

Genome-Wide Analysis of MIKC^C-Type MADS Box Genes in Grapevine^{1[W][OA]}

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MIKC^C-type MADS box genes encode transcription factors that play crucial roles in plant growth and development. Analysis of the grapevine (*Vitis vinifera*) genome revealed up to 38 MIKC^C-type genes. We report here a complete analysis of this gene family regarding their phylogenetic relationships with homologous genes identified in other sequenced dicot genomes, their genome location, and gene structure and expression. The grapevine genes cluster in 13 subfamilies with their *Arabidopsis* (*Arabidopsis thaliana*) and poplar (*Populus trichocarpa*) counterparts. The lack of recent whole genome duplications in grapevine allows assigning the gene diversification processes observed within each subfamily either to an ancestral polyploidization event predating the divergence of those three species or to later duplication events within each lineage. Expression profiles of MIKC^C-type genes in vegetative and reproductive organs as well as during flower and tendril development show conserved expression domains for specific subfamilies but also reflect characteristic features of grapevine development. Expression analyses in latent buds and during flower development reveal common features previously described in other plant systems as well as possible new roles for members of some subfamilies during flowering transition. The analysis of MIKC^C-type genes in grapevine helps in understanding the origin of gene diversification within each subfamily and provides the basis for functional analyses to uncover the role of these MADS box genes in grapevine development.

MADS box genes encode transcription factors that are involved in developmental control and signal transduction in eukaryotes (Riechmann and Meyerowitz, 1997; Messenguy and Dubois, 2003; De Folter and Angenent, 2006). They are defined by the presence of a conserved domain, the MADS box, in the N-terminal region that is involved in DNA binding and dimerization with other MADS box proteins. Two monophyletic lineages, known as MADS type I and MADS type II, which are present in plants, animals, and fungi, can be distinguished (Alvarez-Buylla et al., 2000b; De Bodt et al., 2003). Type II group includes MEF2-like genes of animals and yeast and MIKC-type genes only found in plants. MIKC-type genes received this name because, apart from the MADS (M) domain, they contain three additional conserved domains, the Intervening (I) domain, the Keratin (K) domain, and the C-terminal (C) domain (Theissen et al., 1996; Kaufmann

et al., 2005). The I domain is responsible for specificity in the formation of DNA-binding dimers, the K domain mediates dimerization, and the C domain functions in transcriptional activation and the formation of higher order protein complexes. MIKC-type genes have been further divided into two subgroups, MIKC^C and MIKC*, based on sequence divergence at the I domain (Henschel et al., 2002). The MIKC* group has six genes in *Arabidopsis* (*Arabidopsis thaliana*) that seem to be involved in male gametophyte differentiation (Verelst et al., 2007). The type I lineage groups genes with simpler gene structure and lacking the K domain. Their function is generally not well understood yet in *Arabidopsis* or other species, with some exceptions (Bemer et al., 2008; Colombo et al., 2008). MIKC^C-type MADS box genes (from now on called MIKC genes) are the best characterized group of MADS box genes and have been involved in essential and diverse functions related to plant growth and development (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a; Theissen, 2001; Becker and Theissen, 2003; Kaufmann et al., 2005; Theissen and Melzer, 2007). Extensive analyses of members of this gene lineage in sequenced plant genomes have identified up to 13 gene subfamilies based on protein sequence conservation (Martinez-Castilla and Alvarez-Buylla, 2003; Parenicova et al., 2003; De Bodt et al., 2006; Kater et al., 2006; Leseberg et al., 2006; Arora et al., 2007).

The first plant MIKC genes were identified as floral organ identity genes in *Antirrhinum majus* and *Arabidopsis*. Further genetic and molecular analyses grouped

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their biological functions in flower organogenesis into five classes, A, B, C, D, and E, which are required, in different combinations, to specify the identity of sepals (A + E), petals (A + B + E), stamens (B + C + E), carpels (C + E), and ovules (D + E; Schwarz-Sommer et al., 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Angenent and Colombo, 1996; Pélaz et al., 2000, 2001; Theissen, 2001; Ditta et al., 2004; Theissen and Melzer, 2007). Briefly, Arabidopsis MIKC genes classified in each of those functional classes correspond to *APETALA1* (*AP1*) in class A, *PISTILATA* (*PI*) and *AP3* in class B, *AGAMOUS* (*AG*) in class C, *SEEDSTICK/AGAMOUS-LIKE11* (*STK/AGL11*) in class D, and *SEPALLATA* (*SEP1*, *SEP2*, *SEP3*, and *SEP4*) genes in class E. MIKC genes in the *AG* and *AP1/FRUITFULL* (*FUL*) subfamilies also participate in fruit and seed development (Gu et al., 1998; Ferrándiz et al., 2000b; Pinyopich et al., 2003).

Other MIKC genes were later identified as involved in different regulatory steps of networks controlling flowering time and flower initiation. In this way, MIKC subfamilies like those represented by *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 1999; Ratcliffe et al., 2003; Searle et al., 2006; Reeves et al., 2007), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Lee et al., 2000; Samach et al., 2000; Hepworth et al., 2002; Moon et al., 2003; Schönrock et al., 2006; Liu et al., 2008), and *SHORT VEGETATIVE PHASE* (*SVP*; Hartmann et al., 2000; Yu et al., 2002; Michaels et al., 2003; Lee et al., 2007; Liu et al., 2008) are involved in the regulation of flowering transition by the integration of signals from different flowering pathways. These genes function as either positive (*SOC1*, *AGL24*) or negative (*FLC*, *SVP*) regulators of flower meristem identity genes, which include some MIKC genes belonging to the *AP1/FUL* subfamily (Mandel and Yanofsky, 1995; Ferrándiz et al., 2000a). Regarding the *AGL15* subfamily (*AGL15* and *AGL18* genes; Alvarez-Buylla et al., 2000a; Lehti-Shiu et al., 2005), recent results also suggest their possible role as repressors of floral transition (Adamczyk et al., 2007).

Expression of MIKC genes has also been detected outside reproductive organs, among them those belonging to subfamilies *AGL12* and *AGL17* (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a; Burgeff et al., 2002). Their expression suggested a role for those genes in vegetative development, which has later been evidenced for some of them in root development (Zhang and Forde, 2000; Tapia-López et al., 2008). Notwithstanding, a role for *AGL12* and *AGL17* genes as flowering promoters was also recently proposed (Han et al., 2008; Tapia-López et al., 2008).

The recent generation of the first-draft sequence of the grapevine (*Vitis vinifera*) genome (Jaillon et al., 2007; Velasco et al., 2007) offers the possibility of genome-wide analysis of MIKC genes. In addition, comparative genomics suggest that the grapevine genome has not undergone recent genome polyploidizations (Jaillon et al., 2007), facilitating the study of functional evolution of specific MIKC gene subfam-

ilies. Grapevine has a pattern of organ formation and development distinct from those previously described for annual herbaceous plants or for woody polycarpic plants (Mullins et al., 1992; Boss et al., 2003; Carmona et al., 2007). The presence of tendrils, which in the Vitaceae are considered modified reproductive structures, marks specific developmental differences when compared with other species. Tendril and inflorescences originate from lateral meristems, historically named anlagen or uncommitted primordia (Tucker and Hoefert, 1968; Pratt, 1974; Gerrath and Posluszny, 1988; Gerrath et al., 1998). Flowering transition is induced within summer latent buds of grapevine plants. Induction causes the two to three consecutive lateral meristems produced by the shoot apical meristem (SAM) to follow the fate of inflorescence meristems in place of tendril primordia. Inflorescence meristems divide actively to give inflorescence branch meristems during the summer before the entrance to dormancy. Flower initiation and organogenesis take place the following season, when bud growth resumes (Mullins et al., 1992; Boss et al., 2003; Carmona et al., 2007).

Previous molecular studies of grapevine reproductive development have described the characterization and expression of specific MIKC genes identified on the basis of their sequence homology to known genes in other plant species. This has been the case for several members of the *AP1/FUL*, *AP3/PI*, *AG*, *AGL6*, *SEP*, and *SOC1* subfamilies (Boss et al., 2001, 2002; Calonje et al., 2004; Sreekantan and Thomas, 2006; Sreekantan et al., 2006; Poupin et al., 2007). Based on the availability of the grapevine genome sequence (Jaillon et al., 2007; Velasco et al., 2007), we report here a thorough unbiased identification and analysis of grapevine MIKC genes. We have also analyzed their expression profiles in selected organs during plant development and during the process of flowering induction. Previous genome-wide phylogenetic analyses of these genes have been done in Arabidopsis, rice (*Oryza sativa*), and poplar (*Populus trichocarpa*), three species whose genomes have been sequenced (Martinez-Castilla and Alvarez-Buylla, 2003; Parenicova et al., 2003; De Bodt et al., 2006; Kater et al., 2006; Leseberg et al., 2006; Arora et al., 2007). The grapevine genome has a similar number of MIKC genes as Arabidopsis, which fall within 13 gene subfamilies. The origin of some of these subfamilies can be traced back to an ancestral polyploidization event predating the origin of the sequenced dicot genomes. Gene number within specific grapevine subfamilies varies with respect to what has been described in Arabidopsis and poplar, indicating the existence of specific gene duplication events in each lineage. Interestingly, larger differences in gene number are observed in MIKC gene subfamilies involved in flowering transition. Expression analyses of MIKC genes during grapevine development suggest the recruitment of specific genes to regulate the development of specific grapevine organs, such as tendrils and berry fruits. Altogether,

these results provide a framework for studying the biological function of MIKC genes in grapevine development.

