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VviFSK and *VviFTK*, Two Novel Genes Encoding Putative Non-RD Receptor Kinases Associated with Reproductive Development in Grapevine

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Abstract
Although several genes homologous to those involved in the modulation of reproductive development in the model plant *Arabidopsis thaliana* have been identified in the *Vitis vinifera* genome, the regulatory network associated with pollen development, pollen tube elongation, and fecundation in grapevine is largely unknown. In *Arabidopsis*, receptor kinases play essential roles in pollen tube growth and guidance, leading to proper fertilization and fruit initiation. Comparing the transcriptomic profiles of flowers and early developing berries, two grapevine genes encoding proteins with structural domains corresponding to non-RD receptor kinases were identified. The first of them, *VviFTK* (*Vitis vinifera Flower and Tendril Kinase*), is transcribed in flowers at pre-anthesis and in tendrils and shares high sequence homology with *At3g03770* gene from *Arabidopsis* which encodes a putative phloem-specific receptor kinase of unknown function. The second gene, *VviFSK* (*Vitis vinifera Flower and Seed Kinase*), is mainly expressed in flowers at anthesis stage and in immature seeds and codes for a protein with high similarity to ScORK17, a receptor kinase involved in the ovule and seed development regulation in *Solanum chacoense*. *VviFSK* shows different expression patterns in two cultivars with opposite tendency to parthenocarpic fruit development (PFD) and its transcription is induced in response to exogenously added sucrose. In concordance with the expression mode of *VviFSK*, the in silico analysis of its promoter region indicates the presence of *cis* regulatory sequences recognized by floral homeotic transcription factors as well as elements associated with seed-specific expression and sugar induction of gene transcription. These results suggest that VviFSK protein plays a role in the seed development process in grapevine.

Keywords Atypical kinases · Reproductive development · Parthenocarpy · Grapevine

Introduction

As in others woody perennial crops, grapevine (*Vitis vinifera* L.) sexual reproduction occurs through a peculiar process which takes place over two growing seasons interrupted by a winter dormancy period. In the first season and after induction and differentiation, inflorescence and tendril *primordia* are developed within the latent buds. After dormancy, bud burst begins the second growing season and the induction of floral homeotic organ identity genes triggers the floral organogenesis together with male and female gametophyte development. Upon pollination and fertilization, fruit set takes place and berries develop following a double sigmoid pattern composed of two fast growing phases (Phase I and Phase III) separated by a slow or arrested growth period (Phase II) (Mullins et al. 1992; Carmona et al. 2008; Keller 2010). Pollination promotes an extensive change in phytohormones content by modifying the expression level of hormone biosynthesis-associated genes, leading to an increase in both gibberellin and auxin content, which precedes fruit set and berry initiation 5–7 days after anthesis (Kühn and Arce-Johnson 2012). On the other hand, double fertilization, occurring 2–3 days after anthesis, triggers embryo and endosperm development leading to seed formation along with Phase I (Mullins et al. 1992; Keller 2010).

Some *Vitis vinifera* cultivars (i.e., Carménère, Malbec and Merlot) exhibit a high tendency to develop parthenocarpic

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fruits, yielding seeded and seedless berries in the same cluster (Alva et al. 2015). Such phenomenon has been associated with pollination without effective fertilization event (parthenocarpy) (Mullins et al. 1992). Modifications in polyamine metabolism (Colin et al. 2002), deficiency in essential micronutrients as boron (B) and zinc (Zn) (Gartel 1993; Keller 2005; Vasconcelos et al. 2009), and the application of exogenous gibberellins and auxins (Weaver et al. 1962) have been suggested as causes for PFD. As a rule, PFD appears enhanced under conditions reducing pollen germination potential and sperm cells delivery to the ovules. Supporting this assumption, the development of morphologically abnormal pollen with reduced germination capability shows a straight correlation with a significant increase in PFD (Alva et al. 2015). In the model plant *Arabidopsis thaliana*, MADS box type transcription factors play a central role in the regulation of both male and female gametophyte development (Yan et al. 2016). Anther and pollen development start with the activation of the *NZZ/SPL* (*NOZZLE/SPOROCYTELESS*) gene by the MAD-box transcription factor AGAMOUS (Ito et al. 2004), inducing a regulatory network which appears to be conserved among several cultivated plants analyzed so far (Wilson and Zhang 2009; Fernandez et al. 2015). A relevant role in this regulatory network is played by receptor-like kinases (RLKs), which control the early steps in anther development (Wilson and Zhang 2009). Downstream NOZZLE/SPL, the Leucine-rich repeat RLK (LRR-RLK) EXS/ENS1 (EXCESS SPOROGENOUS CELLS/EXCESS MICROSPOROCYTES 1) together with the SERK1 and SERK2 (LRR-RLKs SOMATIC EMBRYOGENESIS RECEPTOR–LIKE KINASES 1 and 2) controls archesporial cells differentiation in the anther primordium (Canales et al. 2002; Albrecht et al. 2005; Colcombert et al. 2005) while the LRR-RLKs BAM1 and BAM2 (BARELY ANY MERISTEM1 and 2) are negative regulators of sporogenous cell number (DeYoung et al. 2006; Hord et al. 2006). In addition to pollen development, RLKs also play essential roles in ovule fertilization. After pollination, compatible pollen tube bursts in the receptive synergids releasing the sperm cells to accomplish double fertilization. Pollen tube guidance and reception, as well as the intercellular interaction between the pollen tube and female tissues, involve the participation of different RLKs which could be localized either on the pollen tube or on the ovule (reviewed by Kanaoka and Higashiyama 2015; Li and Yang 2016; Muschietti and Wengier 2018). As an example, the RLK FER (FERONIA), which regulates pollen tube reception by the ovule, is localized on the synergid cells. On the other hand, ANX1 and ANX2 (ANXUR1 and ANXUR2) are localized on the pollen tube-growing tip controlling its rupture (Escobar-Restrepo et al. 2007; Miyazaki et al. 2009; Franklin-Tong 2010; Dresselhaus and Franklin-Tong 2013; Li et al. 2016). Ligands of RLKs play essential roles either for pollen tube guidance as to assure specificity in pollen–ovule interaction. Small peptides named as LUREs are secreted diffusing in the path of the pollen tube and acting as attractants (Takeuchi and Higashiyama 2016). Specific receptors in the pollen tube were reported for LURE peptides sensing. A three LRR receptor-like kinases receptor heteromer, MDIS1 (MALE DISCOVERER1)- MIK1-2 (MDIS1-INTERACTING RECEPTOR-LIKE KINASE1-2), has been described as receptor of LURE1, with defects in the guidance of the pollen tube in the mutants *mdis1*, *mik1-2* and a demonstrated direct binding of LURE1 with MDIS1, MIK1-2 (Wang et al. 2016). In a similar way, PRK6 (POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 6), another RLK, also recognizes and binds LURE1, relaying the LURE signal to the pollen tube cytoplasm. Experimental data suggest its participation in a cell-to-cell recognition event conferring specificity to pollen–ovule interaction (Takeuchi and Higashiyama 2016). Recently, the expression profiles for 492 RLK-encoding genes from *Arabidopsis* have been established and a pollen-induced group as well as a stigma-induced group has been determined. With these data, a predicted pollen RLKs interactome has been proposed, generating a complex and yet not well-defined network associated with the regulation of the fertilization event in *Arabidopsis* (Muschietti and Wengier 2018).

