Floral Meristem Identity Genes Are Expressed during Tendril Development in Grapevine[1]

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To study the early steps of flower initiation and development in grapevine (*Vitis vinifera*), we have isolated two MADS-box genes, *VFUL-L* and *VAP1*, the putative *FUL*-like and *AP1* grapevine orthologs, and analyzed their expression patterns during vegetative and reproductive development. Both genes are expressed in lateral meristems that, in grapevine, can give rise to either inflorescences or tendrils. They are also coexpressed in inflorescence and flower meristems. During flower development, *VFUL-L* transcripts are restricted to the central part of young flower meristems and, later, to the prospective carpel-forming region, which is consistent with a role of this gene in floral transition and carpel and fruit development. Expression pattern of *VAP1* suggests that it may play a role in flowering transition and flower development. However, its lack of expression in sepal primordia, does not support its role as an A-function gene in grapevine. Neither *VFUL-L* nor *VAP1* expression was detected in vegetative organs such as leaves or roots. In contrast, they are expressed throughout tendril development. Transcription of both genes in tendrils of very young plants that have not undergone flowering transition indicates that this expression is independent of the flowering process. These unique expression patterns of genes typically involved in reproductive development have implications on our understanding of flower induction and initiation in grapevine, on the origin of grapevine tendrils and on the functional roles of *AP1*-and *FUL*-like genes in plant development. These results also provide molecular support to the hypothesis that *Vitis* tendrils are modified reproductive organs adapted to climb.

The early phases of reproductive development and flower formation have been well characterized in the herbaceous model plants Arabidopsis and *Antirrhinum majus* (Egea-Cortines and Davies, 2000; Theissen, 2001; Mouradov et al., 2002; Simpson and Dean, 2002). In Arabidopsis, transition to flowering is dependent on the activity of floral meristem identity genes such as *LEAFY* (*LFY*), *FRUITFULL* (*FUL*), *APETALA1* (*AP1*), and *CAULIFLOWER* (*CAL;* for review, see Pidkowich et al., 1999; Simpson et al., 1999; Kieffer and Davies, 2001; Zik and Irish, 2003). *LFY* expression in lateral meristems seems to be responsible for their acquisition of flower meristem identity (Blázquez et al., 1997). *FUL*, *CAL*, and *AP1* are closely related MADS-box genes that belong to the *SQUAMOSA* (*SQUA*) gene subfamily, also called *AP1/FUL* gene lineage (Theissen et al., 2000; Becker and Theissen, 2003; Litt and Irish, 2003). Together with *LFY*, they play functionally redundant roles in the control of flower meristem identity (Mandel et al., 1992; Weigel et al., 1992; Mandel and Yanofsky, 1995; Liljegren et al., 1999; Ratcliffe et al. 1999; Ferrándiz et al., 2000). In Arabidopsis, the patterns of expression of these genes relate well with their proposed function. *FUL* is up-regulated in the shoot apical meristem (SAM) around the transition to flowering and before the initiation of flower meristems. *LFY* and *AP1* are expressed in emerging flower meristems, while *FUL* is soon excluded from this region (Weigel et al., 1992; Mandel and Yanofsky, 1995). Apart from their function as floral meristem identity genes, *AP1* and *FUL* also seem to play roles as organ identity genes at later stages of flower development. *AP1* was initially defined as a class A gene involved in sepal and petal identity (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Theissen, 2001). *FUL* has been shown to play a role in carpel and fruit development that affects valve, replum, and style morphology (Gu et al., 1998; Ferrándiz et al., 2000).

Genetic and molecular characterization of the flowering process in different species reveals a conservation of the basic genetic mechanisms controlling the early stages of flower formation (Theissen and Saedler, 1999; Ng and Yanofsky, 2001). However, when homologous genes from species other than Arabidopsis have been

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analyzed, differences—regarding expression patterns and mutant phenotypes—have been found that may reflect distinct roles from those described for the Arabidopsis genes. For instance, the lack of examples of A-function mutants in most angiosperms analyzed questions a general role of *AP1*-like genes as A-function genes (Huijser et al., 1992; Yu et al., 1999; Litt and Irish, 2003). Moreover, in monocots, *SQUA*-like genes do not seem to be always functional orthologs of their Arabidopsis counterparts, based on their relatively large number and expression patterns (Schmitz et al., 2000; Theissen et al., 2000; Yu and Goh, 2000; Gocal et al., 2001). More recent studies have even questioned the existence of the *AP1* gene lineage outside core eudicots (Litt and Irish, 2003). All these data suggest that flower initiation and development may involve common regulatory mechanisms to all the angiosperms as well as species-specific mechanisms whose genetic and molecular bases are yet unknown.

We are studying the reproductive biology of grapevine (*Vitis vinifera*), a woody perennial vine with a pattern of organ formation and development quite distinct to those previously described for annual herbaceous plants (Mullins et al., 1992). Wild grapevines are vigorous climbing plants with pressure-sensitive tendrils that allow them to climb into the forest canopy to a height of 20 to 30 m, supported by the forest trees. When grown from seeds, grapevine seedlings undergo a short-lived juvenile phase during which they produce leaves with a spiral phyllotaxis for the first 6 to 10 nodes. Later on, phyllotaxis changes and this event marks the transition to the adult phase of the plant. In adult plants phyllotaxis is alternate, with the SAM producing a characteristic sequence of leaves and lateral meristems, historically called *uncommitted primordia* (Tucker and Hoefert, 1968; Pratt, 1974; Gerrath and Posluszny, 1988a; Gerrath et al., 1998). Due to unequal internode elongation, lateral meristems that arise alternate to leaf primordia become opposed to leaves in the expanded shoot. Lateral meristems give rise to tendrils for a long period of time (2–5 years) before the plant initiates flowering. Upon flowering induction, inflorescences are formed in place of tendrils from the same lateral meristems (Pratt, 1971; Srinivasan and Mullins, 1981; Posluszny and Gerrath, 1986; Gerrath and Posluszny, 1988a; Morrison, 1991). As they develop from the same meristematic structures, tendrils and inflorescences have been proposed to be homologous organs. Furthermore, inflorescences and tendrils can substitute for each other depending on environmental conditions or hormonal treatments, and occasionally tendrils can give rise to flowers and fruits (Darwin, 1875; Bugnon, 1953; Pratt, 1971, 1974; Srinivasan and Mullins, 1981; Boss and Thomas, 2000, 2002). The presence of lateral meristems that give rise to tendrils, inflorescences, or intermediate organs and the production by the SAM of both leaves and lateral meristems that become opposed at maturity are a special feature of the Vitaceae. Attempts to explain their unusual organs position and their origin at the SAM have generated equally plausible interpretations on shoot formation, either sympodial or monopodial (Gerrath and Posluszny, 1988a; Gerrath et al., 1998).

