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Full Length Research Paper

The expression patterns of the genes *VvTFL1, VFL*, *VAP1*, *VvAG1* and *VvSEP3* during bud and flower development in the "Xiangfei" grapevine (*Vitisvinifera L.*)

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In this study, the differentiation of buds, inflorescences and flowers of the "Xiangfei" grapevine (*Vitis vinifera* L.) was examined, and the roles of the genes *VvTFL1, VFL, VAP1, VvAG1* and *VvSEP3* in the initiation and development of these organs were studied. We found that the structure of burst buds included an apical point, a leaf primordium, an inflorescence or tendril primordium, bract and stipule primordia. The original floral primordium formed lateral meristems for branching inflorescence primordia and continued to differentiate into floret primordia in lateral bud. *VvTFL1* gene expression was detected in the apical meristems of swelling buds, but it was not detected during the entire process of inflorescence initiation and flower development. *VFL* and *VAP1* genes were strongly detected in inflorescences and developing flowers. Furthermore, the *VFL* gene strongly detected in inflorescence meristems and flower organ primordia. *VAP1* expression was detected in the branching of the pedicel region of burst buds, and after petal, stamen and carpel primordium development, *VAP1* transcripts were clearly detected. The expression of *VvAG1* and *VvSEP3* began to be detected in the placental region of anatropous ovules at later stages of flower development, and *VvAG1* had earlier effects than *VvSEP3*. These results suggest that those genes might play a different role in grapevine.

Key words: Grapevine, *VvTFL1* gene, *VFL* gene, *VAP1* gene, *VvAG1* gene, *VvSEP3* gene, bud, flower.

INTRODUCTION

Grapevines are a major global horticulture crop, with a pattern of organ formation and development distinct from other plants. The shoot apical meristems of lateral buds produce both vegetative structures such as leaves and primordia that are capable of forming reproductive organs such as inflorescences and tendrils at regular intervals (Boss and Thomas, 2000). In particular, the morphological structure and development of buds and inflorescences in grapevines are very different in comparison to *Arabidopsis* (Or et al., 2002). Bud structure is completely different because of different bud locations and developmental stages (May, 2000). The tendrils and inflorescences are thought to be homologous structures in grapevines, as both structures are formed from uncommitted primordia (Tucker and Hoefert, 1968). Understanding the initiation and development of buds and inflorescence organ structure in grapevines can be relevant to improving the flower development.

Plant organ initiation and development is a continuous and complicated process, and it requires the functions and interactions of many genes. During the last few years, data relevant to a molecular understanding of floral pathway genes have increased rapidly, making it unwieldy to comprehensively review the field (Jaeger et al., 2006). Genetic and molecular approaches in

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Arabidopsis have allowed the identification of some of the key genes regulating flowering induction and reproductive development (Parcy, 2005). Among them, *TFL1* is expressed at the center of the shoot meristem below the apex, where it inhibits the activity of floral meristem identity genes by both delaying the up-regulation of *LFY* and also preventing a response to these genes in the inflorescence meristems (Bradley et al., 1997). *LFY* plays a key role in the control of flowering in the model species *Arabidopsis* (Weigel et al., 1992). Particularly seem to have a central role in the specification of flower meristem identity. Constitutive expression of *LFY* is sufficient to promote flower initiation and development from shoot apical and axillary meristems in Arabidopsis (He et al., 2001). *AP1* was initially defined as a class A gene involved in sepal and petal identity (Mandel et al., 1992), and it seems to play roles as a floral meristem identity gene and an organ identity gene at later stages of flower development (Coen and Meyerowitz, 1991). The genes *AG* and *SEP* are both floral organ identity genes, and they control flower morphology and development (Gómez-Mena et al., 2005). These genes and the molecular characterization of the flowering process in different species have revealed a degree of conservation of the basic genetic mechanisms controlling early stages of bud and flower initiation (Hayama and Coupland, 2004). However, when homologous genes from species other than *Arabidopsis* have been analyzed, differences regarding expression patterns have been found that may reflect distinct roles from those described for the *Arabidopsis* genes. Thus, studying functions of homologous genes in a different species could greatly expand the theory and molecular mechanisms of flower developmental.

