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The *FT/TFL1* gene family in grapevine

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Abstract The *FT/TFL1* gene family encodes proteins with similarity to phosphatidylethanolamine binding proteins which function as flowering promoters and repressors. We show here that the *FT/TFL1* gene family in *Vitis vinifera* is composed of at least five genes. Sequence comparisons with homologous genes identified in other dicot species group them in three major clades, the FT, MFT and TFL1 subfamilies, the latter including three of the *Vitis* sequences. Gene expression patterns are in agreement with a role of *VvFT* and *VvMFT* as flowering promoters; while *VvTFL1A*, *VvTFL1B* and *VvTFL1C* could be associated with vegetative development and maintenance of meristem indetermination. Overexpression of *VvFT* in transgenic *Arabidopsis* plants generates early flowering phenotypes similar to those produced by *FT* supporting a role for this gene in flowering promotion. Overexpression of *VvTFL1A* does not affect flowering time but the determination of flower meristems, strongly altering inflorescence structure, which is consistent with the biological roles assigned to similar genes in other species.

Keywords Flowering transition · *FT/TFL1* gene family · Grapevine · Inflorescence meristem identity · Juvenile phase

Abbreviations
SAM shoot apical meristem

Introduction

Extensive analyses performed in the facultative long day annual plant *Arabidopsis thaliana* have provided a complex picture of how these plants integrate environmental and endogenous signals to regulate the flowering transition. In this species, several flowering time regulatory pathways function to promote or repress flowering depending on the environmental and endogenous conditions of the plant (Ausín et al. 2005). These pathways regulate the expression of a few genes known as flowering signal integrators such as *SUPRESSOR OF CO1* (*SOC1*) and *FT*, which further promote the expression of genes specifying flower meristem identity (for recent reviews, see Puterill et al. 2004, Boss et al. 2004, Ausín et al. 2005; Parcy 2005). FT belongs to a larger group of plant proteins that share structural similarities to mammalian phosphatidylethanolamine-binding proteins (PEBPs), and have also been found in

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yeast and bacteria (Bradley et al. 1996). Based on these similarities, the plant PEBPs are predicted to play a role in the regulation of signalling cascades controlling diverse processes as has been shown in mammals (Yeung et al. 1999; Banfield and Brady 2000).

The *PEBPs* gene family in *Arabidopsis* includes *FT* as well as five other related genes—*TSF* (*TWIN SISTER OF FT*), *TFL1* (*TERMINAL FLOWER 1*), *BFT* (*BROTHER OF FT AND TFL1*), *ATC* (*ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUE*) and *MFT* (*MOTHER OF FT AND TFL1*); (Kobayashi et al. 1999). Despite their sequence similarities, these genes have antagonistic roles in the regulation of flowering transition acting as either promoters or repressors. FT interacts with FD, a bZIP transcription factor promoting flowering in *Arabidopsis* through direct activation of *APETALA1* (*AP1*) in the SAM (Abe et al. 2005; Wigge et al. 2005). *TSF*, the closest homolog of *FT*, also seems to function as integrator of flowering time pathways and appears to promote flowering redundantly with *FT* (Michaels et al. 2005; Yamaguchi et al. 2005). *MFT* could also have a redundant function in flowering promotion since loss-of-function alleles are aphenotypic whereas its overexpression causes acceleration of flowering time (Yoo et al. 2004). Contrary to the flowering promoting effects shown for *FT*, *TSF* and *MFT*, the phenotype of Arabidopsis *tfl1* mutants suggests that *TFL1* controls the length of the vegetative phase and delays the flowering transition. *TFL1* also maintains the identity of inflorescence meristems, thus playing a role in the control of inflorescence architecture (Shannon and Meeks-Wagner 1993; Bradley et al. 1997; Ratcliffe et al. 1998). *ATC* could be functionally redundant with *TFL1* since loss-of-function mutants show no flowering phenotypes while its overexpression has similar effects as *TFL1* overexpression (Mimida et al. 2001). Finally, nothing is known about the biological role of the *Arabidopsis BFT* gene or its putative orthologs in other species. Sequence comparisons and phylogenetic analyses in diverse plant species group plant PEBP proteins in three main clades: the MFT-, the FT- and the TFL1-like subfamilies (Carmel-Goren et al. 2003; Chardon and Damerval 2005; Ahn et al. 2006). Furthermore, the opposite functional roles of FT and TFL1 proteins have been related to the presence of critical amino acid residues (Tyr85/Gln140 in FT versus His88/Asp144 in TFL1) (Hanzawa et al. 2005; Ahn et al. 2006).

Much less is known about the regulation of this developmental process in woody perennial species or in species with particular growth habits (Martín-Trillo and Martínez-Zapater 2002). However, comparative and functional genomic approaches undertaken in some species are starting to provide information on the conservation of flowering regulatory pathways between woody and herbaceous plant species (Brunner and Nilsson 2004; Böhlenius et al. 2006; Hsu et al. 2006). We are interested in the regulation of reproductive development in grapevine (*Vitis vinifera* L.), a woody perennial vine with a pattern of organ formation and development distinct to those previously described for other species (Mullins et al. 1992; Carmona et al. 2007). Grapevine has a short juvenile phase during which the SAM produces leaves with a spiral phyllotaxis. Transition to the adult phase is marked by changes in leaf shape and phyllotaxis and by the formation of tendrils (Gerrath et al. 1998). In grapevine, these climbing organs develop from lateral meristems, historically called uncommitted primordia, which can also give rise to inflorescences upon flowering induction. The formation of a lateral meristem, which can differentiate as either a sterile (tendril) or fertile (inflorescence) structure, represents a peculiar characteristic of reproductive development in the *Vitaceae*. As in many other woody species, seasonal flowering in grapevine requires two consecutive growing seasons. Flowering is induced in summer latent buds. In these buds, the SAM produces 2–3 consecutive lateral meristems that give rise to inflorescence primordia before entering winter dormancy. Flower initiation and development takes place the following spring when bud growth resumes (Mullins et al. 1992; Carmona et al. 2002).

The peculiarities of grapevine reproductive development as compared to other woody perennials led us to try to understand the role of *FT/TFL1*-like genes along the processes of phase transition and tendril and inflorescence initiation in this species. Some members of this gene family could be involved in the regulation of inflorescence architecture affecting bunch shape and size as well as number and size of the berries, all of which are important traits in crop production. As a first step to understand their developmental role, we isolated and characterized five members of this gene family in grapevine. Their expression patterns and the effects of overexpression on transgenic *Arabidopsis* plants provide the first clues on their possible function in grape. Moreover, the information provided by these *Vitis* orthologs may help to better understand the biological function and evolution of the *PEBPs* family in flowering plants.

