in leaves and roots (Fig. 4). At the shoot apex, detection of *LtMADS1* and *LtMADS2* transcripts was only possible after PCR amplification. Lower bands evident in these PCR products may represent alternative splice forms, but this has not yet

been tested. Figure 4, E and F show that, compared with SD apices, expression of both genes increased within 30 h of the end of a single LD exposure. The expression of a control tubulin gene (see “Materials and Methods”) was not different between day length treatments.

To provide a basis for relating mRNA expression and flowering, the timing of floral development was examined in two independent experiments. The SD apices (solid symbols) remained vegetative over the 30-d period of the experiment (Fig. 5A). The first morphologically definitive sign of flowering was the formation of double ridges, corresponding to developing spikelet sites overtopping regressing leaf primordia (stage 2), on Days V through VII. Thereafter, floral development progressed rapidly at least until floret formation at about Day XIV, timing comparable to our many earlier studies with *L. temulentum* (see McDaniel et al., 1991). In situ hybridization using longitudinal sections of apices showed that changes in *LtMADS1* expression occurred by the time that flower formation was first visible on Day V (Fig. 5B). The three-dimensional characteristics of this early developmental change are illustrated here by a multidimensional reconstruction based on stacking/combining the digitized images of up to five sequential 10- μ m-thick sections.

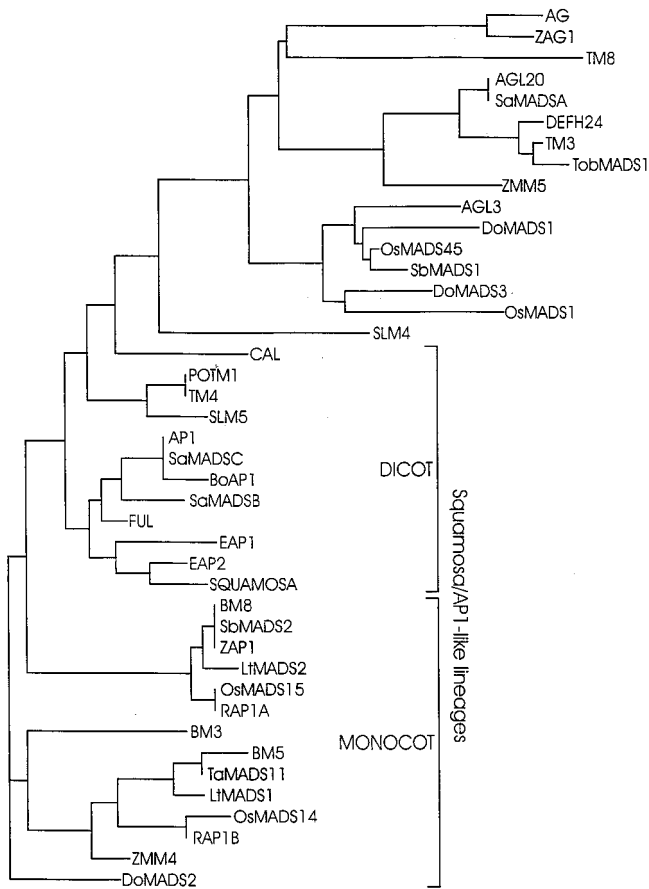


Figure 2. Phylogenetic tree based on complete MADS box domain sequences of selected members of the plant MADS box gene family. Apart from including more recent sequences the analysis and subfamily designations were based on Theissen et al. (1996). Sequences: Arabidopsis (*AP1*, *CAL*, *FUL*, *AGL3*, *AG*, and *AGL20*); snapdragon (*SQUA* and *DEFH24*); barley (*Hordeum vulgare*; *BM3*, *BM5*, and *BM8*); *Brassica oleracea* (*BoAP1*); *Dendrobium globulosum* (*DoMADS1*, *DoMADS2*, and *DoMADS3*); *Eucalyptus* sp. (*EAP1* and *EAP2*); *L. temulentum* (*LtMADS1* and *LtMADS2*); maize (*Zea mays*; *ZAG1*, *ZAP1*, *ZMM4*, and *ZMM5*); potato (*Solanum tuberosum*; *POT1*); rice (*OsMADS1*, *OsMADS14* [also known as *RAP1B*], *OsMADS15* [possibly the same as *RAP1A*], and *OsMADS45*); *Silene latifolia* (*SLM4* and *SLM5*); *Sinapis alba* (*SaMADSA*, *SaMADSB*, and *SaMADSC*); sorghum (*Sorghum bicolor*; *SbMADS1* and *SbMADS2*); tobacco (*Nicotiana tabacum*; *TobMADS1*), tomato (*Lycopersicon esculentum*; *TM3*, *TM4*, and *TM8*), and wheat (*Triticum aestivum*; *TaMADS11*; accession no. AB007504).

A detailed time course of *LtMADS1*, *LtMADS2*, and *LtLFY* expression in the single median longitudinal shoot apex sections is shown in Figures 6 and 7. Up to eight separate apices were sectioned and examined for each gene at each time, the results being reproducible between hybridizations. *LtMADS1* and *LtMADS2* were expressed in the shoot apical meristem prior to and at all stages following LD floral induction (Fig. 6; compare with Fig. 4). In contrast to the wide temporal range during which *LtMADS1* and *LtMADS2* were expressed, *LtLFY* was only expressed at the advanced double-ridge and glume stages of

development (LD XII apex in Fig. 7). *LtLFY* was not detectable at earlier or later times and was not expressed in the vegetative apex (not shown). The expression of *LtMADS1* was greater than that of *LtMADS2* at all stages analyzed, the *LtMADS2* probe requiring 2 d for color development, *LtMADS1* requiring only 8 h at an equal probe concentration (among other things, equal sensitivity of the riboprobes is assumed). There was little or no hybridization with sense control probes for *LtMADS1* (Fig. 6A, SD apex), *LtMADS2* (Fig. 6K, LD VI apex), and *LtLFY* (Fig. 7D, LD XII apex).

Increase in cell density potentially could give apparent increases in expression of these genes. However, this suggestion is clearly inappropriate for *LtLFY* given the limited time of its expression and the fact that cell density altered both prior to and after that time (Knox and Evans, 1966). A similar argument holds for expression patterns of the other two genes.

In the vegetative (SD) shoot apex, *LtMADS1* was predominantly expressed in the epidermal and subepidermal layers (three or four cell layers deep) as well as in the tips of the developing leaf primordia (Fig. 6B), whereas *LtMADS2* was expressed at a low level in the apical dome of the shoot apex (to a depth of three cell layers), but was largely excluded from the leaf primordia (Fig. 6I). Expression in leaf primordia of *LtMADS1* but not of *LtMADS2* is supported by the RNA gel-blot analysis (Fig. 4).

