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Full Length Research Paper

The expression of *VFL* and *VvTFL1* genes in relation to the effects of gibberellins in different organs of "Xiangfei" grapevine

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The effects of gibberellins (GAs) on plant growth and the expression of *VFL* and *VvTFL1* genes in different organs of "Xiangfei" grapevine (*Vitis vinifera* L.) were investigated. The endogenous GA content significantly increased in the apical bud, inflorescence and tendril after GA treatment of grapevine. The expression of the *VFL* gene was obviously reduced in the meristem of apical buds, tendrils with florets and small inflorescences after GA treatment, but *VFL* gene expression was clearly found during the middle and big inflorescence developmental stages. These results suggested that the exogenous GAs delayed floral development of grapevine by inhibiting *VFL* gene expression at the early stage of inflorescence development. *VvTFL1* gene expression was detected in the meristem of apical bud and lateral bud, but not during the process of inflorescence development. After applying GAs on grapevine, *VvTFL1* gene expression decreased in the apical bud. These results suggested that exogenous GA treatments might repress differentiation of the inflorescence meristem primordia by reducing *VvTFL1* expression in the apical bud but have no effect on *VvTFL1* expression during floral development.

Key words: Grapevine, *VFL* gene, *VvTFL1* gene, gibberellins (GAs).

INTRODUCTION

As one of the necessary hormones for plant growth, gibberellins (GAs) regulate several steps in plant growth and development, including seed germination, hypocotyl elongation, leaf growth and the transition to flowering (Peng and Harberd, 2002). GAs have been widely used in vineyards to increase the rate of fruit-set, produce seedless berries, prevent fruit drop and enhance fruit quality (Palma and Jackson, 1989; Weaver and Pool, 1965). In recent years, the extensive research into the floral development of the model plant *Arabidopsis thaliana* has built a complete gene regulatory network controling the flowering process. Among them, the genes in the GA pathway are among the most important for floral induction (Parcy et al., 1998). GAs can obviously promote flowering in *Arabidopsis* (Thomas and Sun, 2004), but they inhibit flower development and delay flowering in grapevine (Boss et al., 2003; Manankov, 1976). This indicates that GAs play important but divergent roles in the flower development of the model plant and the perennial grapevine.

The functions and interactions of genes are necessary for the flowering process and floral development (Jack, 2004; Parcy, 2005). Based on the identification and functional analysis of *Arabidopsis* flowering signal integrators and floral meristem identity genes, the homologous genes in grapevine were analysed (Boss et al., 2006; Calonje et al., 2004; Carmona et al., 2002; 2008; Joly et

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Abbreviations: Gas, Gibberellins; ***gai,*** GA-insensitive mutant; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcriptase-PCR; **PBS,** phosphate buffer saline; **DEPC,** diethylpyrocarbonate; **ELISA,** enzyme-linked immunosorbent assay; **BHC,** 2,6-di-tert-butyl-4-methylphenol; **CTAB,** cetyl trimethyl ammonium bromide.

al., 2004; Sreekantan and Thomas, 2006). *LEAFY* (*LFY*) is the floral meristem identity gene involved in integrating the multiple pathways that promote flowering during the floral transition (Araki, 2001; Weigel et al., 1992). *LEAFY* can promote floral induction. Mutation of *LFY* changes the identity of some flowers to inflorescences (Blázquez et al., 1997; Liljegren et al., 1999). The *TFL1* gene delays the flowering transition, maintains the shoot meristem identity and has an antagonistic relationship with the *LFY* gene (Busch et al., 1999; Shannon and Meeks-Wagner, 1991; Ratcliffe et al., 1998). The juvenile phase of citrus is positively correlated with the transcript level of *CsTFL* and negatively correlated with the mRNA levels of the *LEAFY* and *APETALA1* genes (Pillitteri et al., 2004). *VFL* in grapevine plays a role not only in the development of the floret meristem, but also in the early stages of apical meristem differentiation into flowers, leaves and tendrils (Carmona et al., 2002). However, homologous genes can differ in their temporal and spatial expression in different plant species (Calonje et al., 2004; Joly et al., 2004). Therefore, research on the effects of homologous genes among different species of plants is important to reveal gene functions and divergent floral development mechanisms.