Materials and methods

Plant material

Grapevine (*Vitis vinifera* L. cv. Tempranillo) samples were collected in the fields of Instituto Madrileño de Investigación y Desarrollo Rural Agrario y Alimentario (IMIDRA, Alcalá de Henares, Madrid). Several plant organs at different developmental stages (phenological stages A–I; Baggiolini 1952) were sampled along two consecutive growing seasons. In the first growing season, young buds in the axils of leaves (latent buds) were collected at equivalent branch positions from May to August. For phenological stage A (winter buds), samples were collected in November (dormancy period) of the first season, as well as in February and March of the second season. Along the second growing season, swelling buds were collected during early and advanced phenological stages B and C in April and May. As the shoot began to elongate, expression was analyzed in inflorescences taken at stages E, F, G, H, and I. Four samples were considered for inflorescences in stage H (H10, H25, H40, and H50), the number indicates the length of the shoot in centimeters. Stages E and F correspond to the presence of inflorescences separated from leaves with groups of developing flowers that start to outgrow from protective bracts. Along stage E, sepals develop and petal and stamen primordia become visible. At stages G and H10, the inflorescences are well developed but flowers are still present in compact groups. Later in stage H (H25–H50), the inflorescences display separated flowers that are still undergoing maturation. Development of flower organs span stages E, F, G and H being gynoecium the last organ, which develops along stage H. Stage I corresponds to the beginning of anthesis.

During fruit setting and maturation, berries from stage I to III were analyzed. Stage I corresponds to a rapid growth of the berry; stage II to a slow growth and maturation of the seeds; and stage III to ripening, after the color change or veraison takes place (Mullins et al. 1992). During stage I seeds could not be easily separated from the rest of the fruit. However, for stages II and III, fruit seeds and flesh were collected and analyzed separately. Different organs of the plant from phenological stages E to I were also independently analyzed: shoot apex, young leaves and stem internodes. Roots from in vitro grown plants were also tested. To analyze expression in tendrils, samples were collected from the arms of the first five tendrils of advanced stage H shoots. Tendril number 1 corresponds to the latest developed by the shoot apex. Tendrils in the 5th position were dissected in three regions: the inner and outer arms (a), the branching zone (b) and the hypoclade zone (h). In young plants coming from seeds of selfed Tempranillo plants, tendril sample corresponds to the first formed tendril (TJ).

Cloning of cDNA and sequence analyses

An extensive search for *FT/TFL1*-like genes was performed using a 3′/ 5′-RACE strategy (Frohman et al. 1988) with the Marathon™ cDNA amplification kit (Clontech, Palo Alto, CA, USA). RNA samples extracted from diverse organs were used to isolate as many *FT/TFL1* family members as possible. Such organs included developing buds of phenological stage B, leaves from stages E to I; shoots and apices from stages E to I; and roots from in vitro grown plants. 3′-RACE was performed on double-stranded adaptor-ligated cDNA synthesized from total RNA of the different organs. Three independent amplifications were performed on each RNA sample using the Marathon™ adaptor primer and each of three primers from conserved regions of *FT*- and *TFL1*-like genes:

FamFT1: 5′-TTCTACACTCTGGTTATGGTGGATCCTGA-3′
FT1: 5′-GCAGCGTTGTTGGTGAYGTTYTTGA-3′
TFL1: 5′-GACAGACCCAGATGTTCCTGGTCCTAGTGA-3′

Afterwards, a second PCR amplification was performed on each initial amplification product, using anchored primers to the 3′-end and primers complementary to conserved regions downstream of the first ones:

FamFT2: 5′-GGGAATACCTACACTGGTTGGTGACTGATAT-3′ and FT2: 5′-GTGTATGCACCAGGGTGGCGCCARAAYTT-3′ were used for FamFT1 and FT1 amplified products while two primers, TFL2 and FamTFL1 were used for TFL1 amplified ones:

TFL2: 5′-TATGAGATGCCAAGGCCAAACATTGGGAT-3′
FamTFL1: 5′-CCTTATCTGAGAGAGCACCTGCACTGGAT-3′

Amplified fragments obtained, after both amplification rounds or even after the first one, were cloned in pGEM-T easy vector (Promega, Madison, WI). Two hundred clones corresponding to the 3′ regions of putative grapevine *FT/TFL1*-like genes were sequenced and analyzed. Five different cDNA sequence species were identified and designated as *VvFT*, *VvMFT*, *VvTFL1A*, *VvTFL1B* and *VvTFL1C*. For each cDNA, 5′-RACE amplification was performed

using the Marathon™ adaptor primer and one specific primer from the 3′untraslated region of each sequence species:

5′-CCATTGATTATGATTCTTCGACCACCCGA-3′ for *VvFT*
5′-GCGGTCAATGTTTTCTGTTCGTTCGCTCCT-3′ for *VvMFT*
5′-GCAGCTGTTTCCCTTTGGGCATTGAAGA-3′ for *VvTFL1A*
5′-GCGCTTCTGATCATTCAAGTTACAGGTGT-3′ for *VvTFL1B*
5′-CGGGACCCACGTGTCCCAAAACCTGGTAGA-3′ for *VvTFL1C*

The complete coding region of each cDNA was obtained by RT-PCR with Pfu DNA Polymerase (Stratagene, La Jolla, CA) and the following primer pairs flanking each gene sequence:

5′-CCCCCTCTTGTATTGTATCGGTGAGGTGTGT-3′/5′-GCCTTTGTAAGTCGCAAGGTTGCGTACA-3′ for *VvFT*
5′-AAAGAAGAACGGCAGGCAACAACCACCA-T-3′/5′-GCGGTCAATGTTTTCTGTTCGTTCGCTC-CT-3′ for *VvMFT*
5′-TGTCCAGTCCCACAGCCTCTCCTCGTCTCT-3′/5′-GTTGACCTCTGGGACTCGGGTCTGTTTCT-3′ for *VvTFL1A*
5′-CTCTCTCTCTCTCTCATATGGCAAGAA-3′/
5′-GGAGTTTTATGGTGGGACGCTAGCTA-3′ for *VvTFL1B*
5′-GGAACATGGAGCCTCTCAGTGTA-3′/
5′-CACCCTAGTACAAGTACAGTACGACCTCA-3′ for *VvTFL1C*

The amplified sequences were cloned in pGEM-T easy vector and six independent clones of each amplified fragment were completely sequenced and compared.

All the gene sequences except *VvTFL1B* are represented by partial or complete ESTs sequences in the TIGR database (TC42702 (VvTFL1A), TC51438 (VvTFL1C), TC47473 (VvFT), TC44785 (VvMFT) (http://www.tigr.org)). For *VvTFL1A* there is a previous release of a full size sequence corresponding to Cabernet Sauvignon (Boss et al. 2006). Sequence data of these genes are deposited in the NCBI/GenBank data libraries under the following accession numbers: *VvTFL1A* (DQ871591), *VvTFL1B* (DQ871592), *VvTFL1C* (DQ871593), *VvFT* (DQ871590) and *VvMFT* (DQ871594).

To analyze the genomic structure of these genes, we amplified the genomic sequences using the same PCR primers in single or overlapping amplifications. Amplified genomic fragments were cloned and six independent clones were sequenced and analyzed for each fragment. Sequencing was performed using the Big Dye Terminator Cycle sequencing kit on the ABI Prism 377 sequencer (ABI, Sunnyvale, CA).