In temperate regions, grapevine requires two consecutive growing seasons to flower. A rise in light intensity and temperature seems to be required to promote flowering (Butrosse, 1974; Mullins et al., 1992) that is induced in latent summer buds. Upon flowering induction, the SAM produces a few (2–3) lateral meristems that will give rise to inflorescence meristems. The inflorescence meristems form several inflorescence branch meristems before the bud enters dormancy at the end of the summer. The next spring, additional inflorescence branch meristems are formed before each one divides into a cluster of 3 to 4 flower meristems that develop into flowers (Srinivasan and Mullins, 1981; Gerrath and Posluszny, 1988b; Gerrath, 1993; May, 2000; Carmona et al., 2002).

Because of its particular developmental features, we are interested in the process of flower initiation in grapevine. Furthermore, grapevine belongs to the Vitaceae, a basal family of core eudicots (Judd et al. 1999), and understanding the genetic and molecular control of flower initiation and development in this family can shed light on the evolution of the regulation of flower development in angiosperms. Previously, we isolated *VFL*, the grapevine ortholog of *FLO/LFY*, and described its characteristic expression patterns in meristematic regions (Carmona et al., 2002). We have now investigated the role of other meristem identity genes during flower initiation and development. We have identified two genes within the *SQUA* subfamily, *VFUL-L* and *VAP1*, which could correspond to the grapevine *FUL*-like and *AP1* orthologs. Our results are consistent with *VFUL-L* having a role in floral transition and carpel and fruit development. In contrast, the expression patterns of *VAP1* do not support its role as an A-function gene. Unexpectedly, both genes are highly expressed during tendril development. These unique expression patterns of genes traditionally involved in reproductive development are discussed in relation to the special features of grapevine development and the ontogenetic relationship between tendrils and inflorescences.

RESULTS

Isolation of Grapevine *FUL*- and *AP1*-Like Genes

Genes belonging to the SQUA subfamily of MADS-box transcription factors were isolated using a 3′/5′RACE strategy. Two different cDNA types were identified. One of them (GenBank accession no. AY538747) contained an ORF of 741 bp preceded by a 5′-untranslated region of 83 bp. The 3′-untranslated region showed length heterogeneity, with four polyadenylation sites at positions 910, 997, 1012, and 1029 bp. The other cDNA type (GenBank accession no. AY538746) contained an ORF of 723 bp and the

5′-untranslated region was 166 bp in length. Two polyadenylation sites at positions 1058 and 1075 were detected in its 3′-untranslated region. The encoded MADS-box proteins (247 and 241 amino acids, respectively) aligned well with those from the SQUA subfamily (Fig. 1, A and B) based on sequence conservation in the I-region, which is typical for each MADS-box subfamily (Fig. 1, A and B, continuous line; Elo et al., 2001). Furthermore, differences at the beginning of the K-box and especially at the C terminus, where a prenylation motif (CaaX) is typical of AP1/SQUA-like proteins (Rodriguez-Concepción et al., 1999) but is absent in FUL-like proteins, allowed us to identify the grapevine MADS-box proteins as belonging to the FUL and AP1/SQUA clades (Fig. 1, A and B, dashed lines, dotted line, and white box; Theissen et al., 2000;