RESULTS

Identification and Annotation of Grapevine MIKC Genes

A total of 32 MIKC genes were identified in the grapevine genome and are listed in Table I. Six additional sequences containing MADS domains characteristic of MIKC genes and mapping to defined chromosomal positions were also identified. This suggests that the total number of MIKC genes could rise to 38. Two of those sequences would belong to the *AGL17*, two to the *B-sister* (*BS*), and two to the *SVP* subfamilies based on the available sequence information (see below). Final confirmation of these sequences will require the analysis of a more complete release of grapevine genome sequences. We named the grapevine MIKC genes on the basis of their assignment to the previously established MIKC subfamilies (Becker and Theissen, 2003) followed by a number when several members were identified for a given subfamily. Most of the sequences found by BLAST searches were already annotated in the Genoscope proteome database and are listed in Table I with the corresponding locus tag. References of previously characterized and published genes are also included in Table I. Although the integrated method used by the Genoscope database to deduce proteins is very exhaustive (Howe et al., 2002), some gene annotations were found incorrect regarding the available ESTs as well as Arabidopsis and poplar information. In these cases, the proposed gene structure was deduced by comparison between the genomic and EST sequences and further alignment with Arabidopsis and poplar MIKC proteins. This permitted the identification of possible mistakes based on the expected location of exon-intron junctions in the corresponding subfamily. The gene structure of *VvAGL15.1* found in genomic searches but not annotated in the proteome database was first deduced by FGENESH software and confirmed by alignment with Arabidopsis and poplar MIKC proteins. The gene structure of *VvSVP5*, for which there is an annotated protein in the proteome database, was provided by L. Fernández, L. Torregrosa, G. Segura, A. López, A. Bouquet, and J.M. Martínez-Zapater (unpublished data). Finally, the *VvAG2* gene structure was derived from a tentative consensus (TC) present in the Gene Index database. The deduced protein sequences for all grapevine MIKC genes are included in Supplemental Figure S1.

All identified MIKC genes encode proteins ranging from 198 to 280 amino acids long that possess the modular structure and the conserved motifs of MIKC proteins. Only one gene (*VvSVP3*) presented a stop codon in a position corresponding to amino acid 181 within the K domain (Table I). This stop codon was

detected in all *VvSVP3* ESTs present in the databases. Exon-intron organization was annotated for all of the identified genes (Supplemental Table S1) based on comparison with the corresponding ESTs and Arabidopsis genes (Parenicova et al., 2003). Genes belonging to subfamilies *SEP*, *AGL6*, and *AP1/FUL* as well as *VvTM8*, *VvAGL15.2*, *VvSVP4*, and *VvSVP5* all have eight exons with similar lengths and positions, as in Arabidopsis. The fusion between exons 4 and 5 observed in Arabidopsis *SEP1* and *SEP2* was not found in grapevine, indicating that it took place later in the lineage giving rise to Arabidopsis. The remaining genes have seven exons, with the exception of the two members of the *BS* subfamily, which lack the third intron, like their Arabidopsis counterparts. In grapevine, neither the *PI* fusion of exons 1 and 2 nor the *SVP* exon 5 duplication characteristic of Arabidopsis genes was found. In general, the length of exons 1, 3, 4, 5, and 6 is conserved with respect to Arabidopsis, with the remaining exons being more variable in length, mainly those at the 3' end of the genes.

Phylogenetic Analysis of MIKC Proteins

To examine the phylogenetic relationships among grapevine MIKC proteins and group them within the established subfamilies, we constructed a phylogenetic tree from alignments of full-length grapevine, Arabidopsis, and poplar protein sequences (Fig. 1). The phylogenetic tree revealed 10 major clades grouping 13 subfamilies. One clade grouped the three close subfamilies *AP1/FUL*, *SEP*, and *AGL6*, while another included the *AGL15* and *AGL17* subfamilies. All grapevine MIKC genes were grouped with their Arabidopsis and poplar counterparts, with high bootstrap support. The only exception was the *TM8* subfamily, for which no representative has been found in Arabidopsis (Supplemental Figure S2). Remarkably, in most cases, two poplar genes were found for every homolog in grapevine or Arabidopsis. Although the number of genes grouped in each subfamily was generally similar between grapevine and Arabidopsis, some interesting exceptions could be observed. Grapevine genes outnumber Arabidopsis genes within the *SVP* subfamily, with grapevine having five genes and Arabidopsis having only two. Alternatively, Arabidopsis triples the number of genes in the *FLC* subfamily (six) with respect to grapevine (two).

Chromosomal Location of MIKC Genes

MIKC genes were found to be distributed on at least 13 of the 19 grapevine chromosomes (Fig. 2). Substantial clustering of these genes was evident on several chromosomes. The highest number of genes are located on chromosome 18 (six genes) and chromosome 1 (five genes). Three genes are located on chromosomes 14 and 17, two on chromosomes 10 and 15, and one on chromosomes 2, 4, 7, 8, 12, 13, and 16. The physical positions of contigs containing four of the

Table 1. *Grapevine MIKC genes*

For every gene, locus tag, accession number, protein length, and chromosomal location are listed. Genes in random chromosomes (chr_random) were assigned chromosome numbers but without known relative positions. Genes in chrUn_random are not yet located on chromosomes.