Phylogenetic analyses

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA (Molecular Evolutionary Genetics Analysis) software package version 3.1 (Kumar et al. 2004). To generate a phylogenetic tree, predicted proteins were aligned with ClustalW software package (http://www.ebi.ac.uk/clustaw) using default parameters. Neighbour-joining (NJ), maximum parsimony (MP) and minimum evolution (ME) methods were used to construct different trees. To estimate evolutionary distances, the proportion of amino acids differences were computed using amino acid *p*-distance, Dayhoff PAM matrix algorithm and Poisson-correction models. To handle gaps and missing data, the pairwise-deletion option was used. Reliability of the obtained trees was tested using the bootstrap with 1,000 replicates and interior-branch tests.

RNA blot hybridization analyses

RNA blot analyses were performed on plant materials collected at different developmental stages as described above. Total RNA extractions were performed following the protocol of Chang et al. (1993). For RNA blot hybridization analyses 20 μg of total RNA was loaded per lane of agarose/ formaldehyde gels, electrophoretically separated, and transferred to Hybond-N+ membranes. Membranes were not reused and equal hybridization conditions were used in all cases to avoid spurious differences in hybridization signals. Membranes were hybridized with ^{32}P-radiolabeled probes corresponding to the complete coding region of each gene. The specific activities of the five probes were very similar, and concentrations of 10^6 cpm/ml were always used in a 15 ml hybridization volume. Hybridizations were carried out for 18 h at 65°C as described by Church and Gilbert (1984). Membranes were washed twice in 2× SSC and 0.1% (w/v) SDS for 10 min at 65°C and once in 0.1× SSC and 0.1% (w/v) SDS for 20 min at 65°C. Membranes were exposed to autoradiographic film for 96 h. Longer exposures (20 days) were applied to confirm the lack of gene expression (data not shown).

Generation and analysis of *Arabidopsis* transgenic lines

The complete coding sequences of *VvFT* or *VvTFL1A* were cloned into vector pROK II (Clontech Laboratories, Palo Alto, CA, USA) under the control of the *CaMV* 35S promoter and the NOS terminator. These constructions were introduced into *Agrobacterium tumefaciens* C58 by electroporation and the resultant *Agrobacterium* strains used to transform *Arabidopsis* Columbia plants using the floral-dip method (Clough and Bent 1998). Transgenic plants were selected on MS medium supplemented with 10 g/l sucrose and 50 mg/l kanamycin. For each transformation assay, several independent transgenic plant homozygous for a single insertion locus was selected based on the antibiotic marker segregation in the T3 generation. Homozygous T3 plants were used for all phenotypic analyses.

For phenotypic characterization, plants were grown in pots with soil and vermiculite at 3:1 proportion in a greenhouse at 22°C and under natural spring LD illumination. Three random blocks of transgenic and control lines were planted and analyzed. Flowering time was measured as the number of days from sowing tills the opening of the first flower (FT) and as the total number of leaves produced by the primary shoot before flowering (TLN) distinguishing between rosette leaves (RLN) and inflorescence leaves (ILN). Flowering phenotypes of wild type and transgenic lines were compared by ANOVA using the SPSS v13 statistical package.

Results

Identification of grapevine FT/TFL1 homologous genes

An extensive search for members of the *FT/TFL1* family was performed on RNA samples extracted from different grapevine organs using 3′ and 5′ RACE strategies. Five different cDNA sequence species were identified as unambiguously belonging to this family as revealed by sequence similarities with other family members characterized in flowering plants (Figs. 1 and 2). Isolation of genomic clones revealed that all five genes conserved the characteristic genomic organization for this gene family, with four exons and three introns in identical positions (Fig. 1A and B). Comparisons of deduced protein sequences with FT/TFL1-like sequences from other dicots (Fig. 2A) and the results of the phylogenetic analysis (Fig. 2B, see below) allowed considering one of these sequences as a FT ortholog, another as a MFT ortholog and the rest as TFL1-related proteins. Based on these sequence similarities they were respectively designated as VvFT, VvMFT and VvTFL1A, VvTFL1B and VvTFL1C.

Phylogenetic analyses of grapevine FT/TFL1 homologous genes

To analyze the phylogenetic relationships between grapevine and other dicot *FT/TFL1* homologous genes, we selected those plant species in which at least two related genes had been reported. Phylogenetic analysis was conducted using the complete amino acid sequences and generated an unrooted tree containing three major clades supporting three major subfamilies (Fig. 2B). The FT-subfamily appeared as a well-resolved monophyletic clade, relating VvFT with *Arabidopsis* FT and TSF as well as other FT orthologous proteins identified in other dicot species (Fig. 2B). VvFT, the putative ortholog of FT, displays all the characteristic features of the FT protein subfamily (Ahn et al. 2006), which include the conservation of Tyr85 (Tyr84 in VvFT) and Gln140 (Gln139 in VvFT), the conserved 11 amino acid residues in exon 4, critical for FT activity, as well as the highly conserved LYN triad also present in exon 4 (Fig. 1A and 2A).

A second distinctive monophyletic clade related VvMFT with *Arabidopsis* MFT and its putative orthologous proteins identified in *Populus* and tomato (Fig. 2B). In all of them (Figs. 1A and 2A), the critical Tyr and Gln residues are substituted by residues with similar characteristics (Trp and Ser, respectively). Furthermore, the conserved triad sequence characteristic of the FT group is absent in VvMFT.

The three additional grapevine genes appeared more dispersed in three different subclades related with *Arabidopsis* BFT, TFL1 and ATC (Fig. 2B). All of them bear conserved, charged residues His88 and Asp144 in similar positions as TFL1, as well as the characteristic amino acid triad (ENE, END and DNG respectively for VvTFL1A, VvTFL1B and VvTFL1C) in exon 4 (Figs. 1A and 2A). VvTFL1C grouped with *Arabidopsis* BFT, *Populus* PnFTl1a and *Nicotiana* CET1, in what could represent a clade of BFT orthologous sequences. VvTFL1A was the most closely related protein to the *Arabidopsis* ATC and TFL1 as well as to the *Antirrhinum* CEN, while VvTFL1B appeared in a different subclade associated to TFL1-like proteins less related to those identified in *Arabidopsis* and *Antirrhinum*.