Searching for RLK-encoding genes involved in the regulation of fruit initiation and seed development in grapevine, ESTs libraries from different reproductive organs and tissues were screened (Peña-Cortés et al. 2005). Because of its homology to a putative A. thaliana LRR-RLK coding gene, an EST (VVCCGS2117F10.b) identified in a cDNA library obtained from grapevine fruits at growing phase was selected for further analysis. Two EST-homologous genes were identified in the grapevine genome. In this work, the structural analysis of both genes and their encoded proteins corresponding to putative non-RD receptor kinases, as well as their gene expression pattern throughout reproductive developmental stages and in response to different elicitor molecules like sucrose and hormones, is described.

Materials and Methods

Plant Material

Grapevine plants (*Vitis vinifera* L. vars. Carménère and Cabernet Sauvignon) grown under field conditions in a vineyard located at the Estación Experimental Panguilemo, Universidad de Talca (Maule Valley, Central Chile, 35°22.2′ S, 71°35.39′ W, 121 m.a.s.l.) were used in this study. The region is characterized by Mediterranean climatic conditions with approximately 1800 growing degree days (Winkler III) with a dry season of 6 months and an average rainfall of

550 mm concentrated during the winter period. The vineyard soil has a clay loam texture and a slope of about 1%. The vines were planted in 1998 with a spacing of 1.5 m × 3.0 m (2222 vines/ha) on their own roots and were trained to a vertical shoot positioned system (VSP) with East–West oriented rows and flood-irrigated. Nutritional status of plant leaves was monitored and corrected by foliar spray applications of Zn (45–55 ppm) and B (55–75 ppm) to maintain sufficiency conditions of these critical micronutrients.

Random sampling of different organs was performed starting at early flowering until mature fruit stage (from October to April) from plants grown in the same plot. Stages to be sampled were defined according to the modified Eichhorn-Lorenz system (Coombe 1995). Flowering stages collected were as follows: EL19, inflorescences or little clusters (lc); EL21, pre-anthesis flowers (7F); and EL 23, flowers at full bloom (F). Fruit developmental stages were as follows: EL31, berries at pre-veraison 7 mm in diameter (pvB); EL35, berries at veraison (vB); EL36, berries at post-veraison (Bpv); and EL38, mature berries (M). For sampling, phenological stages were determined for normal seeded berries, and then clusters were collected and seeded and non-seeded berries from the same bunch were separated for further processing. Unless something different is said, whole berries were used. Pollen was obtained from flowers at full bloom stage. Seeds, skin, and pericarp, as well as roots, leaves, and tendrils, were collected from plants at the pre-veraison fruit-growing stage.

Gene Isolation

The Carménère *VviFSK* and *VviFTK* were isolated from a flower full-length cDNA library. PCR primers for ORF isolation were designed from the respective gene sequences identified in the grapevine GENOSCOPE database (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis). Oligonucleotide sequences were as follows: VviFSKfull_Fw, 5′-ATGGGGACACAAATCCTTCCT-3´; VviFSKfull_Rv, 5′-TCATTGTGAAGACAAGTC AGGT-3´; VviFTKfull_Fw, 5′-ATGGCAAAAGGATTCTGCCATT-3´; VviFTKfull_Rv, 5′-TTA ATGAATGTTGAGACGTAGGC-3´.

In Silico Structural Analysis of VviFSK and VviFTK Proteins

The encoded protein sequences were determined by in silico translation with Traslate tool from the Expasy Bioinformatics Resource Portal (Gasteiger et al. 2003). Searching for conserved protein domains was performed by using InterProScan (Hunter et al. 2009), SMART (Schultz et al. 1998), and Scansite 2.0 (Obenauer et al. 2003) algorithms. Transmembrane domains in the encoded proteins were identified by Phobius (Käll et al. 2004) and TMHMM (Krogh et al. 2001) software while LRRFinder (Gong et al. 2010) was used to identify leucine-rich repeats (LRR) motifs. The presence of signal peptides was determined by means of SignalP 3.0 (Bendtsen et al. 2004), while subcellular protein targeting was assessed by SherLoc (Shatkay et al. 2007), CELLO v.2.5: subCELlular LOcalization predictor (Yu et al. 2006) and Wolf PSORT II (Horton et al. 2007) algorithms.

Phylogenetic Analysis and Subdomain Determination of the Catalytic Regions

Protein sequences related to VviFSK and VviFTK were obtained by screening the GENBANK database (http://www.ncbi.nlm.nih.gov/BLAST), with the BLASTp algorithm. Multiple sequence alignment was performed with ClustalX (Thompson et al. 1997) using a full-length sequence of proteins selected by the role described in the literature and with a minimal identity of 55% in a BLAST search (Table S1). The phylogenetic tree was built according to the neighbor-joining method (Saitou and Nei 1987) by using the MEGA 6.0 software (Tamura et al. 2007). The subdomains in the catalytic kinase domain of RLKs were determined according to the definitions by Hanks and Hunter (1995), using multiple alignments and examining the secondary structure with the MINNOU online tool (http://minnou.cchmc.org/). Alignments were manually adjusted to ensure that conserved kinase motifs were accurately aligned. Each protein was verified on the presence or absence of the conserved residues typically required for kinase catalytic activity (Fig. 2). Proteins containing an R and a D in subdomain VIb were classified as RD kinases, and proteins lacking those residues were classified as non-RD kinases.

Promoter Region Analysis

cis regulatory elements in the 5´upstream region were identified by sequence analysis with PLACE (www.dna.affrc.go.jp/htdocs/PLACE/; Higo et al. 1999) and CONSITE (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite; Sandelin et al. 2004) tools.

In Vitro Elicitor Assay

In order to determine whether *VviFSK* and *VviFTK* expressions are affected by some common hormones and elicitors, *V. vinifera* L. cv. Carménère grape clusters were harvested from the above mentioned vineyard at the pre-veraison stage (EL31, pvB). Uniform berries with their respective peduncles were excised under water and were positioned on perforated plastic trays (Kim trak 25 × 14 cm) so that the cut pedicels protruded through the holes into a dish containing the proper solution. The experimental conditions were as follows: temperature at 25 °C and light at 156 Wcm-2. The

dips solutions used throughout the experiment were as follows: 500 µM gibberellin; 200 µM abscisic acid; 500 µM naphthaleneacetic acid and 0.1 M sucrose; using as a control distilled water and Silwet L77 (Sigma-Aldrich, USA) as surfactant. The experiment included three trays per treatment and was repeated twice. Eight random berries from each tray were collected at 6 and 24 h, pooled together, and processed for gene expression analysis.