The grapevine may have the same floral development genes as the model plant *Arabidopsis*, but it has fundamental differences from this herbaceous-annual model system in terms of expression pattern, function and gene regulation (Carmona et al., 2007, 2008; Diaz-Riquelme et al., 2009). Most specific features of the grapevine flowering transition relate to the fact that the vine uses tendrils, which share a common ontogenetic origin with inflorescences, as climbing organs. Several laboratories have reported the isolation of putative grapevine flower signal integrators and flower meristem identity genes and analyzed their expression during reproductive development, such as *VFL,VAP1,VFUL-L,VvTFL* genes (Carmona et al., 2002; Calonje et al., 2004; Boss et al., 2006; Sreekantan and Thomas, 2006). However, information about gene function in the grapevine is still scarce because for most genes, expression information only exists for *Arabidopsis*. Therefore, to better understand the molecular processes involved in initiation, the fate of buds and the development of inflorescences, we investigated the expression of five genes (*VvTFL1, VFL, VAP1, VvAG1* and *VvSEP3*) in swelling buds, bursting buds, apical buds, inflorescences and flowers of different development stages in the “Xiangfei” grapevine (*Vitis vinifera L.*).

MATERIALS AND METHODS

Plant materials

The grapevine variety “Xiangfei’ (*Vitis vinifera L.*) was cultivated in Wenquan nursery, Beijing, during 2007 and 2009. We collected buds and flower from different developmental stages every 2 to 5 days. Part of the material was immediately frozen in liquid nitrogen, kept on dry ice during transport to the laboratory, and stored at -80°C for further analysis. Part of the material was fixed with FAA for paraffin sectioning; another part was fixed with PBS (0.8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter, pH 7.4) containing 4% (w/v) paraformaldehyde and kept in a vacuum at 4°C overnight. After washing, dehydration, placement in transparent wax and embedding, the material was stored at 4°C for in situ hybridization.

Developing buds and flower morphological structure observations

In 2008, the buds and flower were collected from after buds swelling to anthesis stages and then every 2 to 5 days. They were fixed in FAA and then dehydrated. After setting in transparent wax, the materials were embedded and stained, based on a slightly improved traditional method. Paraffin sections were prepared with a slice thickness of 10 μm and then observed using a light microscope (Olympus BX51).

RNA extraction

Total RNA was extracted from various organs in the grapevine using the CTBA method (Murray and Thomposon, 1980). Next, the products were purified with RNase-free DNase I (Takara Bio, Beijing, China). The RNA concentration was determined using an atom UV spectrophotometer ($OD_{260/280}$=1.8 to 2.0). Two micrograms of total RNA was used to synthesize first-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) and an oligo dT primer. The cDNA was used as the template for PCR performed as follows: 30 cycles of 94°C for 30 s, 55 to 58°C for 30 s, and 72°C for 1 min. The specific products were electrophoresed through a 2% (w/v) agarose gel. Primers for the *VvTFL1*, *VFL*, *VAP1, VvAG1* and *VvSEP3* genes were designed based on their ESTs (*VvTFL1* GenBank accession no. AF378127, *VFL* GenBank accession no. AF450278, *VAP1* GenBank accession no. AY538746, *VvAG1* GenBank accession no. AF265562, *VvSEP3* GenBank accession no. AF373602; all were synthesized at the Beijing Sunbiotech Limited Company).

Probe preparation and *in situ* hybridization

The probes were obtained by a PCR approach using previously synthesized cDNA as the template. The sense probe primers were5’-AAGCTTGGCCATGCACTTCTTACTAT-3’ for *VvTFL1*, 5’-GGTACCGGCCAGTTTATTCAAGT-3 for *VFL*, 5’-TGGAGAAGATCCTTGATCGCTATGA-3’ for *VAP1*, 5’-AGCGGATCGAGAACACCACTAAT-3’for *VvAG1* and 5’-AAGCTTGTATCCACAAGGGAGGC-3’ for *VvSEP3*. The anti-sense probe primers were 5’-GGTACCTGATCTTCCCGTTGGTTA-3’for *VvTFL1*, 5’-AAGC TTAAGCACTTAAGCATCGCCATAGCCTC-3’ for *VFL,* 5’-AATATAAGGGGAAACATCTTAA-3’ for *VAP1,* 5’-

GTTTGGTCGTGGCGGCGAGACTAA-3' for *VvAG1* and 5'-GAATTCCACACTGGGGCCTGC -3' for *VvSEP3* The PCR products consisting of cDNA fragments of 800 bp for *VvTFL*1, 870 bp for *VFL*, 720 bp for *VAP1*, 632 bp for *VvAG1* and 466 bp for *VvSEP3* were sequenced. The DNA fragments had 99% similarity to the published sequences. The five products were ligated into the *pMD*-18 vector (Takara Bio, Beijing, China). *VvTFL1* was digested with *Pst* I and *EcoR* I, *VFL* was digested with *Knp* I and *Hind* III, *VAP1* was digested with *Pst* I and *EcoR* I, *VvAG1* was digested with *Pst* I and *Xba* I and *VvSEP3* was digested with *Hind* III and *EcoR* I. Next, they were cloned into the *pSPT*-18 vector (Roche). The anti-sense probes were synthesized using a DIG RNA labeling kit (SP6/T7) according to the manufacturer's instructions (Roche). Sense probes were synthesized as controls. Digoxigenin-labeling of RNA probes, tissue preparation and in situ hybridization were carried out as described by Drews et al. (1991).