The expression of a gene can be affected by many factors, such as nutrition, hormones and external environmental signals (Blázquez and Weigel, 2000; Blázquez et al., 2006). It has been proved that GAs have direct or indirect relationships with the expression of some floral development genes (Blázquez et al., 2002; Huang et al., 2006; Zhang et al., 2009). GAs can regulate the expression of the floral meristem gene *LFY* in *Arabidopsis*. It has been demonstrated that GA_4 is active in regulating *LFY* transcription in *Arabidopsis* (Eriksson et al., 2006; Sumitomo et al., 2009). The GA-insensitive mutant *gai* of grapevine develops inflorescences in sites where it would normally form tendrils in the wild-type grapevines, which proves that GAs can inhibit the differentiation of inflorescences in grapevine (Boss and Thomas, 2002). However, how the GAs regulate the expression of relevant floral genes in the grapevine remains unclear.

In view of the importance of GAs in the regulation of floral gene expression, *VFL* and *VvTFL1* from "Xiangfei" grapevine, which are homologous with *LEAFY* and *TFL1* of *Arabidopsis,* were identified in our laboratory. By comparing their expression pattern in different organs (including the bud, inflorescence and tendril) and the changes in gene expression after the grapevine was treated with exogenous GAs, the effect of GAs on the regulation of *VFL* and *VvTFL1* gene expression in grapevine was further elucidated.

MATERIALS AND METHODS

Plant materials

The grapevine variety "Xiangfei" (*Vitis vinifera* L.) was cultivated in a Beijing hot spring nursery. At different stages, the apical bud, lateral bud, small tendril (0-5 cm), big tendril (5-10 cm), small inflorescence (0 - 6 cm), middle inflorescence (6 -10 cm), big inflorescence (10 - 15 cm) and young leaf were collected. All tissues were immediately frozen in liquid nitrogen, kept on dry ice during transport to the laboratory and stored at -80°C for further analysis.

GAs treatments and content determination

In 2006-2008, after bud burst, the buds were covered with absorbent cotton and soaked in 100 mg/l liquid GAs (GA_3 > 80%) for 2 -3 h. At different stages, the young leaves, apical buds, tendrils and inflorescences were sampled using untreated shoots as a control. Some of the materials were stored at -80°C for semi-quantitative RT-PCR analysis and determination of GA content. The other materials were fixed with PBS (NaCl 0.8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g per litre, pH 7.4) containing 4% (w/v) formaldehyde and kept in a vacuum at 4°C overnight for *in situ* hybridisation experiments. According to published protocols, the fixed tissues were then washed, dehydrated and embedded in paraffin. The water used in all experiments was treated with diethyl-pyrocarbonate (DEPC). Meanwhile, the developmental progress of different organs from normal plants and GA-treated plants was photographed for comparison.

The determination of GA content was carried out by enzyme-linked immunosorbent assay (ELISA) analyses. The GA samples, which were stored at -80°C, were extracted in 80% methanol containing 1mmol/l 2,6-di-tert-butyl-4-methylphenol (BHC) by sonication and freeze-thaw cycles, centrifuged at 4°C and concentrated on C_{18} cartridges, which were then washed with 100% methanol and 100% ethylether prior to elution with methanol. The eluted components were dried by evaporation and then resuspended in 2 ml of dilution buffer (PBS containing 0.1% (v/v) Tween-20). Ten microlitres (10 µl) of supernatant from each sample was used in the enzyme-linked immunosorbent assay (ELISA) analyses. Anti-immune complex ELISA analyses were conducted according to the procedures described by An and Carmichael (1994). The GA content in the supernatants was determined using a GA ELISA kit, which was kindly provided by Professor Baomin Wang in the College of Agronomy and Biotechnology at China Agricultural University.

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was extracted from various tissues from grapevine using the CTAB method (Murray and Thomposon, 1980). After the products were purified by RNase-free DNase I (Takara Bio, Beijing, China), the RNA concentration was determined using an atom UV spectrophotometer ($OD_{260/280}$ = 1.8 - 2.0). Two micrograms of total RNA were used to synthesise the first-strand cDNA using Moloney murine leukaemia virus reverse transcriptase (Promega) and an oligo dT primer. The reaction condition for RT was as follows: samples were incubated at 70°C for 5 min, followed by 37°C for 1 h, and then immediately placed on ice. These cDNA were used as templates for PCR performed as follows: 30 cycles of 94°C for 30 s, 55 - 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 *VFL* and *VvTFL1* genes were designed based on their ESTs (*VFL* GenBank accession no. AF450278, *VvTFL1* GenBank accession no. AF378127) (forward primer: 5'-TGCTATCCTTCGTCTTGA CCTTG-3' for Vv*Actin2*; 5'-GCCAAAGAGCGCGGTGAGAAGTGC-3' for *VFL*; 5'-TGGTAGAGTGATTGGGGATG-3' for *VvTFL1*; reverse primer: 5'-GGACTTCTGGACAACGGAATCTC-3' for Vv*Actin2*; 5'-GGGAATACTACGTGCCATTTAAC-3' for *VFL*; 5'-TGGGACTTGAA GAATACAGC-3' for *VvTFL1*) and were synthesised at the Beijing Sunbiotech Limited Company. The *VvActin2* gene was used as the

