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Manipulation of *VviAGL11* expression changes the seed content in grapevine (*Vitis vinifera* L.)

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ABSTRACT

Seedlessness in grapes is a desirable trait, especially for *in natura* consumption. Previously, we showed that *VviAGL11* is the main responsible gene for seed morphogenesis in grapevine. Here we tested the function of this gene in grapevine with the use of plant plasmids. *VviAGL11* was cloned into silencing and overexpression versions of p28iIR plasmid. Reproductive grapevine bunches from different seeded and seedless cultivars were separately treated with *VviAGL11*-harboring plasmids, along with controls. Plasmids were detected in leaves after a month of treatment, and berries, leaves, stems and seeds were analyzed for ectopic gene expression by RT-qPCR after 90 days of plasmid injection. Fruits from the seedless 'Linda' treated with the VviAGL11-overexpression plasmid showed high expression levels of *VviAGL11* and exhibited small seeds that were not found in the untreated control samples. Mature grapes from seeded 'Italia' and 'Ruby' bunches treated with the VviAGL11-silencing plasmid showed decreased *VviAGL11* expression, reduced number of seeds and increased number of seed traces. The present study confirms that *VviAGL11* is a key master regulator of seed morphogenesis in grapevine and corroborates with the applicability of plant plasmids as promising biotechnological tools to functionally test genes in perennial plants in a rapid and confident way.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the major and oldest cultivated plant species worldwide [1], covering an area of 7.5 million hectares [2–4]. Its world production increased from 59.74 million tons in 1990–73.7 million tons in 2014, of which 2.4 tons were table grapes. The large and increasing consumption of table grapes is accompanied by an increasing demand for better quality, not only relative to the visual aspect of fruits, but also focused on taste, flavour and consistency of berries [5,6]. Moreover, the market for table grapes is expanding its preference for seedless grapes [7–9].

The absence of seeds in grapevine, called apyreny, results from two distinct processes named stenospermocarpy and parthenocarpy. In the process of stenospermocarpy, predominantly selected in breeding programs of table grapes, the development of embryo and endosperm starts after fertilization but the endosperm tissue degenerates prematurely, leading to the production of fruits containing seed traces [10]. In parthenocarpy, the ovary develops into a fruit without fertilization, i.g., fruits develop from maternal tissues only, and therefore have no seeds [11,12]. A plant is considered apirenic when it is capable of producing (i) seedless fruits, (ii) fruits with seed traces or (iii) fruits with seeds reduced in number and size [12]. To determine the phenotype of seedlessness, a particular grapevine cultivar is classified by the International Organization of Vine and Wine (OIV) standard descriptor 243, according to which cultivars with a rate of less than 30 mg/seed are considered apirenic. Other useful scale was proposed by Bouquet and Danglot [11] in which the total seed dry matter percentage should be under 40% to be consider as a seedless cultivar.

In silico analysis of the grapevine genome allowed the identification of a candidate gene related to seed morphogenesis called *Vitis vinifera AGAMOUS-LIKE 11* (*VviAGL11*), mapped at the same *Seed development Inhibitor* (*SdI*) locus where the microsatellite molecular marker VMC7F2 was located. VMC7F2 is a highly polymorphic microsatellite marker associated to seedlessness in grapevines with discrimination efficiency

Abbreviations: OIV, Organisation Internationale de la vigne et du Vin; SdI, seed development inhibitor; STK, seedstick; TYLCV, Tomato Yellow Leaf Curl Virus; RNAi, silencing; OX, overexpression; NT, non-treated; GFP, green fluorescent protein; GUS, beta-glucuronidase protein

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Table 1
Experimental overview of the treated cultivars. The cultivars used in this study, their respective phenotype concerning seed presence and the genotype data for *VviAGL11* alleles. In this work, we used three sets of plant plasmids: the experiment control set (pIR + p1470), the *VviAGL11* overexpression set (pIRVviAGL11OX) and the *VviAGL11* silencing set (pIRVviAGL11RNAi). The number of bunches treated for each cultivar and the final number of bunches evaluated were accounted.