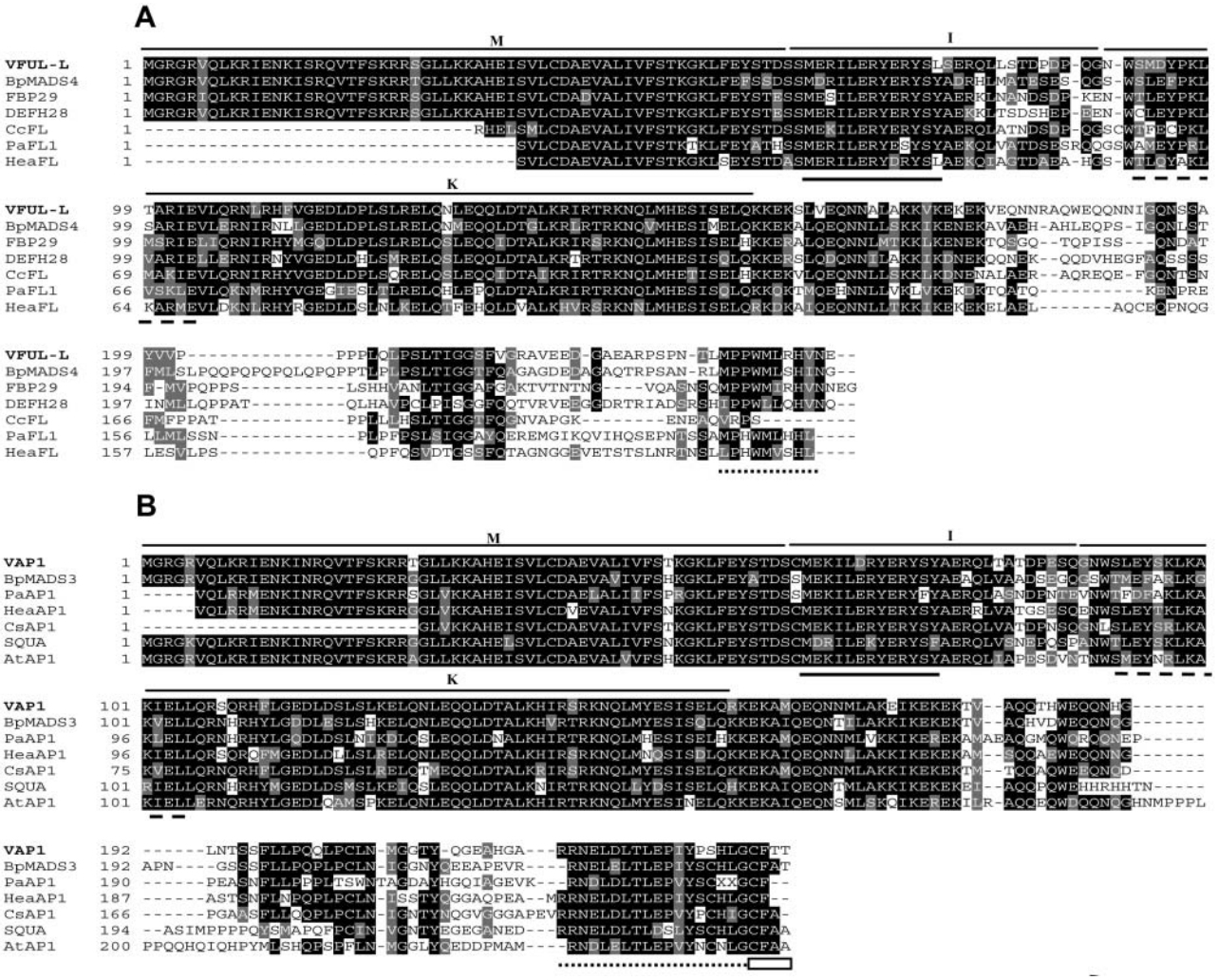

Figure 1. Sequence analysis of VFUL-L, VAP1, and related SQUA-like proteins. A, VFUL-L protein and sequence comparison to other FUL-like proteins. B, VAP1 protein and sequence comparison to AP1/SQUA-like proteins. In both alignments, the MADS-box (M), I-region (I), and K-box (K) are indicated. In the I-region, the sequence highly conserved between both proteins is underlined with a continuous line. Dashed lines indicate distinctive sequences in the K-box. The highly divergent C-terminals show some conserved motives in each group (dotted line), mainly in the AP1 group that includes the prenylation motif (white box). C, Phylogenetic tree of the predicted SQUA-like proteins from different eudicots plant species (accession no. in parentheses): *Antirrhinum SQUA*, *DEFH28*, *AmFUL* (X63701, AY040247, AY306139); Arabidopsis *AP1*, *CAL*, *FUL*, *AtFL* (Z16421, L36925, AY072463, Q9LI72); Apple *MdMADS2, MdMADS5* (U78948, AJ000759); *Betula pendula BpMADS3, BpMADS4, BpMADS5* (X99653, X99654, X99655); Cauliflower *BOAP1* (Z37968); *Clarkia concinna CcFL* (AY306143); *Corylopsis sinensis CsAP1*, *CsFUL* (AY306146, AY306147); *Eucalyptus EAP1* (AF305076); *Heuchera americana HeaAP1, HeaFL, HeaFUL* (AY306148, AY306149, AY306150); *Nicotiana sylvestris NsMADS2* (AF068726); *Nicotiana tabacum NAP1-1, NAP1-2, NTMADS5* (AF009126, AF009127, AF068724); *Petunia PFG*, *FBP26*, *FBP29* (AF176782, AY370517, AF335245); Pea *PEAM4* (AF461740); *Phytolacca americana PaAP1*, *PaFL1*, *PaFL2*, *PaFUL* (AY306160, AY306161, AY306162, AY306163); Potato *POTM1-1*, *SCM1* (U23757, AF002666); *Silene latifolia SLM5* (X80492); *Sinapis alba SaMADSC*, *SaMADSB* (Q41276, Q41274); Tomato *TRD4* (AY098732). Bootstrap support values are indicated when over 50. In order to root the SQUA subfamily, *Arabidopsis* SEP1 (M55551), SEP2 (M55552), and SEP3 (AF015552) were used as outgroup.

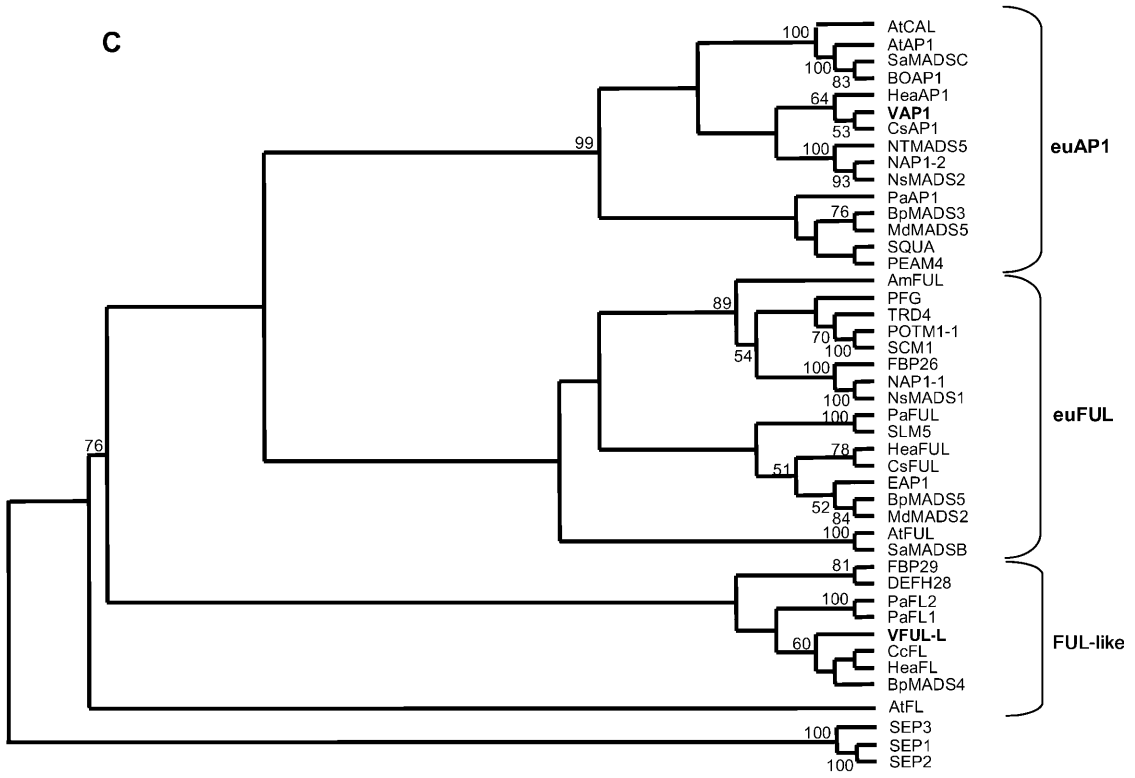

Figure 1. (*Continued.*)

Berbel et al., 2001; Elo et al., 2001; Becker and Theissen, 2003).

Recent phylogenetic analyses have identified two clades of *FUL*-like genes in core eudicots, eu*FUL* and *FUL*-like (Litt and Irish, 2003). To determine the evolutionary relationships between proteins encoded by the isolated grapevine genes and other core eudicots MADS-box proteins of the SQUA subfamily, a phylogenetic tree was constructed using the complete predicted protein sequences (Fig. 1C). This analysis grouped the grapevine genes within the FUL-like and the euAP1 clades, respectively (Fig. 1C), for what we refer to as *VFUL-L* and *VAP1,* respectively. The existence of a putative eu*FUL* gene cannot be ruled out with our experimental approach.

VFUL-L and *VAP1* Are Expressed throughout Grapevine Reproductive Development

The temporal and spatial expression patterns of *VFUL-L* and *VAP1* were analyzed in buds collected during two consecutive years and in developing and mature reproductive structures (Figs. 2 and 3, see "Materials and Methods" and Carmona et al., 2002 for description of developmental stages).