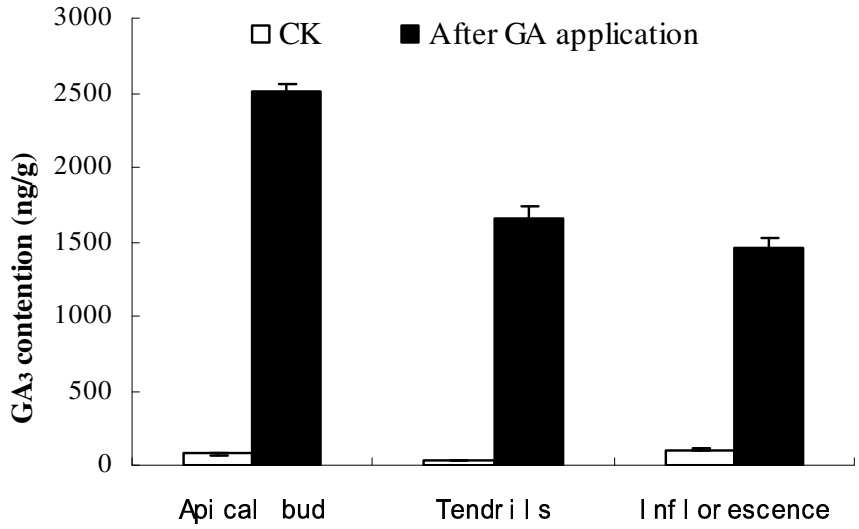

Figure 1. Changes in the GA content of the apical bud, tendril and inflorescence of "Xiangfei" grapevine after GA treatment.

internal control. PCR products were electrophoresed through a 2% agarose gel and visualised after ethidium bromide staining.

Probe preparation and *in situ* hybridisation

The probes were obtained by a PCR approach using previously synthesised cDNA as templates. The sense probe primers were 5'-GGTACCGGCCAGTTTATTCAAGT-3' for *VFL*, and 5'-AAGCTTGG CCATGCACTTCTTACTAT-3' for *VvTFL1*. The anti-sense probe primers were 5'-AAGCTTAAGCATCGCCATAGCCTC-3' for *VFL* and 5'-GGTACCTGATCTTCCCGTTGGTTA-3' for *VvTFL1*. The primer sequences were designed according to published sequences (*VFL* GenBank accession no. AF450278, *VvTFL1* GenBank accession no. AF378127). The PCR products consisting of cDNA fragments of 870 bp for *VFL* and 460 bp for *VvTFL1* were sequenced. The DNA fragments had 99% similarity to the published sequences. The two products were ligated into the pMD-18 vector (Takara Bio, Beijing, China). *VFL* was digested with *Kpn* I and *Xba* I, whereas *VvTFL*1 was digested with *Hind* III and *Xba* I. Then, they were cloned into the pSPT-18 vector (Roche). Antisense probes were synthesised using a DIG RNA labelling kit (Sp6/T7) according to the manufacturer's instructions (Roche). Sense probes were synthesised as controls. Digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridisation were carried out as described by Drews et al. (1991).

RESULTS

The effect of GAs on the growth and development of apical buds, tendrils and inflorescences of the grapevine

After the grapevine was treated with GAs, a substantial increase in the GA content in the apical bud, tendril and inflorescence was detected by ELISA analyses (Figure 1). The GA content in the tendril was 52 times higher than that of control plants; the GA content in the apical bud and inflorescence was 34 and 14 times higher than that of the controls. After applying GAs, the new shoot was thin and delicate (Figure 2A) and the leaf was thin and yellowish-green in colour (Figure 2B). The tendrils of the second section (Figure 2C) and the tendrils of the third section (Figure 2D) below the apical bud obviously began to elongate. The rachis noticeably elongated in both the small and big inflorescences (Figures 2E and F). Moreover, the number of flowers on the rachis was reduced and the transverse diameter of the flower was smaller (Figure 2G).