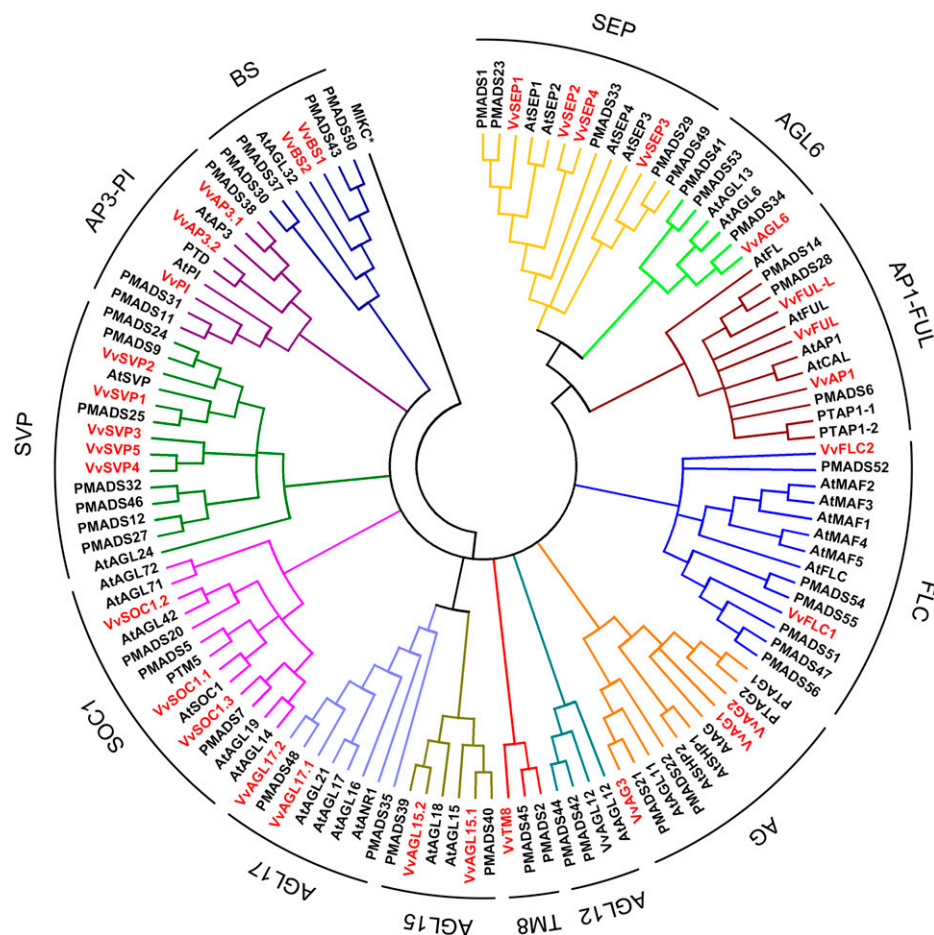
Gene Name	Locus Tag	Nucleotide Accession No.	Reference	Protein Length	Chromosome Location
<i>VvSEP1</i>	GSVIVT00038077001	AF373601	Boss et al. (2002)	244	chr14 from 12561660 to 12577986 strand –
<i>VvSEP2</i>	GSVIVT00000012001			246	chr17 from 5512792 to 5519273 strand –
<i>VvSEP3</i>	GSVIVT00002777001	AF373603	Boss et al. (2002)	242	chr1_random from 4526610 to 4544101 strand +
<i>VvSEP4</i>	GSVIVT00030008001			243	chr1 from 178664 to 206535 strand –
<i>VvAGL6</i>	GSVIVT00026310001	AF373602	Boss et al. (2002)	244	chr15 from 5979042 to 5994124 strand –
<i>VvFLC1</i>	GSVIVT00002779001 ^a	TC52043		210	chr1_random from 4586872 to 4691079 strand +
<i>VvFLC2</i>	GSVIVT00037877001 ^a	TC66735 CB918017		205	chr14 from 14712446 to 14739070 strand +
<i>VvAP1</i>	GSVIVT00030005001	AY538746	Calonje et al. (2004)	241	chr1 from 134931 to 159874 strand –
<i>VvFUL</i>	GSVIVT00000011001			243	chr17 from 5489843 to 5507341 strand –
<i>VvFUL-L</i>	GSVIVT00038079001	AY538747	Calonje et al. (2004)	247	chr14 from 12518771 to 12539672 strand –
<i>VvAGL12</i>	GSVIVT00021903001			198	chr18 from 17934884 to 17950596 strand –
<i>VvSOC1.1</i>	GSVIVT00026312001	DQ504309	Sreekantan and Thomas (2006)	218	chr15 from 5947971 to 5971440 strand +
<i>VvSOC1.2</i>	GSVIVT00010608001			210	chr16_random from 2909756 to 2947079 strand –
<i>VvSOC1.3</i>	GSVIVT00001070001			214	chr2 from 4136531 to 4147445 strand +
<i>VvTM8</i>	GSVIVT00017956001 ^a	TC62855		210	chr17 from 866730 to 869614 strand +
<i>VvAG1</i>	GSVIVT00018932001 ^a	AF265562	Boss et al. (2001)	225	chr12 from 391146 to 400827 strand +
<i>VvAG2</i>		TC62522		226	chr10 from 2636126 to 2645002 strand +
<i>VvAG3</i>	GSVIVT00021934001 ^a	AF373604	Boss et al. (2002)	223	chr18 from 18299365 to 18307245
<i>VvAGL17.1</i>	GSVIVT00015065001			235	chr18 from 6272032 to 6296722 strand +
<i>VvAGL17.2</i>	GSVIVT00008566001			233	chrUn_random from 50773717 to 50814913 strand +
<i>VvAGL15.1</i>			This work ^c	233	chr13 from 13140402 to 13149199 strand +
<i>VvAGL15.2</i>	GSVIVT00025618001			258	chr8 from 21179462 to 21182108 strand –
<i>VvSVP1</i>	GSVIVT00004864001			227	chrUn_random from 96950143 to 96956323 strand –
<i>VvSVP2</i>	GSVIVT00015108001			222	chr18 from 5628524 to 5645310 strand +
<i>VvSVP3</i>	GSVIVT00002394001 ^a	TC54716		181	chrUn_random from 15162252 to 15193980 strand +
<i>VvSVP4</i>	GSVIVT00007548001			259	chrUn_random from 47812625 to 47852333 strand –
<i>VvSVP5</i>	GSVIVP00009443001 ^b		L. Fernández, L. Torregrosa, G. Segura, A. López, A. Bouquet, and J.M. Martínez-Zapater (unpublished data)	218	chrUn_random from 58215171 to 58292578 strand –
<i>VvBS1</i>	GSVIVT00031869001			240	chrUn_random from 121594011 to 121617861 strand –
<i>VvBS2</i>	GSVIVT00030142001			283	chr1 from 1299900 to 1302201 strand +
<i>VvAP3.1</i>	GSVIVT00014506001	EF418603	Poupin et al. (2007)	226	chr18 from 11366453 to 11372200 strand +
<i>VvAP3.2</i>	GSVIVT00036846001	DQ979341	Poupin et al. (2007)	225	chr4 from 14253082 to 14255344 strand +
<i>VvPI</i>	GSVIVT00015451001 ^a	DQ059750	Sreekantan et al. (2006)	212	chr18 from 2253893 to 2256096 strand –
	GSVIVT00015452001 ^a				

^aGenoscope annotation corrected using EST information. ^bGenoscope annotation corrected using genomic information. ^cSequence found by genomic searches and not present in the proteome database.

MIKC genes have not been defined yet by the grape genome sequence projects. Interestingly, different members of many MIKC gene subfamilies are located in chromosomal regions that might represent paralogous segments resulting from ancestral polyploidization events (Jaillon et al., 2007; Velasco et al., 2007). This was observed for the *AP1/FUL*, *SEP*, and *FLC*

gene subfamilies, for which different members are located in chromosomes 1, 14, and 17; the *SOC1* subfamily, with different gene members on chromosomes 2, 15, and 16; the *AGL15* subfamily, with members on chromosomes 8 and 13; the *AP3/PI* and *SVP* subfamilies, with members on chromosomes 4, 7, and 18; and the *AG* subfamily, with two members on

Figure 1. Phylogenetic tree of the MIKC gene family in grapevine, Arabidopsis, and poplar. The tree was generated after sequence alignment with Multalin using the neighbor-joining method. Branches with less than 50% bootstrapping support were condensed. MIKC proteins grouped into 13 subfamilies. MIKC*-type MADS box proteins were used as an outgroup.



chromosomes 10 and 12 (the third one, *VvAG3*, is located on chromosome 18; Fig. 2).

Expression Analyses of MIKC Genes

MIKC genes have mainly been involved in the regulation of flowering time and the specification of reproductive organ identity. In order to further associate their biological function in grapevine with specific developmental processes, we analyzed their expression in eight representative vegetative and reproductive organs of the plant using quantitative reverse transcription (qRT)-PCR. Figure 3A displays the expression patterns of these genes as related to their phylogenetic relationships. As a general rule, gene expression patterns were frequently conserved within subfamilies, although expression levels of specific members could change in different organs. In this way, quantitative differences could be observed among members of the *SEP* and *AG* subfamilies in flowers and fruits. Similarly, within the *AP3/PI* clade, *VvAP3.2*, the closest homolog to tomato (*Solanum lycopersicum*) *TM6*, showed some expression in fruits, while *VvAP3.1* and *VvPI* expression was more restricted to flowers. More important changes in gene

expression within subfamily gene members could be observed in the *SVP* subfamily, where *VvSVP2* and *VvSVP5* were differentially expressed from the other three *VvSVP* genes in shoots and leaves, and within the *AP1/FUL* subfamily, where the different members showed a differential expression in tendril.

A cluster analysis of gene expression patterns allowed the identification of the major developmental processes in which grapevine MIKC genes could be involved (Fig. 3B). Three major clusters of expression patterns were distinguished that corresponded to genes preferentially expressed in vegetative organs, flowers, and flowers/fruits. The first cluster included two expression groups, corresponding to buds and buds/vegetative organs. The first expression group included six genes expressed in buds. Three are members of the *SVP* subfamily (*VvSVP1*, *VvSVP3*, and *VvSVP4*), two others form the *FLC* subfamily (*VvFLC1* and *VvFLC2*), and the sixth gene, *VvFUL*, belongs to *AP1/FUL* subfamily. These genes were preferentially expressed in buds, although they were also detected in vegetative organs and some of them in reproductive organs. The second expression group (five genes) included all three genes belonging to the *SOC1* subfamily and two *SVP* genes (*VvSVP2* and *VvSVP5*).