Gene Expression Analysis

For expression analyses, four randomly chosen clusters from different plants were independently processed for RNA isolation (biological replicates). Total RNA was extracted from 2 to 3 g of frozen material at the stages defined in Plant Material, using the modified CTAB method (Reid et al. 2006). Three independent extractions were made from each sample and RNA integrity analysis and quantification were carried out by using Agilent RNA 6000 Nano Kit for the Agilent 2100 Bioanalyzer System. Following DNase (DNAse I, Ambion) treatment of total RNA, first-strand cDNA synthesis was carried out from 2 µg of total RNA for each sample using oligo (dT) according to the manufacturer's instructions (Affinity Script QPCR cDNA Synthesis Kit, Stratagene, La Jolla, CA). Quantification of transcripts by real-time quantitative reverse transcription–PCR (qRT–PCR) was performed as described previously by Almada et al. (2009). Expression was normalized to the *V. vinifera* glyceraldehyde phosphate dehydrogenase (GAPDH) gene (VvGAPDH; GenBank database accession CN938023) and ubiquitin gene (VvUBQ, TIGR database accession TC32075). Specific primers were designed for each gene with the software PrimerQuest from Integrated DNA Technologies, Inc. (https://www.idtdna.com/Primerquest/Home/Index). Oligonucleotide sequences were as follows: qRTFSK_Fw, 5′-TGCACAATGAACCAAGTGAGAGGC-3′; qRTFSK_Rv, 5′-CTACAGG CTCCTAGGATTACAC-3´; qRTFTK_Fw, 5′-ATCAGATGCCTGCATAAGGACCCA-3´; qRTFTK_Rv, 5′-TGTGGAAGCAGCAAACCTGA-3´; VvGAPDHFwd, 5′-TTCCGTGTTCCTACTGTTG-3′; VvGAPDH Rev, 5′-CTCTGACTCCTCCTTGAT-3′; VvUBQFwd, 5´- GTGGTATTATTGAGCCATCCTT-3´; VvUBQRev, 5´- AACCTCCAATCCAGTCATCTA-3´

Statistical Analysis

Analysis was carried out with R version 3.1.3 (Team RC 2014). The statistical comparison included analysis of variance, standard error, and correlation analysis. Data were compared by Student's t-test. Least significant difference was calculated at $p < 0.05$.

Results

The Grapevine *VviFSK* and *VviFTK* Genes Encode Putative Receptor-Like Kinases

In previous work, a grapevine EST database was obtained by massive sequencing of cDNA libraries from flowers and berries at different developmental stages and bioinformatics analysis and comparison with public databases to assign gene ontologies (Peña-Cortés et al. 2005). A search for putative V. vinifera RLK-encoding genes was carried out. As a result, an EST in RNA samples from small berries at growing phase I (VVCCGS2117F10.b. Fig. S1) was identified. This was then selected for further studies, based on its homology to At3g03770 from A. thaliana, a gene coding for a protein similar to a putative LRR-RLK involved in anther cell differentiation (Wijeratne et al. 2007).

First, *VviFSK* and *VviFTK* were identified by homology search (BLAST) with the EST sequence against the grapevine genome database (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Two homologous putative genes were identified in such analysis. *VviFSK* (LOC100266233), is located on chromosome 13 and is composed of 7 exons with a total size of 5108 bp. The ORF (2346 pb) codes for a 781 aa protein with a calculated molecular mass of 86.2 kDa. *VviFTK* (LOC100243272) is located on chromosome 8, which has 7 exons and 5189 bp in size, comprising an ORF of 2361 bp which codes for a 786 aa protein with a molecular mass of 86.41 kDa (Fig. 1a). In order to identify conserved structural domains, both deduced protein sequences were analyzed in silico. The LRR Finder software revealed the presence of a structural motif composed of 10 leucine-rich repeats at their respective N-terminus moiety (residues 112 to 345 in VviFSK; 107 to 340 in VviFTK). Additionally, **SignalP 3.0** software identified a putative signal peptide (residues 1 a 29 in VviFSK, 1 to 25 in VviFTK) and analysis with **SherLoc**, **CELLO** y **Wolf PSORT II** software predicts a cell membrane localization for both proteins (Table S2). A transmembrane domain was detected in both proteins by **Phobius** and **TMHMM** software (residues 397 tol 419 in VviFSK; 390 to 416 in VviFTK). The protein sequence analysis by using **InterProScan**, **SMART**, and **Scansite 2.0** software recognizes a Ser/Threo kinase-conserved domain at the C-terminus moieties of both proteins (residues 484 to 755 in VviFSK; 477 to 753 in VviFTK), both subdivided in the typical 12 subdomain arrangement (Fig. 2). According to the prediction tool NetPhos 3.1 (Blom et al. 1999) applied at 90% stringency, 7 and 11 phosphorylable Ser and Threo residues were found in VviFSK and VviFTK kinase domain, respectively (Fig. 2).

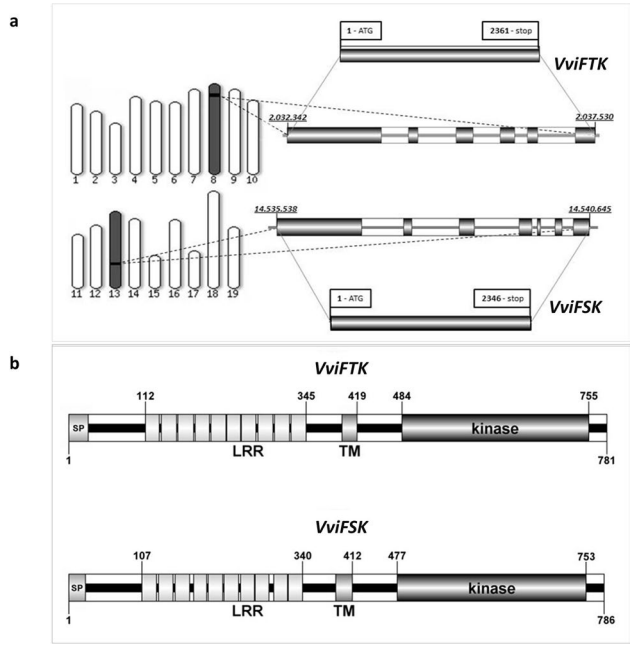

Fig. 1 Schematic representation of *VviFSK* and *VviFTK* genes and their respective encoded proteins. **a** Gene localization on *Vitis vinifera* cv. Pinot Noir karyotype. Position on chromosomes is indicated. The gene exon (in gray)/intron structure is depicted. The ORF size of each gene is also shown. **b** Protein domains and motifs on *VviFSK* and *VviFTK* encoded proteins. *SP* signal peptide, *LRR* leucine-rich repeat domain, *TM* transmembrane domain. Numbers indicate amino acid sequence position

Taken together, the in silico analysis suggests that proteins codified by both genes have structural features similar to receptor kinases (Fig. 1b) but the absence of the conserved arginine and aspartic acid in kinase subdomain VIb classifies these proteins as defective non-RD kinases (a sequence comparison with other atypical RLKs described in plants is shown in fig. S2).