The effect of GAs on the expression patterns of the *VFL* gene in different organs of grapevine

The expression of *VFL* varied between different tissues (Figures 3A and B). The results showed that the transcripts of *VFL* were barely detectable in the young leaves, small tendrils and big tendrils but could be detected in the apical bud. A continuous expression of *VFL* was found in the inflorescence at various development stages. The transcript level of the *VFL* gene accumulated to its maximum level during the stage of small inflorescence formation. However, the transcript level of the *VFL* gene gradually decreased at the later stages of inflorescence development. At the stage of big inflorescence, only a minimal *VFL* gene expression was observed (Figure 3A). After the GA treatment, there was still no expression of the *VFL* gene in the young leaf, small tendril and big tendril. The expression of the *VFL* gene in the apical bud and small inflorescence showed an obvious reduction with no expression of *VFL* gene in the tendril with floret. Interestingly, *VFL* expression progressively increased during the middle and the big inflorescence stages and reached its maximum level at

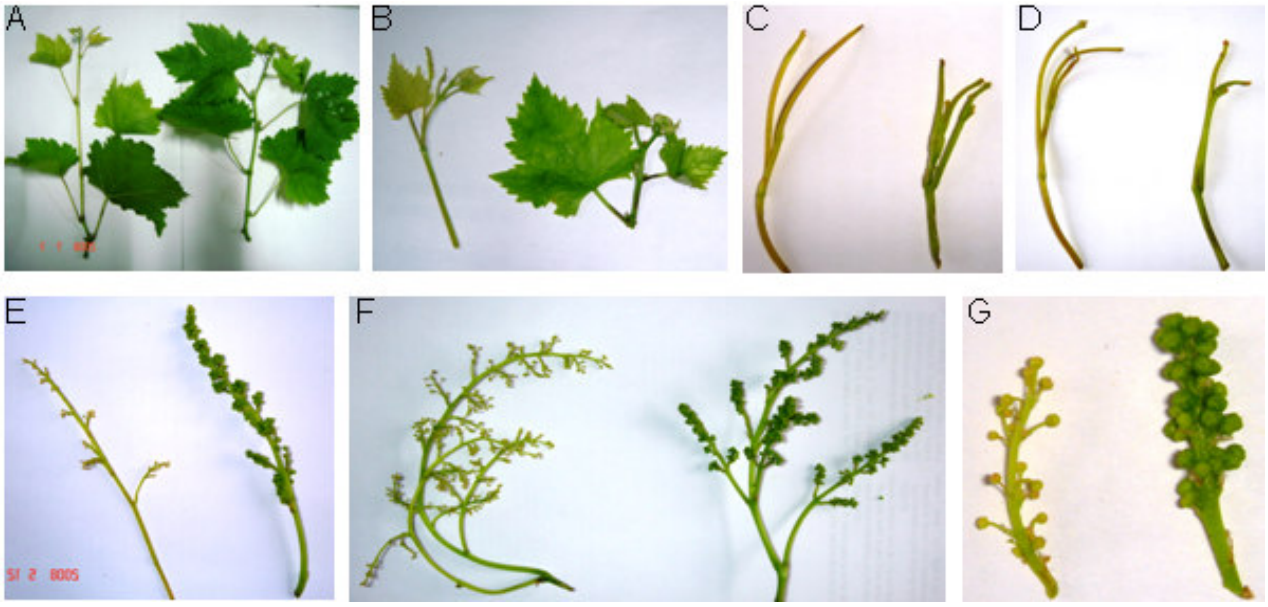

Figure 2. Photographs of different organs in the Xiangfei grapevine in GA-treated plants (right) and controls (left). (A) Shoot, (B) leaf and stem tip, (C) the second tendril below the apical bud, (D) the third tendril below the terminal tendril, (E) small inflorescence, (F) big inflorescence and (G) flower.

the big inflorescence stage (Figure 3B).

In situ hybridisation showed that a strong expression of *VFL* was mainly distributed in the meristem of the apical bud (Figure 4A) and a strong hybridisation signal for *VFL* was found in the apical meristem of the lateral bud (Figure 4B). There was no hybridisation signal for *VFL* in the small and big tendrils (Figures 4C and D). The hybridisation signal could be detected in tendrils with florets, which was mainly expressed in the lateral meristem (Figure 4E). During the process of inflorescence development, the expression of *VFL* in the floret meristem primordia was the strongest (Figure 4F) and the expression signal mainly accumulated in the carpel primordia (Figure 4G). Hybridisation signal was present in the ovule primordia (Figure 4H) and the entire pistil (Figure 4I) with the further differentiation of floral organs. Before flowering, the appearance of *VFL* expression was observed in the calyx and calyptra tissues (Figure 4J).