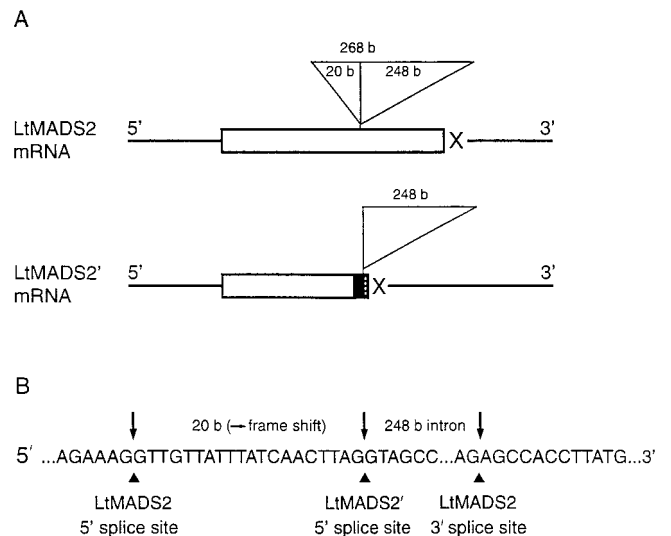


Figure 3. Scheme of alternative splicing events used to generate *LtMADS2* and *LtMADS2'* transcripts. A, The boxed sequence indicates the open reading frame (ORF) with stop codons indicated with an X and UTR as lines. Changes in the amino acid sequence of the putative translated protein from the *LtMADS2'* transcript compared with the *LtMADS2* transcript is indicated by the black (intron) and stippled boxes (frameshifted original ORF). Spliced introns are shown as triangles. B, Nucleotide sequence at the intron/exon 4 junction. Intron sequence was determined by genomic sequencing. Putative splice sites for *LtMADS2* and *LtMADS2'* transcripts are indicated with arrows. b, Bases.

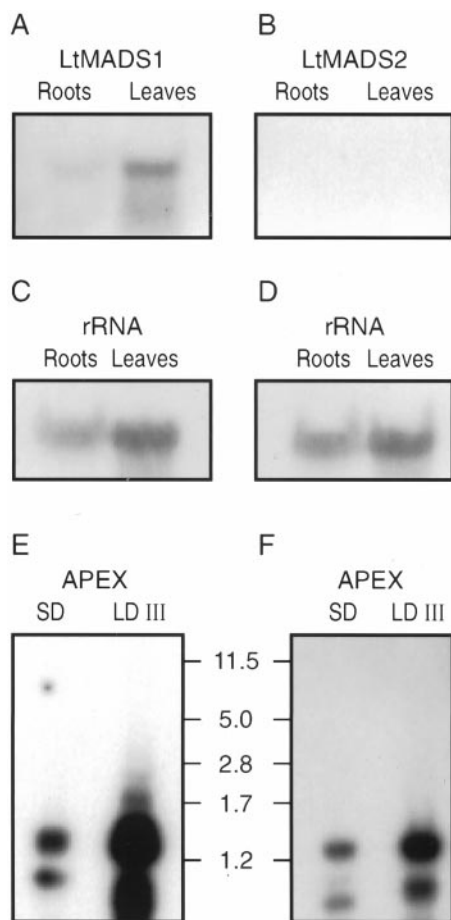


Figure 4. Gel-blot analysis of *LtMADS1* and *LtMADS2* expression in leaves, roots, and shoot apices of *L. temulentum* using the same gene specific probes for *LtMADS1* (A) and *LtMADS2* (B) as for in situ hybridization. Total RNA (20 μ g) from leaves or roots was blotted and hybridized (A and B), then both blots were stripped and reprobred (C) and with a 9-kb wheat 26S rRNA clone, pTA71 (D; Gerlach and Bedbrook, 1979). For analysis of shoot apex mRNAs, PCR-based cDNA product (virtual northern) from mRNA of short day (SD; vegetative) or LD III (florally induced) apices was hybridized with the gene specific probe for *LtMADS1* (E) or *LtMADS2* (F). Sizes in kb are indicated between the blots.

The first changes in expression pattern during inflorescence development were at LD IV for *LtMADS1* and at LD III for *LtMADS2*. *LtMADS1* was detected in spikelet sites forming in the axils of existing leaf primordia, whereas *LtMADS2* expression extended into additional cell layers within the apical dome and into the epidermal and subepidermal layers along the length of the shoot apex. At the early double-ridge stage (Fig. 6C, LD IV), *LtMADS1* expression remained concentrated around the edge of the shoot apical meristem in epidermal and subepidermal layers. However, different levels of *LtMADS1* expression defined three distinct regions within the shoot apex. Highest levels were detected in the dome of the shoot apex as well as the developing spikelet primor-

dia (circular zones indicated with arrowheads), lower levels in the leaf primordia subtending the spikelet, and lowest levels in the center or core of the shoot apical meristem. At a somewhat later stage, *LtMADS2* expression was only detected in the shoot apical meristem and within the spikelet primordia, with little expression in the regressing leaf primordia (Fig. 6L, LD VI apex).

At the advanced double-ridge stage, between LD IX (data not shown) and LD XII (Fig. 6M), each spikelet site (including the terminal dome) was elongated and more like the tip of the original SD apex. At this stage, *LtMADS1* and *LtMADS2* expression became localized to the tip (especially the abaxial side) of each spikelet (Fig. 6, C and M). Also, the expression of both genes was reduced in the region of the spikelet meristem nearest the rachis (adaxial side of the spikelet site; Fig. 6, D and M). From lemma stage until at least anther stage, *LtMADS1* and *LtMADS2* expression was localized to the epidermal and subepidermal layers (about three cell layers deep) of the apical dome of each spikelet (Fig. 6E, LD XXI apex; Fig. 6M, LD XXX apex). *LtLFY* expression was first detectable following the advanced double-ridge stage of development and it colocalized with that of *LtMADS2* on the abaxial side of the spikelet site (Fig. 7B, LDXII apex).

In addition to being expressed in the apical dome of the shoot apex and spikelet sites, *LtMADS1* (Fig. 6E) and *LtMADS2* (Fig. 6M) were expressed at the tips of developing glume primordia, which are likely the counterparts of cauline leaf or bract primordia in dicots. *LtLFY* was also detected in the glume and lemma primordia (see Fig. 7, D and E). All three genes also showed provascular expression, with *LtMADS1* and *LtLFY* being expressed, albeit transiently, in the core of the shoot apex at stages later than advanced double ridges (Figs. 6E and 7E). *LtMADS1* and *LtMADS2* were also detected in the provascular strand of the sterile glumes as they elongated (Fig. 6, F and N).