RESULTS

The structure and different developmental stages of a grapevine bud are shown in Figure 1. Many epidermal hairs are observed in spring on over-winter buds (Figure 1A), and their structure includes a primary bud, a secondary bud and a tertiary bud (Figure 1B). A rise in temperature seems to be required for the beginning of bud swelling (Figure 1C). At this time, their structure includes a apical shoot meristem (SAM) and lateral meristems in the primary buds (Figure 1D). During the continuous development of the swelling spring buds, bud bursting begins with a young leaf produced in the basal part of the shoot (Figure 1E). This budburst structure includes apical and lateral meristems, leaf primordia, inflorescence or tendril primordia and bud scales (Figure 1F). As soon as the apical bud forms, it grows strongly from the spring shoot tip after budburst (Figure 1G). The SAM is visible (Figure 1H), and the primary growing point, bract, tendril or inflorescence primordium and axillary bud primordium are distinctly observable (Figure 1I and J). As the shoot continues to grow, the apical bud appears and rapidly differentiates into shoot tips (Figure 1K). The first apical buds are less structurally differentiated than those that grow later (Figure 1L). The growing point initiates, a new bud primordium begins to form in the axillary leaf (Figure 1M), and the axillary bud primordium, leaf primordium and differentiated inflorescence or tendril primordium gradually develop (Figure 1N).

The structure and different developmental stages of a grapevine inflorescence are shown in Figure 2.The previously dormant winter buds begin to grow and show leaf expansion in the spring (Figure 2A). After the first and second leaves at the basal part of the shoot show complete leaf expansion, the inflorescence appears at the opposite side of the third leaf (Figure 2B), and after it grows from small to large (Figure 2C), it finally forms a flower (Figure. 2D). Our anatomical results show that 5 to 6° more of the inflorescence branch primordium is visible in burst spring bud (Figure 2E). At this time, the inflorescence primordium is still tightly wrapped by the bract (Figure 2F). After the inflorescence primordium goes through development, its lateral meristem region begins to initiate an original floral primordium (Figure 2G), and it initiates 2 to 3 floret primordia from the lateral meristem region of the original floral primordium (Figure 2H). The inflorescence continues to grow toward the tip along the peduncle, and the inflorescence primordium continuously differentiates at its lateral meristem. At this time, the inflorescence peduncle at the bottom grows laterally and differentiates into inflorescence branching primordia with fewer orders (Figure 2I). The floret primordium forms at the lateral meristem of each inflorescence branching primordium and differentiates into flower meristem and develops into flowers (Figure 2J and K). When the whole flower meristem is filled with inflorescence branching, the elongation of the inflorescence stops (Figure 2L).

The expression patterns of the genes *VvTFL1, VFL, VAP1, VvAG1* and *VvSEP3* in grapevine buds are shown in Figure 3. The expression of *VvTFL1* was detected most strongly in the earliest stages of bud swelling in the spring, and it was mainly distributed in the lateral meristems of primary buds and lateral buds (Figure 3A). Transcripts from the *VvTFL1* gene were detected in apical meristems and bract primordia of burst buds, and the hybridization signal was weaker than that in swelling buds (Figure 3B). In the apical buds, the *VvTFL1* gene was mainly detected in the apical meristems of the buds (Figure 3C). Expression of the *VFL* gene was detected from the earliest stages of bud swelling, but the expression pattern of *VFL* was different from that of *VvTFL1*; *VFL* expression was mainly restricted to the scale tissue interval region of swelling buds (Figure 3D). In burst buds, *VFL* mRNA preferentially accumulated at the apical meristems of buds, and then became mainly restricted to the peripheral zone (Figure 3E). The expression of *VFL* was detected most strongly in whole shoot apical meristems (SAM) of apical buds when green shoots began to grow, in both the primary buds of tip shoots and lateral buds (Figure 3F). The expression of *VAP1* was not detected in swelling buds (Figure 3G). In burst buds, the *VAP1* gene began to be expressed in meristems of the rachis form (Figure 3H). In apical buds, the expression of *VAP1* was detected most strongly in SAMs, lateral buds and bracts (Figure 3I).A *VAG1* gene hybridization signal was not detected in swelling buds (Figure 3J). However, a *VAG1* weak signal was detected in apical meristem of the secondary and tertiary buds in burst buds (Figure 3K). The expression of *VAG1* was detected in SAMs of apical buds (Figure 3L).The expression of *VSEP3* was not detected in swelling buds and burst buds (Figure 3M and N), but a hybridization signal was weakly detected in SAM bract primordia of apical buds (Figure 3O).