Phylogenetic Relationship Between VviFSK, VviFTK, and Other RLKs

A phylogenetic tree was obtained compiling deduced VviFSK and VviFTK proteins with other putative receptor kinase sequences available in Genbank (Fig. 3). Since the genes coding these proteins were identified in reproductive tissues and their expression was manifested in flowers and fruit, the tree was made using proteins that are putative homologues and other ones, which are described to participate in development processes and resulted in separated groups in the tree. VviFSK shares 60.6% identity with VviFTK, 57% identity with ScORK17, and 55% identity with the protein coded by *At3g03770*, while VviFTK has identity values of 55.8% with ScORK17 and 65.1% with At3g03770. These results are in agreement with the phylogenetic analysis, where all these proteins were grouped in a common major clade. However, while VviFSK and ScORK17 receptor kinase from *Solanum chacoense,* its closest homologue, were classified in subclade "b" and "a," respectively, VviFTK and the putative receptor kinase encoded by the gene *At3g03770* from *Arabidopsis* were both grouped in subclade "c." This analysis also shows three major clades. The number one is the clade of VviFSK and its putative homologues, the number two contains PRK3-6 and other proteins involved in reproductive development, and the number three is a clade that contains FER (Fig. 3).

VviFSK and *VviFTK* Genes have Different Expression Patterns During Grapevine Reproductive Development

The transcriptional profile of *VviFSK* and *VviFTK* genes was analyzed by qRT-PCR in different organs and tissues from *V. vinifera* cv. Carménère. Since the expression of *VviFSK* gene was higher than *VviFTK* in all analyzed samples (Table S3), the respective gene expression in roots was arbitrarily assigned as 1 relative expression unit for comparing the transcriptional induction levels along reproductive development (Fig. 4). Both genes show a clear increase in their transcription activity in leaves; however, while *VviFTK* expression is strongly induced in tendrils (37.99-fold), little clusters (18.27-fold), and flowers at pre-anthesis (42.53-fold), *VviFSK* expression is enhanced at anthesis (anther and pollen-depleted flowers, 14.76-fold) and in developing seeds from berries at pre-veraison stage (49.67-fold), without detectable induction in unseeded berries and in berry skin or pericarp from normal berries. Taking these results into account, it appears that *VviFTK* and *VviFSK* are not redundant genes as they are expressed at different developmental stages and organs, being *VviFSK* more associated with grapevine seed development and, therefore, we focused especially on this gene for the next analyses.

VviFSK Shows Different Expression Pattern in Cultivars with Opposite Tendency to Abscission and PFD

To further explore about the relation between *VviFSK* expression and seed formation, the transcriptional pattern of this gene was also analyzed at reproductive developmental stages in a low tendency to abscission and PFD grapevine cultivar as Cabernet Sauvignon and compared to the expression profile determined in the same Carménère phenological stages (Fig. 5). Abscission rates (estimated as the ratio between total grapes per cluster at veraison stage against total flowers per cluster at full bloom) and PFD rates (calculated as the ratio between mid-size seedless berries against total grapes in the same bunch) for the vines used in this

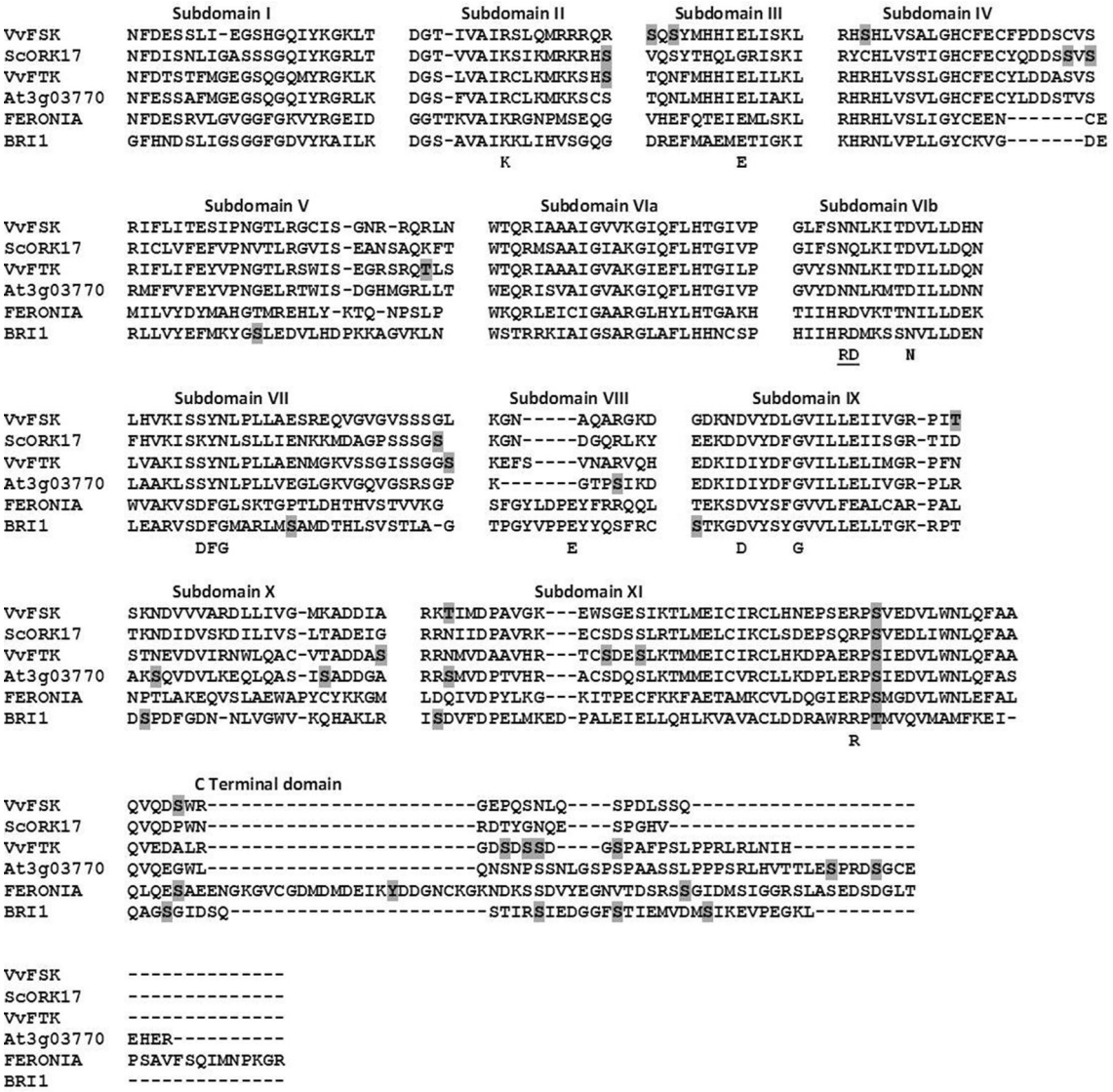

Fig. 2 Sequence comparison of kinase subdomains of VviFSK, VviFTK, and their homologues At3g03770 and ScORK17. Multiple alignment was performed with ClustalW. FER and BRI1, two autophosphorylable RLKs, were used as a reference. The conserved residues reported as essential in active kinases are shown at the bottom. The RD motif is underlined. In gray, the predicted phosphorylation sites (NetPhos 3.1, 95% stringency)

analysis have been already determined. Rates of 41.3% and 57.8% were established for Cabernet Sauvignon and Carménère cultivars, respectively, while PFD rates were 7.8% for Cabernet Sauvignon and 31.2% for Carménère (Alva et al. 2015).