In buds, the RNA levels of *VFUL-L* and *VAP1* were analyzed by RNA-blot hybridization experiments (Fig. 2A). Transcripts of *VFUL-L* and *VAP1* were barely detected in buds of the first season (Fig. 2A, latent buds) and in dormant buds (Fig. 2A, dormancy). During the first season, the SAM produces lateral meristems that generate inflorescence meristems. During the second season, expression of *VFUL-L* and *VAP1* was already detectable in winter buds (end of stage A). The expression of the two transcripts increased significantly in swelling buds (stage B) and decreased in sprouting buds when shoots were beginning to grow out (stage C, Fig. 2A, second season). During stages A, B, and C of the second season, inflorescence meristems divide to generate additional branch inflorescence meristems, which give rise to flower meristems and flowers.

Expression of both genes was also analyzed in developing inflorescences, berries, and seeds of the growing shoots (Fig. 2B). During inflorescence development, the expression patterns of *VFUL-L* and *VAP1* were very similar, but the levels of *VFUL-L* transcripts were higher than those of *VAP1*. Transcripts were detected during flower development (Fig. 2B, stages E-H40). *VFUL-L* levels were particularly high during stage H when carpel development takes place (Fig. 2B, H25 and H40). The expression of the two genes decreased in mature flowers (Fig. 2B, stages H50 and I). *VAP1* expression was not detected during fruit formation and maturation (Fig. 2B, berries and seeds). However, *VFUL-L* transcripts were detected during

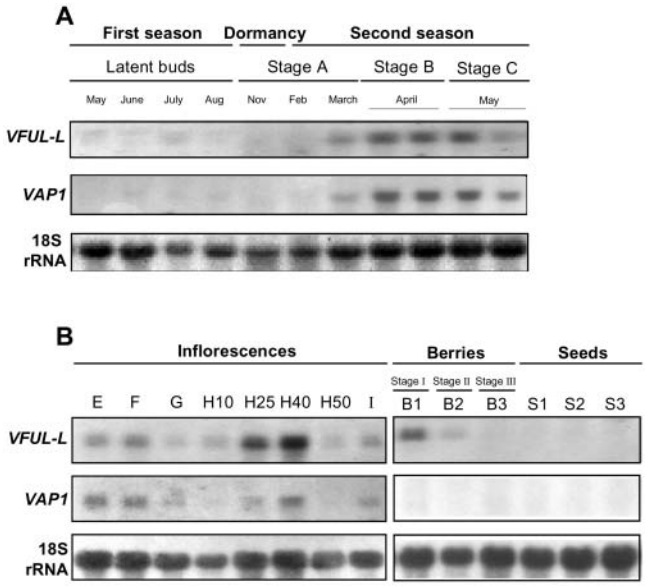

Figure 2. Expression of *VFUL-L* and *VAP1* during reproductive development studied by RNA-blot. A, Expression of *VFUL-L* and *VAP1* in buds during two consecutive growing seasons. Latent buds in the first growing season, winter buds (stage A) and spring buds (stages B and C) from the second growing season. B, *VFUL-L* and *VAP1* expression in developing inflorescences (phenological stages E, F, G, H10, H25, H40, H50, and I), berries, and seeds. 18S RNA was used as a quantitative control of loading.

stage I of berry development. This expression decreased at stage II and disappeared as the ripening of the berry progressed (stage III). *VFUL-L* transcripts were not detected in seeds.

To determine the spatial distribution of *VAP1* and *VFUL-L* transcripts at different stages of reproductive development, we carried out in situ hybridization experiments (Fig. 3). The expression patterns of both genes were very similar during the first season when inflorescence meristems are forming (*VAP1*, Fig. 3, A–C, *VFUL-L*, not shown), but they diverged during the second season when flower initiation and development takes place (Fig. 3, D–I).

In the first season, *VAP1* and *VFUL-L* were detected from the earliest stages of inflorescence development (Fig. 3, A and B). At later stages, *VAP1* and *VFUL-L* were strongly expressed in the inflorescence branch meristems but not in their subtending bracts (Fig. 3C). In the second season, when flower meristems were initiated, *VAP1* and *VFUL-L* expression patterns diverged. *VAP1* was broadly expressed in the newly formed flower meristems (Fig. 3D). As soon as sepal primordia began to grow, *VAP1* was excluded from the sepal-forming region and became restricted to the inner part of the meristem that forms the petals, stamens, and carpels (Fig. 3D). Later, when petals and stamen primordia were visible, *VAP1* mRNA preferentially accumulated at the tips of these growing primordia (Fig. 3, E and F) and then became mainly restricted to the carpel-forming region (Fig. 3F). On the other hand, *VFUL-L* was expressed in a small area of the central part of the meristem already in very young floral meristems, (Fig. 3G). During floral organ formation, *VFUL-L* was not detected in sepal, petal, or stamen primordia but was confined to the prospective carpel-forming region (Fig. 3, H and I).

VFUL-L and *VAP1* Are Expressed during Tendril Initiation and Development

Gene expression was also analyzed during vegetative development by RNA-blot hybridization. *VFUL-L* and *VAP1* transcripts were not detected in the vegetative apices, leaves, or shoots at different stages of cane development (E, G, H50, and I) nor in roots of in vitro grown plants (data not shown). However, high levels of both transcripts were observed in tendrils. To further investigate this expression, three regions were distinguished in tendrils: the hypoclade (h) or basal zone, the branching zone (b) where the hypoclade splits in two arms, and the inner and outer arms (a). First, we analyzed the transcription of both genes in the arms of five consecutive developing tendrils nearest to the shoot apex (Fig. 4, T1–T5a). *VFUL-L* and *VAP1* mRNAs were detected in all five tendrils with highest levels of expression in the most developed one (T5a). Then, we studied the spatial expression of *VFUL-L* and *VAP1* in the three regions of tendril 5 (T5): arms (T5a), branching zone (T5b), and hypoclade (T5h). Expression of *VFUL-L* was high in the three tendril zones, while that of *VAP1* was high in the arms, low in the branching zone, and undetectable in the hypoclade.

In situ hybridization experiments confirmed the results obtained by RNA-blot hybridizations. *VFUL-L* and *VAP1* genes were expressed all along tendril development (Fig. 5). *VFUL-L* and *VAP1* were expressed in the lateral meristem as soon as it became distinguishable in the periphery of the SAM (Fig. 5A for *VFUL-L*, *VAP1* not shown). Both genes continued to be expressed throughout tendril development in different spatial patterns. *VFUL-L* was detected in all the regions of the developing tendrils (Fig. 5, B and C), whereas *VAP1* expression was restricted to the tendril arms (Fig. 5, E and F). Transcripts accumulated in the parenchyma but not in the vascular tissues.

