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RESEARCH PAPER

The MADS-box gene *Agamous-like 11* is essential for seed morphogenesis in grapevine

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Abstract

Despite the wide appreciation of seedless grapes, little is known about the molecular mechanisms that drive the stenospermocarpic seedless-type phenotype in grapevine. In order to address the molecular mechanisms that control seedlessness in grapevine, our study aimed to characterize *VviAGL11*, a class D MADS-box transcription factor gene that has been proposed as the major candidate gene involved in *Vitis vinifera* seed morphogenesis. *VviAGL11* allelic variations in seeded and seedless grapevine cultivars were determined, and its correlations with allele-specific steady-state mRNA levels were investigated. *VviAGL11* relative expression was significantly higher in seeds at 2, 4, and 6 weeks after fruit set, whereas in the seedless grape its transcript levels were extremely low in all stages analyzed. *In situ* hybridization revealed transcript accumulation specifically in the dual endotesta layer of the seeds, which is responsible for elongation and an increase of cell number, a necessary step to determine the lignification and the final seed size. No hybridization signals were visible in the seedless grapevine tissues, and a morphoanatomical analysis showed an apparent loss of identity of the endotesta layer of the seed traces. Ectopic expression of *VviAGL11* in the Arabidopsis *SEEDSTICK* mutant background restored the wild-type phenotype and confirmed the direct role of VviAGL11 in seed morphogenesis, suggesting that depletion of its expression is responsible for the erroneous development of a highly essential seed layer, therefore culminating in the typical apirenic phenotype.

Key words: Apireny, grapevine, *in situ* hybridization, seedlessness, Sultanine, *VviAGL11*.

Introduction

Seedlessness is one of the most appreciated features in table grapes (*Vitis vinifera*) along with enlarged fruit size, both being the main goals in breeding programs (Varoquaux *et al.*, 2000; Cabezas *et al.*, 2006). In the past two decades, the worldwide

market for *in natura* grapes increased ~26% per year, while the production of such grapes grew at a 13% annual rate (FAO, 2014; OIV, 2014). In grapevine, the absence of seeds, also called apireny, can occur by two distinct biological processes. One is known as parthenocarpy, when fecundation does not occur and there is no seed formation (used in production of raisins). The second process is called stenospermocarpy, in which fertilization takes place to form the fruit but is followed by embryo abortion due to the cessation of endosperm development, normally after 4 weeks of fruit growth. The consequence of stenospermocarpy is the production of berries with reduced seed size, known as seed traces (Bouquet and Danglot, 1996; Mejía *et al.*, 2011). The Sultanine cultivar is the main donor of the stenopermocarpy phenotype among breeding programs worldwide, and most of the commercial table grape varieties descend from this cultivar (Di Genova *et al.*, 2014).

In grapevine, several studies have shown that the presence of a dominant allele in the region of the *Seed Development Inhibitor* (*SdI*) locus, located at the distal portion of chromosome 18, has been responsible for 50–90% of the total phenotypic variance of seedlessness (Bouquet and Danglot, 1996; Cabezas *et al.*, 2006; Mejía *et al.*, 2007; Costantini *et al.*, 2008; Doligez *et al.*, 2013). *In silico* analysis allowed the identification of a MADS-box transcription factor gene *AGAMOUS-LIKE 11* (*VvAGL11*; Vv18s0041g01880; Boss *et al.*, 2002), renamed *Vv1AGL11* by Grimplet *et al.* (2014), that mapped to the same locus contig *SdI* (Costantini *et al.*, 2008). The same work also co-located *VviAGL11* with the molecular microsatellite marker VMC7F2, a highly polymorphic microsatellite marker capable of an efficiency of up to 95% in the detection of seedlessness in grapes (Costantini *et al.*, 2008).

The transcription factors of the MADS-box family have extremely important roles in the development of plants by controlling floral organ identity (Smaczniak *et al.*, 2012). In a study covering the MADS-box family in grapevine, it was demonstrated that *VviAGL11* is induced in floral and fruit tissues and it is repressed in roots, branches, leaves, buds, and tendrils (Díaz-Riquelme *et al.*, 2012). More recently another study showed that *VviAGL11* is 25 times more expressed in fruits at the pea size stage compared with flower stages, in seedless grapevines homozygous genotypes, whereas in heterozygous genotypes an intermediate level of *VviAGL11* expression was observed (Mejía *et al.*, 2011).

The *VviAGL11* gene was proposed, by our group and others, as a strong candidate gene involved in the absence of seeds in grapevine (Costantini *et al.*, 2008; Mejía *et al.*, 2011; Revers *et al.*, 2014; Ocarez and Mejía, 2016). This MADS-box gene was shown to be a possible ortholog of *AGL11* (*STK*) of *Arabidopsis thaliana*, which acts on the identity and control of ovule and seed development (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003; Díaz-Riquelme *et al.*, 2012). The *AtAGL11* mutant *SEEDSTICK* (*stk*) presents a reduced number and size of seeds (Pinyopich *et al.*, 2003; Brambilla *et al.*, 2008).

In this work, we identified several polymorphisms in the *VviAGL11* allele sequences in the apirenic genotype background including mutations in the intronic regions and a set of single nucleotide polymorphisms (SNPs) in the coding region of the gene. *VviAGL11* transcript levels were 65-fold higher in grapevine seeds compared with apirenic grapevine fruits. Spatial and temporal analyses of expression patterns in reproductive tissues demonstrate correlation between abnormal development of a specific seed layer and reduction of *VviAGL11* expression. Additionally, *VviAGL11* ectopic expression restored silique and seed development to the wild-type-like standards in the Arabidopsis *stk* mutant, confirming its essential role in seed morphogenesis.

Materials and methods

Plant material

Grapevine samples were harvested from cv. Chardonnay and cv. Sultanine plants located at Embrapa Grape and Wine experimental areas in Bento Gonçalves, Rio Grande do Sul, Brazil (29°09'48"S, 51°31'42"W, and 616 m altitude). The cultivar plants were at the reproductive stage (6–8 years old), planted on a pergola trellising system and managed with conventional annual pruning and control of pests and diseases. Both cultivars were grafted on Paulsen 1103 rootstock and the phenological scale was followed as described by Baggiolini (1952) (Supplementary Table 2 at *JXB* online). The 'Chardonnay' cultivar was chosen for comparison with the 'Sultanine' cultivar because of similar phenological development. For the *VviAGL11* transcriptional profile, 'Chardonnay' (seeded) and 'Sultanine' (seedless) organ and tissue samples were harvested at the stages of flowers at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set in 2010/2011. For *in situ* hybridization and morphological assays, 'Chardonnay' and 'Sultanine' samples were harvested as described by Baggiolini (1952; Supplementary Table S2) at H1, flowers at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set in the 2012/2013 harvest.

Arabidopsis thaliana plants, the wild type (ecotype Columbia) and *stk* mutant, were grown at 21 °C under short-day (8 h light/16 h dark) or long-day (16 h light/8 h dark) conditions. *stk* mutant seeds were kindly provided by L. Colombo (Universitá degli Studi di Milano, Italy).

Sequencing

Genomic DNA was extracted from leaves following the protocol of Lefort and Douglas (1999). Primer sets were designed to amplify sequences in the promoter and in exon/intron regions of *VviAGL11* (see Supplementary Table S1) in order to sequence the whole gene. Overlapping gene fragments were amplified by a primer walking strategy employing six primer pairs that allowed the assembly of six contigs (Fig. 1). Each projected contig contains 300–500 bp of overlapping sequence. PCR amplifications were performed with 10–25 ng of grapevine genomic DNA using Platinum® Pfx DNA polymerase (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. RACE (rapid amplification of cDNA ends) was performed with the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) as recommended by the manufactor. PCR products were cloned into pGEM®-T Easy Vector (Promega, Madison, WI. USA) following the manufacturer's instructions. Plasmid DNA was purified by the boiling miniprep protocol (Sambrook *et al.*, 1987). All PCR products were sequenced with T7 (GTAATACGACTCACTATAGGG) and SP6 (TACGATTTAGGTGACACTATAG) primers and with internal sequence primers (Supplementary Table S1) in an ABI Prism® 310 Genetic Analyser (Applied Biosystems) using standard sequencing protocols described by Falavigna *et al.* (2014). Sequence analysis was carried out with DNA Sequencing Analysis Software v5 (Applied Biosystems) and MEGA7 software (http://www.megasoftware.net/home). The six contigs were assembled manually and