Whereas *LtLFY* expression was not detectable in either vegetative apices (not shown) or during and following floret meristem initiation (Fig. 7F), *LtMADS1* and *LtMADS2* were expressed throughout floret meristems, which form alternately along the rachilla axis of the spikelet (Fig. 6, F, G, and N, LD XXX apices). Both transcripts were present in the lemma, stamen, and carpel primordia. *LtMADS1* expression persisted in the reproductive organ primordia, but at a lower level, until the latest stage examined (anther stage; see LD XXX apices in Fig. 6H). In contrast, *LtMADS2* expression was first excluded from the carpel primordia (Fig. 6P) and later from the stamens, but persisted in the lemmas (Fig. 6O). It was difficult to adequately determine gene expression patterns in the palea and lodicule primordia because of their small size.

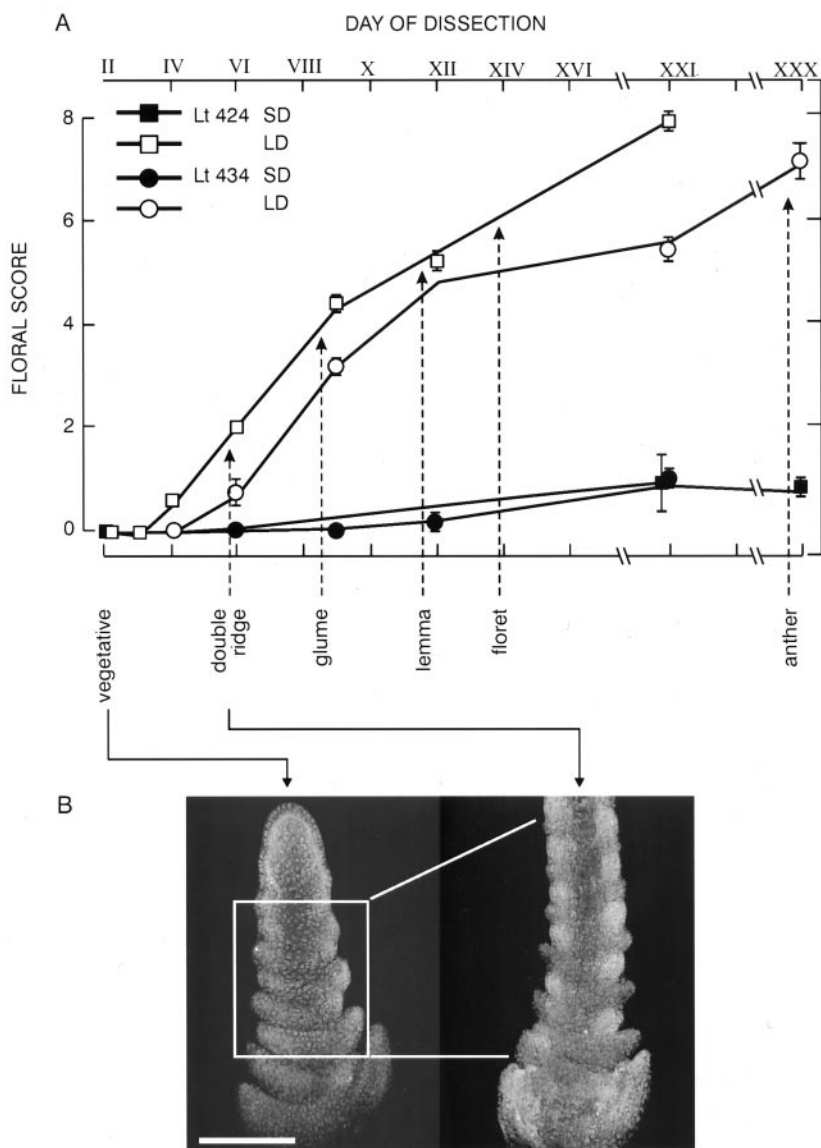


Figure 5. A, Floral score, assessed by dissection, of *L. temulentum* shoot apices as a function of time after a single inductive LD (Day II). Values are means \pm SE, $n = 10$; some error bars are hidden by the symbols. Vertical arrows are positioned to indicate developmental stages. A three-dimensional pattern of expression of *LtMADS1* mRNA (B) obtained by in situ hybridization was generated by apex reconstructions obtained by stacking/combining the images from four to five longitudinal sections up to the median one for a vegetative (SD) or LD IV early double-ridge stage apex (single median images shown in Fig. 6, B and C, respectively). Images were digitized and cropped using Adobe Photoshop and stacked using Confocal Assistant 4.02. The bar is 200 μ m.