After applying GAs, the hybridisation signal weakened and the *VFL* expression in the meristem of apical bud obviously decreased (Figure 4K). The signal was still barely detectable in the small or big tendrils and tendrils with florets (Figures 4L, M and N). Very little signal accumulated in the floret meristem primordia at the initial stage of inflorescence development (Figure 4O). When the floret organs developed, the *VFL* hybridisation signal became visible at the middle inflorescence stage, especially in the ovule primordia and calyptra (Figures 4P and Q). Before flowering, *VFL* expression was detected in the calyx, calyptra, stigma, anther, entire ovary wall and ovule (Figure 4R) and the expression in the nucellus tissue was more obvious (Figure 4S).

The effect of GAs on the expression patterns of *VvTFL1* gene in different organs of grapevine

The expression levels of *VvTFL1* gene changed drastically among all the observed developmental stages (Figures 3C and D). The results showed that the expression of the *VvTFL1* gene was strongest in the apical bud and was also strong in the small tendril. The *VvTFL1* transcripts could not be detected in the big tendril or throughout the inflorescence growth (Figure 3C). After the grapevine was treated with GA, *VvTFL1* gene expression decreased in the apical bud and small tendril and was very weak in the tendril with floret. At the same time, the transcript levels of the *VvTFL1* gene in the big tendril and inflorescence was barely detectable (Figure 3D).

Through *in situ* hybridisation, *VvTFL1* expression patterns between normal plants and plants treated by GAs were found to be different. A substantial hybridisation signal for *VvTFL1* was concentrated in the meristem of apical bud (Figure 5A) and a certain quantity of *VvTFL1* expression was found in the meristem of lateral buds (Figure 5B). The *VvTFL1* expression also appeared in the growing tips of small tendrils (Figure 5C), but not in the big tendrils (Figure 5D). The *VvTFL1* expression could not be detected in the tendril with floret (Figure 5E) or throughout the developmental phases of inflorescence growth (Figures 5F and I).

After applying GAs, the hybridisation signal of the *VvTFL1* gene decreased in the meristems of apical buds (Figure 5J). The hybridisation signal of the *VvTFL1* gene still appeared in the meristems of lateral buds (Figure 5K). The hybridisation signal of the *VvTFL1* gene in the

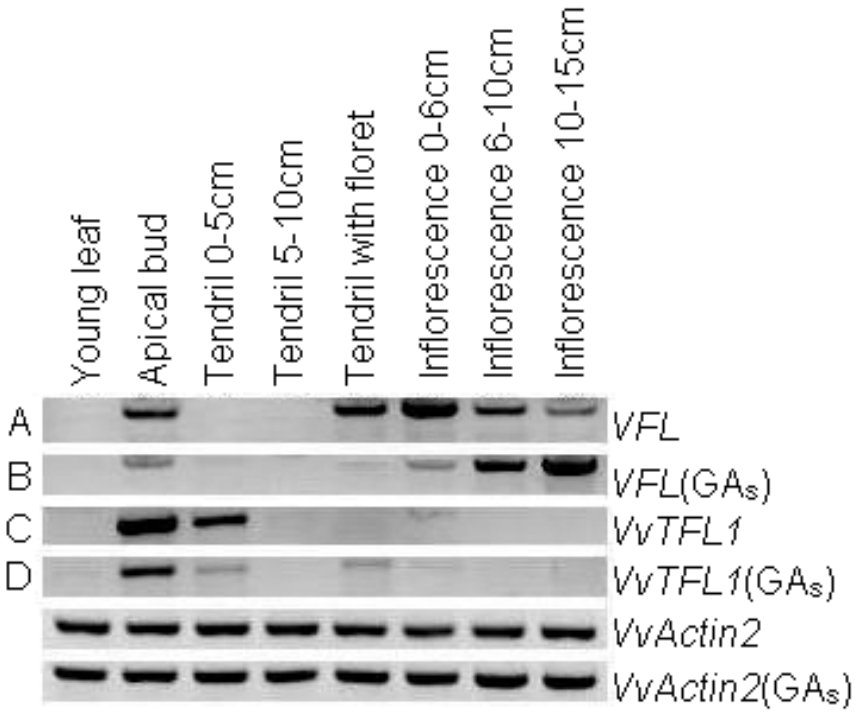

Figure 3. Expression patterns of *VFL* and *VvTFL1* genes in different organs detected by semi-quantitative RT-PCR in "Xiangfei" grapevine. From left to right: young leaf, apical bud, small tendril, big tendril, tendril with floret, small inflorescence, middle inflorescence and big inflorescence.

small tendril showed a reduction compared with that of untreated tissues (Figure 5L). There was still no expression of the *VvTFL1* gene in the big tendril tissues (Figure 5M) and a very weak signal was observed in the tendril with floret (Figure 5N). There was no expression of *VvTFL1* gene in the small, middle and big inflorescences (Figures 5O and R).