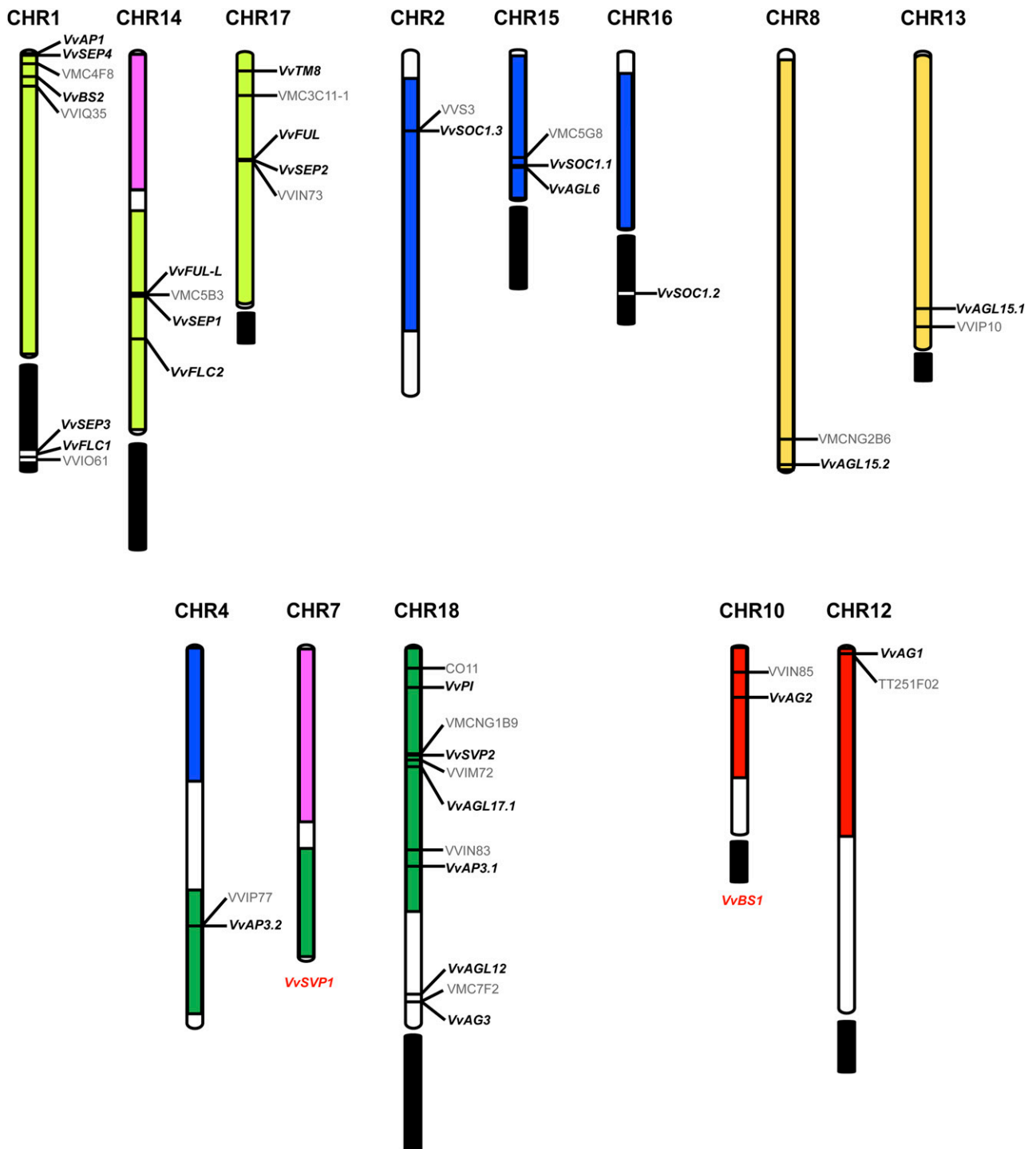


Figure 2. Chromosomal locations of grapevine MIKC genes. Only those chromosomes bearing MIKC genes (13) are represented. Paralogous regions in the putative ancestral constituents of the grapevine genome are depicted in the same color following Jaillón et al. (2007). Black chromosomal regions correspond to random chromosome sequences that are assigned to chromosomes but without a specific physical position. The chromosomal locations of genes *VvSVP1* and *VvBS1* were derived from NCBI information and appear in red at the bottoms of the assigned chromosomes. Molecular markers to help position the genes are listed in gray.

They were mainly expressed in buds and vegetative organs such as leaves and shoots. The second cluster included three major expression groups. The first

group contained two genes expressed in root *VvAGL12* and *VvAGL17.2*. *VvAGL17.2* was also detected in buds. The second group contained two genes of the *AP1/FUL*

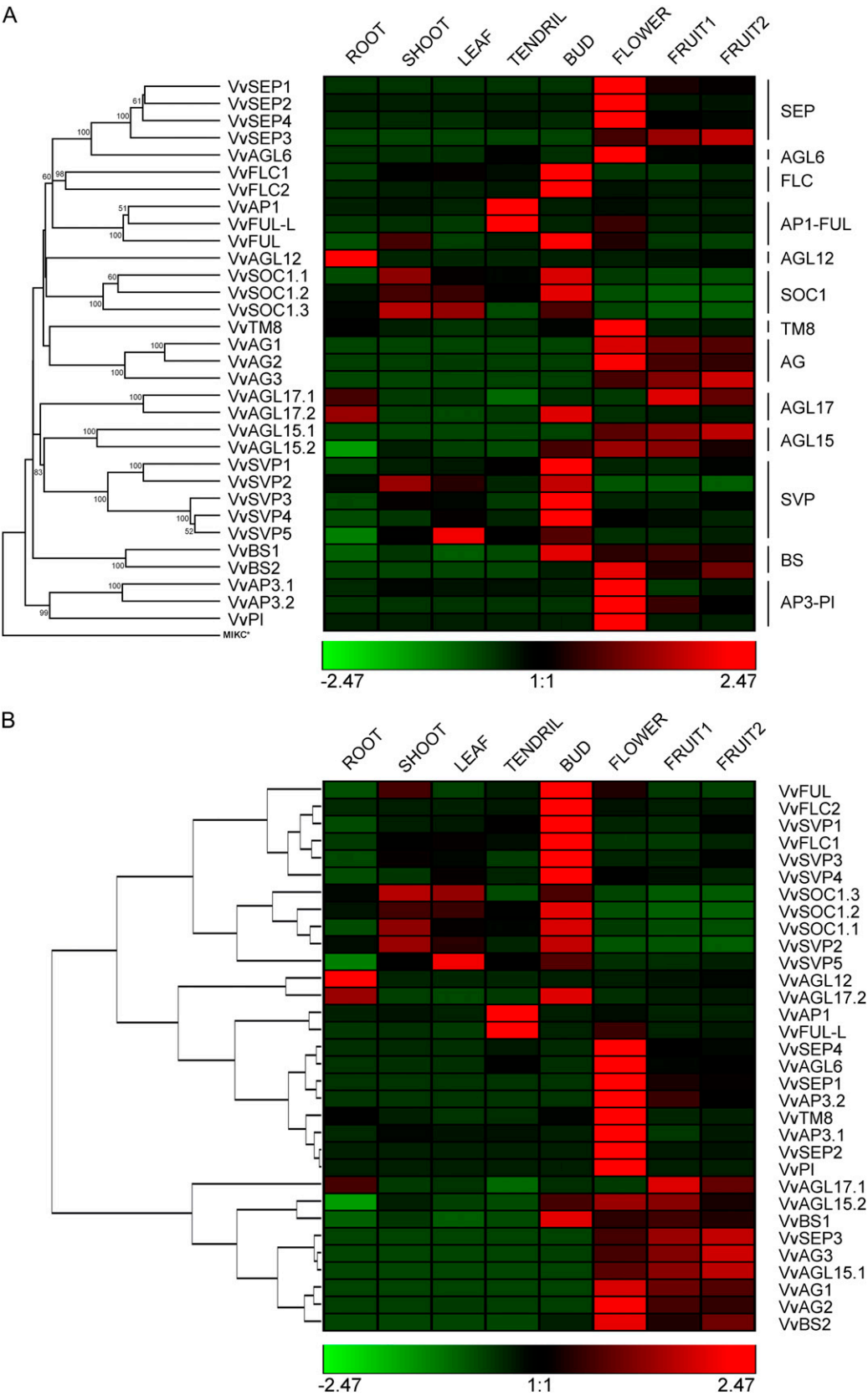


Figure 3. Expression profiles of grapevine MIKC genes in vegetative and reproductive organs. Expression analyses were performed by qRT-PCR, and relative gene expression data were gene-wise normalized. A, Expression pattern related to

subfamily, *VvAP1* and *VvFUL-L*, with a characteristic tendril expression. The third group contained eight genes expressed in flowers and whose Arabidopsis homologs have been involved in the specification of flower organ identity. They belonged to MIKC subfamilies *SEP* (*VvSEP1*, *VvSEP2*, and *VvSEP4*), *AGL6* (*VvAGL6*), *AP3/PI* (*VvAP3.1*, *VvAP3.2*, and *VvPI*), and *TM8* (*VvTM8*). Some of them were also detected, at lower levels, during fruit development (*VvSEP1*, *VvSEP4*, *VvAP3.2*, and *VvAGL6*). The third cluster included genes expressed in both flowers and fruits and grouped in three major expression groups. The first one contained a gene, *VvAGL17.1*, expressed during fruit development and also detected in roots. The second one comprised two genes, *VvAGL15.2* and *VvBS1*, both expressed in flowers and fruits and also detected in buds. The third one contained six genes belonging to four different subfamilies that were mainly detected in flowers and during fruit development. Among them, *VvSEP3*, *VvAG3*, and *VvAGL15.1* seemed to increase their expression levels from flowers to mature fruits, while *VvAG1*, *VvAG2*, and *VvBS2* followed a reverse kinetics.