Activity of *LtMADS1* and *LtMADS2* in Arabidopsis

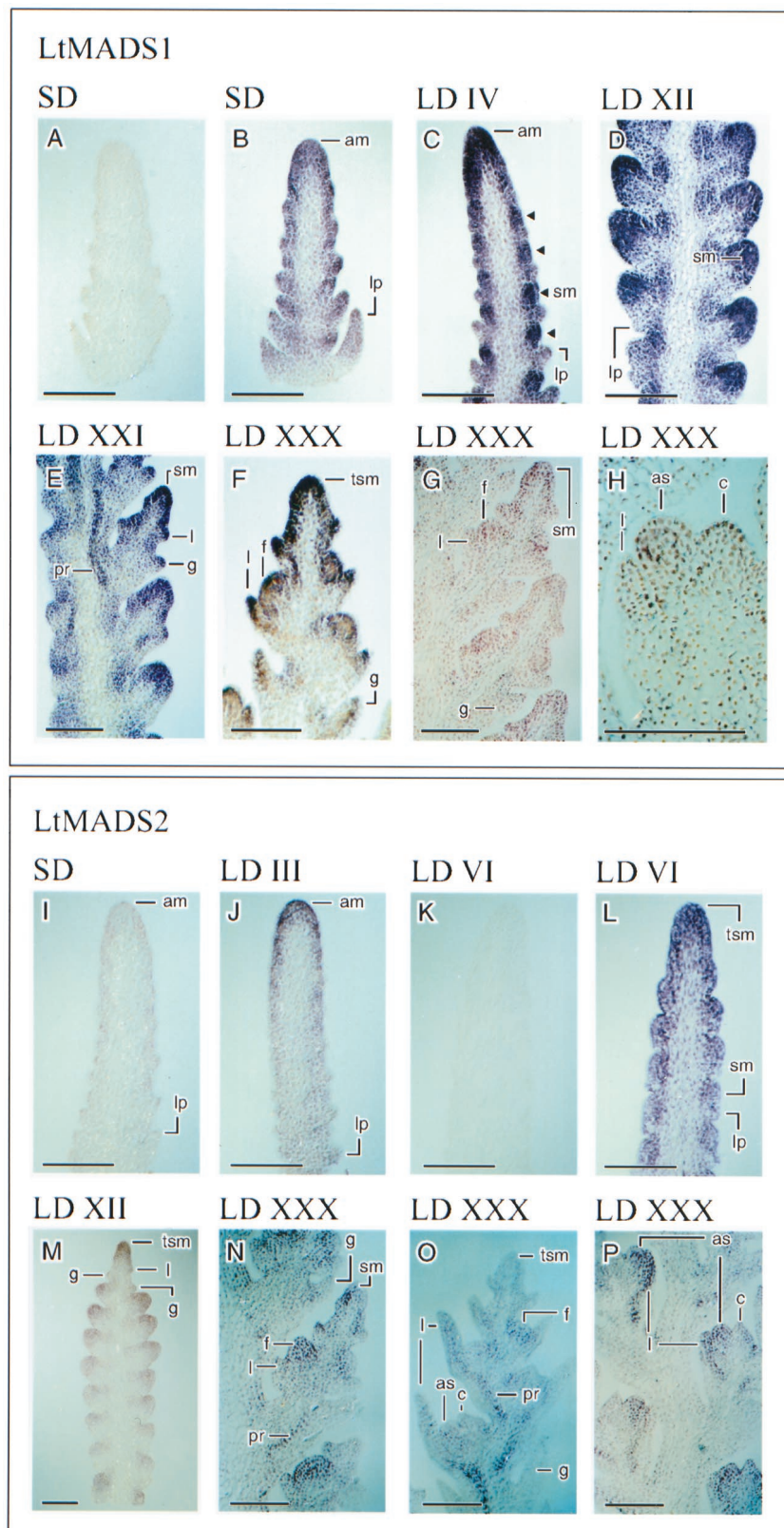
To determine whether the *L. temulentum* AP1-related genes have similar activities as Arabidopsis AP1 in vivo, we transformed into the Arabidopsis *ap1-15* mutant *LtMADS1*, *LtMADS2*, and *LtMADS2'* cDNAs under control of the 1.7-kb Arabidopsis AP1 promoter. The 1.7-kb promoter fragment has been previously shown to drive reporter gene expression that mimics activation of the endogenous gene upon floral induction (Hempel et al., 1997, 1998).

Wild-type flowers have four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl, and a gynoecium comprised of two fused carpels in the fourth whorl. *ap1-15* is a likely null allele because it has a deletion within the MADS box that should truncate the protein (M. Yanofsky, personal communication). The *ap1-15* mutant flowers typically have two or three leaf-like bracts in the first

whorl. Second-whorl organs are either absent or petaloid bracts. The third whorl is largely normal, but there are often fewer than the canonical number of six stamens. The fourth whorl is the only whorl in which organ number is unaffected, and it consists as in wild type of two fused carpels. In addition, first-whorl bracts may produce secondary flowers, or occasionally secondary inflorescences, in the axils of first-whorl bracts (Table I; Bowman et al., 1993).

Only *LtMADS2* had an obvious effect on the *ap1-15* phenotype, and increased organ number in the first, second, and third whorls, whereas the number of axillary flowers and inflorescences was reduced (Table I). Most plants had four or five bracts or sepaloid bracts in the first whorl, and one to three bracts or petaloid bracts in their second whorl (Fig. 8B; Table I). It is interesting that these plants showed gain-of-function phenotypes similar to those of plants that

Figure 6. Expression of *LtMADS1* and *LtMADS2* RNA in *L. temulentum* shoot apices, assessed by in situ hybridization. All sections were hybridized with the antisense riboprobe except for A and K, which show representative sections hybridized with a sense control riboprobe. All sections were hybridized and processed together; therefore, the intensity of antisense probe staining reflects the expression of the genes. Apex stages: vegetative shoot apices (SD; A, B, I, and J); early double ridge (LD IV; C, K, and L); lateral spikelet sites on the flanks of the shoot apex at glume/lemma stage (LD XII; D and M) or the floret stage (LD XXI; E); terminal spikelet site subtended by two glume primordia with three floret meristems positioned alternately along the terminal spikelet axis subtended by lemma primordia (LD XXX; F and O); three lateral spikelet sites at floret stage (LD XXX; G and N); and a lemma, anterior stamen, and carpel primordium (left to right) on the flank of a floret site (LD XXX; H and P). The bars are 50 μ m. am, Apical meristem; as, anterior stamen primordium; c, carpel; f, floret; g, glume; l, lemma; lp, leaf primordium; pr, provascular strand; sm, spikelet meristem; tsm, terminal spikelet meristem.



overexpress *AP1* under the control of the constitutive cauliflower mosaic virus 35S promoter (Mandel and Yanofsky, 1995a), suggesting that sequences in the

LtMADS2 cDNA interacted with the *AP1* promoter to cause ectopic expression. *LtMADS2* plants flowered early, with 7.1 ± 1.0 total leaves compared with