In Arabidopsis and in other herbaceous and woody species, expression of *AP1*-and *FUL*-like genes has been associated with developing reproductive structures. Detection of their grapevine homologs in tendrils, considered to be vegetative climbing structures, raised the question of whether this was a consequence of the maintenance, in tendrils, of an expression induced in inflorescences upon flowering. This could reflect the homology between tendrils and inflorescences, but it might not have a functional relevance. Alternatively, this expression could be independent of the flowering process and have a role on its own. To test whether *VFUL-L* and *VAP1* expression in tendrils was related to the reproductive state of the plant, we analyzed their expression in the earliest formed

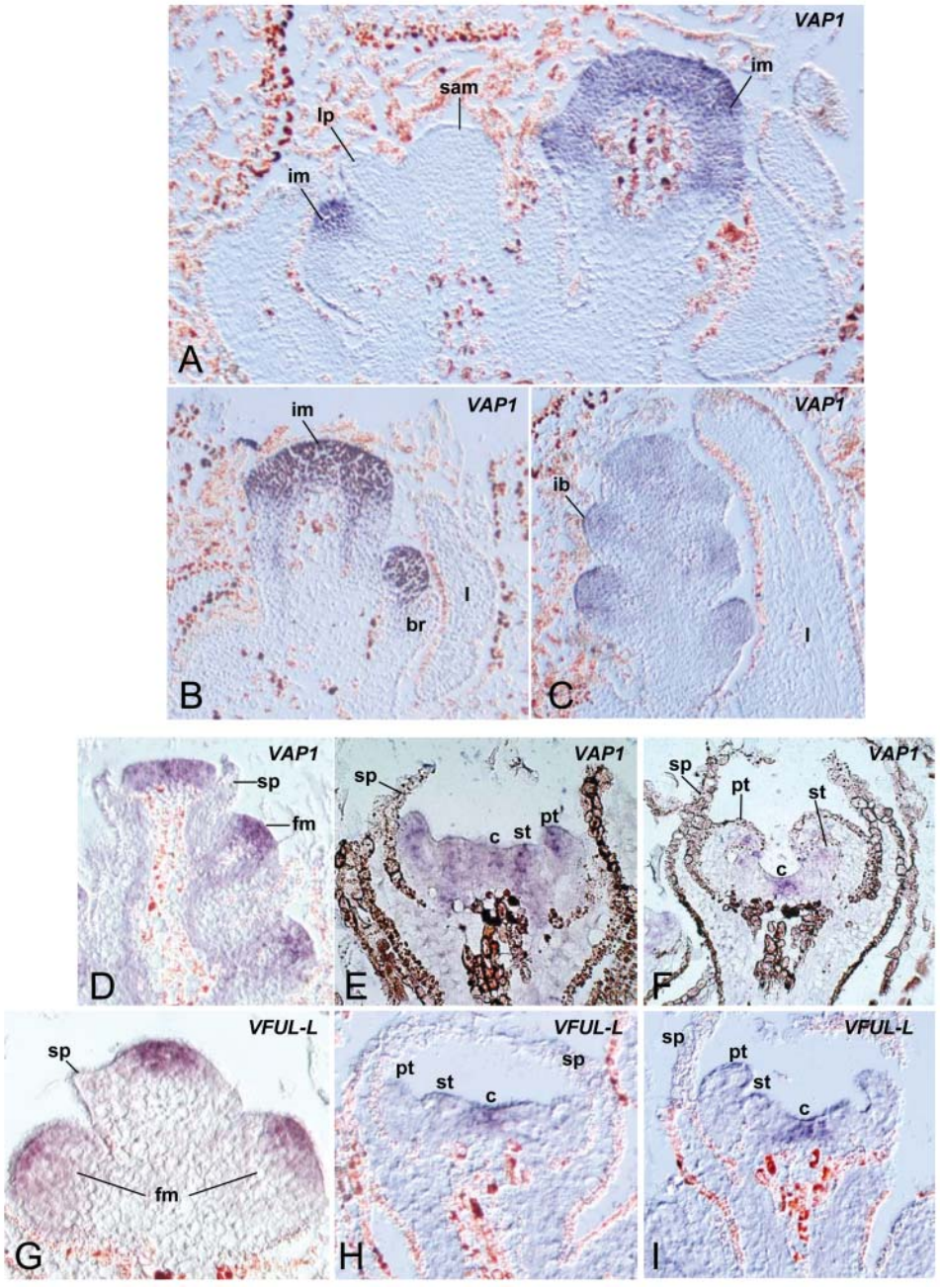

Figure 3. *VFUL-L* and *VAP1* spatial expression patterns during reproductive development. A to C, First season, *VAP1* expression during inflorescence development. D to I, Second season, *VAP1* and *VFUL-L* expression patterns during early flower development. A, *VAP1* expression in young inflorescence meristems. *VAP1* is not expressed in the SAM and leaf primordia. B, Expression of *VAP1* in inflorescence meristems. C, Expression of *VAP1* in inflorescence branch meristems at a later stage of development. D, Close-up of an inflorescence branch where flower meristems have just formed. *VAP1* mRNA accumulates in young flower meristems and becomes excluded from the sepal-forming region. E, *VAP1* mRNA preferentially accumulates at the tip of petal, stamen, and carpel primordia during organogenesis. F, *VAP1* expression at a slightly later stage of flower development when transcripts are mainly detectable at the prospective carpel-forming region. G, Expression of *VFUL-L* in young flower meristems during the second season. Notice that this expression is more spatially restricted than that of *VAP1* (D). H, *VFUL-L* expression in flower meristems of a stage similar to E. I, *VFUL-L* expression in the carpel-forming region of slightly more advanced flowers comparable to F. im, inflorescence meristem; br, bract; lp, leaf primordium; l, leaf; ib, inflorescence branch meristem; fm, flower meristem; sp, sepals; pt, petals; st, stamens; c, carpel.

tendrils of young plants that had not undergone flowering induction. Expression of both genes was already detected in the first formed tendril of plants grown from seeds that would still grow vegetatively for 2 to 5 more years before initiating flowering (Fig. 4, TJ). This indicates that this expression is not dependent on flowering induction but is specifically associated to tendril development. Other genes involved in the flowering process such as *VFL* (Carmona et al., 2002) or *VSOC1*, the putative ortholog of *SOC1* (Samach et al., 2000), were not expressed at this early stage (data not shown), indicating that these expression patterns were not common to all genes involved in flowering initiation but typical of *VFUL-L* and *VAP1*.