DISCUSSION

In grapevine, GA_3 could promote the growth of the tendril, inhibit the formation of the blossom bud, reduce the number of inflorescence anlage and increase the merithal length (Manankov, 1976; Palma and Jackson, 1989). These effects might be caused by the increase in GA signal in the corresponding organs by exogenous GA treatment (Zhang et al., 2008). In this research, after treatment with exogenous GAs during the grapevine germination period, the GA content in the corresponding organs obviously increased. Meanwhile, the internodes and tendrils elongated and the number of flowers in the inflorescence decreased. This indicates that GA treatment at an early stage could promote the growth of vegetative organs and inhibit the development of flowers in the grapevine.

In *Arabidopsis*, flower initiation requires a certain quantity of *LFY* gene expression (William et al., 2004). GAs promote flowering by activating the promoter of *LEAFY* in *Arabidopsis* (Blázquez et al., 1998). In grapevine, *VFL* expression reached its highest level in the floral meristem of bursting buds (Carmona et al., 2002; Joly et al., 2004). The *VvGAI* mutant showed a correlation between inflorescence development and increased *VFL* expression (Boss and Thomas, 2002) and *VFL* was found to be the target gene in the gibberellin-mediated flowering pathway (Carmona et al., 2007). In our research, both semi-quantitative RT-PCR analysis and *in situ* hybridisation results showed that the *VFL* gene is expressed during the entire process of floral development and the signal reached its maximum in the floret meristem primordia at the early stage of inflorescence development. This suggests that the *VFL* gene plays an important role in the induction of floret formation and differentiation at the early stage of inflorescence development. After the grapevine was treated with GAs, the expression of *VFL* significantly decreased in the apical bud and the small inflorescence at the early stage, indicating that the regulation of GAs on *VFL* gene in the grapevine might occur mainly at early stages. In other words, the exogenous GA delays the floral development of grapevine by inhibiting the expression of *VFL* gene at the early stage of inflorescence development.

In *Arabidopsis*, the *TFL1* gene promotes inflorescence meristem identity and is involved in the formation of the inflorescence meristem (Shannon and Meeks-Wagner,