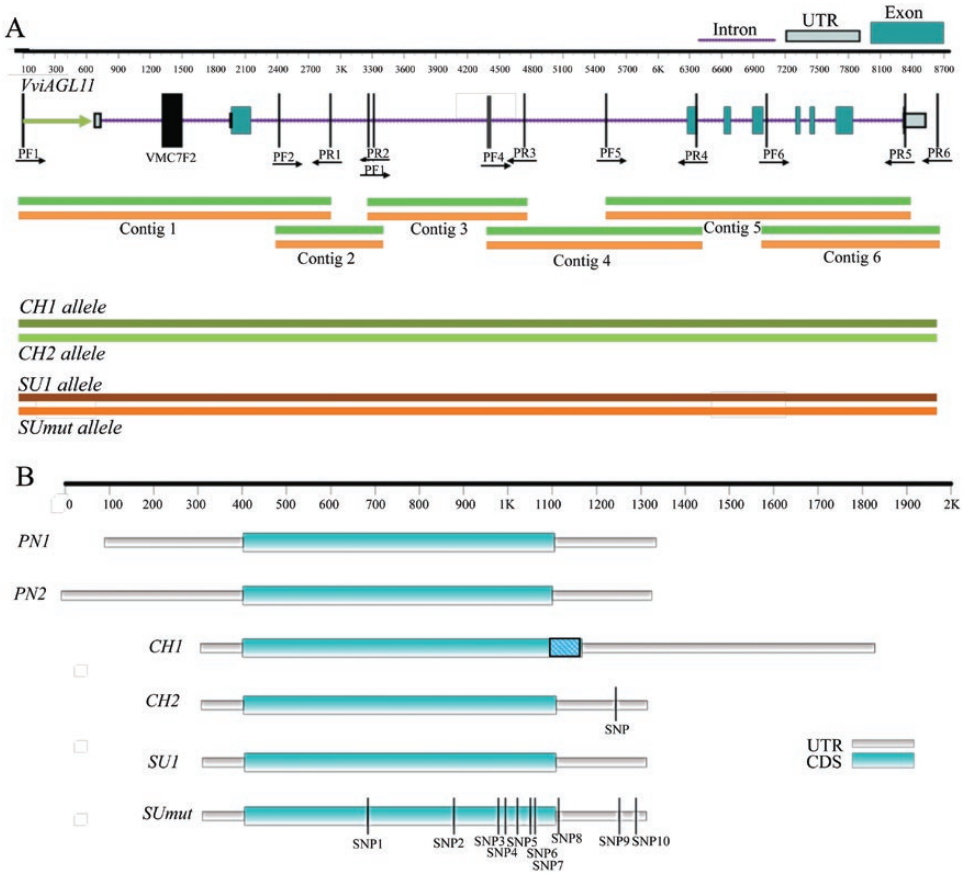

Fig. 1. *VviAGL11* allelic features. (A) Structural representation of *VviAGL11*. DNA structure is composed of a promoter region, 5'-UTR, eight introns, eight exons, and 3'-UTR. The black lines represent the localization of the primers used for allele amplification. Below, the contigs for each cultivar and their assembly, to produce 'Sultanine' and 'Chardonnay' *VviAGL11* alleles. (B) Comparison between *VviAGL11* mRNA alleles. *PN1* and *PN2* represent mRNA versions of *VviAGL11* from the Pinot Noir reference cultivar (VIT_218s0041g01880.1 and VIT_218s0041g01880.2, respectively). 'Chardonnay' allele 1 (*CH1*), 'Chardonnay' allele 2 (*CH2*), and 'Sultanine' allele 1 (*SU1*) are compared with the 'Sultanine' mutant allele (*SUmut*). The box in *CH1* represents the 20 amino acids derived from the 5' alternative splice. SNPs are represented by black stripes (GenBank KM401845, KM401846, KM401847, and KM401848). (This figure is available in colour at *JXB* online.)

with Codon Code Aligner 6.0. (www.codoncode.com/). The contigs from the same alleles were identified by specific polymorphisms in overlapping sequences. The online program Fancy Gene v1.4 (http://bio.ieo.eu/fancygene/) was used to draw the gene and mRNA representations (Fig 1A, B). Sequences were compared with the grapevine reference ('Pinot Noir' PN40024) genome and with the whole-genome sequence from cv. Sultanine (Di Genova *et al.*, 2014).

Identification of putative CArG sequences

Genomic regions located 5 kb upstream of the *VviAGL11* ATG start codon, 377 bp downstream of the stop codon, and exons and introns were analyzed to identify CArG-Box sequences with the New Place Bioinformatic Program 26.0 available at the Sogo website (http://sogo.dna.affrc.go.jp).

RNA extraction

Total RNA samples were extracted from frozen material (~200 mg) by LiCl precipitation using the protocol of Zeng and Yang (2002) scaled to 2 ml microcentrifuge tubes. Each sample extraction was performed in triplicate, and their volumes were pooled before the LiCl precipitation step. Genomic DNA in total RNA samples was removed using the TURBO DNA-free Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA integrity and quantity were monitored by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

Quantitative reverse transcription–PCR (RT–qPCR)

cDNAs were synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's instructions. Gene-specific primer pairs were designed for the two alleles of the *VviAGL11* gene (*VviAGL11F* 5'-CACTTAATGGGT GATTCCTTGGC-3', *VviAGL11R* 5'-AGCAACTCATG CTTCTTCGACC-3'; and *VvAGL3F* 5'-ATTGTTCATCTGGG CATTTCG-3', *VvAGL3R* 5'-GGAGATGAAGTTGGCGGATA-3') and evaluated by Oligo Analyzer 3.1 (IDT, http://www.idtdna.com), with the standards settings of 0.2 µM oligo concentration, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. The reproductive tissues evaluated were divided into initial stages of development, represented by flowers at pre-anthesis and fruit set, and by fruit stages at 2, 4, and 6 weeks of development after fruit establishment (fruit set). Seeds were dissected from the pulp in 'Chardonnay' berry samples and the whole berry was evaluated in 'Sultanine' samples. RT–qPCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems). SYBR Green (Invitrogen, Carlsbad, CA, USA) was used to monitor dsDNA synthesis and ROX (Invitrogen) was employed as a passive fluorescence reference. Each biological sample was analyzed in technical quadruplicates. Cycling consisted of one step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and finished by a dissociation curve between 60 °C and 95 °C. The specificity of PCR amplifications was assessed by the presence of a single peak in melting curves, visualization of single amplification products of the expected size in 1% ethidium

bromide-stained agarose gel electrophoresis, and sequencing of the amplicons. Primer efficiency was calculated by LinRegPCR (version 11.0, Ruijter *et al.*, 2009). Mean relative gene expression was calculated by the method of Pfaffl (2001) employing *ACTIN* (GenBank EC969944) as reference gene (Reid *et al.*, 2006). For the evaluation of *A. thaliana* transformants with the *VviAGL11* coding region, RNA was obtained from leaves, flowers, and siliques, with the same protocols as described above. The expression of *VviAGL11* was analyzed by a specific pair of primers (VviAGL11OX_F 5'-AACAAACGAATCTCAAGCAATCAAGC-3' and VviAGL11SOX_R 5'-TGATCTCGATCTTTCCTCTCCCC-3'). Relative enrichment of *VviAGL11* transcripts was calculated normalizing the amount of mRNA against an *ACTIN* fragment (AtACT2 RTqPCR For 5'-GACCTTGCT GGACGTGACCTTAC-3' and AtACT2 RTqPCR Rev 5'-GTAGTCAACAGCAACAAAGGAGAGC-3', Locus AT3G18780).

Seed morphoanatomical assay

Seeds and seed traces were transferred into a fixation solution under vacuum (Mcdowell and Trump, 1976). Thereafter, samples were dehydrated in an increasing gradient of ethanol as described by Gabriel (1982), and embedded in 2-hydroxyethyl methacrylate resin according to Gerrits and Smid (1983). The 5 µm sections were obtained in a Leica RM 2255 microtome. The metachromatic reagent Toluidine Blue O (Feder and O'brien, 1968) was used to determine seed structure. For the staining procedure, slides with sections were submerged in the reagent for 1 min, washed with water, and dried on a plate at 45 °C. The count of the number of cells in the endotesta (ET) layer was performed with support of ZEN microscope software from Zeiss.

In situ *hybridization analysis*

'Chardonnay' and 'Sultanine' samples were fixed in 4% formaldehyde for 16 h. Around 20 samples of the same stage were used from each cultivar. To submerge the samples, a vacuum was applied (~600 mmHg) for 20 min. The material was stored under refrigeration (4–10 °C) after fixation. Thereafter, the tissues were dehydrated in an ethanol series and embedded in paraffin. Longitudinal and transversal sections (8–10 µm) were prepared with a Leica RM 2255 (Leica, Wetzlar, Germany) microtome and mounted on silanized microscope slides. Gene-specific sense and antisense probes corresponded to 185 nucleotides of the 3'-untranslated region (UTR) of the *VviAGL11* gene, which were amplified using VvAG3F (5'-ATTGTTCATCTGGGCATTTCG-3') and VvAG3R (5'-GGAGATGAAGTTGGCGGATA-3') as primers. Hybridizations were performed with non-radioactive probes (Dornelas *et al.*, 2000). Labeled probes were generated by digoxigenin (DIG) labeling using T7 or SP6 RNA polymerase of the DIG RNA Labeling Kit (Roche, Basel, Switzerland). The hybridization signal was detected by a colorimetric assay in which an anti-DIG antibody coupled with alkaline phosphatase and NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine) served as a substrate. After the detection of the hybridization signals by immunostaining, slides were washed, dehydrated, and mounted using Entellan® (Merck, product accession code 107961). We performed *in situ* hybridization analysis for two consecutive years.