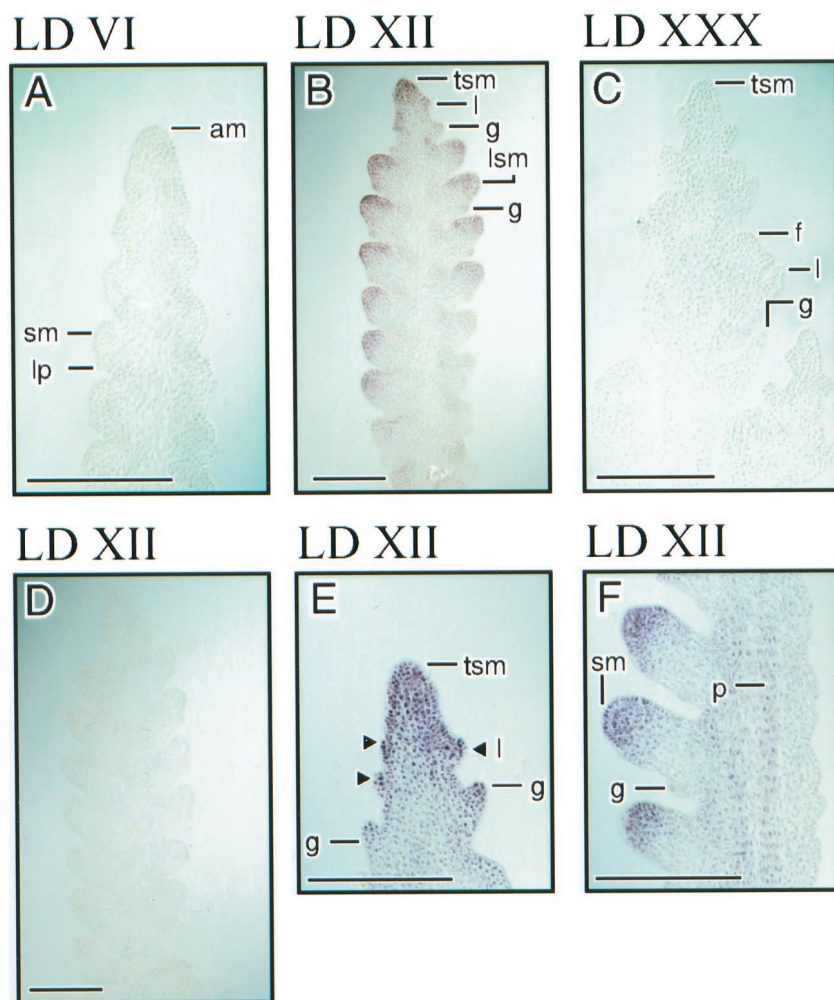


Figure 7. Expression of *LtLFY* RNA in *L. temulentum* shoot apices assessed by in situ hybridization. Sections A through C, E, and F were hybridized with the antisense riboprobe. A representative section hybridized with the sense control riboprobe is shown in D. Magnified images of B are shown in E and F. The bars are 50 μm . Apex stages and other conditions as for Figure 6.

11.9 \pm 1.1 in untransformed *ap1-15* plants (average \pm 2 \times SE of the mean). In addition, side and main shoots formed terminal flowers, similar to *35S::AP1* plants (Mandel and Yanofsky, 1995a). The effect of *LtMADS1* was more subtle, but about 10% of flowers had normal sepals, which were never observed in *ap1-15* mutants (Fig. 8A; Table I).

Floral organs of *ap1-15* mutants carrying the *LtMADS2'* transgene were similar to untransformed *ap1-15* plants. One noteworthy feature was that eight out of 230 bracts were fused, which is normally not observed in *ap1* mutants. The most obvious effect of *LtMADS2'* was that almost one-third of structures in the axils of first-whorl organs developed into indeterminate inflorescences instead of secondary flowers. In addition, secondary flowers of *LtMADS2'* plants had abnormally long pedicels as well as ectopic flowers which developed on their pedicels (Fig. 8C). Although a similar effect was not observed in *LtMADS2* plants or the *ap1-15* control population, it is unclear how significant this observation is because axillary inflorescences, albeit at a lower frequency, were also seen in *LtMADS1* flowers.

DISCUSSION

The ABC model of floral organ development has been very successful in explaining floral development of higher dicots (Coen and Meyerowitz, 1991). Many of its tenets appear to apply to monocots as well (Ambrose et al., 2000), even though there may be some divergence in lower eudicots (Kramer and Irish, 1999). The function of the floral meristem identity gene *FLO/LFY* similarly is conserved in several dicots, as revealed by similar loss-of-function mutant phenotypes (Hofer et al., 1997; Souer et al., 1998; Molinero-Rosales et al., 1999) and by the ability of constitutively expressed *LFY* to promote the conversion of shoots into flowers (Weigel and Nilsson, 1995). Less is known, however, about the role of floral meristem identity genes in monocots. Although overexpression of Arabidopsis *LFY* reduces flowering time of rice, the effects are modest compared to those seen in dicots overexpressing *LFY* (Weigel and Nilsson, 1995; He et al., 2000). Constitutive overexpression of the *LFY* homolog *RFL* similarly has no

Table 1. Phenotype of *ap1-15* mutants expressing *LtMADS1*, *LtMADS2*, or *LtMADS2'* under the control of the *AP1* promoter

Averages with $2 \times \text{SE}$ of the mean are given. Increased organ no. in whorls 1 and 2 and reduced no. of secondary flowers/inflorescences in *LtMADS2*, when compared with the other three genotypes, are statistically significant (Student's *t* test, $p < 0.001$).

	Transgene			
	None	<i>LtMADS1</i>	<i>LtMADS2</i>	<i>LtMADS2'</i>
Whorl 1				
Total organs	2.2 ± 0.3	2.5 ± 0.9	4.5 ± 0.3	2.8 ± 0.3
Sepals	0.0	0.2 ± 0.8	0.0	0.0
Bracts	2.0 ± 0.2	2.2 ± 1.0	4.3 ± 0.2	2.8 ± 0.3
Filamentous organs	0.1 ± 0.1	0.1 ± 0.3	0.2 ± 0.3	0.1 ± 0.1
Whorl 2				
Total organs	1.0 ± 0.3	1.2 ± 1.1	2.6 ± 0.6	1.0 ± 0.2
Petals	0.0	0.0	0.0	0.0 ^a
Petaloid bracts	1.0 ± 0.3	1.2 ± 1.1	1.3 ± 0.6	1.0 ± 0.2
Leaves	0.0	0.0	1.3 ± 0.7	0.0
Whorl 3				
Total organs	5.1 ± 0.3	5.2 ± 1.1	6.6 ± 0.5	5.7 ± 0.2
Stamens	4.1 ± 0.4	4.8 ± 1.1	5.7 ± 0.8	4.8 ± 0.3
Mosaic stamens	0.9 ± 0.4	0.3 ± 0.7	1.0 ± 0.7	0.9 ± 0.3
Filamentous organs	0.1 ± 0.1	0.2 ± 0.5	0.1 ± 0.2	0.1 ± 0.1
Flower No.	28	86	22	84
2° Flowers/inflorescences	1.6 ± 0.3	1.6 ± 0.1	0.3 ± 0.3	1.8 ± 0.1

^a One petal in 84 flowers.

dramatic effects on flowering of *Arabidopsis* (Kyo-zuka et al., 1998).

Here for the grass *L. temulentum*, we have taken advantage of extensive prior knowledge of the timing of its flowering in response to a single LD to examine expression of *LFY*- and *AP1*-related genes at different steps in floral evocation and development. Based on their expression early and in perianth-type organs, the *L. temulentum* *AP1*-like genes fit the concepts developed for dicots but there are difficulties when considering our findings for *LtLFY*, whose expression is not inextricably linked with the first events of floral evocation at the shoot apex.

LtMADS1 and *LtMADS2* and Comparison with Other *AP1/SQUA* MADS Box Genes

The two *L. temulentum* MADS box genes, *LtMADS1* and *LtMADS2*, share greatest similarity with the *AP1/SQUA* clade of the plant MADS box gene family and particularly with other monocot members of this branch (Figs. 1 and 2). The extensive similarity of *LtMADS1* with *LtMADS2* suggests that they have redundant functions, similar to *AP1* and the two *AP1*-related genes in *Arabidopsis*, *CAULIFLOWER* (*CAL*) (Kempin et al., 1995) and *FRUITFULL* (*FUL*; Ferrándiz et al., 2000). There are also two *AP1*-like genes in rice (Moon et al., 1999; Kyo-zuka et al., 2000) and three in barley (Schmitz et al., 2000).