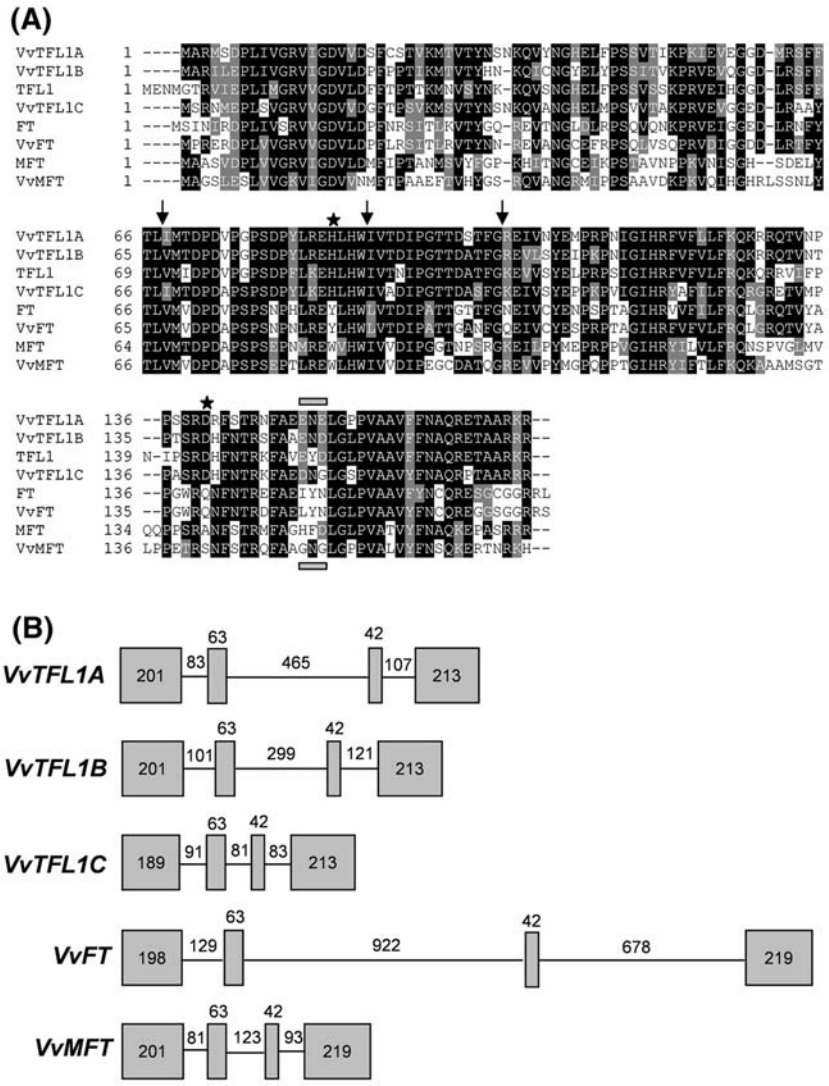

Fig. 1 The FT/TFL-1 grapevine gene family. (**A**) Comparison of the deduced amino acid sequences of VvTFL1A, VvTFL1B, VvTFL1C, VvFT, VvMFT from grapevine and FT, TFL1 and MFT from *Arabidopsis*. Dashed lines indicate gaps introduced to obtain maximum alignment. Intron positions are indicated by black arrows above sequences. Asterisks indicate amino acids that are critical to define FT or TFL1-like proteins. The two grey boxes indicate the amino acid triad in exon four. (**B**) Genomic organization of the five genes. Coding regions (grey boxes) and introns (lines) are illustrated. Numbers represent the length of exons and introns (bp)

Expression pattern of grapevine *FT/TFL1* homologous genes

As a first approach to understand the function of these genes in grapevine development, we studied their temporal and spatial pattern of expression during vegetative and reproductive development using RNA blot hybridization. During reproductive development, all genes were expressed in latent buds of the first season with the exception of *VvMFT* (Fig. 3A). *VvFT* and *VvTFL1B* transcripts were barely detectable in very young latent buds (May), while *VvTFL1C* and *VvTFL1A* reached higher levels of expression in these buds. *VvTFL1C* expression increased progressively during latent bud development, whereas *VvTFL1A* reached its highest level of expression during the first developmental stages. No expression was detected during the dormancy period. However, during the second season, when bud development was resumed, all genes were expressed with the exception of *VvTFL1C*. At these stages (A, B, and C), inflorescence meristems divide to generate additional branch inflorescence meristems, which finally give rise to flower meristems and flowers. *VvFT* expression was detected at low levels during stages B and C. *VvMFT* was also expressed at low level but it was detected earlier, at the end of stage A and during stage B. Expression of *VvTFL1B* was barely detected during the end of stage A and at the beginning of stage B. Again, the highest expression level detected corresponded to *VvTFL1A*, which transcript levels progressively increased reaching a maximum in swelling buds (stage B) and decreasing in sprouting buds when shoots start to grow out (stage C, Fig. 3A, second season).

Expression of the five genes was also analyzed in developing inflorescences, berries and seeds (Fig. 3B).