Functional complementation analysis in Arabidopsis

In order to evaluate the competence of *VviAGL11* to complement the *stk* phenotype, *stk* plants were transformed with overexpression plasmids carrying the *VviAGL11* coding region. Two constructs were generated: (i) pVviAGL11-OX, an overexpression plasmid harboring the *VviAGL11* 'Chardonnay' allele; and (ii) pVviAGL11-OX^{mut}, an overexpression plasmid harboring the *VviAGL11* 'Sultanine' mutant allele (*SUmut*). Both constructions had the same coding region size (672 bp) besides the SNPs in the *SUmut* allele (Supplementary Fig. S2). *VviAGL11* was amplified as described previously and cloned into pENTER/D-TOPO (Invitrogen). The PCR product was obtained with the primers *VviAGL11CDSF* 5'-CACCATGGGGAGAGGAAAGATCG-3' and *VviAGL11CDSR* 5'-TTACCCGAGATGGAGGACCTTCTTATC-3'. Employing the Gateway® LR Clonase TM II Enzyme Mix recombination system (Invitrogen), the insert was transferred into the pH7WG2D vector (Karimi *et al.*, 2002). The T-DNA fragment also encodes a visible selection marker [green fluorescent protein (GFP)] under the control of the promoter RolD (Karimi *et al.*, 2002). Final constructs were verified by sequencing and used to transform thermocompetent *Agrobacterium tumefaciens* strain EHA105. The floral dip method (Clough and Bent, 1998) was used to transform *stk* plants, and T_1 seeds were initially germinated in Phytagel™ BioReagent culture medium (Sigma, St Louis, MO, USA) and selected for hygromycin resistance (25 µg ml^{-1}). Two-week-old putative transformants were screened for GFP fluorescence, and the T-DNA insertion of the *VviAGL11* alleles on T_1 plants was analyzed by PCR using specific primers targeting the plasmid promoter and the *VviAGL11* coding region (TransF 5'-CTGGGAACTACTCACACATTA-3' and TransR 5'-CATTTGGAGAGGACTCCG-3'). Only T_1 lineages positive for these three steps of selection were used for the phenotype assay: (i) silique length; (ii) seed number in siliques; (ii) seed size; (iv) funiculus thickness; and (v) funiculus length. For each plant, five well-developed siliques at the same stage were used for measurements/counting, and the final score for each plant was composed of their average. Columbia-0 and *stk* plants were used as controls for all measurements. Statistical analysis was performed using Prism 5.1 with one-way ANOVA and Tukey test.

Accession numbers

The nucleotide sequences reported in this paper have been submitted to GenBank under the accession numbers KM401845, KM401846, KM401847, and KM401848.

Results

Sequencing of the VviAGL11 *locus*

To distinguish the structural organization of the *VviAGL11* gene in apirenic and seeded grapevines, allelic variations were amplified by PCR with specific primers from genomic DNA extracted from the apirenic 'Sultanine' and from the seeded 'Chardonnay' as previously described. A total of six contigs were assembled for each *VviAGL11* allele as shown in Fig. 1A. The complete *VviAGL11* allelic sequences constituted by their promoter, coding, and intragenic regions (eight exons and eight introns) were sequenced. After allele-specific isolation and sequencing, we identified two alleles for each cultivar. Because 'Chardonnay' is a direct offspring of Pinot Noir, we were able make comparisons with the 'Pinot Noir' (PN40024) sequence in the publicly available genome database (http://genomes.cribi.unipd.it/gb2/gbrowse/public/vitis_vinifera/).

The two *VviAGL11* alleles from 'Chardonnay' showed 99% sequence identity to the corresponding 'Pinot Noir' genome sequence for *VviAGL11*. One allele from 'Sultanine' also exhibited 99% identity with PN40024. However, the second 'Sultanine' *VviAGL11* allele, linked to the molecular microsatellite marker VMC7F2 198 bp allele (Costantini *et al.*, 2008), presented a different group of polymorphisms when

compared with all the other alleles. This allele was named as *SUmut* ('Sultanine' mutant allele) and contains a group of exclusive polymorphisms composed of 28 INDELs (insertions/deletions) and 105 SNPs (Supplementary Fig. S1).

The sequence alignment of the four *VviAGL11* alleles sequenced within this work, from the PN40024 reference genome and from the newest sequences of the *VviAGL11* locus from 'Sultanine' described by Di Genova *et al.* (2014) is presented in Supplementary Fig. S1. Among the observed alterations in *SUmut*, two SNPs give rise to two amino acid substitutions (R590L and T628A) as shown in Supplementary Fig. S2. We also searched the sequences for MADS-box protein complex-binding sites (CArG-box sequences: perfect CArG-boxes and CArG-boxes with one mismatch deduced by a probability matrix; Smaczniak *et al.*, 2012). We were able to identify 10 putative CArG-box sequences within the *VviAGL11* gene structure, one located in the promoter region, eight in the second intron, and one in the 3'-UTR. The seventh CArG-box putative sequence of 'Sultanine' *VviAGL11 mut* has a nucleotide modification (Supplementary Fig. S1, black arrow).

Allele-specific transcription profiles of VviAGL11 *during fruit development*

The 3'- and 5'-RACE experiments allowed the identification of two distinct mRNA alleles of *VviAGL11* in apirenic ('Sultanine') when compared with seeded ('Chardonnay') grapevines. Two amplicons of different sizes were obtained from 'Chardonnay' and one amplicon was obtained from 'Sultanine'. After sequencing them, it was possible to confirm the specificity of the amplicons and to characterize each allele transcript present in both cultivars.

The results revealed that 'Chardonnay' has two alleles of *VviAGL11* mRNA. The allele isoform, named *CH1*, spans 1454 bp and contains a 732 bp coding region. This alternative form is longer and has an increased coding region of 60 nucleotides (20 amino acids when compared with the *VviAGL11* gene model in the PN40024 reference genome (Jaillon *et al.*, 2007). The second Chardonnay allele, named *CH2*, spans 958 bp and contains an ORF of 672 bp. 'Sultanine' also has two transcript alleles of the same total length (958 bp) and coding sequence (672 bp), respectively. The differences between the two *VviAGL11* alleles derived from 'Sultanine' are the presence of 10 SNPs found in the mutant allele (*SUmut*). The coding regions of *CH2* and 'Sultanine' allele 1 (*SU1*) are identical to those of the *VviAGL11* gene predicted in the Pinot Noir reference genome (Jaillon *et al.*, 2007); however, none of the 'Sultanine' alleles is identical to the 'Pinot Noir' gene due to the different 5'-UTR sizes, as shown in Fig. 1B.

To evaluate the relative expression of *VviAGL11* alleles during fruit development, RT–qPCR analysis with mRNA samples extracted from the reproductive organs of 'Chardonnay' and 'Sultanine' at critical fruit development stages was performed. Two sets of primers were used in this approach in order to differentiate *VviAGL11* mRNA isoforms. The first *VviAGL11* primer set was designed to amplify the longer allele (*CH1*) (see the Materials and methods). The second pair of primers was designed to anneal in the first (forward) and in the second (reverse) exons that allowed us to amplify simultaneously the two *VviAGL11* alleles (the shorter *CH1* and longer *CH2*). Considering that in 'Sultanine' the alleles are differentiated only by 10 SNPs, we employed primers flanking the first and second exons, covering both alleles (*SU1* and *SUmut*).

The results showed that *VviAGL11* relative expression was similarly low in all stages sampled from the 'Sultanine' variety (Fig. 2). 'Chardonnay' flowers and berry pulp also presented low transcription levels of *VviAGL11* for isoforms *CH1* and *CH2*. RT–qPCR with primers designed for the intronic region was also performed with 'Sultanine' mRNA samples, and a minor level of expression was detected, probably due to residual pre-mRNA in the samples. Nevertheless, seeds of 'Chardonnay' from fruits at 2, 4, and 6 weeks after fruit set exhibited much higher accumulation of *VviAGL11 CH2* transcripts, 15-fold higher than *CH2* transcripts when compared with berries at fruit set and 65-fold higher when compared with 'Sultanine' whole berries. The 'Chardonnay' *CH1* allele relative expression fluctuated from 5- to 11-fold higher throughout seed stages compared with fruit set (Fig. 2).