For comparison, relative expression in Carménère little clusters was arbitrarily assigned as 1 unit. Significant differences were established between both cultivars, being *VviFSK* relative expression much higher either in developing flowers as well as developing berries from Cabernet Sauvignon. While in Carmenère maximum expression was observed in flowers at anthesis, in Cabernet Sauvignon, the highest expression level was in pre-veraison berries where seed development occurs. Furthermore, in this later cultivar, the transcriptional activity remains high in fruits at veraison and post-veraison stages where seed maturation takes place. The above results are in agreement with a role for the VviFSK protein in grapevine seed development.

VviFSK and *VviFTK* Expressions in Berries at Veraison are Induced by Exogenously Added Sucrose

The ability of *VviFSK* and *VviFTK* promoters for driving gene expression in response to hormones and signaling molecules associated with the fruit initiation event in grapevine was analyzed in isolated fruits. Because

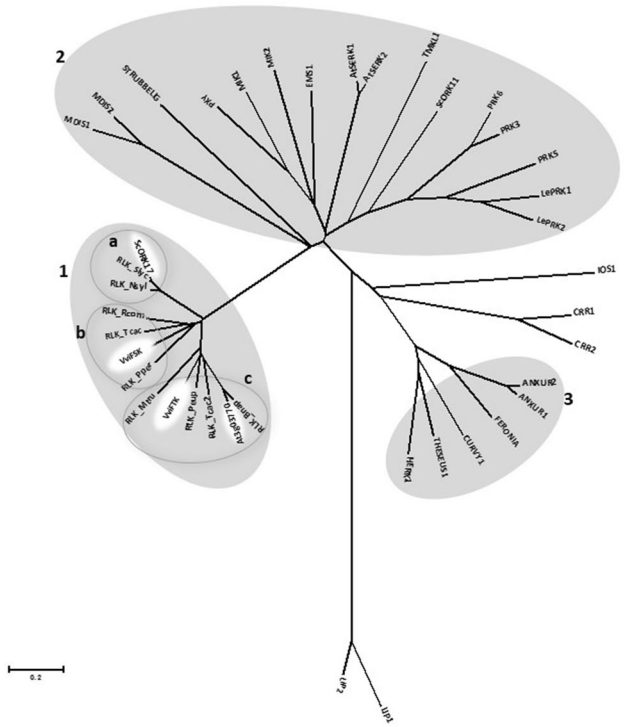

Fig. 3 Phylogenetic relationship of VviFSK and VviFTK with RLKs from other plant species. The length of the branches is proportional to phylogenetic distances. The protein sequences other than VviFSK and VviFTK were obtained from Genbank. Main clades are in gray. Subclades a, b, and c are circled. Slyc, *Solanum lycopersicum*; Nsyl, *Nicotiana sylvestris*; Rcom, *Ricinus communis*; Tcac, *Theobroma cacao*; Pper, *Prunus persica*; Mtru, *Medicago truncatula*; Peup, *Populus euphratica*; Bnap, *Brassica napa*

of their low expression level, berries at veraison stage from the Carménère cultivar were selected for this study and exposed to ABA (200 µM), GA (500 µM), NAA (500 µM), and sucrose (0.1 M) treatments. To reproduce *in planta* situation, exogenous compounds were added by generating a capillary flux to the sink tissues through the berry peduncle and quantification of *VviFSK* and *VviFTK* was performed after 6 and 24 h of treatment (see "Materials and Methods" section). Results are shown in Fig. 6. Compared to their respective controls, no significant difference in gene expression was detected in ABA, GA, or NAA-treated berries after 6 h of exposure and a moderate upregulation (2- to 2.5-fold) was determined only after 24 h of treatment. On the other hand, a significant transcriptional activation of *VviFSK* and *VviFTK* was observed in response to sucrose treatment ranging from 1.85- to 9.46-fold induction after 6 and 24 h of treatment, respectively. These results suggest that sugar signaling pathway could be relevant regulation of *VviFSK* expression.

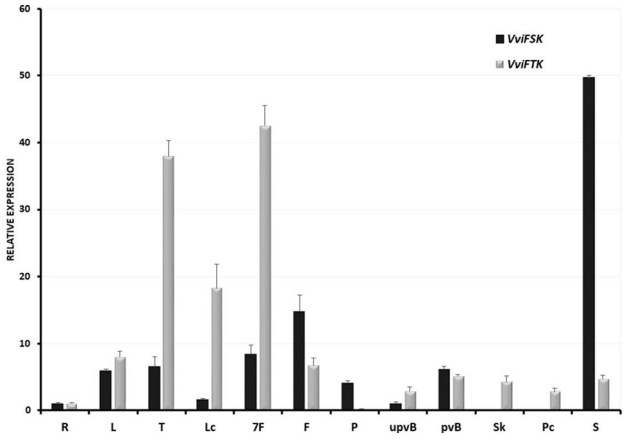

Fig. 4 Gene expression analysis of *VviFSK* and *VviFTK* in different Carménère grapevine tissues and developmental stages. Relative expression was determined in *R* roots at pre-veraison; *L* leaves at pre-veraison; *T* tendrils at pre-veraison; *Lc* little clusters (14 day before anthesis, DBA); *7F* flowers 7 DBA; *F* anther and pollen-depleted flowers at full bloom (anthesis); *P* pollen (at anthesis); *upvB* unseeded pre-veraison berries; *pvB* pre-veraison berries; *Sk* skin from pre-veraison berries; *Pc* pericarp from pre-veraison berries; *S* seed from pre-veraison berries. Unless a particular tissue is mentioned, whole berries were used. For qPCR analyses, *VviFSK* and *VviFTK* expression was normalized against the expression level of *VviUBQ* and *VviGAPDH*. Expression of each gene in root samples was adjusted to 1 relative unit. Error bars represent means ± SD ($n = 3$)

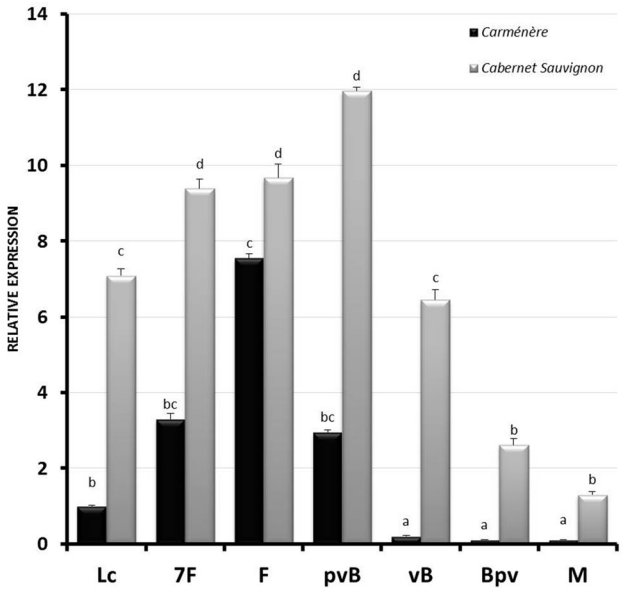

Fig. 5 *VviFSK* gene expression in Carménère and Cabernet Sauvignon grapevine cultivars. Lc: little clusters 14 DBA; *7F* flowers 7 DBA; *F* anther and pollen-depleted flowers at full bloom (anthesis); *pvB* pre-veraison brerries; *vB* veraison berries; *Bpv* berries at post-veraison; *M* mature berries. qPCR analysis of *VviFSK* expression was normalized against the expression level of *VviUBQ* and *VvGAPDH*. Expression in Lc of Carménère was adjusted to 1 relative unit. Error bars represent means ± SD ($n = 3$)