The expression patterns of the genes *VvTFL1, VFL, VAP1, VvAG1* and *VvSEP3* in grapevine inflorescences and flowers are shown in Figure 4. *VvTFL1* transcripts

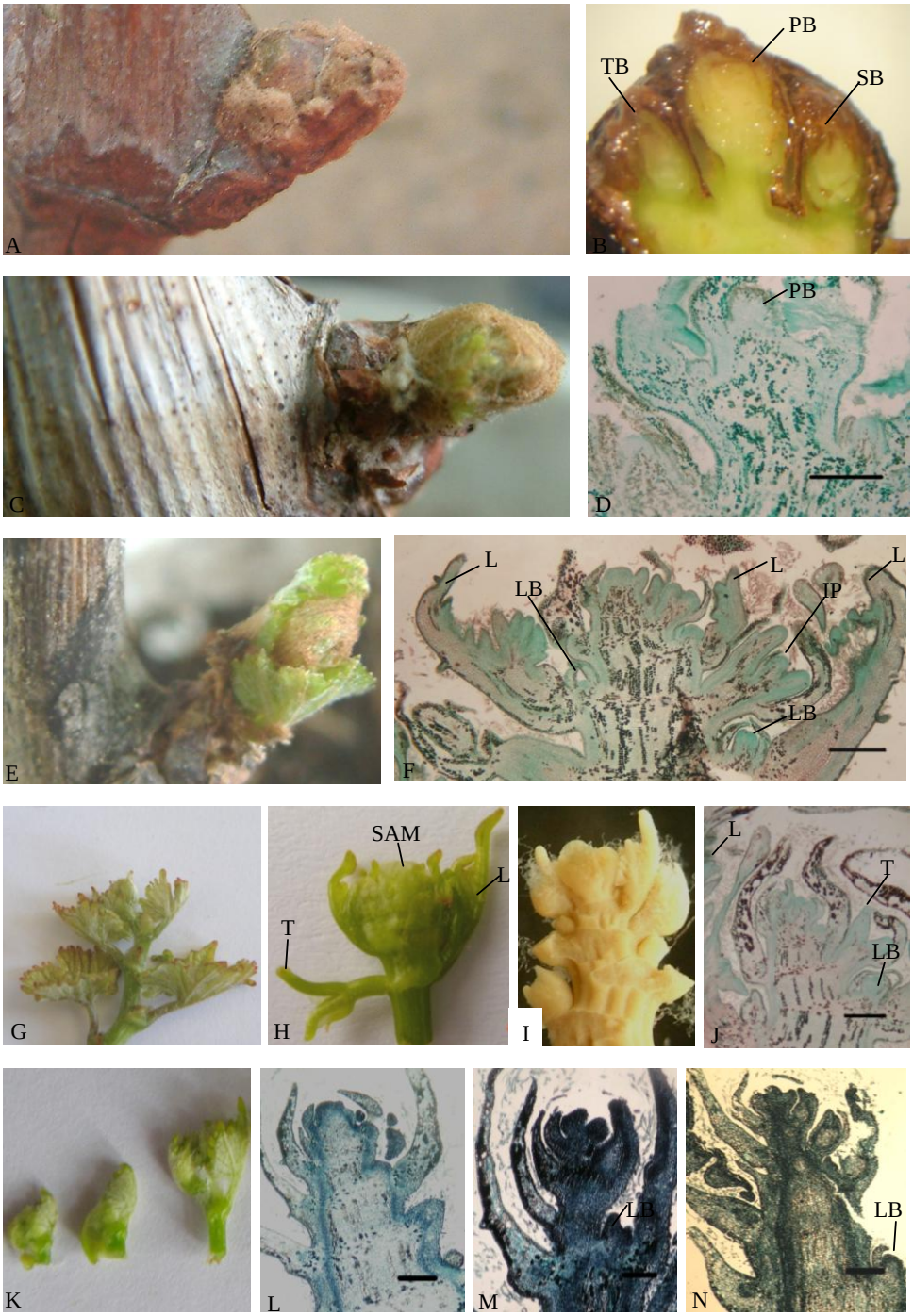

Figure 1. The developmental status and structure of differentiating "Xiangfei" grapevine buds: (A, B) Structure of buds before swelling; (C, D) structure of swelling buds; (E, F) structure of burst buds; (G) status of shoot growth in a winter bud; (H) developmental status of a green apical bud; (I, J) structure of an apical bud; (K) different developmental stages of apical buds; (L-N) structure of apical buds. IP: inflorescences primordium; L: leaf; LB: lateral bud; PB: primary bud; SAM: shoot apical meristem; SB: secondary bud; T: tendril; TB: tertiary bud. The scale bar indicates 20 μm.

were not detected in differentiating inflorescences or floral primordia (Figure 4A and B). *VvTFL1* expression could not be detected in developing