Morphoanatomical analysis of seeds from apirenic and seeded grapevines

Grapevine seeds have a particular shape resembling a pear, being triangular in cross-section (Fig. 3A, B). The seeds are composed of a cuticle, an epidermis, two layers making up the outer integument (exotesta and ET), both forming the seed coat at the ripening stage, and three inner integument layers that are positioned around the endosperm and embryo (Ribereau-Gayon and Peynaud, 1980). The structure of the seeds from 'Chardonnay' and seed traces from 'Sultanine' was found to be very different in shape, size, and hardness (Fig. 3C). For that reason, a morphoanatomical analysis was conducted to better characterize the development of endosperm, embryo, and seed layers of both cultivars at 2, 4, and 6 weeks of fruit development.

A normal and complete development of the endosperm and the embryo was observed in 'Chardonnay' seeds after 6 weeks of fruit development. 'Sultanine' seed traces showed an undeveloped endosperm, and no embryo could be distinguished at any stage. When analyzing the integuments, three layers of inner integuments were observed in the seeds at the initial stage of fruit development in both varieties (Fig. 3D, E), although only two layers were present at later stages in 'Chardonnay' (Fig. 3F, H) and in 'Sultanine' (Fig. 3G, I).

The epidermis covers the outer integument in both varieties. Parenchyma cells are the main constituent of the outer integument, and cell layers are thicker in the ventral face of the seed. At the ripening stage there is an increase in parenchyma cell volume in 'Chardonnay', but the number of cells does not change. We could also observe the lignification process of the seed (Fig. 3D, F, H). However, in 'Sultanine' seed traces, this layer had no visible development (Fig. 3E, G, I).

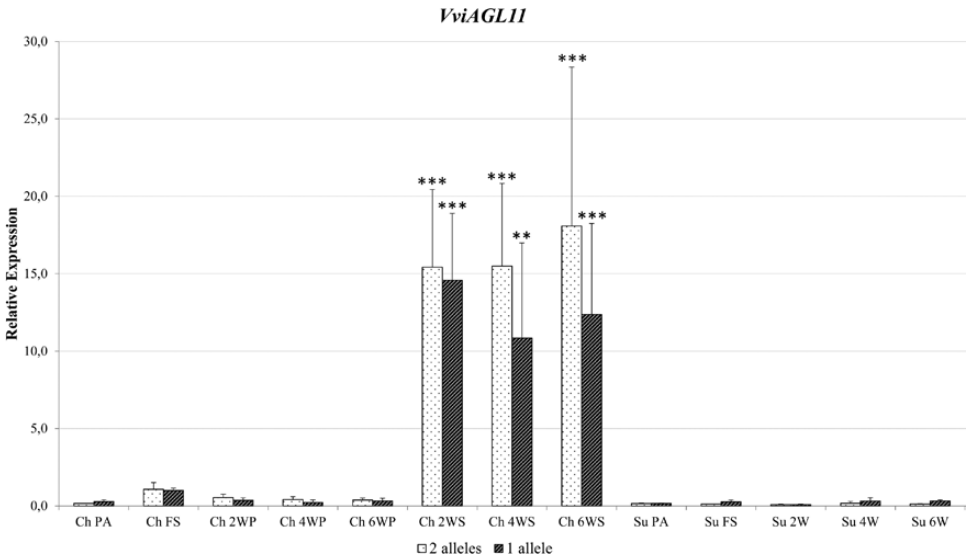

Fig. 2. Relative gene expression of *VviAGL11* alleles from 'Chardonnay' and 'Sultanine' via RT–qPCR. Two alleles correspond to *CH1* and *CH2*, and also *SU1* and *SUmut*. One allele corresponds to *CH1*. The developmental stages sampled are shown on the *x*-axis: PA, pre-anthesis flower; FS, fruit set; 2WP-4WP-6WP, pulp from fruits at 2, 4, and 6 weeks after fruit set; and 2WS-4WS-6WS, 'Chardonnay' seed stages. 'Sultanine' stages evaluated at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set (2W-4W-6W). 'Chardonnay' fruit set was used as reference. The relative expression expressed on the *y*-axis was calculated by the method of Pfaffl (2001). The SD is shown for each stage and sample. Asterisks indicate statistical significance between 'Chardonnay' alleles in seed stages compared with other stages evaluated for the 'Chardonnay' and 'Sultanine' alleles (Tukey test: $^{**}P<0.01$, $^{***}P<0.001$).

The most interesting morphoanatomical results were found in the ET layer, the inner layer of the outer integument. After 2 weeks of fruit development, one layer of rectangular palisade cells was observed in the seeds from both varieties (Fig. 3D, E). After 4 weeks, two layers were observed in 'Chardonnay' ET seeds (Fig. 3F) after a periclinal division, but this duplication was not observed in 'Sultanine' seed traces (Fig. 3G). After 6 weeks of fruit development, 'Chardonnay' seeds showed a sclerified and thicker ET (Fig. 3H), unlike 'Sultanine' seed traces that exhibited the same structures as its initial stage of development, with no lignification of this integument (Fig. 3I). The number of cells making up the ET layer was higher in 'Chardonnay', demonstrating a periclinal development. In contrast, this characteristic was not observed in 'Sultanine' (Fig. 4).

Spatial and temporal accumulation of VviAGL11 *transcripts*

In situ hybridization (ISH) with the *VviAGL11* complementary (antisense) probe reveals no expression at the stages H1 (Fig. 5B), pre-anthesis flower (Fig. 5C), fruit set (Fig. 5D), and at 6 weeks of fruit development (Fig. 5J) in 'Chardonnay'. *VviAGL11* transcripts exhibited a high accumulation in seeds after 2 (Fig. 5E, F) and 4 weeks (Fig. 5G–I) of development in 'Chardonnay'. The ISH signal was clearly visible in the ET layer of the seeds (Fig. 5E, I). In 'Sultanine', no gene expression was observed at reproductive tissue/organ or stage of development (Fig. 6A–F). The ISH slides with the antisense probe were compared with slides hybridized with the sense *VviAGL11* probe as the control in the analysis of 'Chardonnay' and 'Sultanine' samples (Supplementary Figs S3 and S4, respectively). At the time of ISH assays, the transcript isoforms were not known and the probe used for hybridization was designed according to the *VviAGL11* gene predicted model (GSVIVT01025945001) available at http://www.genoscope.cns.fr. Therefore, the whole probe (185 bp) hybridizes with the *CH1* mRNA isoform while 38 nucleotides still hybridize with *CH2*, *SU1*, and *SUmut* mRNA isoforms. Therefore, despite the differences within the genomic organization of the locus, the probe employed in the ISH assays is still capable of generating reliable data.

Functional analysis of VviAGL11

In order to confirm the role of *VviAGL11* as a key gene for seed morphogenesis in grapevine, we generated Arabidopsis *stk* mutant plants overexpressing *VviAGL11*. Two constructs were made with (i) a *VviAGL11* 'Chardonnay' allele (*VviAGL11 CH*); and (ii) a *VviAGL11* 'Sultanine' mutant allele (*VviAGL11 SU*). The 'Sultanine' mutant allele (*SUmut*) was used to evaluate the importance of the two SNP mutations conferring amino acid substitutions (*VviAGL11* R590L and T628A; Supplementary Fig. S2). Thirty-six well-developed plants were used for transformation with each construct, including controls. The reporter gene contained in the plasmid construct (GFP) was visualized in 3-week-old plants (Supplementary Fig. S5). The GFP-positive selected T_1 plants were tested by PCR for the presence of the complementation construct and the presence of the *VviAGL11* alleles, and resulted in 16 independent lineages transformed with *VviAGL11 CH* and 21 with *VviAGL11 SU*. In general, transformed plants had an increase of 12-fold in *VviAGL11* expression, compared with the wild type (Fig. 7C). Remarkably, an *stk* plant complemented with *VviAGL11* from the 'Sultanine' mutant allele presented a relative expression of 400-fold.