DISCUSSION

We have isolated and characterized two grapevine MADS-box genes belonging to the *SQUA* subfamily or *AP1/FUL* gene lineage (Theissen et al., 2000; Becker and Theissen, 2003; Litt and Irish, 2003). Conservation of typical protein motifs in the deduced proteins grouped them as members of the *FUL*-like and *AP1/SQUA*

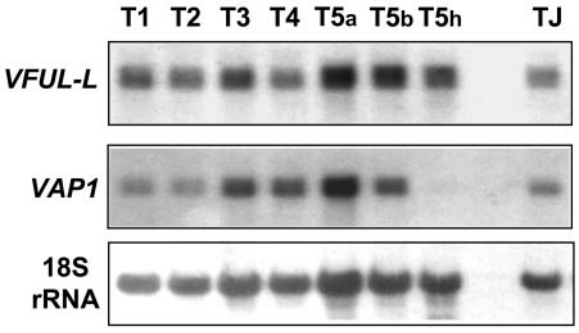

Figure 4. *VFUL-L* and *VAP1* expression during tendril development studied by RNA blot. Expression in the arms of five consecutive tendrils numbered from the shoot apex (T1, T2, T3, T4, and T5a) of an elongated shoot. Expression in three regions of tendril T5: the arms (T5a), the branching zone (T5b), and the hypoclade zone (T5h). Expression in the first formed tendril of a young plant (TJ). 18S RNA was used as a quantitative control of loading.

(eu*AP1*) gene clades, and consequently they were named as *VFUL-L* and *VAP1*. In spite of some peculiarities, their expression patterns also support that they are the putative orthologs of previously described *FUL*-like and *AP1*-like genes in other plant species.

VFUL-L and *VAP1* expression in grapevine is related to reproductive development and spans two growing seasons as previously described for *VFL* (Carmona et al., 2002). Their expression coincides with the time of flowering induction and inflorescence development during the first season and flower initiation and development during the second season (Srinivasan and Mullins, 1981; Gerrath and Posluszny, 1988b; Gerrath, 1993; May, 2000; Carmona et al., 2002). Such a bimodal pattern of expression has also been observed in other temperate woody perennials like kiwifruit (Walton et al., 2001) and apple trees (Sung et al., 1999; Kotoda et al., 2000). In contrast to the expression of *VFL*, *VFUL-L* and *VAP1* transcripts were not detected in the SAM or in leaf primordia.

VFUL-L and *VAP1* are initially coexpressed in lateral meristems and are maintained at high levels in their derived structures. Coexpression of *VFUL-L* and *VAP1* is in contrast to what has been found in herbaceous species like Arabidopsis and *Antirrhinum* where *AP1*- and *FUL*-like genes display mutually excluding spatial patterns of expression. *AP1* and *SQUA* are specifically expressed in floral meristems (Huijser et al., 1992; Mandel et al., 1992), whereas *FUL* and *DEFH2* are expressed in the SAM after flowering transition (Gu et al.,1998; Müller et al., 2001). Coexpression of both genes in reproductive meristems has also been described in *Silene latifolia* and *Malus domestica* (Hardenack et al., 1994; Sung et al., 1999). Since *AP1*- and *FUL*-like genes were originated by duplication from a common ancestral *AP1/FUL* gene (Becker and Theissen, 2003; Litt and Irish, 2003), their similar expression patterns might reflect an ancestral condition related to their common phylogenetic origin.

During flower development, expression patterns of *VFUL-L* and *VAP1* are not coincident. Very early, expression of *VFUL-L* becomes restricted to the carpel-forming region at the central part of the flower meristem and continues to be expressed at high levels through the early stages of fruit development. This expression pattern suggests that *VFUL-L* may play a role in carpel and fruit development in a similar way to what has been described for eu*FUL* in Arabidopsis (Mandel and Yanofsky, 1995; Gu et al., 1998). A similar role has been pointed out for *DEFH28*, another *FUL*-like gene from *Antirrhinum*, based on sequence and expression pattern similarities as well as on functional analyses in transgenic Arabidopsis (Müller et al., 2001). These results support the suggestion that eu*FUL* and *FUL*-like genes might be involved in similar functional roles (Litt and Irish, 2003). On the other hand, expression of *VAP1* during grapevine flower development differs substantially from that of *AP1* (Mandel et al., 1992). Grapevine *VAP1*, initially expressed in the young flower meristem, soon becomes excluded from the sepal-forming region. Later, its expression is preferentially detected in the tip of the growing petals and stamens and in the developing carpel. Carpel expression has also been reported for the *Antirrhinum SQUA* gene (Huijser et al., 1992) and *Silene AP1*-like gene (Hardenack et al., 1994). In contrast, Arabidopsis *AP1* is only expressed in sepal and petal primordia and the phenotype of *ap1* mutants suggested that it could be responsible for the A-function in the ABC model (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Theissen, 2001). The lack of expression of *VAP1* in the sepal whorl is not consistent with a function of this gene in the specification of sepal identity. These results are in agreement with previous observations which questioned a role for the *Antirrhinum SQUA* gene (Huijser et al., 1992) and the *Gerbera hybrida AP1* ortholog (Yu et al., 1999) in the specification of sepal identity and provide additional arguments to revise the concept of the A-function in flower organ identity (Litt and Irish, 2003).

The strong and distinctive expression of *VFUL-L* and *VAP1* in developing tendrils and the fact that their expression is independent of the flowering induction suggest a relevant role of these genes in tendril development. This could represent a novel role for these genes that would have been recruited for the development of tendrils in *Vitis*. Examples of gene recruitment to carry out different developmental functions have been previously described. For instance, *UNIFOLIATA* (*UNI*) and *PEA FIMBRIATA* (*PEAFIM*), the pea orthologs of Arabidopsis *LEAFY* and *UNUSUAL FLORAL ORGANS*, do not only participate in flower initiation and development in pea but are also required for the development of its compound leaves (Gourlay et al., 2000; Taylor et al., 2001). The legume-*Rhizobium* symbiosis is another example of recruitment of genes for the evolution of a new developmental pathway (Szczyglowski and Amyot, 2003). Among them, several MADS-box genes expressed in flowers, such