flower organ primordia of petals, stamens or carpels (Figure 4C), nor at any time throughout flower development before blooming (Figure 4D). *VFL* expression was strongest in the apical

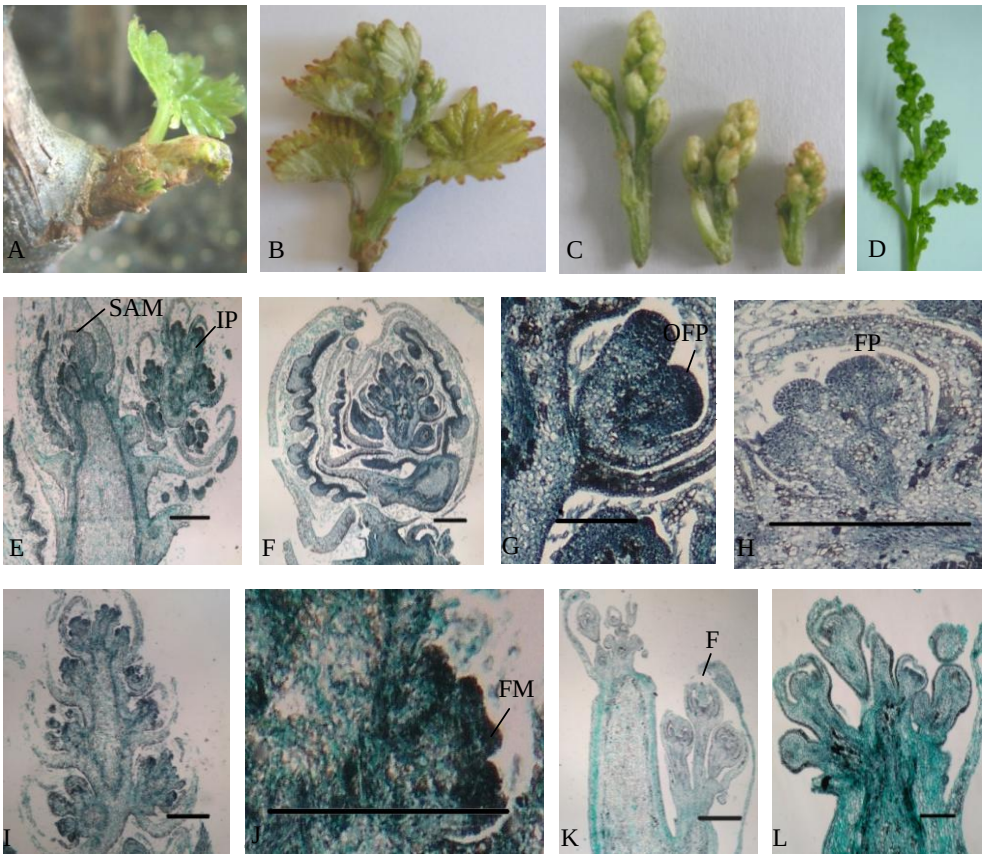

Figure 2. The initiation and development of inflorescences in the "Xiangfei" grapevine. (A) Beginning of leaf expansion; (B) appearance of the first inflorescence; (C) inflorescences at different developmental stages; (D) inflorescence before blossoming; (E, F) branching inflorescence primordium; (G, H) floral rudiment; (I) differentiating inflorescence; (J) differentiating floral primordium; (K) differentiating floret; (L) the differentiation of inflorescences stops when the whole growth point is filled with differentiated flower. F: flower; FM: flower meristem; FP: floret primordial; IP: inflorescences primordium; OFP: original floral primordium; SAM: shoot apical meristem. The scale bar indicates 20 μm.