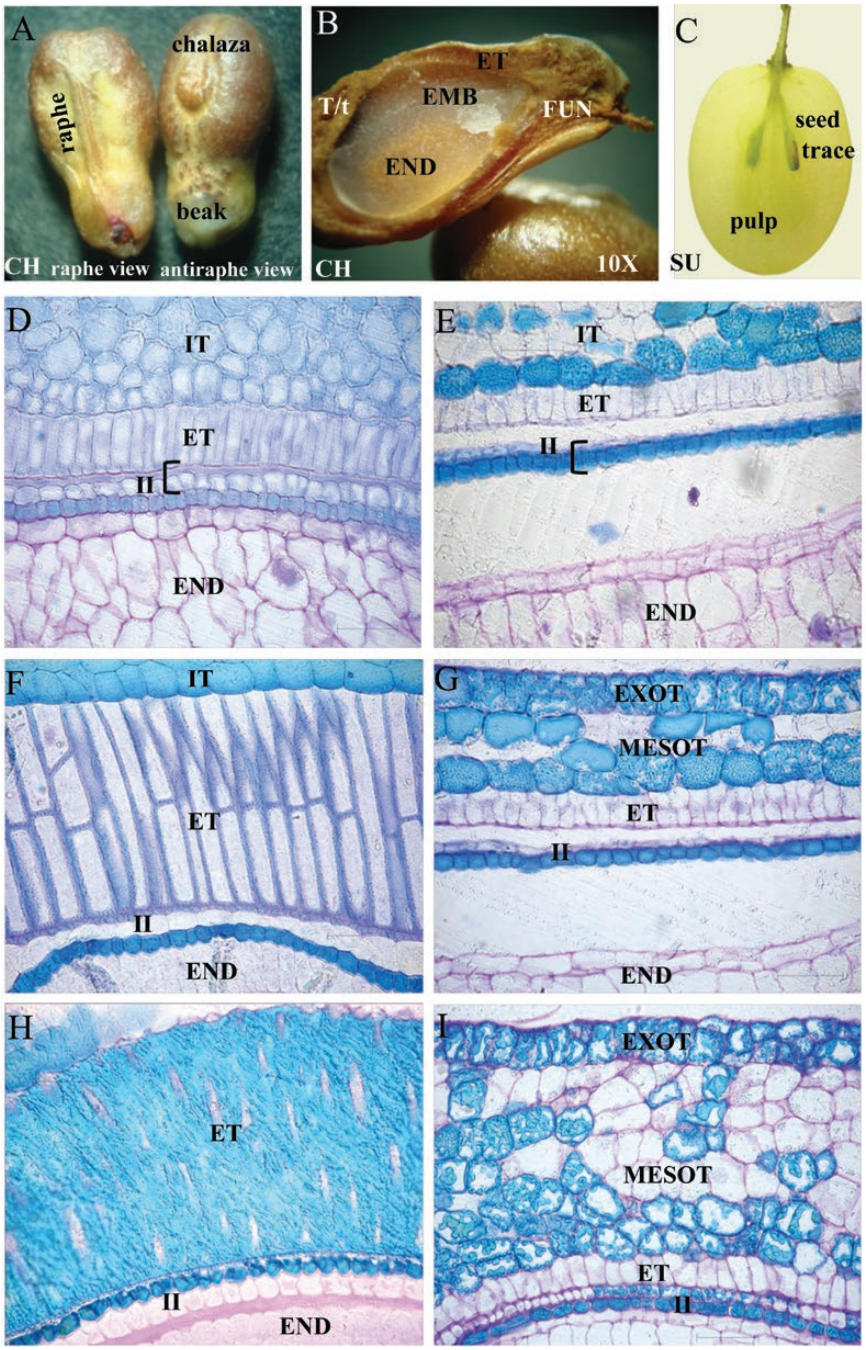

Fig. 3. Seeds and seed traces in *V. vinifera*. (A) Mature 'Chardonnay' (CH) seeds at their raphe and antiraphe view. (B) CH seed longitudinal section, with the view exhibiting the testa/tegmen (T/t), endotesta (ET), endosperm (END), embryo (EMB), and funiculus (FUN). (C) Mature 'Sultanine' (SU) berry showing its seed traces. Grapevine seed structure development from 2 to 6 weeks after fruit set (D–I). Light micrographs of grape seed cross-sections were stained with Toluidine Blue O. The cross-sections in the median area of the seeds of 'Chardonnay' and seed traces of 'Sultanine' enable the visualization of two defined integuments. 'Chardonnay' seed: (D) 2 weeks, (F) 4 weeks, and (H) 6 weeks. 'Sultanine' seed trace: (E) 2 weeks, (G) 4 weeks, and (I) 6 weeks. Endosperm (END), exotesta (EXOT), mesotesta (MESOT), endotesta (ET), inner integument (II), integuments (IT). Scale bar=200 μm.

The Arabidopsis *stk* mutant presents five distinct phenotypes when compared with the wild type: (i) the number of seeds is reduced to half; (ii) the size of the seeds is diminished (including many seed abortions); (ii) the silique length is reduced; (iv) the funiculus shows a thicker conformation; and (v) the funiculus shows an elongated conformation (Pinyopich *et al.*, 2003; Matias-Hernandez *et al.*, 2010). The siliques of T_1 lines present reduced seed abortion, increased seed size, silique length enlargement, and modifications in the funiculus resembling the wild-type phenotype (Fig. 7C). Among the *VviAGL11 CH*-transformed plants, six showed wild-type-like developed siliques and increased seed number, eight demonstrated wild-type seed size, seven plants showed reduction of the funiculus thickness, and eight showed a shortened funiculus (Fig. 7A, D). Among the *VviAGL11 SU* plants, nine presented wild type-like developed siliques, six plants showed increased seed number, 10 had wild-type seed size, 10 plants presented reduced

funiculus thickness, and nine showed a shortened funiculus (Fig. 7B, D).

These results demonstrate that the ectopic expression of *VviAGL11* is capable of restoring silique and seed morphogenesis in the Arabidopsis *stk* background and that very similar genes have a relatively well conserved function *in planta*, gathering evidence to support an evolutionary orthology relationship between *AtAGL11* and *VviAGL11*.

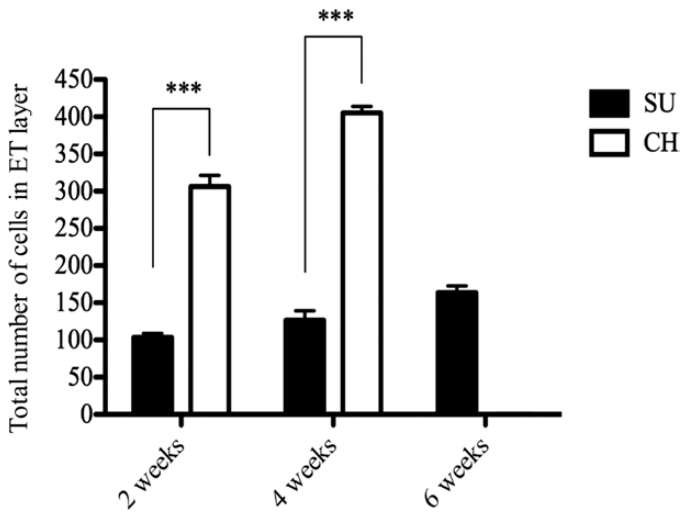

Fig. 4. Analysis of the endostesta (ET) layer of the seed of 'Chardonnay' and the seed trace of 'Sultanine'. The SD is shown for each stage and sample. Asterisks indicate statistical significance between the number of cells in the ET layer of 'Chardonnay' compared with 'Sultanine' (two-way ANOVA with Bonferroni post-test to compare replicate means by row. ***P<0.001).

Discussion

Spontaneous somatic variants have been selected through grapevine domestication, creating new varieties and cultivars with interesting traits such as berry size, taste, color, and seedlessness (This *et al.*, 2006). A collection of reports in the literature indicated *VviAGL11* as the major candidate involved in the absence of seeds (Costantini *et al.*, 2008; Mejía *et al.*, 2011; Revers *et al.*, 2014; Ocarez and Mejía, 2016). Despite that, no specific and direct functional evidence was reported concerning the role of *VviAGL11* during seed development in grapevine. Therefore, our approach was to explore a combination of methods such as *de novo* sequencing, gene expression, and ISH, combined with anatomical examination and complementation analysis of the Arabidopsis *stk* mutant to gather evidence to address the function of *VviAGL11* during seed morphogenesis.