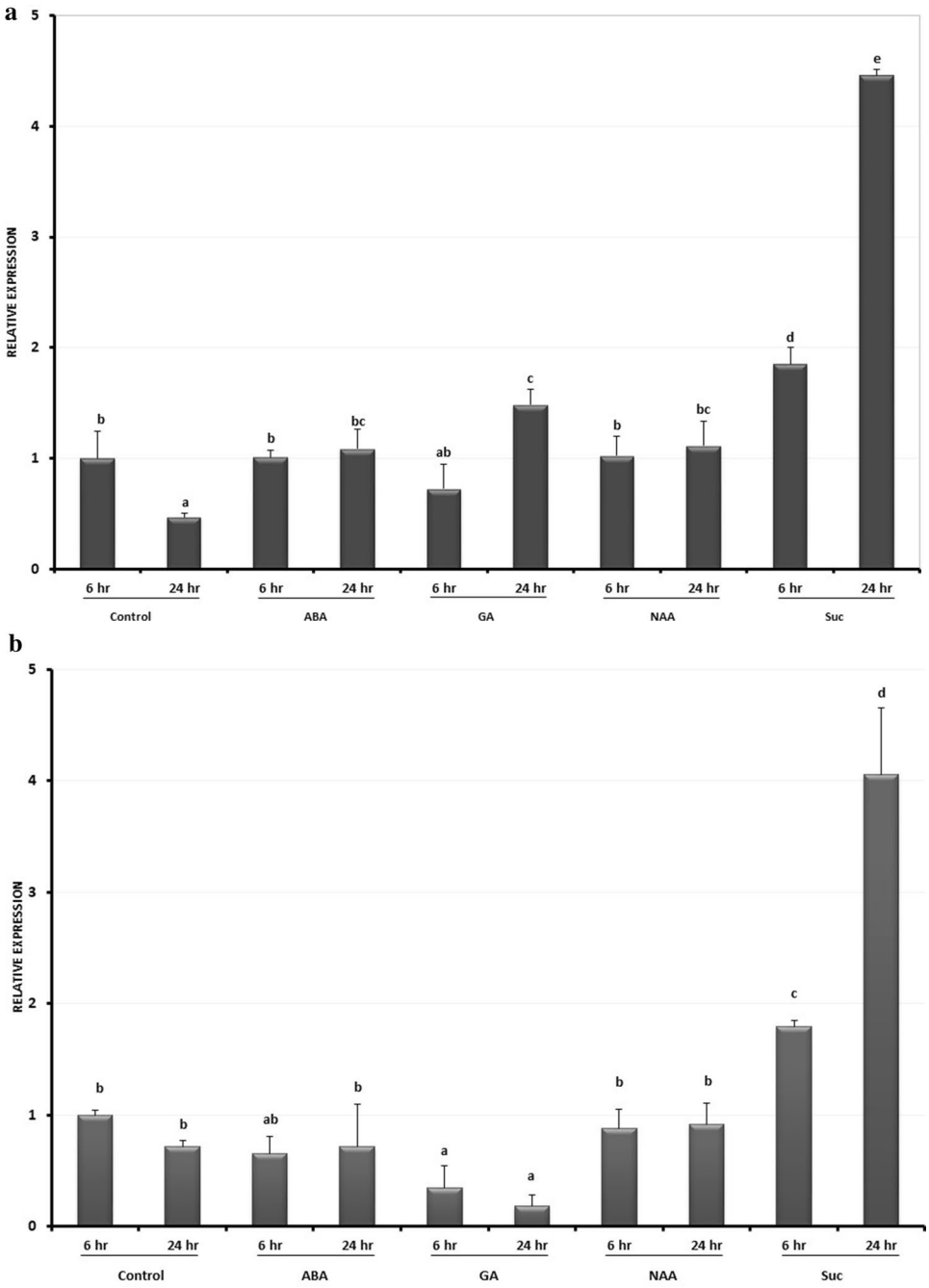

Fig. 6 *VviFSK* (**a**) and *VviFTK* (**b**) response to treatment with hormones and elicitors. Expression analysis in veraison berries after 6 h and 24 h of treatment with 200 µM ABA; 500 µM GA; 500 µM NAA; and 0.1 M sucrose (Suc). qPCR analysis of *VviFSK* expression was normalized against the expression level of *VviUBQ* and *VviGAPDH*. Expression in control (distilled water and the surfactant) at 6 h was adjusted to 1 relative unit. Error bars represent means ± SD ($n = 3$)

The *VviFSK* Promoter Contains Putative Cis-Elements in Agreement with its Expression Mode

In silico analysis of the promoter of *VviFSK* was made by CONSITE tool identifies the CArG BOX, a cis-element relative to TF of the MADS family (AG, SQUAMOSA y AGL3), associated with floral induction and morphogenesis (Fig. 7). The software PLACE identifies the following *cis*-elements associated with the regulation of the genetic expression by sugar, or sugar-responsive elements (SREs): the amylase and G boxes, the CMSRE-1 and the SP8 B elements, the pyrimidine box, SURE1, the SRE ATMSD element, and several copies of the W box, among other elements. PLACE also found elements driving specific expression in seeds, as the ACGT motif, the DPBF motif, a RY repeat, and the SEF3-4 motives. All the significant elements found by PLACE and their prevalence are shown in Table 1. The aforementioned elements are found 2000 bp upstream of the ATG start codon of the gene and could be involved in its transcriptional regulation. Additionally, these findings were compared with the promoter of the homologues genes *VviFTK* and *At3g03770*, where the

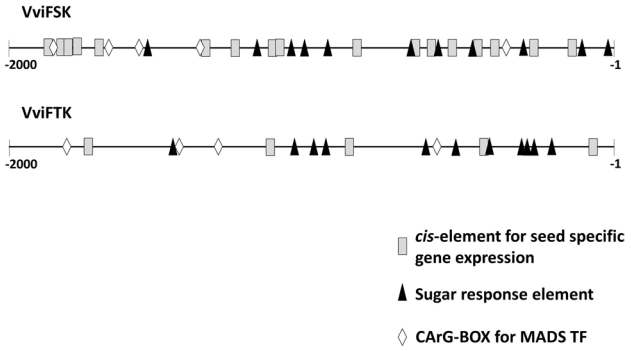

Fig. 7 Schematic representation of the promoter regions of *VviFSK* and *VviFTK* genes. 2000 bp upstream the ATG start codon was considered for in silico analysis using Consite and PLACE tools