Whereas the M-I-K regions of members of the *AP1/SQUA* clade of plant MADS domain proteins share greater than 80% sequence identity, the carboxy terminus is less conserved. In addition to a Ser-rich motif in the monocot members of this clade, the

sequence "LPPWMLSHL/IN" is conserved. A similar sequence "LPAWML" is found in *FUL* and *SaMADSB* from *S. alba* (Menzel et al., 1996). None of the monocot proteins contain the CaaX box that is present in several *AP1*-related proteins of dicots, and that allows prenylation of the protein by farnesyl-transferases (Yalovsky et al., 2000).

Aside from similarity of the gene sequences, a function similar to *SQUA* and *AP1* in dicots is supported by our studies of expression patterns of the two genes. In the shoot apex, *LtMADS1* and *LtMADS2* were detected in the apical dome of vegetative plants and, as for all *AP1/SQUA*-related genes, expression is strong in the floral meristem early in floral development. In common with maize (Mena et al., 1995) and rice (Kyo-zuka et al., 2000), later expression of *LtMADS1* and *LtMADS2* became restricted to perianth primordia, including the glumes, lemma, palea, and probably the lodicules. Expression in the epidermal and subepidermal layers and its exclusion from the central core of the spikelet sites indicates further similarity between *LtMADS1*, *FUL*, and *SaMADSB*, which are excluded from the pith of the inflorescence apex (Mandel and Yanofsky, 1995b; Menzel et al., 1996). Elsewhere in the plant the expression of *LtMADS1* in leaves and roots is more similar to that of *FUL* and *SaMADSB* than to that of *AP1*, *SQUA*, or *ZAP1*. In contrast, there was no expression of *LtMADS2* in leaf and root tissue that fits with RNA-blot data for *ZAP1* (Mena et al., 1995), *RAP1B* in rice (Kyo-zuka et al., 2000), and BM8 in barley (Schmitz et al., 2000). The implication that *LtMADS1* and *LtMADS2* function in floral evocation

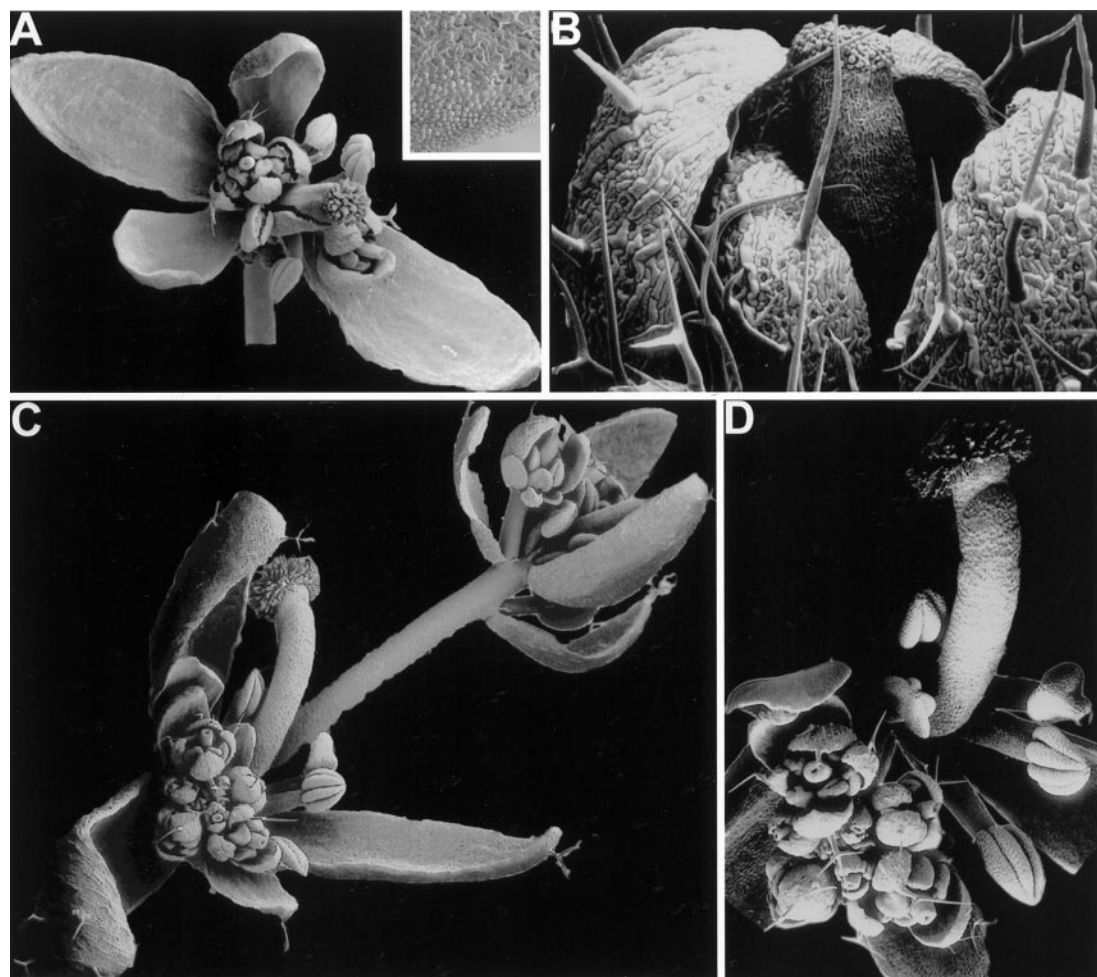


Figure 8. Scanning electron micrographs of flowers from plants expressing *LtMADS1*, *LtMADS2*, and *LtMADS2'* under the control of a 1.7-kb fragment of the AP1 promoter. A, Flower from *LtMADS1* transgenic plant with two petaloid bracts in the perianth (arrows). The inset in A shows petaloid cells on the margin of the lower bract-like petal at high magnification (size bar = 100 μ m). B, Flower from *LtMADS2* transgenic plant. A first-whorl bract has been removed to reveal a second-whorl bract (arrow). C, Inflorescence from *LtMADS2'* transgenic plant. An axillary flower with an abnormally long pedicel is visible in the upper right hand corner. D, Enlargement of flower in lower left hand corner of C. Size bars are 1 mm, except 200 μ m in C.

and development is further supported by the ability of *LtMADS2* to partially complement *ap1-15* organ number defects as well as to transform the shoot into floral meristem (Fig. 8; Table I).