De novo sequencing based on a PCR strategy of *VviAGL11* allelic variants from 'Sultanine' and 'Chardonnay' was performed, allowing the identification of a group of polymorphisms that allowed the identification of four *VviAGL11* alleles (Fig. 1; Supplementary Fig. S1). No transposon or large insertion or deletion sequence was found in 'Sultanine' that could explain the gene loss of transcription observed in previous data (Mejía *et al.*, 2011). We found two amino

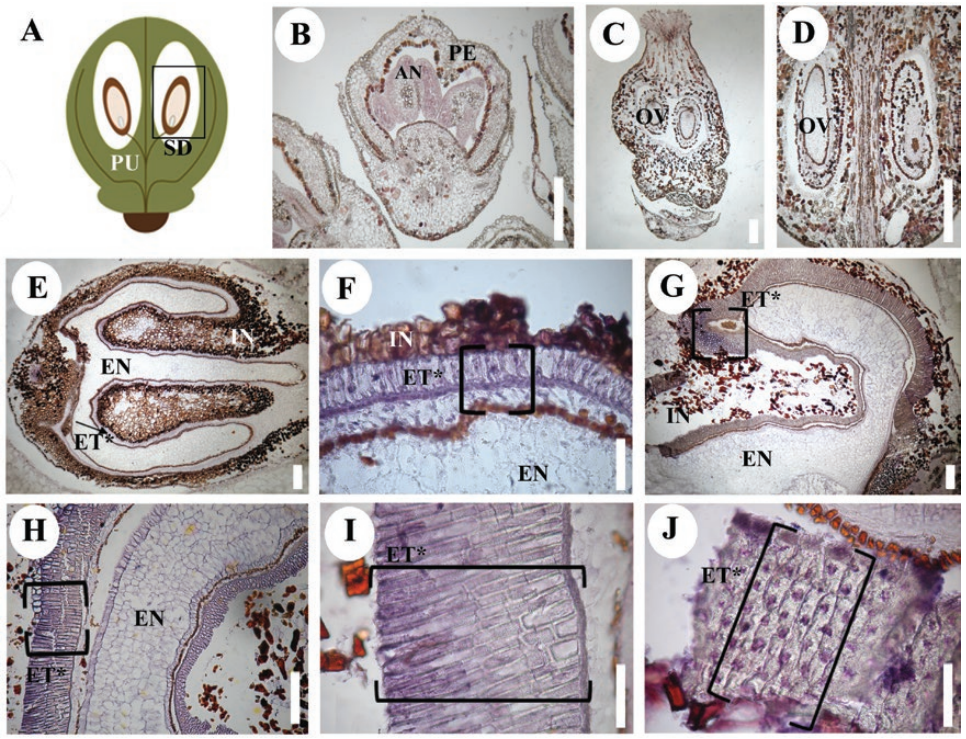

Fig. 5. *In situ* hybridization of *VviAGL11* in 'Chardonnay' reproductive tissues and organs, from flowers to seeds after 6 weeks of fruit development. (A) Schematic berry. No hybridization signal is visible in (B) H1 flower stage, (C) pre-anthesis flower stage, and (D) fruit set flower stage. *VviAGL11* expression is detectable in the endotesta of (E and F) seeds at 2 weeks of development and (G–I) seeds at 4 weeks of development (black arrows). No clear hybridization signal is visible in seeds at 6 weeks of development (J) when compared with the control (Supplementary Fig. S3H). Anther (an), endosperm (en), endotesta (et), integuments (it), petal (pe), pulp (pu), seed (sd). Scale bar=200 μm.

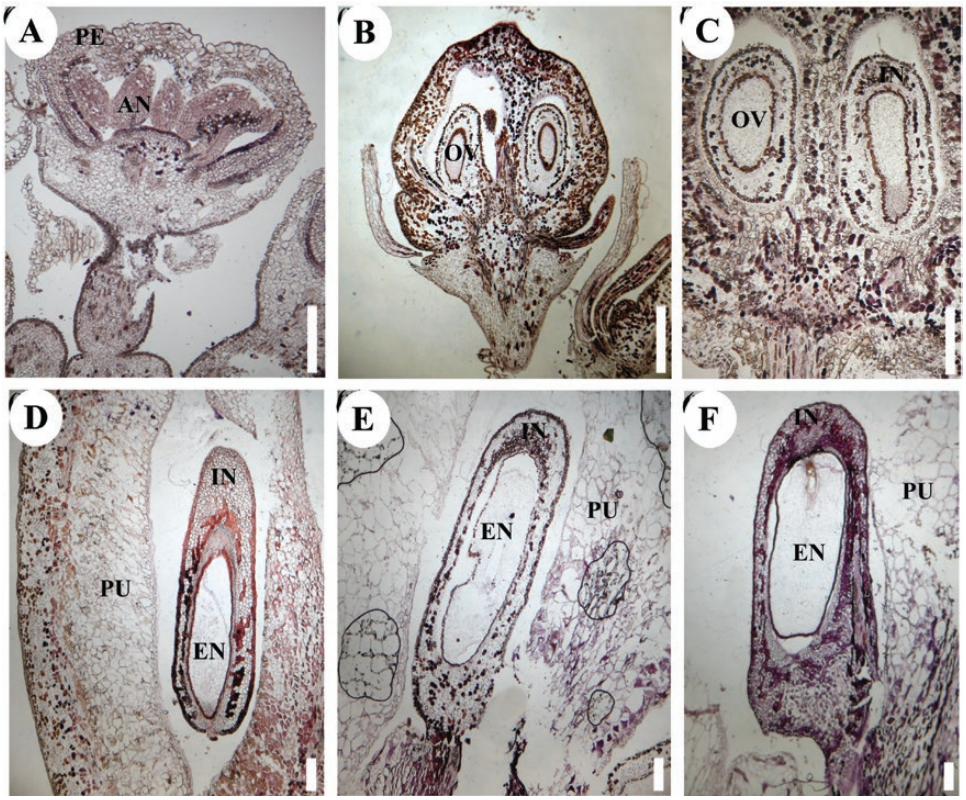

Fig. 6. *In situ* hybridization of *VviAGL11* in 'Sultanine' reproductive tissues and organs, from flowers to fruits and seed traces after 6 weeks of development. No hybridization signal is visible in (A) H1 flower stage, (B) pre-anthesis flower stage, (C) fruit set stage, and in seed traces from fruits of (D) 2, (E) 4, and (F) 6 weeks of development when compared with control slides (Supplementary Fig. S4). Anther (an), endosperm (en), endotesta (et), integuments (it), petal (pe), pulp (pu). Scale bar=200 μm.

acid modifications in 'Sultanine's' *VviAGL11* coding region (Supplementary Fig. S2). Arginine and threonine were substituted by two more hydrophobic amino acids, leucine and alanine, respectively. These amino acid substitutions were localized in the C-terminal portion of the VviAGL11 protein, a well-known region responsible for activity and ternary complex formation (Favaro *et al.*, 2003; Matias-Hernandez *et al.*, 2010). Furthermore, 17 INDELs and 53 additional SNPs were identified in the second intron of the 'Sultanine' mutant allele (Supplementary Fig. S1). The second intron of MADS-box AG (AGAMOUS) subfamily members is recognized by the presence of a quite large (*AtAG*. ~3 kb; *AtAGL11*, ~1.8 kb; and *VviAGL11*, ~4 kb) region with important *cis*-elements that may control tissue-specific expression of AG and AG-like genes (Deyholos and Sieburth, 2000; Hong and Hamaguchi, 2003). Interestingly, MADS-box proteins form complexes of high order, often in tetrameric junctions made by two dimers that bind to two DNA consensus sequences called CArG-boxes [CC(A/T)$_6$GG] separated by up to 300 bp (Liu *et al.*, 2008). A CArG-box modification was observed in the 'Sultanine' mutant (*SUmut*) allele, in the seventh putative sequence. This *cis*-element is only 84 bp distant from its putative partner CArG-box, which would suggest their relevance in *VviAGL11* gene regulation (Supplementary Fig. S1).

The transcription profile of *VviAGL11* isoforms provided complete data for each expression isoform during the development of flowers, fruits, and seeds. 'Sultanine' alleles presented a low level of expression in all organs and stages of development, including berries, while the 'Chardonnay' alleles were expressed at very low levels in the berry pulp, but highly expressed in seeds (Fig. 2). Of the two 'Chardonnay' alleles, *VviAGL11-CH1* possesses a larger coding region, caused by alternative splicing, resulting in 20 additional amino acids. This alternative splicing is characterized as a 5' splice site and occurs in 7.5% of the Arabidopsis genome (Eckardt, 2013). The sequence of the *CH1* allele transcript was also found in a 'Cabernet Sauvignon' cDNA library derived from early stages of berry development (GenBank CB974197.1). This could cause conformational changes in the C-terminal portion of VviAGL11 protein, yet no abnormal seed morphogenesis is observed in Chardonnay or Cabernet Sauvignon cultivars. Thus it is clear that these 20 additional amino acid residues are not relevant to seed development.

The relative expression of the *CH1* allele in seeds showed a fluctuation between 5- and 11-fold when compared with fruit set (Fig. 2). These results suggest that *VviAGL11* indeed has a major role in seed development. Without its expression (transcript accumulation) during early fruit developmental stages, there is no normal seed formation.