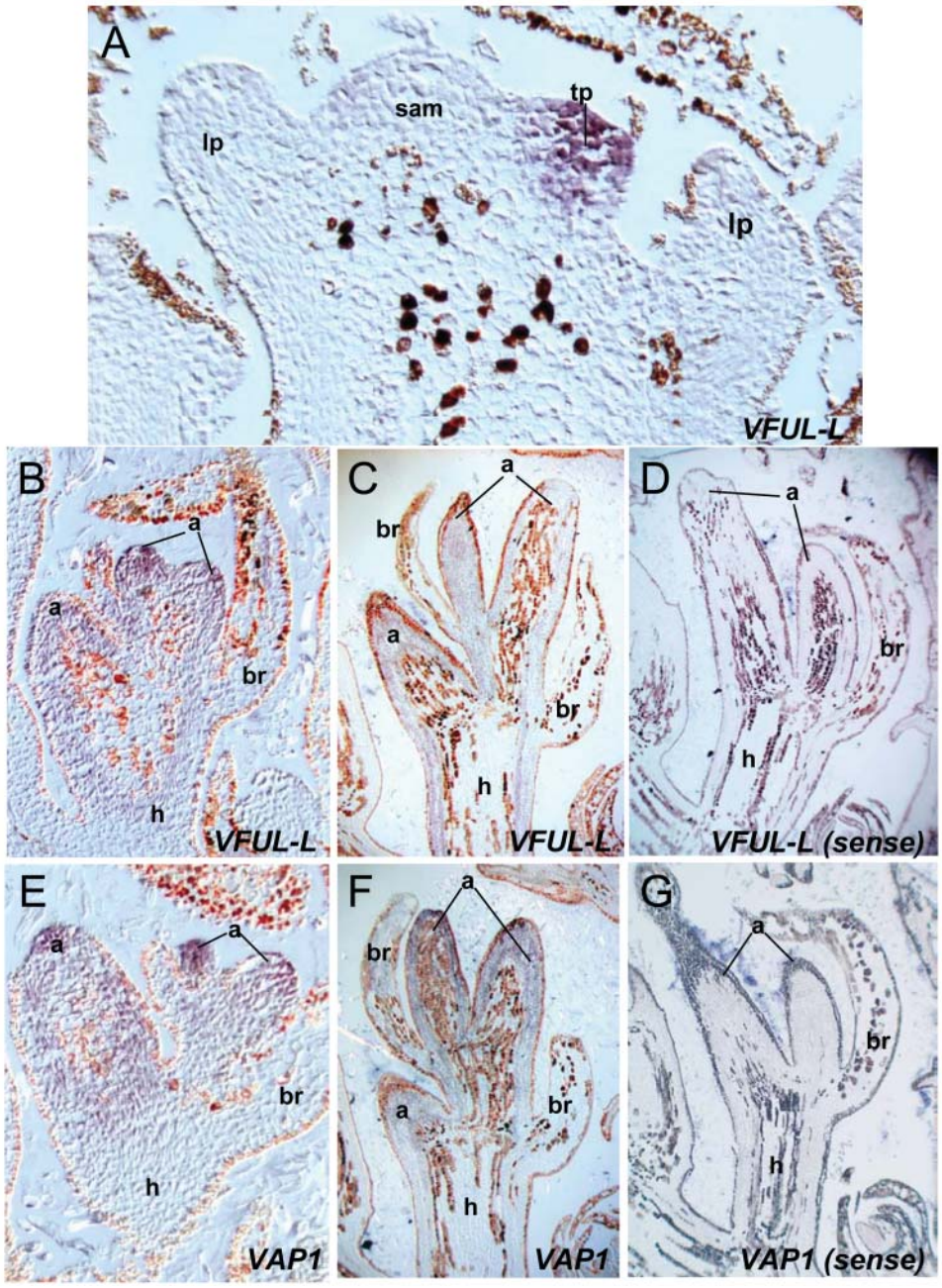

Figure 5. *VFUL-L* and *VAP1* expression in tendrils studied by in situ hybridization. A to C, *VFUL-L* expression. E to F, *VAP1* expression. A, *VFUL-L* is transcribed in the lateral meristems formed in the second season that will give rise to a tendril but not in the leaf primordia. B to C, *VFUL-L* is expressed throughout developing tendrils. D, Tendrils of a similar stage hybridized with a sense *VFUL-L* probe. E to F, *VAP1* expression is restricted to the apical region (arms) of the developing tendril. G, Tendrils of a similar stage hybridized with a sense *VAP1* probe lp, leaf primordium; tp, tendril primordium; br, bract; a, tendril arm; h, tendril hypoclade.

as Alfalfa *nmh7* and *ngl9* genes, are also expressed in infected nodule cells (Zucchero et al., 2001) and could have a role in the development of nodules. The expression of *VFUL-L* and *VAP1* in the grapevine lateral meristems and later in tendrils and inflorescences could, on the other hand, reflect an ancestral role of both genes in the specification and proliferation of these meristems and their derivatives, as has been previously proposed for the ancestral *AP1* and *AG*-related gene lineages (Irish, 2003). Moreover, we cannot completely rule out that tendril expression represents a residual expression without functional significance in a structure that has a common origin with reproductive organs.

Transition from juvenile to adult phase in grapevine is marked by the initiation of lateral meristems at the flanks of the SAM. In vegetative growing plants, these structures differentiate as tendrils. However, upon flowering induction, several consecutive lateral meristems generate inflorescence meristems. Which are the genetic bases of this pattern of growth in which equivalent primordia give rise to either reproductive (inflorescence) or vegetative (tendril) structures? We have shown that *VFUL-L* and *VAP1* as well as *VFL*

(Carmona et al., 2002) are expressed in the lateral meristems independently of their fate. However, *VFL*, *VFUL-L*, and *VAP1* show differential expression in the derived structures. The three genes are highly expressed throughout inflorescence development. However, in tendril primordia, *VFL* is only transiently expressed, whereas *VFUL-L* and *VAP1* are maintained until late developmental stages. This differential expression of *VFL* in the two structures might suggest that a threshold level of *VFL* expression is required for the development of inflorescence and flower meristems instead of tendrils. A threshold level of *LFY* has also been shown to be necessary to induce flower initiation in Arabidopsis (Blázquez et al., 1997). This could be responsible in part for the generation of two different types of structures from a common primordium. The plasticity of grapevine lateral meristem development could represent an adaptation to a climbing habit based on the conversion of a reproductive organ (the inflorescence) into a climbing organ (the tendril). Additional functional experiments will be required to confirm this hypothesis.

MATERIALS AND METHODS

Plant Material

Grapevine (*Vitis vinifera* L. var Tempranillo) samples were collected in the fields of Instituto Madrileño de Investigaciones Agrarias (Alcalá de Henares, Madrid). RNA-blot and in situ hybridization analyses were performed on plant materials collected and fixed at different developmental stages during two growing seasons.

