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 apiernity, 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; Mejia et al., 2011). The Sultanine cultivar is the main donor of the stenospermocarpy 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; Cabezás et al., 2006; Mejia 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 VviAGL11 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 (Mejia 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; Mejia et al., 2011; Revers et al., 2014; Ocañez and Mejia, 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 apieneric 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 Baggioni (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 Baggioni (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 (Università 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 manufacturer. 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 (GAATACGACTCATAAGG) 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
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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 GATTCCCTGGC-3', VviAGL11R 5'-AGCAACTCATG CATTCTTCGACC-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 MgCl₂, 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.
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. Of \( VviAGL11 \) transcripts was calculated normalizing the amount of mRNA against an \( ACTIN \) fragment (\( \text{TransF} \) 5'-CTGGGAACTACTCACACATTA-3' and \( \text{TransR} \) 5'-TTACCCGAGATGGAGGACCTTCTTAC-3'). Relative enrichment of \( VviAGL11 \) transcripts was calculated by the method of Pfaffl (2001) employing \( ACTIN \) as reference.

Seed morphoanatomical assay

Seeds and seed traces were transferred into a fixation solution under vacuum (Medowell 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 \( 5\mu \)m 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 submersed 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 (\( \sim 600 \text{mmHg} \)) 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 \( \mu \)m) 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′-ATTGTTCATCTGGGGACCTTCTTAC-3′) and \( VvAG3R \) (5′-GGAGATGAAGTTGCGGATATG-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 phosphatase 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 \textit{in situ} hybridization analysis for two consecutive years.

Functional complementation analysis in Arabidopsis

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) \( pVviAGL11-OX_{\text{mut}} \), an overexpression plasmid harboring the \( VviAGL11 \) ‘Sultanine’ mutant allele (\( S\text{mut} \)). Both constructions had the same coding region size (672 bp) besides the SNPs in the \( S\text{mut} \) 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 \( VviAGL11\text{CDSF} \) 5′-CACCATGGGGAGAGAATCGTCTG-3′ and \( VviAGL11\text{CDSR} \) 5′-TTACCCGAGATGGAGGACCTTCTTAC-3′. Employing the Gateway® LR Clonase TM II Enzyme Mix recombination system (Invitrogen), the insert was transferred into the pHWG2D 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 \( T1 \) 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 \( T1 \) plants was analyzed by PCR using specific primers targeting the plasmid promoter and the \( VviAGL11 \) coding region (\( \text{TransF} \) 5′-CTGGGAACTACTCACATTA-3′ and \( \text{TransR} \) 5′-CATTTGGGAGAACCTCGG-3′). Only \( T1 \), 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 apicenric and seeded grapevines, allelic variations were amplified by PCR with specific primers from genomic DNA extracted from the apicenric ‘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 VMCTF2 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 pro-
tein 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 seven-
th 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 identifica-
tion 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 nucle-
otides (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); how-
ever, 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 sam-
pies extracted from the reproductive organs of ‘Chardonnay’
and ‘Sultanine’ at critical fruit development stages was per-
formed. 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 com-
pared with berries at fruit set and 65-fold higher when com-
pared 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).
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. 5I) 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 T1 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.
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); (iii) 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 T1 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
These results demonstrate that the ectopic expression of \textit{VviAGL11} is capable of restoring siliqua and seed morphogenesis in the Arabidopsis \textit{stk} background and that very similar genes have a relatively well conserved function \textit{in planta}, gathering evidence to support an evolutionary orthology relationship between \textit{AtAGL11} and \textit{VviAGL11}.

**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 \textit{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 \textit{VviAGL11} during seed development in grapevine. Therefore, our approach was to explore a combination of methods such as \textit{de novo} sequencing, gene expression, and ISH, combined with anatomical examination and complementation analysis of the Arabidopsis \textit{stk} mutant to gather evidence to address the function of \textit{VviAGL11} during seed morphogenesis.

\textit{De novo} sequencing based on a PCR strategy of \textit{VviAGL11} allelic variants from ‘Sultanine’ and ‘Chardonnay’ was performed, allowing the identification of a group of polymorphisms that allowed the identification of four \textit{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
acid modifications in ‘Sultanine’ 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)6GG] 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
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 (Diaz-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 *SEPALATA* genes of grapevine (*VvSEP1*, *VvSEP2*, *VvSEP3*, or *VvSEP4*) as previously described by Boss et al. (2002) and by Diaz-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 Peyraud, 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 in 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 (Mejia 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 absorption. 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 (T₃ generation) with pVviAGL11-OX and pVviAGL11-OX<sup>mut</sup> 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 T₁ stk mutant plants.

The results showing that *VviAGL11* is down-regulated in the apirenic genetic background despite having a wild-type-like
allele raises questions about its regulation and indicates that the mutant allele \((VviAGL11 \text{ } S\text{ } \text{Smut})\) 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.

![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.)](jxb.oxfordjournals.org/content/suppl/2017/04/12/oxy045.7.Tblifecols4182.s4.D11x1551fig8.png)
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, MPP, GP, and JEAM supervised the experiments; JM and VB performed most of the experiments; MCD, MG, 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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