The interaction of proteins encoded by MADS-box genes of classes A, B, C, D, and E forms the widely accepted model of floral quartets (Theissen *et al.*, 2000; Dornelas and Dornelas, 2005). The AGAMOUS subfamily of *MADS-box* genes is known to have redundant functions in *A. thaliana*, as is the case for *AtAGL11* which is the major gene involved in the regulation of ovule development along

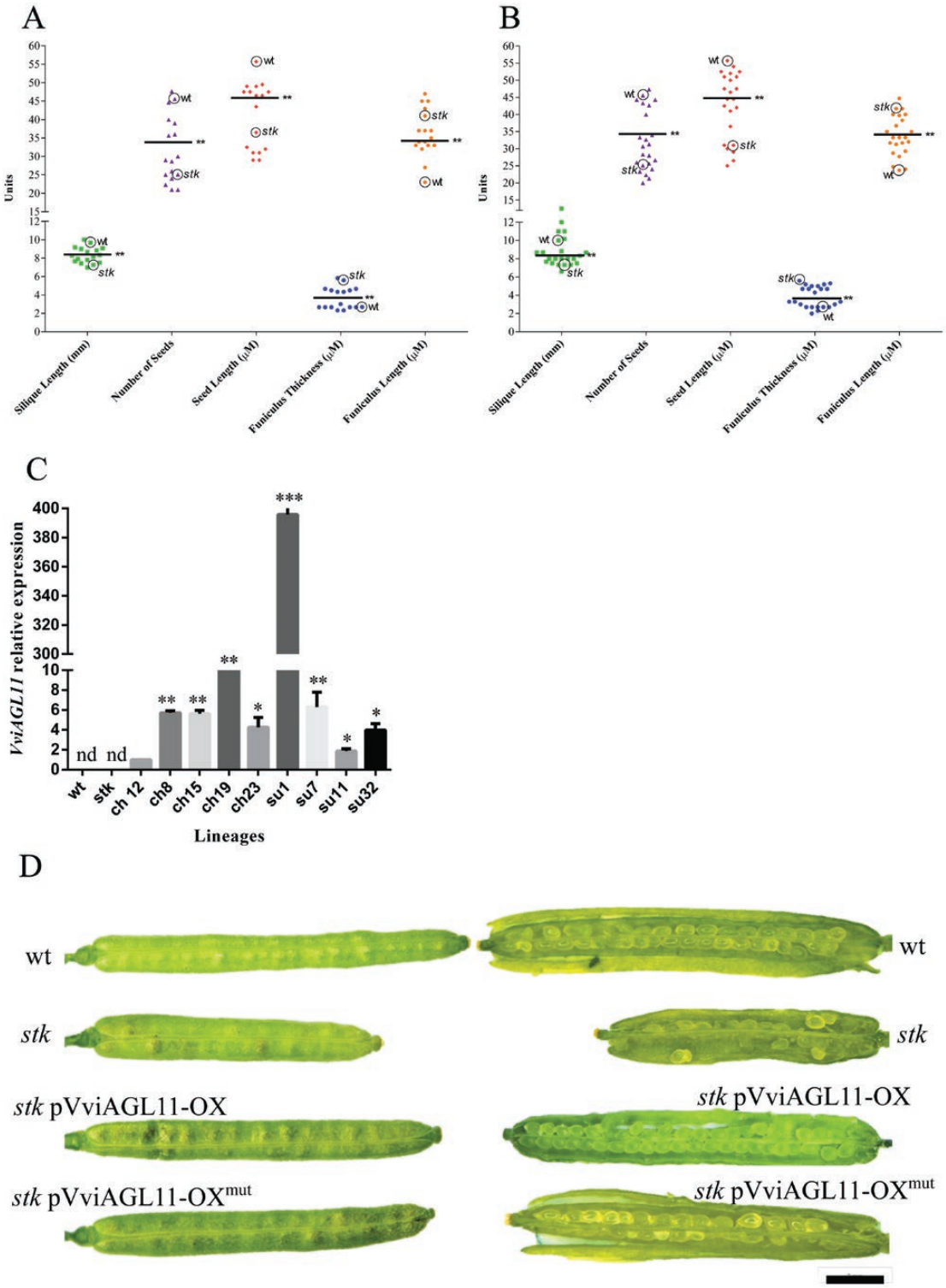

Fig. 7. Complementation analysis in the Arabidopsis *stk* mutant. (A) pVviAGL11-OX T_1 distribution by phenotype. (B) pVviAGL11-OX^{mut} T_1 distribution by phenotype. (C) *VviAGL11* relative expression analyzed in wild-type (wt), *stk*, and *stk*-transformed plants with *VviAGL11* constructions. (D) Comparison between controls and *stk*-complemented constructions. A wild-type silique showing full seed set (wt); siliques of *stk* plants containing aborted ovules and aborted seeds; siliques of *stk* pVviAGL11-OX plants complemented with the genomic region of the *VviAGL11* gene from Chardonnay, and siliques of *stk* pVviAGL11-OX^{mut} plants complemented with the genomic region of the *VviAGL11* gene from Sultanine. For each plant, five well-developed siliques at the same stage were evaluated, and the final score was composed of their average. Columbia-0 and *stk* plants were used as controls for measurements. Scale bar=2 mm.

with other two genes of class D, *SHATERPROOF1* and *SHATTERPROOF2* (Colombo *et al.*, 1995; Pinyopich *et al.*, 2003). Our evidence together with data from the literature support that the *VviAGL11* gene can be considered as an ortholog of *AtAGL11*; however, no gene has been identified yet as orthologous to *SHP1* or *SHP2* in

grapevine (Díaz-Riquelme *et al.*, 2012). These data suggest that *VviAGL11* might be the unique class D gene present in grapevine. Therefore, a possible model of the floral quartet could be proposed to be formed by *VvAG1* and *VvAG2* (*AGAMOUS* genes), *VviAGL11* (*VvAG3*), and one of the *SEPALLATA* genes of grapevine (*VvSEP1*, *VvSEP2, VvSEP3*, or *VvSEP4*) as previously described by Boss *et al.* (2002) and by Díaz-Riquelme *et al.* (2012). Additional studies of the VviAGL11 protein, such as employing fluorescence resonance energy transfer (FRET) (Sekar and Periasamy, 2003), are alternatives to confirm this hypothesis.

Previous studies have described grapevine seed anatomy as well as the changes of internal layers in the process of seed ripening; however, no study has demonstrated detailed morphological aspects in seeds of stenospermocarpic grapevines, especially seed traces (Pratt, 1971; Ribereau-Gayon and Peynaud, 1980; Cadot *et al.*, 2006). We compared normal seeds from 'Chardonnay' with seed traces from 'Sultanine', and the results showed that the most substantial difference was at the ET of the seed coat, which did not develop normally in 'Sultanine' seed traces, preserving its initial features until the complete maturation of the fruits. As expected, 'Chardonnay' seeds developed correctly with the elongation and cell duplication of the ET cells through periclinal divisions along with normal endosperm growth (Fig. 3). Our complete analysis of seed development showed that grapevine seeds have only two integuments, outer and inner (Figs 3, 5, 6), instead of three integument layers as previously described (Pratt, 1971; Cadot *et al.*, 2006).

Garcia *et al.* (2005) have previously shown that there is a strong maternal effect of the integuments in the control, and determination of final seed size, due to the elongation of these layers, and, in the case of absence of elongation, there is a compensation effect promoted by the duplication of these layers. Furthermore, the nutrient flux in seeds occurs in a centripetal way, from the outer integument to the inner, going through apoplasts to the endosperm and to the embryo (Stadler and Lauterbach, 2005). Formation of seed traces is likely to originate due to disruption of normal development of seed tissues. It is possible that the erroneous formation of the ET seed layer that occurs in stenospermocarpic grapevines could interfere in seed nutrient intake and cause the degeneration of some tissues during seed development, such as the endosperm. Therefore, the crosstalk between seed coat and endosperm could be compromised by the defects on the ET, affecting the normal development of the seed (Figueiredo and Köhler, 2014).

The accumulation of transcripts of *VviAGL11* was localized in the ET layer of 'Chardonnay' seeds at 2 and 4 weeks (Fig. 5E–I) of development, while no signal of transcripts was detected in 'Sultanine' seed traces (Fig. 6). When 'Chardonnay' seeds at 6 weeks after fruit set were examined, it was assumed that no hybridization signals was detected (Fig. 5J). However, *VviAGL11* expression at this stage cannot be excluded because RT–qPCR data indicate that at least until 6 weeks after fruit set *VviAGL11* is still expressed in seed tissues (Fig. 2). Unambiguous interpretation of the hybridization signals (Fig. 5J) during this stage was difficult to confirm probably due to the initiation of the lignification process that will be part of the mature and hard seed coat (Haughn and Chaudhury, 2005). A large amount of tannins and phenolic compounds turn brown, tending to darken throughout treatments during slide processing, giving a very strong background, which may impede clear signal identification in grapevine ISH analysis from tissues prone to lignification (Fernandez *et al.*, 2007; Colas *et al.*, 2010).