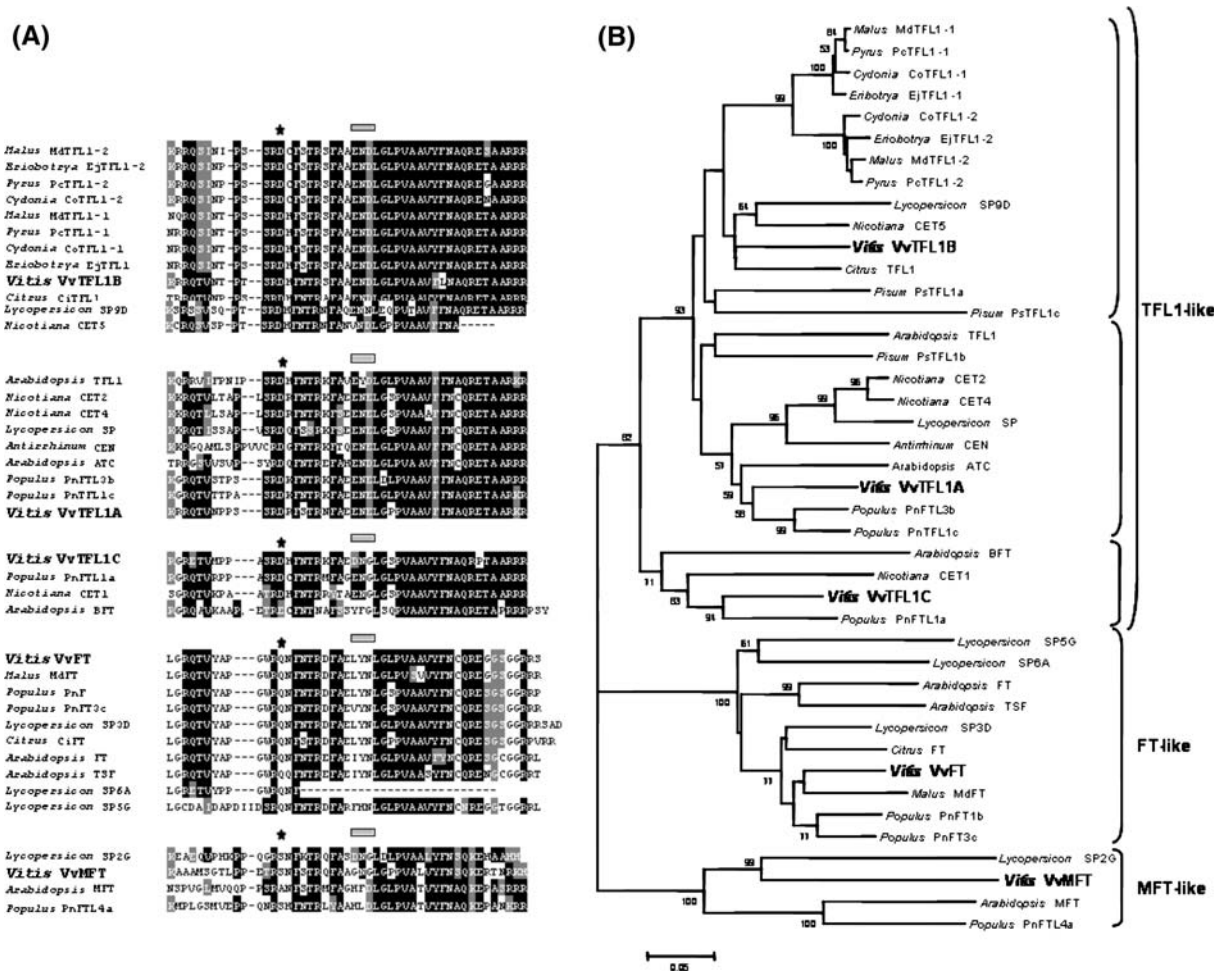

Fig. 2 Comparison of the FT/TFL1 grapevine proteins with homologous proteins of dicots plants. (**A**) Comparison of the deduced amino acid sequences at critical positions of the fourth exon. Asterisks indicate a critical amino acid to define FT or TFL1-like proteins. Grey boxes indicate the amino acid triad. (**B**) Phylogenetic tree of FT/TFL1- proteins. Bootstrap support values are indicated when over 50. The three subfamilies are indicated on the right under parentheses. The scale indicates the average substitutions per site. Accession numbers are the following: *Antirrhinum* CEN (S81193); *Arabidopsis* FT (AF152096), TSF (AF152907), TFL1 (U77674), MFT (AF147721), ATC (AB024714) and BFT (NM_125597); *Citrus* CiFT (AB027456) and CiTFL1 (AY344245); *Cydonia* CoTFL1-1 (AB162043) and CoTFL1-2 (AB162049); *Eriobotrya* EjTFL1-1 (AB162045) and EjTFL1-2 (AB162051); *Lycopersicon* SP (U84140), SP6A (AY186737), SP3D (AY186735), SP9D (AY186738), SP2G (AY186734) and SP5G (AY186736); *Malus* MdFT (AB161112), MdTFL1-1 (AB052994) and MdTFL1-2 (AB162046); *Nicotiana* CET1 (AF145259), CET2 (AF145260), CET4 (AF145261) and CET5 (AF145262); *Pisum* PsTFL1a (AY340579), PsTFL1b (AY340580) and PsTFL1c (AY343326); *Populus* PnFTL1a (AB181183), PnFTL4a (AB161108), PnFT1b (AB161109), PnFTL3b (AB181240), PnTFL1c (AB104629) and PnFT3c (AB110009); *Pyrus* PcTFL1-1 (AB162042) and PcTFL1-2 (AB162048)

VvFT expression was detected all along inflorescence development, with the exception of stage H50. All other genes were mainly detected at the end of flower development (stages H40 and H50, Fig. 3B) just before anthesis (stage I), with *VTFL1A* expression being particularly high in stage H50. During fruit development and ripening, the detection of *VvFT* in stage I and preferentially in the seeds of stage II and III suggests that it is mainly expressed in seeds. Low level of expression was detected for *VvTFL1C* during stage III of berry development both in flesh and seeds, as well as for *VvMFT* in seeds for late stage III. No expression was detected for *VvTFL1A* and *VvTFL1B* even after longer exposures.

During vegetative development, the pattern of expression of the five genes differed significantly. *VvFT* was detected in shoots along their development (stages E–I) as well as in the apex of H50 shoots (Fig. 3C). It was also detected during tendril development mainly in the arms of well-developed tendrils (T5a) as well as in tendrils of young plants grown from seeds, which would still grow vegetatively for 2–5 more years before