Table 1 Sugar-responsive elements (SRE) and seed-specific elements in the promoters of *VviFSK*, *VviFTK*, and *At3g03770* obtained from an in silico analysis

	Sequence	*VviFSK*	*VviFTK*	*At3g03770*
SRE				
Amylase box 1	TAACARA	1	2	2
Amylase box 2	TATCCAT	2	0	0
CMSRE-1	TGGACGG	1	0	0
Pyrimidine box	CCTTTT	3	7	2
SRE ATMSD	TTATCC	3	1	0
S-box	CACCTCCA	1	0	0
SP8 B	TACTATT	2	1	0
SURE 1	AATAGAAAA	1	2	0
TATCCAC box	TATCCAC	1	1	0
WB box	TTTGACY	1	1	0
W box	TGAC	10	7	2
Seed-specific elements				
AACA motif	AACAAAC	2	2	4
ACGT motif	GTACGTG	3	0	1
DPBF	ACACNNG	2	0	1
RY repeat	CATGCA	5	1	2
SEF1 motif	ATATTTAWW	0	1	1
SEF3 motif	AACCCA	2	1	1
SEF4 motif	RTTTTTR	4	1	6

abovementioned binding sites were found, but less abundantly (Table 1).

Discussion

VviFSK and *VviFTK* Genes Encode Putative Non-RD RLKs

Proteins with receptor kinase activity are involved in several intracellular signaling systems relevant for developmental processes (Nodine et al. 2011). More than 600 genes encoding RLKs have been identified in the model plant *Arabidopsis* (Shiu et al. 2004), but only a small number of these have been fully characterized. In reproductive development, they have been associated with a number of signaling pathways including those regulating tapetum and pollen development (Albrecht et al. 2005; DeYoung et al. 2006); ovule development (Hecht et al. 2001; DeYoung et al. 2006; Germain et al. 2007, 2008); pollen–pistil interactions (Miyazaki et al. 2009; Li and Yang 2016; Li et al. 2016; Takeuchi and Higashiyama 2016; Wang et al. 2016) and embryo and seed development (Canales et al. 2002; Germain et al. 2008). In order to better understand the role of RLKs in seed development on grapevines, an EST collection from grapevine reproductive tissues (Peña-Cortés et al. 2005) was used to compare the transcriptomic profiles of normal seeded and parthenocarpic seedless berries. Because of its strong downregulation in seedless berries (91-fold), the EST VVCCGS2117F10.b, representing a gene coding for a protein similar to the putative receptor kinase encoded by the *At3g03770* gene from *Arabidopsis*, was selected for this study. BLAST analysis identified two genes in the grapevine genome, *VviFSK* and *VviFTK*. A bioinformatic analysis revealed that both encoded proteins share structural features with RLKs containing copies of leucine-rich repeat (LRR). In plants, the members of the LRR-RLKs family are located in the plasma membrane and have three main constituents: an extracellular domain containing between 1 and 32 tandem copies of LRR which allow this protein to form heterodimers with other similar proteins generating an active receptor (Li 2011); a transmembrane

domain and an intracellular kinase domain composed of 250–300 residues generally subdivided in 12 conserved subdomains (Hanks et al. 1988). The kinase function is generally controlled by phosphorylation or autophosphorylation of serine and threonine residues (Becraft 2002; Gish and Clark 2011). VviFSK and VviFTK share structural features with Ser/Thr kinases. Analysis with different software indicates that both RLKs have a signal peptide for plasma membrane localization at their N-terminus and they possess an extracellular domain composed of 10 LRR motifs, a transmembrane segment and an intracellular kinase domain containing the typical 12 subdomain arrangement at the C-terminus moiety. According to the prediction tool NetPhos 3.1 (Blom et al. 1999) applied at 90% stringency, 7 and 11 phosphorylable Ser and Thr residues in VviFSK and VviFTK were found, respectively (Fig. 2). However, some amino acids, which are normally present in the autophosphorylable FER and BRI1 RLKs and are reported as essential for catalytic activity (Castells and Casacuberta 2007), were substituted in subdomains II, VIb, VII, and VIII from both proteins (Fig. 2). Hence, they could correspond to defective kinases without autophosphorylation capacity or could be phosphorylated by another kinase. In the first case, these proteins could be involved with phosphorylation-independent signaling pathways, but further studies are required to obtain evidence. In this sense, change of a key arginine (R) and an aspartic acid (D) residue that facilitates phosphotransfer in subdomain VIb in both grapevine kinases is particularly interesting. This allows classify them into a group referred to as non-RD RLKs. These non-RD RLKs are often found in association with cell-to-cell communication, including plant–pathogen interactions (Dardick et al. 2012) and pollen–pistil interaction (Takeuchi and Higashiyama 2016; Wang et al. 2016).

Expression Profile Analysis Suggests that *VviFSK* is Associated with Grapevine Seed Development

As a first approach to determine the role of *VviFSK* and *VviFTK* encoded proteins in reproductive development, the phylogenetical relationships with other reported RLKs and the expression profiles of both genes were determined. When the amino acid sequences of VviFSK and VviFTK were compared with RLKs involved in the regulation of reproductive development through phylogenetical analysis and multiple sequence alignment, they do not appear closely related to RLKs involved in pollen–pistil interactions as ANX 1 and 2 (Miyazaki et al. 2009), FER/SIRENE (Escobar-Restrepo et al. 2007), or PRK6 (Takeuchi and Higashiyama 2016). Both grapevine RLKs are grouped in a clade with At3g03770-putative receptor kinase, a poorly characterized protein localized in the phloem of leaves of *Arabidopsis* (Ruiz-Medrano et al. 2011) and ScORK17 a receptor kinase involved in ovule and seed development in *S. chacoense* (Germain et al. 2008; Fig. 3). Based on the identity percentage, the main clade could be divided into three subclades, with VviFSK and ScORK17 (57% identity) in subclade "**a**" and "**b,**" respectively, while VviFTK and At3g03770 protein (65.1% identity) were together within subclade "**c.**"

Differences were also found in the expression mode of the genes encoding the respective grapevine RLKs. VviFTK is clearly induced at inflorescences reaching their maximum expression level in tendrils and flowers at pre-anthesis (7DBA) stage (Fig. 4). Because their common origin—the anlagen or uncommitted primordia—tendrils and inflorescences are considered homologous organs (Tucker and Hoefert 1968; Mullins et al. 1992; Boss and Thomas 2000). A global transcriptomic analysis indicates that even when both organs share a common transcriptional profile at early developmental stages, organ-specific gene expression programs are activated at later developmental stages, being most of the genes that codes for reproductive development-associated transcription factors specifically expressed in inflorescences (Díaz-Riquelme et al. 2014). However, a gene expression program associated with basal developmental processes is common to both organs even at later developmental stages. This group of genes includes the grapevine ortholog to *Arabidopsis* MADS box gene *APETALA1*, *VAP1*, which is strongly expressed in tendrils and inflorescences (Calonje et al. 2004; Zhang et al. 2015) and the gene *VvFT* which is involved in floral induction in latent buds during the first growing season and in developing inflorescences and flowers during the second growing season (Díaz-Riquelme et al. 2014). Since in *Arabidopsis* both genes are part of the photoperiod floral induction pathway, being *AP1* direct target for FT transcription factor (Wigge et al. 2005) is plausible that this induction pathway could be operative in both organs. In this sense, it is important to note that in silico analysis identified four cis-elements CArG box type (target sites for MADS box transcription factors) in the *VviFTK* promoter region, suggesting that this gene could be part of this regulatory network, acting downstream of *VAP1*.