To further characterize those genes involved in tendril and flower development, we analyzed the expression of MIKC genes during tendril (tendrils 1 and 5) and flower (from stage B2 buds, bearing only inflorescence meristems, to preanthesis flowers) development. Using a similar approach of qRT-PCR and gene-wise expression normalization, a cluster analysis of gene expression (Fig. 4) allowed the identification of two major clusters of expression patterns. The first cluster corresponded to genes expressed in first season latent buds (Fig. 3). They could still be detected in stage B2 of the second season, but their expression was decreasing during flower meristem initiation (stage D) and flower development (stage G to flowers, Fig. 4). These genes were detected in tendril 1 at low levels, with the exception of *VvSOC1.1*, whose expression increased in tendril 5. The second expression cluster included three major expression groups. The first group (*VvAP1*, *VvFUL-L*, and *VvFUL*) corresponded to genes expressed in tendrils and during flower meristem initiation and flower development, in agreement with their previously described expression patterns (Calonje et al., 2004). The second group corresponded to genes expressed during the differentiation of the outer flower whorls, including *VvFLC1* and *VvFLC2*. *FLC* expression has also been detected in developing anthers in Arabidopsis as well as in zygotes and during embryo development, which has been related to the resetting process of *FLC* activity in the next generation to exert its repressive role on

flowering (Sheldon et al., 2008). The third expression group contained genes mostly expressed at later stages of flower development, likely related to reproductive organs and ovule development. Among them, *VvTM8* was also detected in tendril 5.

To identify those MIKC genes whose function could be associated with the regulation of flowering transition, we further analyzed the expression of genes detected in latent buds as well as a few related ones (Fig. 5). Using qRT-PCR and gene-wise expression normalization, it was possible to identify four gene expression groups related to the chronological stages of bud development, corresponding to early, intermediate, late, and very late expressed genes. The early expressed group included three genes, *VvFUL*, *VvSOC1.1*, and *VvFLC1*. Their expression was already detected in May, when inflorescence meristems are not yet initiated, and showed a peak in June or July (*VvFUL*), when inflorescence meristems are actively proliferating. The intermediate expression group included six genes (*VvTM8*, *VvSOC1.3*, *VvSVP1*, *VvSOC1.2*, *VvSVP5*, and *VvSVP2*), whose expression could already be detected in June. Among them, *VvSOC1.2*, *VvSVP5*, and *VvSVP2* seemed to accumulate at similar levels in July and August, while *VvTM8*, *VvSOC1.3*, and *VvSVP1* further increased their expression between July and August. The late expression group contained seven genes. Four of them (*VvSVP4*, *VvSVP3*, *VvBS2*, and *VvAGL17.2*) were first detected in July, at their highest levels, and were still expressed in August. *VvFLC2* showed a similar pattern of expression but was also detected in June. The last two genes (*VvBS1* and *VvAGL15.2*) differed from the rest in showing a relevant expression in August. Finally, two genes, *VvFUL-L* and *VvAP1*, showed very late expression, since they started to be detected in July and were expressed at stage B2, in the case of *VvFUL-L*, at highest intensity.

DISCUSSION

The search for MIKC genes in the grapevine genome allowed the identification of 32 genes belonging to this family of transcription factors. Six additional partial MADS box sequences could represent additional genes. Similar truncated sequences have also been found in Arabidopsis. They could correspond to transcribed pseudogenes or sequences playing a regulatory role, as proposed by Parenicova et al. (2003). Thus, the total number of MIKC genes detected in grapevine is similar to that observed in Arabidopsis (39), while in poplar the number rises to 55. MIKC genes detected in

Figure 3. (Continued.)

phylogenetic relationships. Bootstrap support values are shown when over 50. The corresponding subfamilies of each group of genes are indicated at right. B, Cluster analysis of gene expression patterns. Color scales, representing signal values, are shown at bottom.

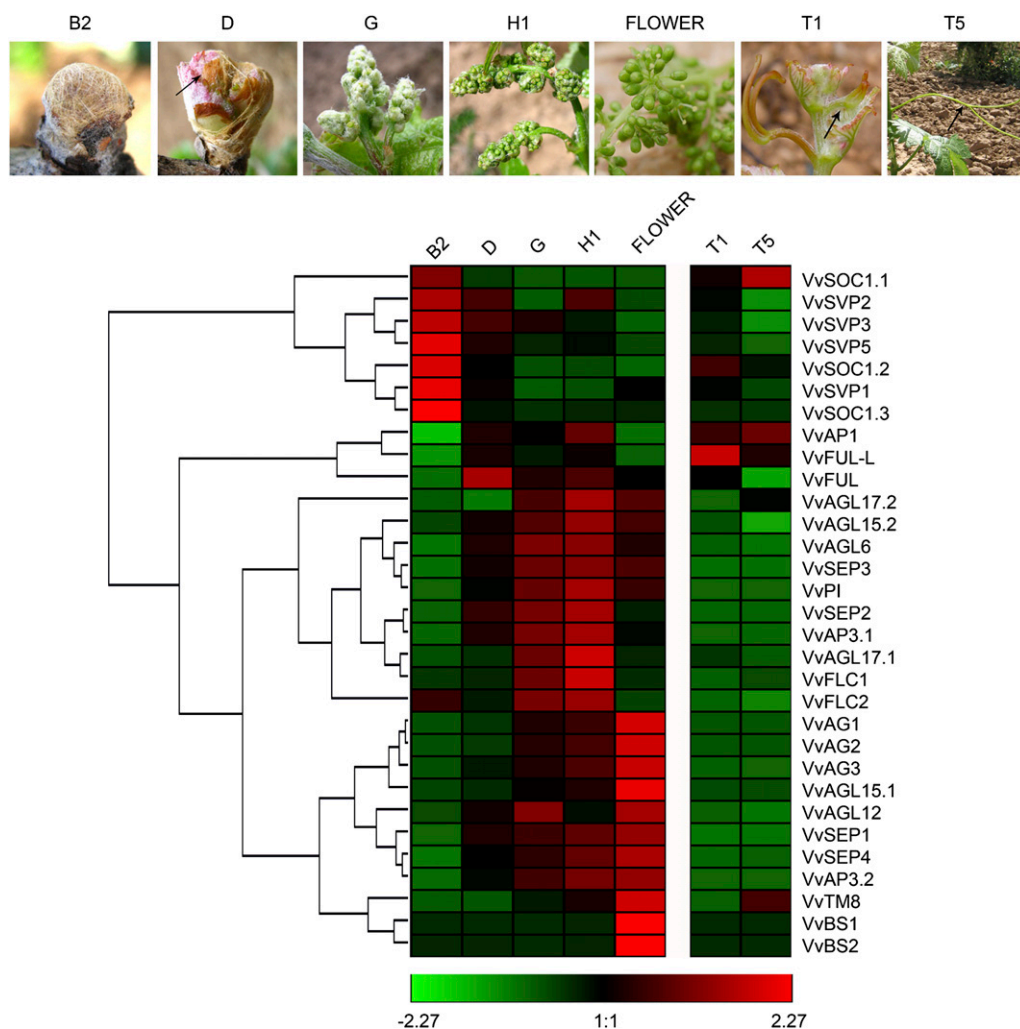


Figure 4. Expression profiles of grapevine MIKC genes during flower and tendril development. Expression analyses were performed by qRT-PCR, and relative gene expression data were gene-wise normalized. Expression of *VvSVP4* was not detected at any developmental stage. Color scale, representing signal values, is shown at bottom. At top, photographs from the different developmental stages are shown. Developmental stages correspond to buds from advanced stage B (B2), inflorescences of stage D (D, arrow), and flowers from inflorescences at stage G (G) and early stage H (H1). Tendril 1 (T1, arrow) and tendril 5 (T5, arrow) correspond to the most recently formed tendril by the shoot apex and that in the fifth position from the apex, respectively.

grapevine belong to the 13 subfamilies so far identified in other angiosperms. They include the *TM8* subfamily (Pnueli et al., 1991; Becker and Theissen, 2003) not present in *Arabidopsis*. Chromosomal location of MIKC genes in the grapevine genome follows the pattern expected for the existence of an ancestral polyploidization event in a common ancestor to these three dicot species (Jaillon et al., 2007; Velasco et al., 2007). The location of genes belonging to the *SEP*, *AP1/FUL*, *FLC*, *SOC1*, *AGL15*, *SVP*, *AP3*, and *AG* subfamilies in paralogous chromosomal segments (Fig. 2) suggests that many of them originated in the same polyploidization event. This ancestral polyploidization event could have been the basis for the functional diversification observed in some subfamilies. This could be the case of *VvAP3.1* and *VvAP3.2*, derived

from an ancestral *AP3* lineage whose duplication gave rise to *euAP3* and tomato MADS box gene 6 (*TM6*) sublineages (Kramer and Irish, 1999).