Cloning of *VFUL-L* and *VAP1* and Sequence Analyses

Cloning of *VFUL-L* and *VAP1* was performed using a 3′/5′-RACE strategy (Frohman et al., 1988), following the instructions of a commercial RACE kit (Roche Diagnostics GmbH, Mannhein, Germany). 3′-RACE was performed with oligo(dT)-primed single-stranded cDNA synthesized from total RNA of developing buds from phenological stage B (Baggiolini, 1952). Further PCR amplification was performed with anchored primers to the 3′-end and degenerate primers from highly conserved regions of SQUA-like genes. These primers were 5′-AAGAAAGCTCAT/CGAGATCTCT/CGT-3′ from the MADS-box for the first amplification and 5′-ATGGAA/GAGGATA/TCTTGAAC/AGGTATGA-3′ from the highly conserved motif of the I-region (Fig. 1, A and B) for the reamplification. Amplified fragments from two independent experiments were cloned in pGEM-T easy vector (Promega, Madison, WI). Thirty clones corresponding to the 3′ region were sequenced to analyze their sequence diversity. Two different cDNA sequences were found, which, based on sequence similarities, were designated as *VFUL-L* and *VAP1*. For 5′-RACE, specific single-stranded cDNAs were synthesized using a *VFUL-L* or *VAP1* specific-primers (5′-ATCTTCTCCCACAAAGTGCCTCAGGTTCCT-3′ and 5′-ATGTTTAGACAGGGAAGCTGCTGTCGCAGT-3′, respectively). Then, terminal transferase was used to add a homopolymeric A-tail to the 3′ end of the cDNAs. Tailed cDNAs were amplified with a *VFUL-L* specific primer (5′-AGCCAGTTTCCCTGTGAGTCAGGAT-3) or a *VAP1* specific primer (5′-ATCTTCCCCCAAAAAGTGCCTTTGGCTTCT-3′), respectively, and an oligo(dT)-anchor primer. The resulting products were reamplified in a second PCR using nested *VFUL-L* (5′-AGAGAAGCTGCTTCTCTCCGAAAGTGAG-3′) or *VAP1* (5′-CAGTCAGCTGCCTCTCTGCATAAGAA-3′) specific primers and an anchor primer. The gene sequences were completed after two successive 5′-RACE experiments, which allowed the isolation of the middle part and the 5′end of the genes. The complete coding region of the cDNAs were obtained by reverse transcriptase-PCR with primers flanking the *VFUL-L* sequence (5′-AGCAAACCACTCTTCCCACACATA-3′ and 5′-TCGCGTAAAAAATTCTCACCAGG-3′) or the *VAP1* sequence (5′-CGCAAAACCAAAAGATGGGAAGAGGT-3′ and 5′-CTATAAGTCCATATTCACATGGAACT-3′), respectively, using Pfu DNA Polymerase (Stratagene, La Jolla, CA). These cDNAs were cloned in pGEM-T easy vector. Six clones of each cDNA were completely sequenced and compared. For sequencing, the Big Dye Terminator Cycle sequencing kit and a sequencer (Prism 377, ABI, Sunnyvale, CA) were used.

Phylogenetic Analyses

To generate a phylogenetic tree, predicted proteins were aligned with ClustalW. Using this original data set, 100 data sets were generated by bootstrap resampling using SEQBOOT program. Distance matrices were made for each bootstrap data set using the PRODIST program-Dayhoff PAM matrix algorithm. The distance matrices obtained were used to construct 100 unrooted trees by the neighbor-joining method using the NEIGHBOR program. A consensus tree was obtained using CONSENSE. SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE programs belong to the PHYLIP program (Phylogeny Inference Package, version 3.57c, Department of Genetics, University of Washington, Seattle). Arabidopsis SEP1, SEP2, and SEP3 (Pélaz et al., 2000) were used as outgroup members.

RNA-Blot Hybridization Analyses

Plant material was collected from organs and tissues at different developmental stages (phenological stages A–I; Baggiolini, 1952). In the first growing season, young buds in the axils of the leaves (latent buds) were collected at equivalent branch positions from May to August (Fig. 2A, latent buds). For phenological stage A (winter buds), samples were collected in November (dormancy period), February, and March (Fig. 2A, stage A). In the second growing season, swelling buds were collected during early and advanced phenological stages B and C in April and May (Fig. 2A, stages B and C). As the shoot began to elongate, expression was analyzed in inflorescences taken at the stages E, F, G, H, and I. Four samples were collected from inflorescences during stage H (H10, H25, H40, and H50), the number indicating the length of the shoot in centimeters (Fig. 2B, inflorescences). Stages E and F correspond to inflorescences separated from leaves in the shoot and with developing flowers that begin to grow out from beneath protective bracts. Along stage E, sepals develop and petals and stamens primordia become visible. At stages G and H10, the inflorescences are well developed, but not the flowers, which are still present in compact groups. Later in stage H (H25–H50), the inflorescences posses separated flowers which are still undergoing maturation. Development of flower organs span stages E, F, G, and H, being gynoecium initiated last and developing along stage H. Stage I correspond to the beginning of anthesis. During fruit setting and maturation, berries and seeds from stage I to III (Fig. 2B, berries and seeds) were separately analyzed. Stage I correspond to a rapid growth of the berry, stage II to a slow growth and maturation of the seeds, and stage III to the ripening, when the color change or veraison takes place (Mullins et al., 1992). Different organs of the plant from phenological stages E to I were also independently analyzed: shoot apex, young leaves, and shoots. Leaves, shoots, and roots from in vitro grown plants were also tested (data not shown). To analyze expression in tendrils, samples were collected from the arms of the first five tendrils of advanced stage H shoots (Fig. 4, T1–T5). Tendril number 1 corresponds to the last formed by the shoot apex. Tendrils number 5 were dissected in three regions: the inner and outer arms (a), the branching zone (b), and hypoclade zone (h; Fig. 4, T5a, b, and h). In young plants coming from seeds, tendril samples correspond to the first formed tendril (Fig. 4, TJ). Total RNA extraction was performed following the protocol of Chang et al. (1993). For RNA-blot hybridization analyses, 15 μg of total RNA was loaded per lane of agarose/formaldehyde gels, electrophoretically separated, and transferred to Hybond-N^+ membranes. To avoid cross hybridization with other MADS-box genes, 3′-end probes excluding MADS-box sequences were used. Filters where hybridized with a ^{32}P-radiolabeled *VFUL-L* probe (734 bp) or *VAP1* probe (720 bp), corresponding in both cases to the 3′end of the gene and the 3′ untranslated region, obtained with specific primers for *VFUL-L* (5′-TGGAAAGGATCCTTGAACGATATGA-3′ and 5′-AGAAGGCACATGTGCCAAAATA-3′) and for *VAP1* (5′-TGGAGAAGATCCTTGATCGCTATGA-3′ and 5′-AATATAAGGGGAAACATCTTAA-3′). Hybridization was performed overnight at 65°C as described by Church and Gilbert (1984). Filters were washed several times in 2× SSC and 0.1% (w/v) SDS and once in 0.1× SSC and 0.1% (w/v) SDS for 20 min at 65°C.

RNA In Situ Hybridization

In situ hybridizations were carried out on plant tissue collected and fixed during two growing seasons at the developmental stages described above. Late stages of flower and fruit development could not be analyzed by this method due to the high levels of background obtained in this plant material. Digoxigenin labeling of RNA probes, tissue preparation, and hybridization were performed as described by Coen et al. (1990). The templates for the *VFUL-L* and *VAP1* riboprobes were the 734-bp and 720-bp fragments, respectively, containing the 3′region of the genes as described above, cloned in pBlueScript KS vector. The hybridized sections were visualized with Nomarski optics in a Leica DMR microscope (DMR, Leica, Wetzlar, Germany).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY538747 and AY538746.

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