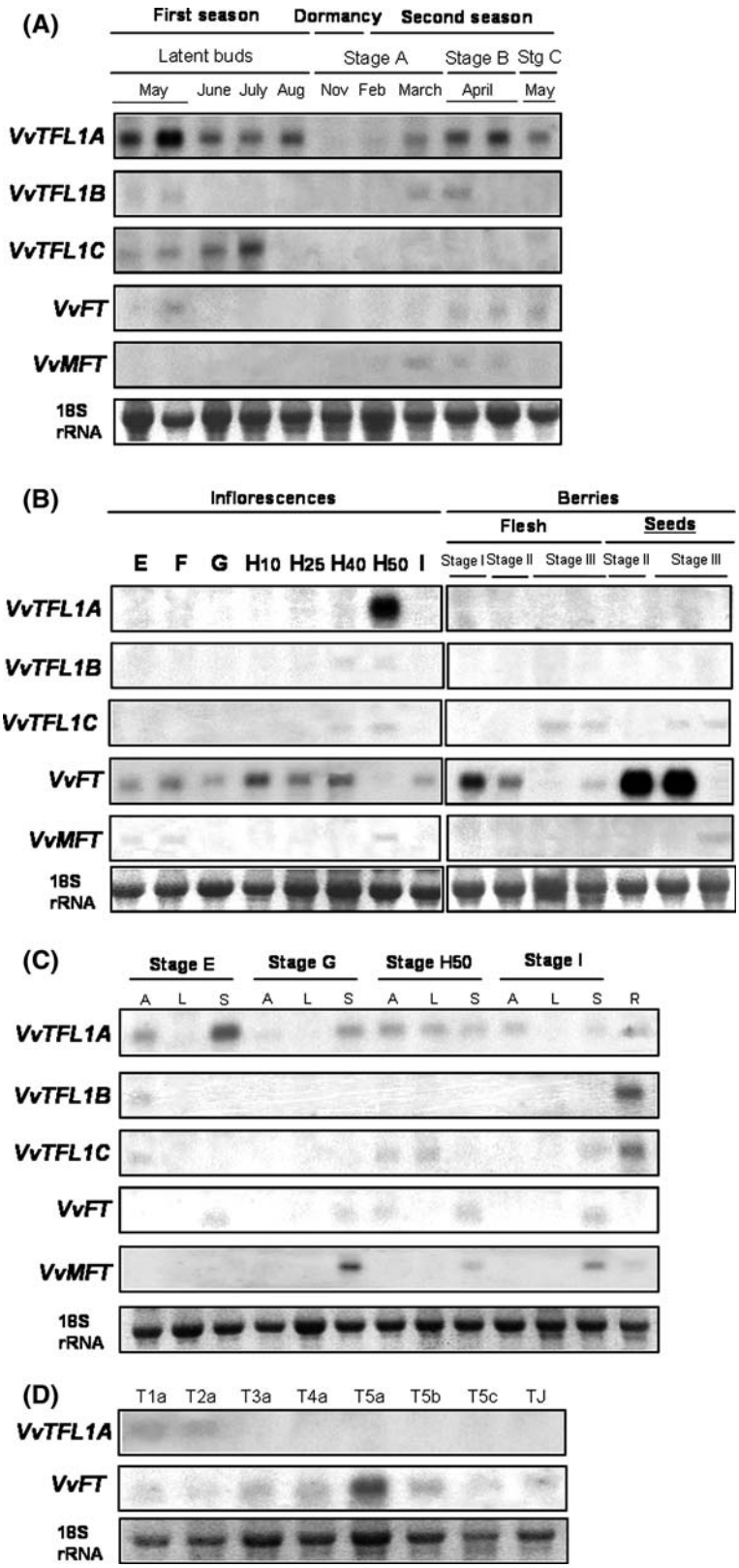

Fig. 3 Expression analyses of the *FT/TFL1* gene family in grapevine. (**A**) Expression in buds during two consecutive growing seasons. Latent buds in the first growing season, winter buds (stage A) and second season buds (stage B and C). (**B**) Expression in developing inflorescences. Inflorescences belonging to phenological stages E, F, G, H10, H25, H40, H50 and I, flesh (stages I, II and III) and seeds (stages II and III) of developing berries. (**C**) Expression in vegetative organs. Shoot apex (A), leaves (L) and shoot internodes (S) at phenological stages E, G, H50 are shown and roots (R) from in vitro grown plants. (**D**) Expression of *VvTFL1A* and *VvFT* during tendril development. Expression in the arms of four consecutive tendrils of an elongated shoot numbered from the shoot apex (T1, T2, T3, T4) and in three regions of tendril T5: the arms (T5a), the branching zone (T5b) and the hypoclade zone (T5h). Expression in the first tendril of a young plant (TJ). 18S RNA was used as a quantitative control of loading

initiating flowering (Fig. 3D). Expression of *VvMFT* was also detected in shoots at higher level than *VvFT*; it was observed in shoots of stages G-I but not in younger shoots (stage E). Among the *TFL1* related genes, *VvTFL1A* showed the broadest expression pattern with transcripts detected in all the shoot stages, mainly in shoot apices and shoot internodes but also in H50 leaves (Fig. 3C). *VvTFL1A* transcripts were also detected in the arms of tendrils at their first stages of development (T1a, T2a, Fig. 3D). *VvTFL1C* was also detected in the shoot apices of stages E and H50, leaves of stage H50 and stage I shoots, but was not found in tendrils. In contrast, *VvTFL1B* expression *w*as restricted to the apex of young shoots (stage E, Fig. 3C). Finally, expression of all genes but *VvFT* was detected in roots with *VvTFL1B* and *VvTFL1C* displaying the highest levels.

Effects of the ectopic expression of *VvFT* and *VvTFL1A* in transgenic arabidopsis plants

Sequence similarities and expression pattern analyses suggested that *VvFT* and *VvTFL1A* could be orthologs of *Arabidopsis FT* and *TFL1* respectively. In order to test their possible functional relationship, we ectopically expressed these *Vitis* genes in transgenic *Arabidopsis* plants using transcriptional fusions to the CaMV 35S promoter. More than 20 kanamycin-resistant transgenic plants were selected from five independent transformation experiments performed for each construct. Among them, five homozygous T3 lines were randomly selected for each construct to carry out a phenotypic analysis.

Three transgenic lines overexpressing *VvFT* flowered significantly earlier than wild type plants (Table 1 and Fig. 4A–F). These lines showed a reduction in their flowering time of up to one week and flowered with approximately half the number of leaves than the wild type plants (Table 1 and Fig. 4B), showing a significant reduction in leaf number both in rosettes and inflorescences. Leaves of transgenic plants were generally narrower and smaller than wild type leaves and displayed a curled phenotype (Fig. 4C–D). Furthermore, plants from the earliest *35S::VvFT415* transgenic line often showed the development of terminal flowers resulting from the differentiation of inflorescence meristems as flower meristems (Fig. 4F).

Four transgenic *Arabidopsis* lines ectopically expressing *VvTFL1A* were significantly delayed ($P < 0.01$) in flowering time with respect to wild type plants (Table 1 and Fig. 4G–N). However, contrary to what has been reported (Boss et al. 2006), only one line showed a significant increase ($P < 0.05$) in the total number of leaves, as a consequence of a significant higher number of inflorescence leaves ($P < 0.01$) (Table 1). Thus, the observed delay in flowering time could be more related to an increase in the plastochron length than to the production of additional leaves. In many transgenic plants, overexpression of *VvTFL1A* caused a reduction of apical dominance promoting the

Table 1 Flowering phenotype of transgenic Arabidopsis lines expressing either *VvFT* or *VvTFL1A* constructs

	n	RLN x ± SD	ILN x ± SD	TLN x ± SD	FT x ± SD	Comments
Col wild type	25	11.2 ± 1.1	2.9 ± 0.3	14.2 ± 1.2	29.0 ± 1.9	
35S::VvFT112	19	10.6 ± 1.2	2.7 ± 0.6	13.4 ± 1.3	29.4 ± 3.3	
35S::VvFT157	20	10.1 ± 1.4*	3.0 ± 0.6	13.1 ± 1.4	29.6 ± 1.9	
35S::VvFT415	15	4.4 ± 0.7**	1.5 ± 0.7**	5.9 ± 0.9**	23.1 ± 3.1**	tfl
35S::VvFT441	28	4.6 ± 1.1**	1.6 ± 0.6**	6.2 ± 1.6**	23.2 ± 3.3**	
35S::VvFT451	14	5.4 ± 0.9**	1.6 ± 0.6**	7.0 ± 1.3**	26.1 ± 3.7**	
35S::VvTFL1A122	14	11.3 ± 0.7	2.9 ± 0.5	14.2 ± 0.9	33.5 ± 3.2**	
35S::VvTFL1A274	15	10.9 ± 1.3	2.7 ± 0.6	13.6 ± 1.7	31.8 ± 2.2	
35S::VvTFL1A313	12	10.8 ± 1.1	3.3 ± 0.5	14.2 ± 1.3	38.7 ± 5.5**	fmi
35S::VvTFL1A416	14	11.6 ± 1.1	4.0 ± 0.7**	15.6 ± 1.5*	39.9 ± 6.1**	fmi
35S::VvTFL1A417	15	11.3 ± 1.4	3.1 ± 1.1	14.4 ± 2.1	34.2 ± 4.5**	fmi

RLN: Rosette leaf number in the main stem

ILN: Inflorescence leaf number

TLN: Total leaf number (Rosette + Inflorescence)