It is interesting that both *LtMADS1* and *LtMADS2* are expressed within the provascular strands in the central core of the meristem and in the glumes and lemmas. Provascular expression is also observed for *SaMADSB* (see Menzel et al., 1996). Any *LtMADS2* expression in the carpel and stamen primordia was only transient early during their initiation. Overall, in *L. temulentum*, the intensive expression of *LtMADS1* and *LtMADS2* within the dome of the floral shoot apex, as well as in spikelet and floret meristems indicates that these genes may have a floral meristem identity function as well as a function in floral organ identity.

Comparison of *LtLFY* with Other *LFY* Homologs

Kyozuka and colleagues (1998) have previously described the expression pattern of *RFL*, the rice *LFY* homolog. Like *LtLFY*, *RFL* expresses in young panicles but in contrast, *RFL* is also expressed vegetatively. In this respect, *RFL* is similar to *LFY* and its dicot orthologs, with the exception of *FLO* from snapdragon (Coen et al., 1990; Bradley et al., 1996; Blázquez et al., 1997; Hofer et al., 1997; Pnueli et al., 1998; Souer et al., 1998). Vegetative expression has also been found for *NEEDLY* (*NLY*), a *LFY* homolog from gymnosperms. It is interesting that although *NLY* is more divergent from *LFY* than *RFL* both with respect to sequence similarity and origin, when ectopically expressed in *Arabidopsis* *NLY* is much more potent than *RFL* in transforming shoots into flowers (Kyozuka et al., 1998; Mouradov et al., 1998).

Although we have not performed this experiment for *LtLFY*, its high sequence similarity with rice *RFL* suggests a similar outcome.

Timing of Expression of *LtMADS1*, *LtMADS2*, and *LtLFY* during Inflorescence Initiation

Characterization of the events of *L. temulentum* floral induction over the past 40 years has precisely defined the timing of biochemical changes at the shoot apex. These changes occur within hours to days of a single inductive LD and morphological change is first evident within several days (Fig. 5). Early expression of the *L. temulentum* *AP1*-like genes can now be added to this sequence of events then, much later, *LtLFY* expresses (compare Fig. 6 with Fig. 7). Up-regulated expression of *LtMADS1* and *LtMADS2* in lateral and terminal spikelet sites was evident by the afternoon of LD III (30 h after the end of the LD; Figs. 5 and 6) and this correlates with the time of commitment of the shoot apical meristem to flower as assessed by culturing induced apices (McDaniel et al., 1991). At the time of increased *AP1* expression at the *L. temulentum* apex on LD III, there are also increases in cell division at the apical dome (Ormrod and Bernier, 1990) and RNA and DNA staining increases dramatically in the terminal and lateral spikelet sites (Knox and Evans, 1966) where *LtMADS1* and *LtMADS2* express (Figs. 5 and 6). In Arabidopsis *AP1* is also expressed early during the floral transition (within 2 d) as it is in *S. alba* where, because the latter species flowers with exposure to a single LD, it is clear that, expression of the two *S. alba* *AP1* genes correlates with commitment to flower (Menzel et al., 1996), as also in *L. temulentum*.

The much later expression of *LtLFY* compared with *LtMADS1* and *LtMADS2* contrasts with snapdragon and Arabidopsis, where increased *FLO/LFY* expression precedes *AP1* activation (Coen et al., 1990; Huijser et al., 1992; Bradley et al., 1996; Blázquez et al., 1997; Hempel et al., 1997). Further, at least in early arising flowers, *LFY* is required for *AP1* up-regulation (Liljegren et al., 1999) and *LFY* protein has been shown to be a direct regulator of *AP1* (Parcy et al., 1998; Wagner et al., 1999). Our findings indicate, therefore, that this hierarchy of regulation involving floral meristem identity genes may not be conserved in monocots. As an alternative, however, when *LtLFY* does express it may act directly on the *L. temulentum* *AP1*-like genes but that, at earlier times during floral evocation, these same *AP1*-like genes may be acting independently of *LtLFY*.

MATERIALS AND METHODS

Plant Material

Lolium temulentum strain Ceres plants were grown as described elsewhere (Evans et al., 1990) in natural daylight of 8-h SD for 5 weeks. Tillers were removed and the plants

transferred to SD in cabinets illuminated with fluorescent and incandescent lamps providing a total irradiance of 250 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. One week later the plants were exposed to a single inductive LD, designated as LD I, imposed as a 16-h extension of the 8-h SD photoperiod at a low irradiance (10–12 $\mu\text{mol m}^{-2} \text{s}^{-1}$) from incandescent lamps. Two directly related measures of inflorescence development were employed: floral stage and shoot apex length (Evans et al., 1990). To allow comparison of the timing of floral development across experiments, floral stage was determined by daily dissections until at least Day XII.

For analysis of changes in mRNA, shoot apices (0.8 mm long on Day I) were harvested at various times following the single LD exposure (floral) or in parallel for the SD (vegetative) controls. The apices were immediately placed on dry ice and later stored at -70°C prior to RNA extraction.

Construction of PCR-Based cDNA Libraries

As described in Gocal (1997), PCR-based cDNA libraries were made from shoot apices from either SD vegetative or LD III plants (30 h after the end of the single LD). Primary libraries consisted of between 2 and 4×10^6 plaques with the portion of empty vector being less than 5%, and the average insert size being 1.2 kb. Although each library was constructed from a small amount of tissue (about 10 mg fresh weight), multiple PCR products from the same transcript were never isolated in our library screens. Of 27 full-length clones isolated to date from *L. temulentum* shoot apex PCR-based libraries, their poly-A tails ranged from four to 22 nucleotides and their 5'-UTRs range from 80 to 140 nucleotides.

Generation of MADS Box Fragments

A 129-bp fragment of the MADS box was PCR amplified from *L. temulentum* genomic DNA using a touchdown program with a degenerate oligonucleotide primer pair. MADS-1 [5'-d(CGGAATTCATGGGNMGNGGNAARRT)-3'] was identical to nucleotides 1 through 17 (underlined; restriction endonuclease sites indicated in bold), with MADS-43 [5'-d(CGGGATCCACIACYTGIGCRCTCRCAIARIA-C)-3'] being complementary to nucleotides 111 through 129 (underlined; designed by Dr. Steve Strauss, Oregon State University, Corvallis). Single bands representing putative *L. temulentum* MADS box gene fragments were gel purified and cloned as *EcoRI/BamHI* fragments into pBluescript SK⁺ plasmid. The four inserts sequenced were from two genes.