The ISH data are in agreement with the RT–qPCR transcriptional profile, where 'Sultanine' shows almost no expression of *VviAGL11* in the stages evaluated. The particular accumulation of *VviAGL11* transcripts in a specific layer of the seed coat in 'Chardonnay', combined with the morphological differences in this same layer when comparing 'Chardonnay' and 'Sultanine', suggests that this gene is essential for the elongation and duplication of the ET of the seed coat. Furthermore, seed growth is dictated by the coordinated development of endosperm and integuments and, hence, a spatial and temporal co-ordination of endosperm and integument growth is critical for normal seed development (Garcia *et al.*, 2005). These results showed the similarities between the expression pattern of *AtAGL11* and *VviAGL11* in the fruit tissues, both being expressed in the ovule integuments. *AtAGL11*, however, has a spatial–temporal expression in the funiculus and in the ovule integuments mainly before fertilization, while *VviAGL11* expression is observed in ovule integument after fertilization (Mejía *et al.*, 2011; Fig. 3).

The overexpression of *AtAGL11* in the Arabidopsis *AGAMOUS* mutant (*ag*) promotes homeotic conversions of sepals into carpeloid structures, achieving ovule development (Favaro *et al.*, 2003). More recently, Mizzotti *et al.* (2014) expressed *AtAGL11* in the mutant *stk* background and observed the complete complementation of the wild-type phenotype, with normal seeds and correct seed abscission. In order to drive the expression of *VviAGL11*, the *Cauliflower mosaic virus* (CaMV) 35S promoter instead of the endogenous promoter of *AtAGL11* was used in the constructions. The *stk* transformants (T_1 generation) with pVviAGL11-OX and pVviAGL11-OX^{mut} restored the wild-type phenotype with both constructions (Fig. 7). The number and seed size increased, the silique achieved its normal size, and the typical morphology of the funiculus was also restored (Fig. 7D). Studies with the *stk* single mutant demonstrated that the funiculus is longer and thicker, wherein *AtAGL11* is also responsible for controlling cell expansion and cell division in this structure (Pinyopich *et al.*, 2003). *VviAGL11* overexpression in *stk* plants demonstrates that the ectopic expression of this gene was able to restore *stk* to the wild-type phenotypes for siliques, seeds, and funiculus (Fig. 7C). These results show that *VviAGL11* is responsible for the control of seed morphogenesis in a similar manner to Arabidopsis *AtAGL11*, providing evidence of an orthology relationship between them. The differences in the coding region conferred by *VviAGL11 SUmut* (*SUmut*) had no effect during silique and seed development in the transformed T_1 *stk* mutant plants.

The results showing that *VviAGL11* is down-regulated in the apirenic genetic background despite having a wild-type-like

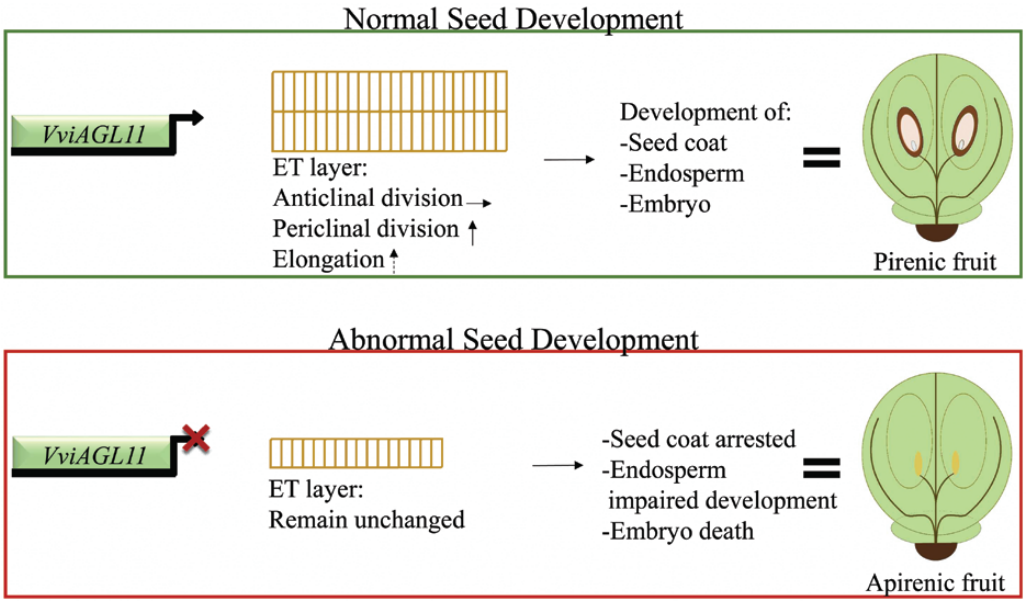

Fig. 8. The effect of *VviAGL11* on grapevine seed development. When *VviAGL11*expression is increased in the ovule after fertilization, the endotesta (ET) layer undergoes several modifications such as cell elongation,and cell duplication in anticlinal and periclinal directions. The correct development of this layer directs the further development of the seed coat, the endosperm, and, consequently, the embryo. Therefore, the seeds have a normal development, resulting in a seeded fruit. However, when *VviAGL11* expression remains low, the ET layer remains without the expected modifications, affecting the development of the seed coat and the endosperm, and causing embryo death. Thus, the final seed have an abnormal development, referred to as seed trace, resulting in an apirenic (seedless) fruit. (This figure is available in colour at *JXB* online.)

allele raises questions about its regulation and indicates that the mutant allele (*VviAGL11 SUmut*) has a dominant effect in the resulting phenotype, excluding the possibility that the seedless phenotype might be caused by variations in its promoter region as previously proposed by Mejía *et al*. (2011). Based on the general gene structure of the MADS-box genes reported in the literature and the data gathered in this work, our hypothesis is that the CArG-boxes present in the second intron may play a role in *VviAGL11* regulation. In addition, based on the genetic data obtained from sequencing of the *VviAGL11* alleles and in the transcription profile of this gene, we suggest that VviAGL11 protein may self-regulate *VviAGL11* transcription. *AtAGL11* direct targets are VDD (VERDANDI), BAN (BANYULS/ANTHOCYANIDIN REDUCTASE), ABS (ARABIDOPSIS B SISTER), and EGL3 (ENHANCER OF GLABRA3) genes, but unfortunately there are still no data referring to or testing this class D gene self-regulation (Matias-Hernandez *et al.*, 2010; Mizzotti *et al.*, 2014). Experiments involving the functional study of the polymorphisms between 'Chardonnay' and 'Sultanine' and analysis of VviAGL11 proteins, derived from different alleles of *VviAGL11*, are under way in order to test the *VviAGL11* self-regulation hypothesis.

We propose that the lack of increased expression of *VviAGL11* during berry development is responsible for the abnormal development of the seeds. The extremely small final seed size results from the non-differentiation of the ET seed layer, causing the cessation of elongation, division, and duplication of this layer and, consequently, of the lignified seed coat. Moreover, with the loss of identity of the ET layer, the endosperm ceases its growth, causing embryo death and seed trace formation (Fig. 8). Our results are in agreement with the hypothesis of Bouquet and Danglot (1996) for seed development, in which the inheritance of seedlessness in grapevine is based on a complex system whereby the expression of three independently inherited recessive genes is controlled by a dominant major regulator gene from a single dominant locus named *SdI*. The previous evidence reported by Mejía *et al*. (2011), Revers *et al.* (2014), and Ocarez and Mejía (2016) is now reinforced by the data gathered in this work, allowing affirmation that the *SdI* locus phenotype effect is derived from the *VviAGL11* function during seed morphogenesis. Taken together, our results provide functional evidence to show that *VviAGL11* transcription is essential for seed morphogenesis in grapevine during berry development. Despite the valuable literature dedicated to seedlessness in grapevine, our work presents new and solid data towards the understanding of the molecular mechanism driving grapevine seed development with high potential in agriculture use in the generation of seedless grape varieties.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Complete alignment of *VviAGL11* alleles.

Fig. S2. Alignment of the coding regions of *VviAGL11* alleles.

Fig. S3. *In situ* hybridization of the *VviAGL11* sense probe (control) in 'Chardonnay'.

Fig. S4. *In situ* hybridization of the *VviAGL11* sense probe (control) in 'Sultanine'.

Fig. S5. Plants of *Arabidopsis thaliana* under GFP evaluation.

Table S1. Primers designed for the amplification and sequencing of *VviAGL11*.

Table S2. Stages of development of grapevine reproductive organs.

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Author contributions

LFR and JM conceived the original screening and research plans; LFR, VB, MCD, MMP, GP, and JEAM supervised the experiments; JM and VB performed most of the experiments; MCD, MLG, and JEAM provided technical assistance to JM and VB; JM, VB, LFR, and MCD designed the experiments and analyzed the data; JM conceived the project and wrote the article with contributions of all the authors; LFR supervised and complemented the writing.

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