FT: Flowering time (days) from sowing to the opening of the first flower

tfl: Approximately 20% of the plants develop terminal flowers

fmi: Flower meristem identity defects

* Significantly different from wild type Columbia plants at $P < 0.05$

** Significantly different from wild type Columbia plants at $P < 0.01$

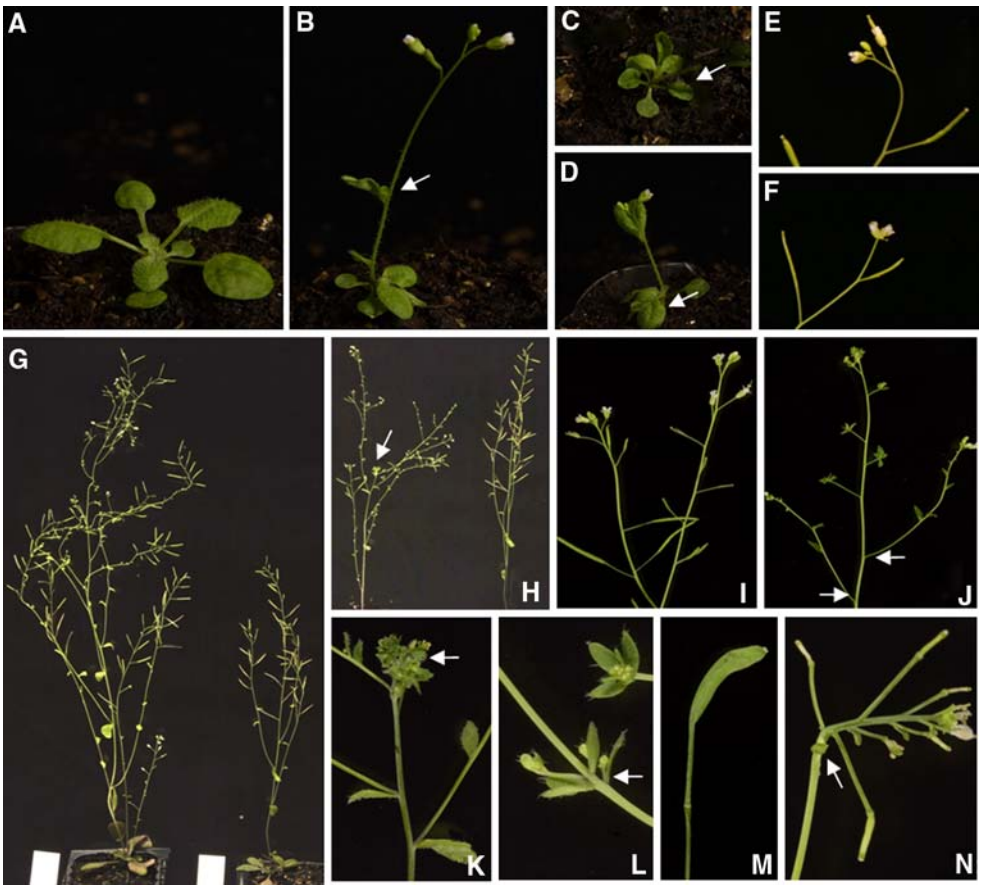

Fig. 4 Reproductive phenotype of transgenic Arabidopsis plants expressing either *VvFT* or *VvTFL1A* genes from grapevine. (**A**) Wild type Arabidopsis Columbia plant three weeks after sowing. (**B**, **C** and **D**) Transgenic *35S::VvFT415* plants initiating flowering three weeks after sowing. The arrows indicate the formation of small curly leafs. (**E**) Inflorescence apex in a flowering wild type plant. (**F**) Inflorescence apex in a *35S::VvFT415* transgenic plant showing the development of a terminal flower. (**G**) Transgenic *35S::VvTFL1A416* (left) and wild type plant (right) eight weeks after sowing. (**H**) Detail of transgenic (left) and wild type (right) inflorescence. The arrow indicates the abortion of the inflorescence apex in the transgenic line. (I) Close-up of wild type inflorescence. (**J**) Close-up of *35S::VvTFL1A416* inflorescence. Inflorescence-like structures develop in place of flowers. The arrows indicate the development of coflorescences not subtending by leaves. (**K**) Close-up of an aborted inflorescence apex in a *35S::VvTFL1A416* transgenic plant. (**L**) Close-up of inflorescence-like structures formed in *35S::VvTFL1A416* transgenic plants showing whorled phyllotaxis of leaf-like organs and the development of leaf axillary meristems as a new round of flower meristems (arrow). (**M**) A *35S::VvTFL1A416* fruit developed from a non-completely determinate flower that frequently breaks out giving rise a short inflorescence. (**N**) Inflorescence developing from a broken fruit in a *35S::VvTFL1A416* transgenic plant. The arrow indicates the place where the silique broke

growth of lateral inflorescences from rosette leaves axillary meristems (Fig. 4H and K). These lateral meristems produce additional rosette leaves that would yield a high leaf number score if considered together with the main stem ones. Apart from this delay, three transgenic lines showed an inflorescence phenotype consistent with a delay in the establishment of flower meristem identity and the production of a high number of coflorescences not subtended by leaves (Table 1 and Fig. 4I–L). As exemplified for line *35S::VvTFL1A416* in Fig. 4, these transgenic lines show a lack of determination of flower meristems resulting in the frequent development of inflorescence branches or coflorescences in place of flowers as well as the differentiation of additional inflorescences within the siliques (Fig. 4I–J and L–N).

Discussion

We have identified five members of the grapevine *FT/TFL1* gene family as revealed by the comparison of sequences and genomic organization. Only one of these sequences, *VvTFL1A*, has previously been reported and partially characterized (Joly et al. 2004; Boss et al. 2006). Additional sequences belonging to

this family could still be found in the grapevine genome when the whole sequence is completed. Phylogenetic analyses group these genes in three clades or subfamilies, as has been previously shown in other species (Carmel-Goren et al. 2003; Chardon and Damerval 2005; Ahn et al. 2006). As in *Citrus* (Endo et al. 2005) and apple trees (Kotoda and Wada 2005) only one *FT* related sequence has been found in grapevine, whereas duplication and divergence of this sequence has been frequently observed in other botanical families such as *Brassicaceae* (Kobayashi et al. 1999), *Solanaceae* (Carmel-Goren et al. 2003; Lifschitz et al. 2006) or *Salicaceae* (Brunner and Nilsson 2004). Similarly, a single *MFT* orthologous gene was found in grapevine in agreement with the presence of single genes within this subfamily in fully sequenced genomes such as *Arabidopsis* or *Populus*. Regarding TFL1 proteins, the picture observed in grapevine agrees with what has been observed in other plant species. TFL1 proteins do not appear as monophyletic, likely due to the existence of a large divergence among the different sequences as a result of accelerated evolution (Ahn et al. 2006).