meristems of inflorescence primordia at the early stage of development (Figure 4E). During inflorescence primordium growth and development, the expression of *VFL* was detected in flower organ primordia, including those of petals, sepals and stamens (Figure 4F). When pistil primordia began to initiate, *VFL* was strongly expressed in the meristem primordia of carpel and sepals (Figure 4G). Before blooming, *VFL* yielded little hybridization signal in the inner ovarian wall of flowers (Figure 4H). Expression of *VAP1* was detected starting from floral organ primordium initiation (Figure 4I), and its hybridization signal was found in the primordia of petals, stamens and pistils (Figure 4J). As soon as ovule primordia began to grow, *VAP1* was excluded from the ovule-forming region (Figure 4K). Before blooming, *VAP1* mRNA visibly accumulated at the inner integument, ovarian walls and styles of flowers (Figure 4L).The *VvAG1* gene was not expressed during early inflorescence differentiation (Figure 4M). Later, anatropous ovules development process were visible, the expression of *VAG1* was detected in the flower placenta (Figure 4N and O). Before blooming, *VvAG1* was highly expressed in the placenta, inner ovarian wall, stigma and style tissue (Figure 4P).The *VvSEP3* gene was not expressed in early inflorescence differentiation and floral organ initiation (Figure 4Q and R). When compound ovules formed, the *VvSEP3* mRNA hybridization signal was found in the placenta and inner ovarian wall (Figure 4S). Before blooming, the expression of *VSE31* was detected in the placenta, diaphragm, style and inner ovarian wall (Figure 4T).

DISCUSSION

The winter bud of the grapevine looks like a simple structure, but it is actually a compound bud consisting of three growing points: the primary, secondary and tertiary