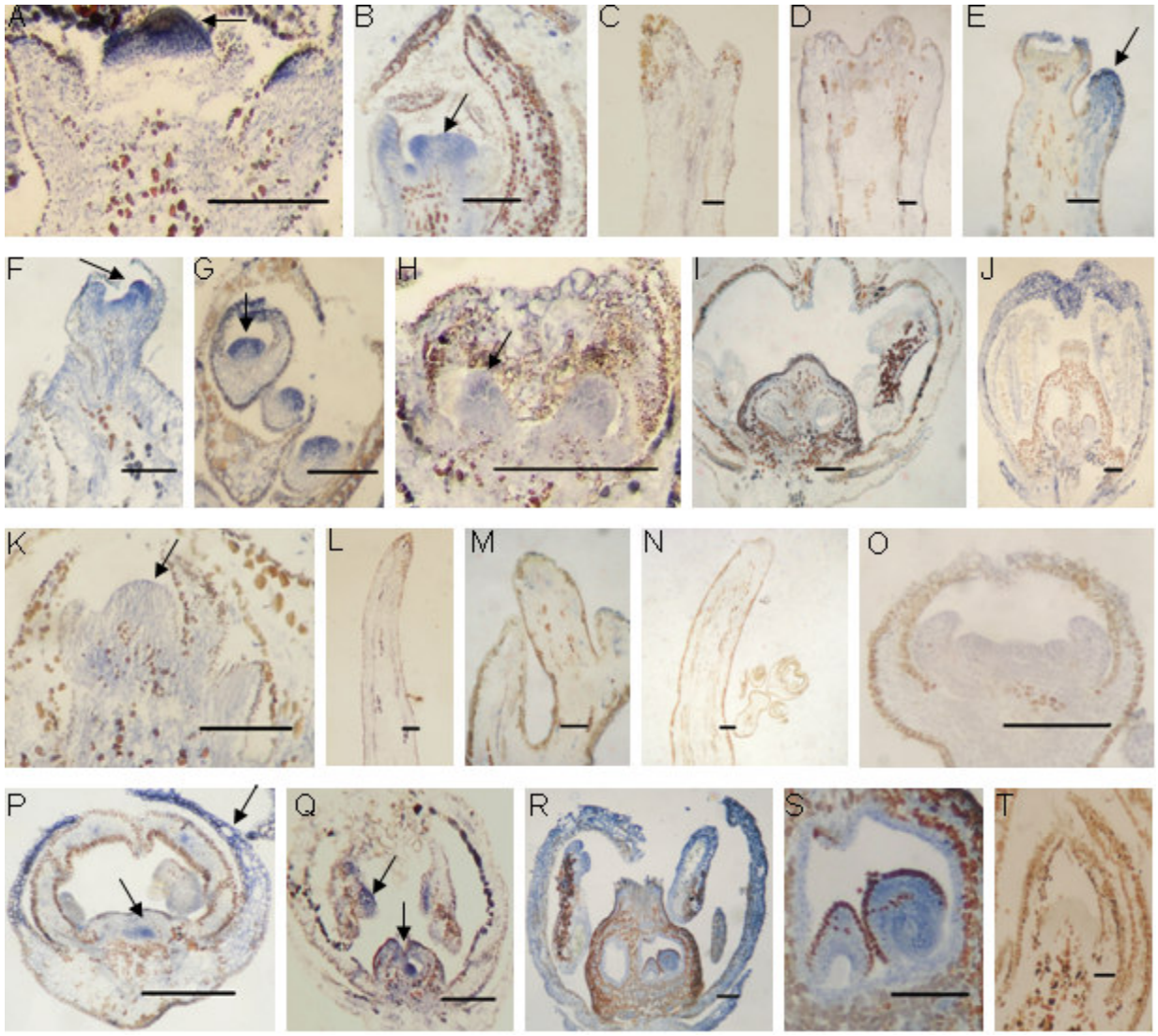

Figure 4. *In situ* expression pattern of the *VFL* gene. A - J shows the *VFL* gene expression in different organs under normal developmental conditions; K - S is the *VFL* gene expression in different organs after GA treatment. (A) Apical bud; (B) lateral bud; (C) small tendril; (D) big tendril; (E) tendril with floret; (F - G) small inflorescence; (H) middle inflorescence; (I - J) big inflorescence; (K) apical bud after GA treatment; (L) small tendril after GA treatment; (M) big tendril after GA treatment; (N) tendril with floret after GA treatment; (O) small inflorescence after GA treatment; (P - Q) middle inflorescence after GA treatment; (R - S) big inflorescence after GA treatment; and (T) control of *in situ* hybridisation. The scale is 20 µm.

1993). The overexpression of the *VvTFL1* gene in transgenic *Arabidopsis* resulted in inflorescence development in the place of siliques and flowers, which confirmed that *VvTFL1* is functionally similar to the *TFL1* gene (Boss et al., 2006). In our research, the *VvTFL1* gene is strongly expressed in the meristem of apical buds, revealing that the *VvTFL1* gene might participate in the process of inflorescence meristem differentiation during apical bud development. After GA treatment, the reduction of *VvTFL1* expression in the apical bud suggested that GAs might be able to inhibit inflorescence meristem differentiation by decreasing *VvTFL1* expression in the apical bud. Meanwhile, the *VvTFL1* gene did not act in inflorescence development and floral growth and exogenous GA treatment had no effect on *VvTFL1* expression during floral development.

The research on the model plant *Arabidopsis* revealed an antagonistic relationship between the *TFL1* and *LFY* genes (Ahn et al., 2006; Boss et al., 2004; Ratcliffe et al., 1999). This regulation mechanism has also been verified in various fruit trees. The homologous genes of *TFL1* in both Japanese pear and quince are expressed during the development of the bud. Furthermore, the expression significantly decreases before flower differentiation (Esumi et al., 2007). The transcript level of *CsTFL* in citrus is positively correlated with the juvenile phase (Pillitteri et