Library Screening

Aliquots of the *L. temulentum* LD III apex-specific PCR-based cDNA library were screened with the two *L. temulentum* MADS fragments (see above) after ³²P-dCTP labeling (Megaprime DNA labeling system, Amersham, Sydney). Hybridization was overnight in 5× sodium chlo-

ride/sodium dihydrogen phosphate/EDTA buffer (SSPE) and 0.5% (w/v) SDS, 5× Denhardt's solution, and 25 $\mu\text{g mL}^{-1}$ sheared salmon sperm DNA at 65°C. Washes were for 30 min at 65°C in 2× SSPE and 0.1% (w/v) SDS (two washes) and a final high stringency wash in 0.1× SSPE and 0.2% (w/v) SDS.

Isolation of *L. temulentum* LEAFY (*LtLFY*)

Two approaches were used to isolate *LtLFY*. First, a 206-bp fragment of *LFY* was amplified from *L. temulentum* genomic DNA using the degenerate primers *Glfy1* [5'-d(CGGAATTCATGCGVCACTACGTG/TCACTG)-3'] and *Glfy2* [5'-d(CGGGATCCACATACCARATVGMGAGRC-GVGG)-3'] from the conserved C terminus (amino acids 310–377 from exon 3) of *LFY*. A single band representing a putative *L. temulentum* *LFY* gene fragment was gel purified and cloned as an *EcoRI/BamHI* fragment into pBluescript SK⁺ plasmid. Second, a UniZAP Custom cDNA library (Stratagene, La Jolla, CA) was made from *L. temulentum* using mRNA isolated with Dynal beads. The source tissue included inflorescences, leaves, and stems. The library was screened at medium stringency (0.1× SSC, 0.1% [w/v] SDS; 2 × 30 min at 65°C) with the ORF from an *LpLFY* cDNA clone previously isolated from *Lolium perenne* (C. Andersen, unpublished data).

Sequencing and Sequence Analysis

Cloned DNA fragments were sequenced on both strands with either Dye Primer or DyeDeoxy Terminators using sequencers (PRISM 310 or 377, ABI, Foster City, CA). Sequence analysis was done using the University of Wisconsin GCG version 8 software package (Devereux et al., 1984), or Sequencer v4.0.2 software (Gene Codes Corporation, Ann Arbor, MI). PCR-based errors were detected by comparing the sequence for at least three clones over the entire ORF.

To determine the phylogenetic relationship of *LtMADS1* and *LtMADS2* within the plant MADS box gene family, protein sequences were first aligned using the PILEUP program of the GCG version 8 package. The various sequences examined were: (a) the MADS domain alone; (b) the MADS domain and I region; (c) the MADS domain, I region, and K domain; and (d) the entire protein sequence. Partial sequences were completed with ?s. Phylogenetic trees were constructed for each data set using the PROTPARS program of the phylogenetic inference package (PHYLIP) as described in Theissen et al. (1996). The input order of the sequences was randomized using the jumble option in the PROTPARS program and repeated ten times. Multiple most parsimonious trees were found in each instance and the consensus tree was determined by using the PHYLIP program CONSENSE. The consensus most parsimonious tree was drawn for each data set using the PHYLIP program DRAWTREE.

PCR-Based (Virtual) Northern-Blot Analysis

All PCR-based northern blots presented here were prepared from a single cDNA synthesis using 250 shoot apices

from plants in SD or LD (Day III) Apices from this experiment (Lt424) reached the double-ridge stage on LD VI. The PCR-amplified cDNA was suspended in 65 μL of Tris/EDTA (pH 7.6) and 10 μL of each of the SD and LD III pools were electrophoresed in parallel on six 1.2% (w/v) agarose gels, blotted, hybridized, and washed as above. Virtual northern blots produced from different amplifications hybridized with the same probe always yielded similar results. To compare loading, each set of blots was also probed with a fragment of an α -tubulin gene from *L. temulentum* (not shown; G.F.W. Gocal, unpublished data).

RNA Gel-Blot Analysis

Leaf and root RNA were isolated by the method of Chandler et al. (1983). For RNA gel-blot analysis, 20 μg of total RNA was size fractionated by gel electrophoresis under denaturing conditions in a 1% (w/v) agarose gel with formaldehyde, transferred to a Hybond-N membrane (Amersham), and hybridized as above. To compare RNA loading across lanes, following autoradiography, RNA gel blots were stripped and reprobed with a 9-kb wheat rRNA clone, pTA71 (Gerlach and Bedbrook, 1979).

In Situ Hybridization

Shoot apices were processed according to Gocal (1997). To determine the temporal (vegetative through LD XXX) and spatial localization of the *LtMADS1*, *LtMADS2*, and *LtLFY* mRNAs within the apex following floral induction, 10- μm longitudinal sections were prepared. In vitro transcribed Digoxigenin-labeled riboprobes were synthesized for a 3' gene-specific fragment of *LtMADS1* (MADSc1/2; nucleotide 650–1062) or *LtMADS2* (MADSr1/2; nucleotide 568–874) and for the conserved C-terminal region of *LtLFY*. Sense and antisense riboprobes for each gene were generated from the T7 promoter using PCR-generated 3' gene-specific fragments, which for *LtMADS1* and *LtMADS2* were cloned as *EcoRI/HindIII* fragments and for *LtLFY* as an *EcoRI/BamHI* fragment in pBluescript SK⁺ or pBluescript KS⁺. Hybridizations were with an equivalent sense or antisense probe concentration and were performed simultaneously on each tissue set. Most of the in situ results presented were from material collected in one experiment (Lt434).

T-DNA Constructs

cDNAs of *LtMADS1*, *LtMADS2*, and its alternatively spliced version *LtMADS2'*, were cloned between a 1.7-kb fragment of the Arabidopsis *APETALA1* promoter upstream of the ATG (*HindIII/EcoICR* I; Hempel et al., 1997) and the pea (*Pisum sativum*) RBCSE9 terminator (accession no. M21375) in a pBluescriptKS⁺ (Stratagene) derivative termed pGG62. Each *pAPI:LtMADS:RBCSE9* construct was shuttled as a *HindIII* fragment into pCGN1547 (McBride and Summerfelt, 1990). These constructs were introduced via *Agrobacterium tumefaciens*-mediated vacuum infiltration into *apetala1-15* mutant plants (Columbia ecotype;

Bechtold et al., 1993). Transformants were selected on 0.5× Murashige and Skoog plates containing 50 μg mL⁻¹ kanamycin. At least 20 T1 lines were obtained per construct and between four and six were chosen randomly for subsequent analysis. For *LtMADS1* and *LtMADS2'*, floral organs were counted for the first four to eight flowers and for *LtMADS2* all flowers were counted (up to four per plant). Inflorescences were fixed and examined using scanning electron microscopy as described (Weigel et al., 1992).

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