On the other hand, the *VviFSK* gene is induced later than *VviFTK* in flower development with expression peaks in the female tissues of anther and pollen-depleted flowers at anthesis stage and, after pollination and fruit setting, in developing seeds of pre-veraison berries. As expected, no upregulation was observed in parthenocarpic berries where seed formation does not take place (Fig. 4). The expression mode agrees with *VviFSK* promoter structure determined by in silico analysis (Fig. 7). CArG boxes required for transcriptional regulation by floral homeotic MADS-type transcription factors as well as *cis*- elements associated with seed-specific expression were identified in the *VviFSK* upstream region suggesting that this gene could be directly regulated

by *VvMADS1*, the grapevine class C transcription factor homolog to AGAMOUS (Carmona et al. 2008).

In order to explore if *VviFSK* takes part in the grapevine regulatory network of reproductive development, two grapevine cultivars with contrasting tendency to develop fruitlet abscission and parthenocarpic fruit development (PFD) were selected. In a previous work, it was found that Cabernet Sauvignon shows low abscission and PFD rates, while the Carménère cultivar exhibits moderate to high tendency to abscission and high PFD rates. Malformations in pollen grains, which restrict the germination potential, seem to be responsible for these phenomena (Alva et al. 2015). When the expression level of *VviFSK* was compared between both cultivars, transcriptional activity of this gene was higher in Cabernet Sauvignon than in Carménère. In addition, in C. Sauvignon *VviFSK* transcription remained at significant levels along all berry developmental stages, including seed formation to seed maturation. On the other hand, in Carménère, *VviFSK* expression was detected until berries at pre-veraison stage, before seed formation (Fig. 5).

Thus, a negative correlation between *VviFSK* expression and PFD occurrence was established. Since this gene was still expressed in pollen-depleted flowers, a role for *VviFSK* protein in seed formation but not in pollen development is suggested. Differences between cultivars could be associated with VviFSK protein potentially playing a role in female floral organogenesis and, after pollination, in the regulation of seed development. Therefore, the absence of transcripts in the later developmental berry formation could lead to PFD. In fact, *VviFSK*'s expression mode is similar to the transcriptional profile of *At3g03770* (Fig. S3) and *ScORK17* from *S. chacoense*, its closer homolog. This later gene is specifically transcribed in the female reproductive tissues, being induced after pollination in ovaries and placenta with a maximum expression level four days after pollination, which correlates with the early steps of embryo development. It is proposed that this gene fulfils roles either in ovule development at pre-fertilization stages or in seed development (Germain et al. 2008). Similarly, the expression of genes coding for HD-Zip type transcription factors in *V. vinifera* was detected in ovules and developing embryos of two cultivars with different reproductive development: Thompson Seedless, a stenospermocarpic variety that do not develop seed endosperm, and Pinot Noir, a seeded variety. These differentially expressed genes have regulatory elements related to seed expression in their promoter region, as well as hormone-response elements. These genes are differentially expressed in both cultivars, with a higher expression in Thomson Seedles, where the ovule abortion takes place. That indicated they have a role in seed formation and suggesting a role for *VvHD-Zip* in the regulation of reproductive development in grapevine (Li et al. 2017).

To determine if repression of *VviFSK* transcription is a cause of PFD or a consequence of seed absence in parthenocarpic berries should be investigated in more detail in further studies.

Floral to fruit development transition after pollination is a crucial step in reproductive development. In grapevine, this process involves the downregulation of ABA-signaling and the onset of GA and auxin signaling pathways which regulates fruit setting and initiation through a cross-talk mechanism (Kühn and Arce-Johnson 2012; Jung et al. 2014). To determine if such hormones could be involved in the upregulation of *VviFSK* in developing seeds, their ability to modulate *VviFSK* transcriptional rate was analyzed by exposing isolated berries at veraison stage to solutions containing ABA (200 µM), GA (500 µM), NAA (500 µM), and sucrose (0.1 M). Only moderate upregulation after 24 h exposure (two–threefold) was observed under the assayed conditions, suggesting that mechanisms other than hormonal pathways should be also involved in regulating the expression of this gene at fruit setting and initiation stages. In this sense, it is important to note that sugar signaling could be also involved in the fruit setting regulation in grapevine. Decrease in photoassimilates and sucrose promotes fruitlet abscission, while induction of setting implies an increase in sucrose content (Aziz 2003). *VviFSK* transcription is strongly upregulated in response to sucrose (9.4-fold at 24 h exposure; Fig. 6) and several sugar-responsive *cis*-elements were found in the *VviFSK* promoter (Fig. 7). Since sucrose participates as mobile signal between source (i.e., leaves) and sink (i.e., flowers) organs in sugar signaling regulatory network (Li and Sheen 2016), it is tempting to speculate that this could be the mechanism for inducing *VviFSK* expression in response to photoassimilates availability in early fruit development. Additional studies should be done to elucidate this assumption.

In plant reproductive development, ovule fertilization is a strictly regulated key process that involves the participation of several RLKs (reviewed by Li and Yang 2016; Li et al. 2016). In this regard, it has been reported that events as pollen tube growth, pollen tube guidance to the embryo sac, pollen tube reception by the synergids, and sperm cells release for double fertilization implicate the participation of a specific group of peptides called LURE that act as signal molecules in this process, acting as ligands for specific RLKs located in the tip of the pollen tube (Kanaoka and Higashiyama 2015). Mechanisms similar to those described for the plant–microbe interaction and innate immunity, in which a subgroup of receptor kinases functions as "pattern recognition receptors" (PRRs; Dardick and Roland 2006; Dardick et al. 2012), are also regulating the pollen–pistil interaction (Kessler et al. 2010). Supporting this idea, in *Arabidopsis*, the species-specific pollen–ovule interaction is mediated by the RLKs PRK6 and MDIS1—localized

in the pollen tube—which recognizes and binds their specific LURE ligands in the ovule secretome (Takeuchi and Higashiyama 2016; Wang et al. 2016). Most of pattern recognition events so far described involve the participation of defective non-RD kinases (Dardick et al. 2012). Examining amino acid sequence of the kinase subdomain VIb of RLKs participating in reproductive development regulation, we observe that RLKs involved in determining specificity for pollen–ovule interaction as PRK3, PRK5 and PRK6 or MDIS1 and MDIS2, also correspond to non-RD RLKs (Fig. S2). Based on these antecedents, it is possible to speculate a role for VviFSK in a not yet described PRR-triggered mechanism implicated in the reproductive development in *V. vinifera*, leading to fruit and seed formation. Further studies should be conducted to provide experimental evidence to support this speculation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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