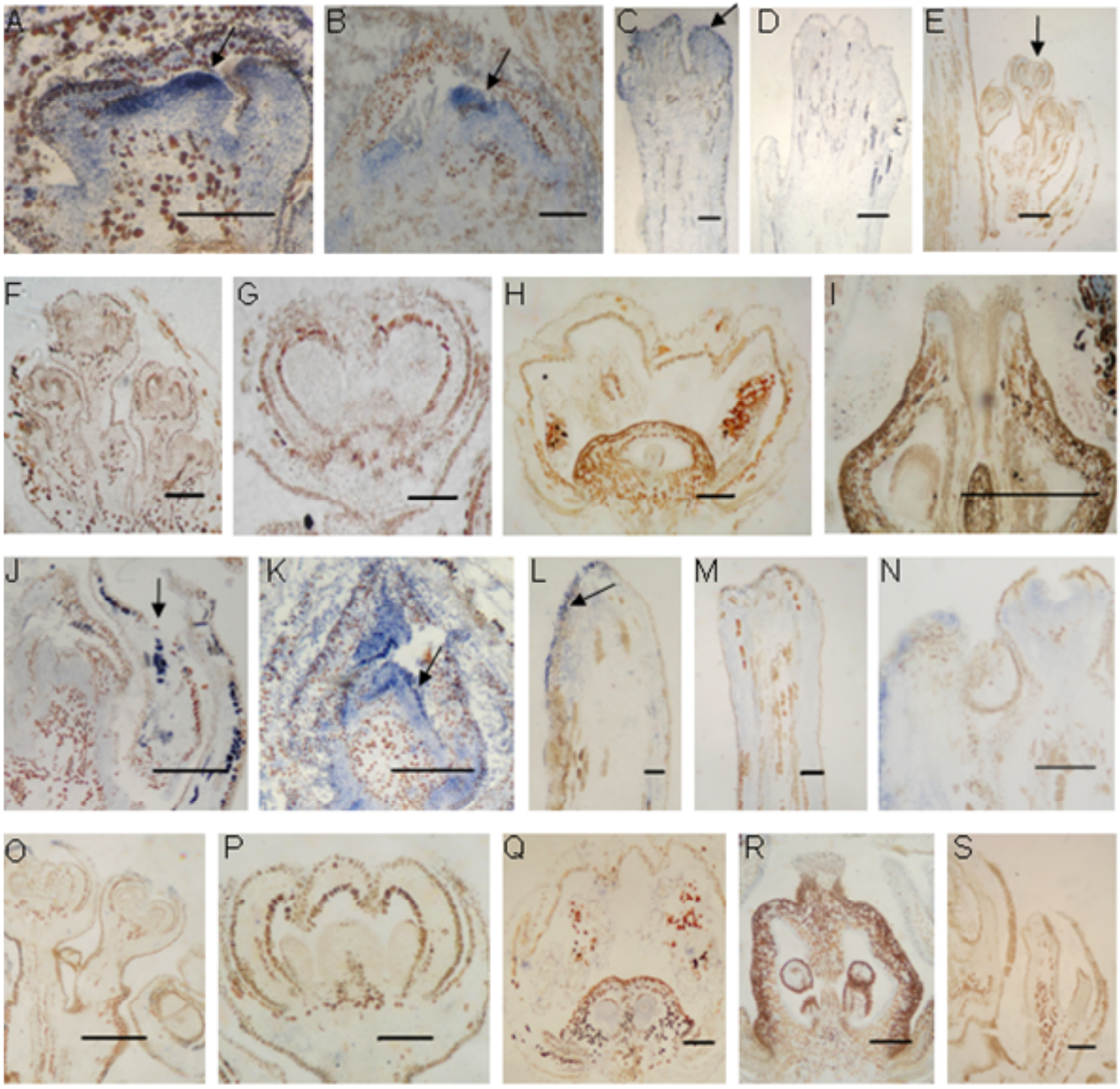

Figure 5. *In situ* expression pattern of the *VvTFL1* gene. A - I shows the *VvTFL1* gene expression in different organs under normal developmental conditions; J - R is the *VvTFL1* gene expression in different organs after GA treatment. (A) apical bud; (B) lateral bud; (C) small tendril; (D) big tendril; (E) tendril with floret; (F - G) small inflorescence; (H) middle inflorescence; (I) big inflorescence; (J) apical bud after GA treatment; (K) lateral bud after GA treatment; (L) small tendril after GA treatment; (M) big tendril after GA treatment; (N) tendril with floret after GA treatment; (O - P) small inflorescence after GA treatment; (Q) middle inflorescence after GA treatment; (R) big inflorescence after GA treatment; (S) control of *in situ* hybridisation. The scale is 20 µm.

al., 2004). In our study, we found that the *VFL* gene has an important role in inflorescence and floral development, but the *VvTFL1* gene only affects inflorescence meristem differentiation in apical buds and does not affect inflorescence and floral development. These results suggest that the *VFL* gene and the *VvTFL1* gene might have different functions in the inflorescence differentiation and floral development of grapevine.

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