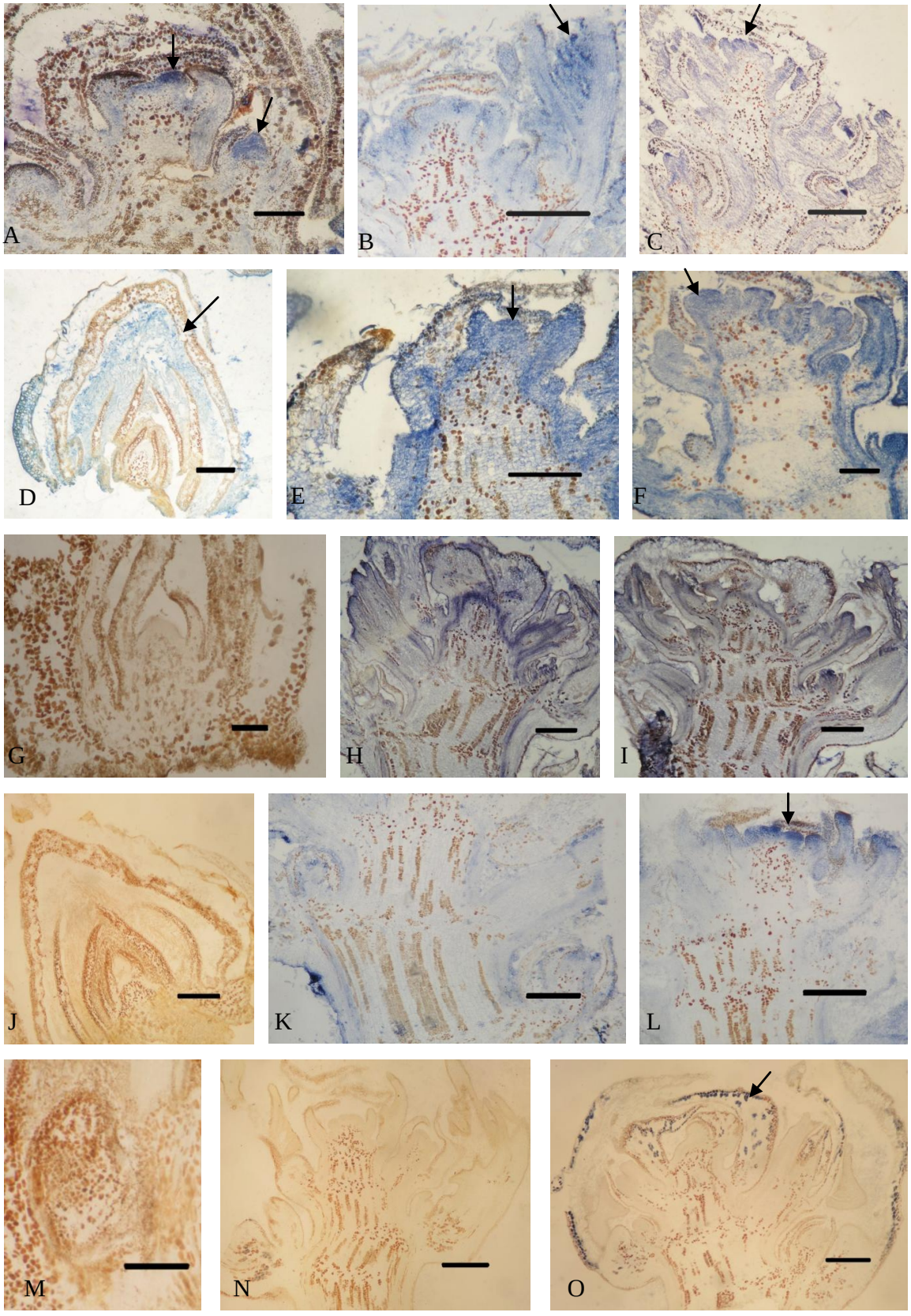

Figure 3. Expression patterns of the genes VvTFL1, VFL, VAP1, VvAG1 and VvSEP3 in different types of 'Xiangfei' grapevine buds.(A-C) Expression patterns of VvTFL1 in swelling, burst and apical buds; (D-F) expression patterns of VFL in swelling, burst and apical buds; (G-I) expression.

Figure 4. Expression patterns of the genes *VvTFL1, VFL, VAP1, VvAG1* and *VvSEP3* during the development of 'Xiangfei' grapevine inflorescences and flowers. (A-D) expression patterns of *VvTFL1* during inflorescence and flower development; (E-H) expression patterns of *VFL* during inflorescence and flower development; (I-L) expression patterns of *VAP1* during inflorescence and flower development; (M-P) expression patterns of *VvAG1* during inflorescence and flower development; (Q-S) expression patterns of *VvSEP3* during inflorescence and flower development. (T) is the sense *VvSEP3*probe control. The scale bar indicates 20 μm.

buds. The compound bud initiates at the axil of each leaf of a shoot during the first growing season and produces various organ primordia before becoming dormant. In the second growing season, only the primary bud grows in most cases, producing a primary shoot, and an apical bud forms in its growing tip. Sometimes, the secondary and tertiary buds germinate with the primary buds, producing double shoots and triple shoots in the same grapevine shoot node (May, 2000; Srinivasan and Mullins, 1976). In our study, the differentiation degree and structure were differences in swelling buds, burst buds and apical buds of grapevine. The structure of burst buds is very complex, as it includes an apical meristem region, a leaf primordium, an inflorescence or tendril primordium and bud scales. The structure of green apical buds is less differentiated than that of burst buds in the spring growth of shoots. Later, the shoots grow fast, and with the gradually increasing length of the shoot, the differentiation signals are too low in the tip shoot to produce an apical bud. These results are relevant to the environmental conditions and nutrient supply of grapevine trees.

Inflorescence development in the grapevine requires two consecutive growing seasons (Pratt, 1971; Gerrath, 1993). In the first year, an inflorescence primordium appears, and the inflorescence meristem forms several inflorescence branch meristems before the bud enters dormancy at the end of the summer. The next spring, additional inflorescence branch meristems are formed before each one divides into a cluster of three to four flower meristems that develop into flowers (Gerrath and Posluszny,1988).In grapevines, shoot growth is described as monopodial or sympodial (Gerrath et al., 2004). Our results showed that the inflorescence branching primordium was formed within lateral buds at bud bursting and gave rise to inflorescence branch meristems that can form additional inflorescence meristems in a spiral phyllotaxis. At the lateral meristem of the inflorescence, an original floral primordium first initiates and continues to differentiate into floret primordia. Subsequently, each floral primordium divides into a cluster of three to four flower meristems arranged as a dichasium. When the whole flower meristem is filled with inflorescence branching, the elongation of the inflorescence stops. Thus, the growth and development of the inflorescence axis is more like monopodium growth.