VvFT is associated with reproductive development in grapevine

Three lines of evidence support *VvFT* as the *FT* grape ortholog. First, VvFT shows a strong conservation of all the amino acid residues and regions characteristic of this subfamily and critical for their function. Second, the *VvFT* expression pattern is associated to seasonal flowering induction in latent buds and to the development of inflorescences, flowers and fruits (Fig. 3A and B), similarly to what has been described for *FT* (Kobayashi et al. 1999; Kardailsky et al. 1999; Takada and Goto 2003; Yamaguchi et al. 2005). Third, the overexpression of *VvFT* in transgenic *Arabidopsis* plants causes similar effects as *FT* overexpression in flowering promotion (Kobayashi et al. 1999; Kardailsky et al. 1999) including the production of terminal flowers in the most extreme lines.

In *Populus*, the function of the *FT* ortholog *FT2* has been related with phase change (Hsu et al. 2006), since a critical level of *FT2* expression is associated with flowering initiation. Juvenile phase is very short in grapevine and transition to adult vegetative phase is marked by the development of tendrils. *VvFT* could play a similar function in grapevine and, in fact, its expression is already detected in those first tendrils marking the juvenile to adult phase transition. Further expression analyses in juvenile grapevine plants will be required to elucidate the role of *VvFT* in this transition as well as its environmental or developmental regulation.

Tendrils and inflorescences share a common ontogenetic origin in grapevine (Pratt 1971) and we have previously shown that *VAP1*, the putative grapevine *AP1* ortholog, is expressed in all the tendrils of the plant (Calonje et al. 2004). Detection of *VvFT* expression in tendrils and inflorescences additionally supports the homology between those two organs. *AP1* was shown to be a downstream target of *FT* in *Arabidopsis* (Ruíz-García et al. 1997, Wige et al. 2005) and the observed parallelism between the expression of *VvFT* and *VAP1* in grapevine could suggest the conservation of a similar regulatory network. Expression of *VvFT* and *VAP1* in tendrils and inflorescences associates with the formation of grapevine reproductive structures but additional functions should be required to trigger inflorescence development and flower initiation. Finally, the high *VvFT* expression during fruit development and especially in developing seeds could suggest a role for this gene during those processes, not yet reported in other systems. It would be interesting to check whether this is a particular feature of grapevine or common to other plant species.

VvMFT, as other putative *MFT* orthologs (Figs. 1A and 2B), shows sequence differences in critical residues that are conserved in *FT* and is related to the tomato *SP2G* and *Populus PnFTL4a* (Fig. 2). *VvMFT* expression displays a certain parallelism with the expression pattern to *VvFT*, especially during flower initiation (stages A–B of the second growing season). However, it does not seem to be associated with flowering induction during the first season. These results could be in agreement with a possible role as flowering promoter as has been suggested for the *MFT* gene of *Arabidopsis* (Yoo et al. 2004). Detection of *VvMFT* and *VvFT* transcripts in shoots parallels the detection of *FT* in vascular tissues of *Arabidopsis* where it has been associated with its role in flowering induction (Huang et al. 2005). Further in situ hybridization experiments will be required to support these possibilities.

At least three *TFL1*-like genes with divergent expression profiles are present in grapevine

Three grapevine proteins group within the TFL1 clade. Both VvTFL1A and VvTFL1B are related to *Arabidopsis* ATC and TFL1, with VvTFL1A showing more sequence similarity to ATC than to TFL1. Although VvTFL1C, the third member of TFL1-like subfamily, groups together with *Arabidopsis* BFT, *Nicotiana* CET1 and *Populus* FTL1a in a separated clade, it is not clear whether they can be considered BFT orthologs or can represent another type of TFL1-like proteins.

Considering the critical residues and the amino acid triad characteristic of FT, BFT shows sequence features that all together make it more similar to the FT subfamily than to the TFL1 subfamily. However, the rest of the genes of the cluster including *VvTFL1C* accomplish all the characteristics of the TFL1 subfamily.

Expression of the three *TFL1*-like genes in latent buds of the first season and during the initial stages of inflorescence development (Fig. 3A) and their absence along flower development (Fig. 3B) is compatible with a role of these genes in maintaining meristem indeterminacy (SAM, lateral meristems and inflorescence meristems) within the bud. The initiation of flower meristems and flower development is coincident with their lack of expression that is only resumed in the late stages of flower development, perhaps coincidentally with ovule development. Similarly, expression of *VvTFL1A* but not the others in the first stages of tendril development could also be compatible with the maintenance of meristematic activity in the initial stages of tendril development. Whether these *TFL1*-like genes are functionally redundant or have specific roles in different meristems awaits further characterization. The meristem maintenance role of *VvTFL1A* is consistent with the phenotype observed in transgenic *Arabidopsis* plants, where the expression of *VvTFL1A* prevents flower meristem determination (Fig. 4). Although *TFL1* has been shown to regulate both flowering time and inflorescence meristem indeterminacy in *Arabidopsis* (Bradley et al. 1997), both functions do not seem to be controlled by the same gene in other species. For example, *CEN* in *Anthirrinum* (Bradley et al. 1996) is mainly expressed in the inflorescence meristems and loss-of-function mutants do not show a flowering time phenotype but the formation of a terminal flower with radial symmetry in place of the inflorescence meristem. In pea (Foucher et al. 2003) two *TFL1* homologs have been found. One of them, known as *DETERMINATE* (*DET*, *PsTFL1a*), acts to maintain the identity of inflorescence meristem and its expression is limited to the shoot apex after floral initiation. The other homolog, known as *LATE FLOWERING* (*LF*, *PsTFL1c*), seems to control the length of the vegetative phase by delaying floral initiation. Duplication of genes and further subfunctionalization could explain the existence of two genes exerting each function (Foucher et al. 2003).

Expression of grapevine *TFL1*-like genes during vegetative development also follows a common pattern. *VvTFL1A* is more highly expressed than the two other genes and found in the roots, in the shoot apex and in the stems probably associated to apical and intercalary meristematic regions. This expression is followed by the two other genes in a pattern that is not always completely overlapping. This similar expression pattern could also suggest certain level of functional redundancy among the grapevine *TFL1*-like genes. As a whole, this expression pattern is also consistent with a role of these genes in the maintenance of meristematic functions during vegetative development. Some *TFL1*-like genes such as *CsTFL1* of *Citrus* (Pillitteri et al. 2004) and *MdTFL1* of *Malus* (Kotoda et al. 2006) have been involved in the regulation of juvenile phase. Further analyses of *TFL1*-like gene expression along juvenile phase in grapevine could provide information about their involvement in the juvenile to adult phase transition.

When expression patterns of grapevine *FT/TFL1*-like genes are considered as a whole, *VvFT* and *VvMFT* are found associated with meristem determination and differentiation of organs such as inflorescences, flowers or tendrils; whereas *TFL1*-like genes expression associates to proliferative stages and organs, such as shoot apices and roots. These expression patterns are in agreement with the biological function proposed for these genes subfamilies in other species (Bradley et al. 1997; Pillitteri et al. 2004; Ahn et al. 2006) and could suggest a basic role for the gene family in meristem maintenance and determination (Lifschitz et al. 2006). Further functional analyses will be required to precisely establish the biological function of each of these genes in grapevine.

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