MIKC Genes and Flowering Transition in Grapevine

The study of MIKC gene expression profiles in different vegetative and reproductive organs of grapevine plants and during the process of flowering transition and flower development allows the preliminary association of these genes with specific grapevine developmental processes. MIKC genes regulating flowering transition in *Arabidopsis* mainly belong to the *FLC*, *SVP*, and *SOC1* subfamilies (Gregis et al., 2006; Schönrock et al., 2006; Searle et al., 2006; Lee et al., 2007; Liu et al., 2007, 2008). Among them, the

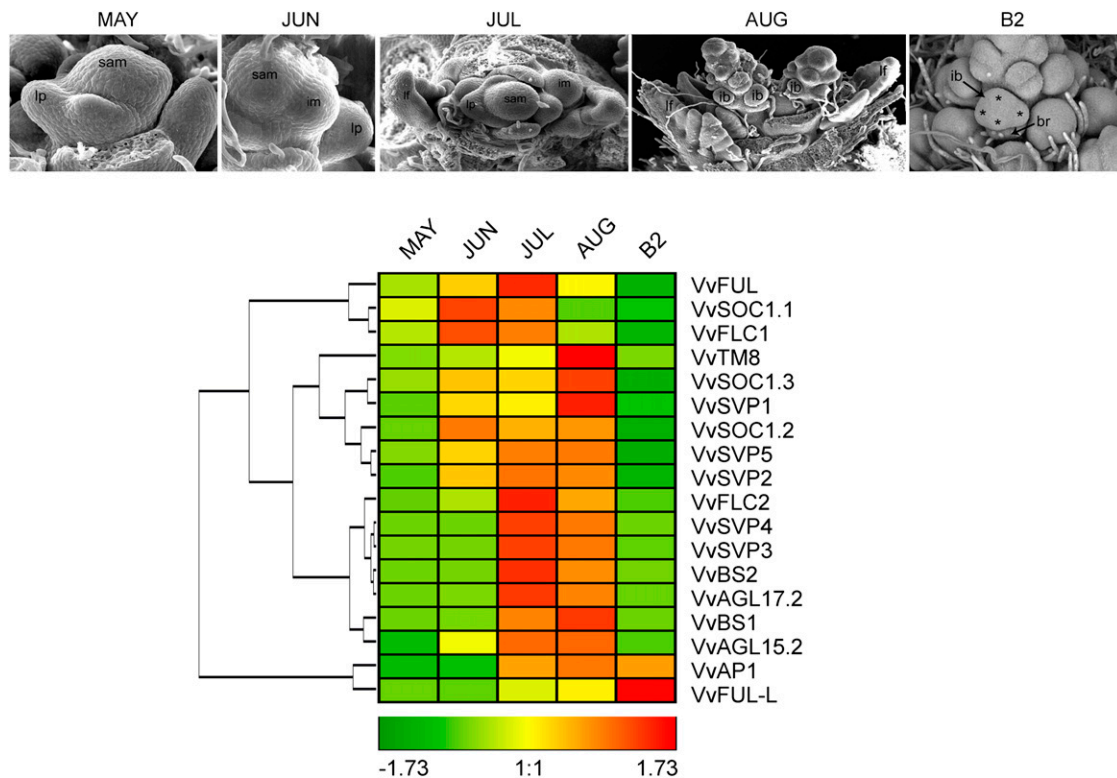


Figure 5. Expression profiles of representative MIKC genes in latent buds during flowering transition. Expression analyses were performed using qRT-PCR, and relative gene expression data were gene-wise normalized. Color scale, representing signal values, is shown at bottom. At top, the developmental stages of the shoot apex and derived structures within each bud are illustrated by scanning electron microscopy micrographs. Developmental stages correspond to buds from May to August in the first season as well as advanced stage B (B2) during the second season. In August, the two-branched inflorescence meristems are shown. In stage B2, only a detail of an inflorescence branch meristem, subtended by a bract in which the four flower meristems (asterisks) start to be outlined, is shown. br, Bract subtending each inflorescence branch meristem; ib, inflorescence branch meristem; im, inflorescence meristem; lf, leaf; lp, leaf primordium; sam, shoot apical meristem.

FLC subfamily, with six gene members, has been involved in the negative regulation of flowering induction. Only two genes, *VvFLC1* and *VvFLC2*, belong to this subfamily in grapevine, and partial ESTs were identified for both of them (Reeves et al., 2007). The reduced sequence similarity among *FLC*-like genes of grapevine, *Arabidopsis*, and poplar precluded the identification of closer homologies (Fig. 1). Higher sequence divergence among *FLC* homologs has also been reported between poplar and *Arabidopsis* (Leseberg et al., 2006) as well as in an extensive analysis of *FLC* homologs belonging to the three main eudicot lineages (Reeves et al., 2007). This high sequence divergence has been proposed to result from the existence of different amplification events in different lineages and evolution under positive Darwinian selection (Martinez-Castilla and Alvarez-Buylla, 2003). The two grapevine *FLC*-like genes show their highest expression in buds and are detected throughout bud development at stages coincident with the active proliferation of inflorescence branch meristems. This pattern of expression is distinct from what has been described for *Arabidopsis FLC*, whose expression in the apex precedes the

flowering transition and is also widely expressed in roots and leaves (Michaels and Amasino, 1999; Searle et al., 2006). Given the complexity of the analyzed buds, we cannot determine whether these genes play a negative role in the control of flowering transition in grapevine. Moreover, their sequence divergence with respect to *Arabidopsis* genes and their different expression pattern could be related to playing different roles in other species.

In contrast to the *FLC* subfamily, the *SVP* subfamily is particularly overrepresented in grapevine with respect to *Arabidopsis*. This subfamily could even be larger in grapevine, since two other partial and related sequences have been detected. The phylogenetic analysis indicates that grapevine and poplar genes are more related to *SVP* than to *Arabidopsis AGL24*. Overrepresentation of putative *SVP*-like genes and the lack of putative *AGL24* homologs in the two woody species analyzed are remarkable differences from *Arabidopsis*. Grapevine genes *VvSVP1* and *VvSVP2* and poplar *PMADS9*, *PMADS24*, and *PMADS25* are more closely related to *Arabidopsis SVP* at the protein sequence level, although their gene structure is not

completely conserved in grapevine (Supplemental Table S1). The remaining *VvSVP* genes do not have close counterparts in Arabidopsis or in other studied plant species. Expression of *SVP*-like genes in grapevine was observed in latent buds and in vegetative and reproductive organs such as roots, leaves, stems, flowers, and fruits, similar to Arabidopsis, in which *SVP* and *AGL24* have been detected in many vegetative and reproductive organs (Hartmann et al., 2000; Yu et al., 2002; Michaels et al., 2003; Liu et al., 2007). In Arabidopsis, *SVP* seems to mediate in ambient temperature signaling by interacting with *FLC* to negatively regulate the expression of the floral pathway integrators *SOC1* and *FLOWERING LOCUS T* (*FT*; Lee et al., 2007; Li et al., 2008). Given the relevance of high temperature in promoting flowering transition in grapevine latent buds, it is tempting to speculate that this gene subfamily could play a similar role in grapevine. Furthermore, the seasonal separation between inflorescence and flower meristem formation in grapevine could require the participation of transcriptional repressors, such as members of the *FLC* and *SVP* subfamilies, to prevent the development of flower meristems before the dormancy period. Nevertheless, *SVP* genes could play new roles in grapevine. In this way, *JOINTLESS*, a tomato *SVP* homolog, is involved in the development of the pedicel abscission zone (Mao et al., 2000).

Relationships among members of the *SOC1* subfamily in the three dicot species compared seem closer than within *FLC* or *SVP* subfamilies. *VvSOC1.1*, previously reported as *VvMADS8* (Sreekantan and Thomas, 2006), is more closely related to Arabidopsis *SOC1*, *VvSOC1.2* is more closely related to *AGL42*, and *VvSOC1.3* is more closely related to *AGL14* and *AGL19*. No grapevine or poplar genes were found related to the Arabidopsis pair *AGL71* and *AGL72*. The three grapevine *SOC1* genes show parallel expression patterns in vegetative organs and latent buds. They fit well with the expression patterns described for members of this subfamily in other species, where the major expression domains are not the floral organs (Lee et al., 2000; Samach et al., 2000; Schönrock et al., 2006). *SOC1* and *AGL19* have been shown to function as flowering promoters integrating flowering signals from different pathways (Lee et al., 2000; Samach et al., 2000; Hepworth et al., 2002; Moon et al., 2003; Schönrock et al., 2006; Liu et al., 2008) and positively regulating downstream targets like flower meristem identity genes *AP1* and *LEAFY* (*LFY*; Schönrock et al., 2006). *VvSOC1.1* is one of the earliest MIKC genes detected in latent buds, which fits well with a putative role as a flowering promoter.