In the grapevine, *VvTFL* is a repressor of floral development that is expressed in shoot apices early in lateral bud development and in buds soon after bud bursting (Boss et al., 2006;Yao et al., 2010), but over-expression of *VvTFL1A* in transgenic *Arabidopsis* seems to delay flowering and the initiation of flower meristems (Boss et al., 2006). Recently, it was reported that over-expression of *VvTFL1A* does not affect flowering time but does affect the determination of flower meristems, strongly altering inflorescence structure (Fernandez et al., 2010). We found that *VvTFL1* was expressed in the apex meristems of swelling buds, burst buds and apical buds, and its effects were evident earlier than those of *VFL* and *VAP1*. However, expression of *VvTFL1* was not detected during the development of inflorescences or flowers.

These results suggest that *VvTFL1* does not affect reproductive development in the grapevine, although it might delay the transformation of inflorescence meristems and play roles in vegetative development and the maintenance of meristem indetermination.

Concerning *VFL* as a putative grapevine flower meristem identity gene, its expression was detected in lateral meristems prior to any commitment, and later in inflorescence and tendril meristems. *VFL* expression increases in the proliferating inflorescence meristems that are dividing to generate inflorescence-branch meristems in lateral buds. Furthermore, its expression reaches the highest levels in the floral meristems that develop into bursting buds (Carmona et al., 2002). However, overexpression of *VFL* in transgenic *Arabidopsis* promotes the rapid transformation of inflorescences to flower meristems similarly to the effect of overexpressing the endogenous *LFY* gene (Carmona et al., 2007). In our study, it was shown that *VFL* expression was detected in swelling buds, sprouting buds, apical buds, initiating inflorescences and all flower organ primordia, and it was weakly detected during late flower organ development. It has been suggested that *VFL* plays an extended role during the reproductive development of buds and flowers at early growth stages. Furthermore, VFL was expressed in the peripheral zone of SAMs in swelling buds, all meristem zones of growth points in apical buds and inflorescence meristem primordia, and it was strongly expressed in petals and stamen primordia. These results suggest that *VFL* plays important roles in the initiation of inflorescence meristem, the flowering transition and the morphological formation of floral organs.

VAP1 is expressed throughout flower development (Calonje et al., 2004) and is broadly expressed in newly formed flower meristems, but it then becomes excluded from the sepal-forming region and restricted to the inner part of the meristem that forms the petals, stamens and carpels (Carmona et al., 2007). Furthermore, GAs regulated the transcripts of *VAP1* during the development of latent or apical buds and floral induction in grapevine.(Zhang et al., 2008). We found that the expression of *VAP1* was later than that of *VvTFL1* or *VFL*, and it was expressed in branching pedicels, petals, stamens and carpel primordia. These results suggest that *VAP1* might influence the initiation of inflorescence rachis and the size of inflorescences in the grapevine. Due to its high expression in flower organ primordia, especially that in carpel primordia, it was demonstrated that the *VAP1* gene is not an A-type gene, in agreement with studies by Calonje (2004) and Zhang (2008).

In *Arabidopsis*, the *AG* gene participates in the development of stamens and petals (Theissen, 2001),

and the *SEP* gene influences the development of ovules (Corley et al., 2005). In the grapevine, the *VvAG1* gene is usually thought to play a role in floral development and fruit development during, before and after fertilization. *VvSEP3* plays an important role in fruit development and maturation (Diaz-Riquelme et al., 2009). In our research, neither *VvAG1* nor *VvSEP3* were expressed in swelling buds or sprouting buds. However, hybridization signals from the *VvAG1* gene were detected in the meristem region of apical buds, and the *VvSEP3* gene was expressed faintly in bract primordia. The results demonstrated that the *VvAG1* gene might be expressed earlier than the *VvSEP3* gene, and it may influence the morphological formation of organs in the grapevine. Neither *VvAG1* nor *VvSEP3* expression was detected in initiating inflorescences or differentiating flowers during early development. However, *VvAG1* expression began to be detected in the placenta at the anatropous ovule development stage, and *VvSEP3* began to be expressed in the placenta after the end of the anatropous ovule stage. These results suggest that both the *VvAG1* and *VvSEP3* genes might be relevant to ovule development, seed formation and fruit setting.

Of the five genes studied, *VvTFL1, VFL* and *VAP1* play important roles during the early development of buds and flowers in the grapevine. Moreover, *VvTFL1* acted earlier than either *VFL* or *VAP1*, and *VFL* acted earlier than *VAP1*. The three genes might play important roles in SAM differentiation, inflorescence meristem initiation, inflorescence branching primordium differentiation and flower organ primordium formation in early differentiating buds and flowers. The other two genes studied (*VvAG1* and *VvSEP3)* were found to play important roles in the late-mid-stage of flower development, and the effect of the *VvAG1* gene was earlier than that of the *VvSEP3* gene.

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