MIKC Genes and Tendril Development

Grapevine tendrils and inflorescences are considered homologous organs with a common ontogenetic origin. Two MIKC genes belonging to the *AP1/FUL* subfamily, *VvAP1* and *VvFUL-L*, were previously

shown to be expressed in the grapevine tendril, supporting its consideration as a sterile reproductive organ (Calonje et al., 2004). Our genomic survey of MIKC genes allowed the identification of a third member of this subfamily, *VvFUL*, which was identified as the closest *FUL* homolog (Supplemental Fig. S3). Interestingly, the expression analyses performed for the entire MIKC family showed that only the two previously analyzed genes *VvFUL-L* and *VvAP1* were highly expressed in tendrils, while *VvFUL* was highly expressed in latent buds and during flower meristem initiation and flower development. The early and high expression of *VvFUL* in latent buds during flowering transition suggests a role in this process, as has been proposed for *VvAP1* and *VvFUL-L* based on in situ hybridization experiments (Calonje et al., 2004). This is consistent with the role proposed for *AP1* and *FUL* in the specification of inflorescence and flower meristem identity in Arabidopsis (Mandel and Yanofsky, 1995; Ferrándiz et al., 2000a). Thus, the evolution of tendrils as climbing organs could have conditioned functional divergence within this subfamily. Further functional analyses will be required to characterize the extent of this subfunctionalization in tendril and inflorescence development. Members of the *VvSOC1*, *VvAGL17*, and *VvTM8* subfamilies were also differentially expressed between tendril and flowers, suggesting a possible involvement in tendril development.

MIKC Genes and Grapevine Flower and Fruit Development

Grapevine flower development shows extensive similarities with what has been described in Arabidopsis and other plant species when the ABCDE model is considered. With the exception of *VvAP1*, whose role in function A in grapevine has been questioned on the basis of its expression pattern (Calonje et al., 2004), all other MIKC subfamilies involved in the functions required to establish flower organ identity are detected in flowers (Fig. 3). Regarding function B, three *AP3/PI* subfamily members were detected, all of them previously characterized as *VvMADS9* (*VvPI*), *VvAP3* (*VvAP3.1*), and *VvTM6* (*VvAP3.2*; Sreekantan et al., 2006; Poupin et al., 2007), close homologs of Arabidopsis *PI* and *AP3* and tomato *TM6*, respectively. Our results show that *VvAP3.2* is also detected during fruit development, which is consistent with the results of Poupin et al. (2007), who showed that *VvAP3.2* is more highly expressed in carpels, fruits, and seeds than in petals. This differential expression of *VvAP3.1* and *VvAP3.2* (*VvTM6*) suggests their possible subfunctionalization in grapevine, similar to what has been proposed in Solanaceae (De Martino et al., 2006; Rijpkema et al., 2006), where *euAP3* could play a more direct role in petal development and *TM6* could play a more direct role in stamen differentiation. Detection of *VvAP3.2* in carpels and during berry development and ripening suggests a new role for this gene in grapevine fruit development.

Genes involved in C and D functions form the monophyletic AG subfamily. In grapevine, this subfamily contains three members, two of them (*VvAG1* and *VvAG2*) more related to AG and the third one (*VvAG3*) more related to *STK/AGL11*. Two of these AG-like grapevine genes were previously characterized, and their reported expression patterns fit well with those found in this work (Boss et al., 2001, 2002). These expression patterns correspond to what could be expected based on their proposed function in Arabidopsis. AG specifies the identity of stamens and carpels, and it is also required, together with D function genes such as *STK/AGL11*, *SHATTERPROOF1 (SHP1)*, and *SHP2*, for ovule identity. These D function genes also participate in the regulation of fruit development (Pinyopich et al., 2003). Interestingly, no genes related to Arabidopsis *SHP1* and *SHP2* were identified in poplar or grapevine.

The *SEP* subfamily in grapevine has four members, as in Arabidopsis (Fig. 1; Supplemental Fig. S4). Our results show that *VvSEP1* (previously described as *VvMADS2*; Boss et al., 2002) is the closest homolog to tomato *TM29* and Arabidopsis *SEP1* and *SEP2*, whereas *VvSEP3* (previously known as *VvMADS4*; Boss et al., 2002) is closely related to *SEP3*. No close homologs could be identified in Arabidopsis to *VvSEP2* and *VvSEP4*. *VvSEP2* seems more related to the *FBP9/FBP23* subclade (Immink et al., 2003; Vandebussche et al., 2003; Malcomber and Kellogg, 2005) that is present in several species but absent in Arabidopsis. *VvSEP4* could be related to the *SEP4* group, which shows diverse patterns of expression in different plant species, suggesting a wider sequence and functional divergence within this subclade (Supplemental Fig. S4). All four *VvSEP* genes are expressed in flowers and fruits, as described in other species (Malcomber and Kellogg, 2005). However, *VvSEP3* was detected at a lower level in flowers than in fruits (Fig. 3). Arabidopsis *SEP* genes are responsible for function E and play redundant roles with other MIKC genes in floral meristem determinacy and organ identity in the four whorls (Pélaz et al., 2000, 2001; Ditta et al., 2004). The involvement of *SEP* homologs in other roles is becoming progressively evident in other plant species (Malcomber and Kellogg, 2005). Expression of *VvSEP* genes and especially *VvSEP3* during fruit development and ripening suggests a role for these genes in those processes. Similarly, two tomato *SEP* genes, *TM29* and *LeMADSRIN*, seem to play a role in tomato fruit development (Ampomah-Dwamena et al., 2002; Vrebalov et al., 2002). Finally, detection of *VvFUL-L* expression in fruits is also consistent with a role in fruit development, as evidenced for Arabidopsis *FUL* (Gu et al., 1998; Ferrándiz et al., 2000b).

Other MIKC Subfamilies in Grapevine

Apart from the mentioned MIKC subfamilies, six additional ones were identified in grapevine for which

functional information is so far more restricted in plants. Among them, the *TM8*, *AGL6*, and *BS* subfamily members show expression patterns related to the development of reproductive organs. *VvTM8* is the unique grapevine representative of the *TM8* subfamily (Becker and Theissen, 2003), with two members in poplar as well as homologous genes in monocots and gymnosperms (Supplemental Fig. S2). *VvTM8* expression is detected late in latent buds during flowering transition as well as in late stages of flower development, suggesting a role in the development of reproductive organs. Similarly, expression of tomato *TM8* was detected in the three inner tomato flower whorls (Pnueli et al., 1991). *VvAGL6* belongs to the *AGL6* subfamily, with two members in Arabidopsis, *AGL6* (Ma et al., 1991) and *AGL13* (Rounsley et al., 1995), and three in poplar. *VvAGL6* expression was detected during flower development, in agreement with what has been reported in Arabidopsis, where *AGL6* is expressed in all floral organs while *AGL13* expression is restricted to ovules. Their specific biological functions are still unknown. The *BS* subfamily (Becker et al., 2002) has two members in grapevine, *VvBS1* and *VvBS2*, although the presence of additional genes cannot be disregarded given the identification of related partial sequences in the genome. Four homologous genes have been identified in poplar and one closer homolog in Arabidopsis, *AGL32* (also known as *ABS* or *TT16*; Nesi et al., 2002). The expression patterns observed for these genes in grapevine suggest a role in reproductive development, with *VvBS2* more related to flower and fruit development and *VvBS1* more related to processes taking place in buds.

Members of the *AGL12*, *AGL15*, and *AGL17* subfamilies display more divergent expression patterns and were recently found to be involved in the regulation of flowering in Arabidopsis. The *AGL12* subfamily has a single member in grapevine (*VvAGL12*) and Arabidopsis and two in poplar. Expression of *VvAGL12* was detected in roots and fruits and during flower development, while the Arabidopsis homolog is expressed in roots, the leaf vascular system, and flower meristems (Rounsley et al., 1995; Burgeff et al., 2002; Tapia-López et al., 2008). Roles for this gene in the regulation of the cell cycle in root meristems and as a promoter of flowering transition through up-regulation of *SOC1*, *FT*, and *LFY* have been shown (Tapia-López et al., 2008). The *AGL15* subfamily has two members in grapevine (*VvAGL15.1* and *VvAGL15.2*) and poplar that are close homologs of *AGL15* and *AGL18*, respectively. *VvAGL15.1* expression was restricted to flowers and fruits, while *VvAGL15.2* was also detected in buds. These expression patterns are more restricted than those of their Arabidopsis homologs, which are broadly expressed in vegetative and reproductive organs (Alvarez-Buylla, 2000a; Lehti-Shiu et al., 2005; Adamczyk et al., 2007). *AGL15* and *AGL18* are proposed to function as repressors of the floral transition, acting upstream of *FT* and probably in combination with other floral repressors like *SVP* or *FLC* (Adamczyk

et al., 2007). Two members of the *AGL17* subfamily have so far been identified in grapevine, although two additional MADS box partial sequences detected in the genome could correspond to the same subfamily. Two genes have been described in poplar and four in Arabidopsis (Alvarez-Buylla et al., 2000a; Becker and Theissen, 2003). As previously shown for *FLC* and *SVP*-like genes, there is not enough sequence homology among the three species to establish closer relationships. Expression of *VvAGL17.1* and *VvAGL17.2* was detected in roots, as in their Arabidopsis counterparts (Rounsley et al., 1995; Burgeff et al., 2002), as well as during reproductive development. Recently, a flowering promoter role has been reported for *AGL17*, which could participate in the photoperiodic induction of *AP1* and *LFY* independent of *FT* (Han et al., 2008).

In summary, the global analysis of grapevine MIKC genes reveals a basic conservation of the number of gene subfamilies and their corresponding expression patterns. Over this basic pattern, there is variation in the number of gene members in some specific subfamilies as well as expression pattern divergence in a few others, which suggests the existence of subfunctionalization. Remarkably, larger variation in gene members is observed in MIKC subfamilies putatively involved in flowering transition, such as the *SVP* and *FLC* subfamilies, than in those subfamilies involved in the specification of organ identity (e.g. *AP1/FUL*, *AP3/PI*, *AG*). Whether these differences relate to the different evolutionary forces acting on different traits remains to be analyzed through the study of MIKC gene family organization in additional plant genomes.

The developmental particularities of grapevine are reflected in the specific expression of members of the *AP1/FUL* subfamily in tendril development, which suggests the recruitment of these genes for a new function. Furthermore, in parallel to what is observed in tomato fruits, several members of different gene subfamilies (such as *SEP* and *AP3/PI*) are detected during fruit development and ripening, whereas Arabidopsis *SHP*-related genes are not found in both berry-bearing species. Whether these differences reflect existing developmental differences between dry fruits such as siliques and fleshy berry fruits remains to be studied. Further functional analyses of grapevine MIKC genes will be required to advance the understanding of their biological roles in this species.

MATERIALS AND METHODS

Plant Materials

Grapevine (*Vitis vinifera* 'Tempranillo') samples were obtained from an experimental plot at the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (Alcalá de Henares, Madrid). Samples were collected from at least 20 independent plants per data point, frozen in liquid nitrogen, and stored at -80°C before RNA extraction. Expression analyses were performed on plant organs collected at different developmental stages during two consecutive growing seasons. Developmental stages were classified following the developmental series of Baggiolini (1952). Organs selected

for gene expression analysis corresponded to the following: roots (Root) obtained from in vitro-grown plants of the same cultivar; young apical shoot internodes (Shoot), leaves (Leaf), and developing tendril 1 (Tendril) from plants of advanced stage H just before anthesis; latent July buds (Bud), in which flower transition has already taken place (inflorescence meristems are differentiated but flower meristems are not yet present); flowers from advanced stage H (Flower), just before anthesis; green fruits of 4 to 7 mm, stages J to K (Fruit1), and fruits at veraison, stage M (Fruit2). To analyze gene expression during flower and tendril development, the following stages were considered: swelling buds collected in April during advanced phenological stage B (B2) bearing inflorescence meristems that are initiating the differentiation of flower meristems; small inflorescences from stage D (D), in which flower meristems are already formed; developing flowers from stage G (G) and early stage H (H1), which correspond to the development of flower organs, with gynoecium initiated at the latest during stage H; flowers from advanced stage H (Flower), just before anthesis; and developing tendrils 1 and developed tendril 5, corresponding to the first and fifth tendrils respectively. Tendril 1 corresponds to the most recently formed tendril by the shoot apex in plants of advanced stage H, just before anthesis, and tendril 5 corresponds to tendril 5 of the branch. Gene expression during the flowering transition was examined in first season latent buds and in the second season. First season latent buds were collected at equivalent branch positions every month. May buds (May) correspond to very young buds in which the SAM has not yet differentiated any inflorescence meristem; June buds (Jun) are buds in which the SAM has initiated the production of the first inflorescence meristems; in July buds (Jul), two or three inflorescence meristems that will give rise to different clusters of grapes in the branch can be found; August buds (Aug) corresponds to buds in which the inflorescence meristems have proliferated, giving rise to inflorescence branch meristems subtended by a bract and organized in a spiral phyllotaxis. In the second growing season, swelling buds were collected in April, during B2, which corresponds to buds in which the inflorescence branch meristems display further proliferation and flower meristems start to be initiated.

Database Search, Gene Structure Determination, and Chromosomal Locations of Grapevine MIKC Genes

Protein sequences encoded by MIKC genes in grapevine were searched using BLAST (Altschul et al., 1990) at the Genoscope BLAST server (http://www.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) and at the EST databases of The Institute for Genomic Research (<http://www.tigr.org/>) and the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Prediction of gene structure from genomic contigs was performed using FGENESH software in the Softberry server (<http://www.softberry.com/berry.phtml>). In addition, we carried out an HMM (for hidden Markov model) search in the proteome database of the Genoscope Genome Project (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast-info.pl) using two different HMM profiles. One was constructed with the MADS box domain of MIKC genes of Arabidopsis (*Arabidopsis thaliana*) and poplar (*Populus trichocarpa*), while the other domain profile (serum response factor) was obtained from Pfam (Finn et al., 2006). Profile generation and searches were performed using the HMMER 2.3.2 software package (Eddy, 1998). Sequences were edited and analyzed using BioEdit version 7.0.9 software (Hall, 1999). Gene structure was deduced from Genoscope gene annotations or from manual annotation based on the genomic sequence provided by Genoscope and its comparison with the corresponding ESTs and the deduced protein sequences for homologous MIKC genes of Arabidopsis and poplar. Chromosomal locations of MIKC genes were obtained using the BLAT server and additional physical localization tools at the Genoscope Genome Browser. Two genes, *VvBS1* and *VvSVP1*, contained in ChrUn_random (ultracontigs whose physical positions on specific chromosomes have not been defined) were assigned to their corresponding chromosome by analyzing the available information at NCBI from the IASMA sequencing project (Velasco et al., 2007). Genes *VvSVP3*, *VvSVP4*, *VvSVP5*, and *VvAGL17.2* have not yet been assigned to any chromosome.

Phylogenetic Analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). To generate a phylogenetic tree, complete MIKC^C-type predicted proteins of Arabidopsis, poplar, and grape-

vine were aligned in the Multalin server (Corpet, 1988). The neighbor-joining method was used to construct different trees. To estimate evolutionary distances, the proportions of amino acid differences were computed using amino acid p-distance. To handle gaps and missing data, the pair-wise deletion option was used. Reliability of the obtained trees was tested using bootstrapping with 1,000 replicates. Additional phylogenetic trees were built for MIKC proteins belonging to the TM8, AP1/FUL, and SEP subfamilies, including additional proteins from plant species other than *Arabidopsis* and poplar in the case of TM8 and SEP proteins.

Gene Expression Analyses

Total RNA was extracted from frozen tissues according to Reid et al. (2006). DNase digestion of contaminating DNA in the RNA samples was carried out with the RNase-Free DNase Set (Qiagen). Final RNA purification was performed using the RNeasy Mini Kit (Qiagen) according to standard protocols. Total RNA (1 µg) was reverse transcribed in a reaction mixture of 20 µL containing 1× PCR buffer II (Applied Biosystems), 5 mM MgCl₂, 1 mM deoxynucleoside triphosphates, 20 units of RNase inhibitor, 50 units of murine leukemia virus reverse transcriptase (Applied Biosystems), 2.5 µM oligo(dT)₁₈ and diethyl pyrocarbonate-treated water. Transcript levels were determined by qRT-PCR using a 7300 Real-Time PCR System (Applied Biosystems) and SYBR Green dye (Applied Biosystems). Reactions were performed in a final volume of 15 µL containing 7.5 µL of 2× Power SYBR Green PCR Master Mix (including AmpliTaq Gold DNA Polymerase-LD, deoxynucleoside triphosphates, and SYBR Green dye), 333 nm of forward and reverse specific primers, and a 1:10 dilution of cDNA. After enzyme activation at 95°C for 10 min, amplification was carried out in a two-step PCR procedure with 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing/extension. Gene-specific primers were designed using the Oligo Explorer 1.2 software (Gene Link). Gene primer sequences used in the qRT-PCR analyses are listed in Supplemental Table S2. No-template controls were included for each primer pair, and each PCR was performed in triplicate. Data were analyzed using 7300 SDS software 1.3 (Applied Biosystems). Dissociation curves for each amplicon were analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60°C to 95°C. Transcript levels were calculated using the standard curve method and normalized against the grapevine *EF1-α* gene (BQ799343) for organ expression analyses and the grapevine ubiquitin gene (*VtUB*; CF406001) for bud expression analyses as reference controls. Relative data of gene expression with respect to control genes were gene-wise normalized using Genesis software (Sturn et al., 2002). Hierarchical clustering of gene expression data was performed using the same software.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Grapevine MIKC protein deduced sequences used in this analysis.

Supplemental Figure S2. Phylogenetic relationships among MIKC proteins belonging to the TM8 subfamily.

Supplemental Figure S3. Phylogenetic relationships among MIKC proteins belonging to the AP1/FUL subfamily.

Supplemental Figure S4. Phylogenetic relationships among MIKC proteins belonging to the SEP subfamily.

Supplemental Table S1. Gene structures of grapevine MIKC genes.

Supplemental Table S2. Primers used for qRT-PCR expression analysis of MIKC genes.

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