Cultivar	Phenotype	Plasmids	Number of treated bunches	Number of evaluated bunches
Prosecco	Seeded	pIR + p1470	6	6
Alvarinho	Seeded	pIRVviAGL11RNAi + p1470	12	12
Chardonnay	Seeded	pIRVviAGL11RNAi + p1470	13	10
Italia	Seeded	pIRVviAGL11RNAi + p1470	10	10
Moscato giallo	Seeded	pIRVviAGL11RNAi + p1470	19	12
Pinot Noir	Seeded	pIRVviAGL11RNAi + p1470	24	15
Ruby	Seeded	pIRVviAGL11RNAi + p1470	9	9
Trebbiano	Seeded	pIRVviAGL11RNAi + p1470	15	14
Clara	Seedless (scar trace)	pIRVviAGL11OX + p1470	9	5
Linda	Seedless (scar trace)	pIRVviAGL11OX + p1470	13	13
Sultanine	Seedless (seed trace)	pIRVviAGL11OX + p1470	3	0

[13–15]. The *VviAGL11* gene (genome access http://genomes.cribi.unipd.it/grape/- Vv18s0041g01880) demonstrated to be an ortholog of the *AtAGL11/STK* gene [12], known to have an important role in seed development in *Arabidopsis* [16,17]. The analysis of the *VviAGL11* transcriptional profile in grapevine showed a large accumulation of transcripts in developing seeds at two, four and six weeks after fruit set, whereas in apirenic varieties, there was an extreme depletion of its expression in all berry tissues evaluated [12,18,19]. *VviAGL11* seems to regulate the differentiation of the endotesta layer of the seed coat. In the absence of *VviAGL11* expression, the endotesta duplication and elongation fail to occur, and the seed development is arrested, forming a seed trace-like structure [12].

Fruits are a major source of fibre, nutrients, and antioxidants which are essential for a healthy diet [3,8,20]. Therefore, fruit crops play a key role in the economy of many countries. Efforts have been made to improve the quality of fruits and crops by using both conventional breeding and genetic transformation approaches [21,22]. Genetic engineering and other biotechnologies to improve plants are mostly dependent on efficient procedures of cell and tissue culture, especially for the obtainment of regenerants via organogenesis or somatic embryogenesis. Tissue culture is laborious and time-consuming. It typically allows the regeneration of only a few lines of genetically modified plants [23]. The grapevine breeding and improvement of commercial cultivars are based on sexual reproduction, vegetative propagation, and somatic mutations [1]. The genetic breeding of grapes is highly dependent on genetic markers and genome association studies which take years in order to obtain segregant generations by crossings. The selection of a specific phenotype, particularly a berry trait, is typically a long process considering the juvenile period (three-to-five years) of grapevine plants. Breeding tools that may be readily applied to cultivated varieties are therefore of great interest, reducing the need for developing new cultivars by crossings that inevitably decharacterizes the original genotype. Grapevine genetic transformation is very inefficient, leading to success rates lower than 10% [22,24,25], and it is highly dependent on the genotype used.

Advances have been made in the plant biotechnology field that may help to circumvent many limitations of the traditional techniques. One of the most interesting and promising new tools, especially for perennial plants, is the expression of genes based on episomal plasmids, such as the IL-60 platform [26]. This platform consists of a universal system of virus-derived vectors for the overexpression or silencing of genes directly in adult plants, opening possibilities for trait delivery in woody species. The IL-60 system is derived from the Tomato Yellow Leaf Curl Virus (TYLCV), a dsDNA that is able to replicate in plant cells and spread to other tissues after inoculation. This so called “plant plasmid” is mechanically introduced into plant tissues and the expression and replication of the episomal DNA occur in a few days. It becomes stable and systemic, eliminating the need for selectable markers, being useful for treating grafting stocks prior to transfer to the field [26–28]. The system is compatible with many species, including woody fruit trees such as grapevine, citrus, and olive [28,29]. Due to the lack of heritability of the episomal DNA, biosafety control might be easier than that advised to conventional genetically modified plants that undergo pollen-mobile genetic transmission.

In the present study, we aimed to functionally characterize the *VviAGL11* gene in grapevine by its overexpression in seedless cultivars and via silencing through RNAi in seeded genotypes, demonstrating its key role as a determinant of the fruit seed/seedless trait. We describe the successful use of virus-derived plant plasmids as a powerful tool for gene functional characterization and as a fast delivery method of valuable traits in grapevine. With this strategy, we were able to detect the systemic presence of the plant plasmids in grapevine plants and observe changes in *VviAGL11* expression pattern on treated plants. More interestingly, we showed that the overexpression of the *VviAGL11* in apirenic grapevine cultivars was able to partially restore seed development and morphogenesis whereas inhibition by *VviAGL11* silencing in pirenic cultivars resulted in the opposite effect, i.g., seeds were abnormally formed, reduced both in number and size.

2. Methods

2.1. Plant material

Six-to-eight years old grapevine plants of 11 cultivars were selected in fields from the Serra Gaúcha region, Rio Grande do Sul, Brazil. Three plants of each cultivar were transferred into greenhouse pots, after top and root pruning, during winter time. The greenhouse experimental areas were located at Embrapa Uva e Vinho in Bento Gonçalves, Rio Grande do Sul, Brazil (29°09′48″S, 51°31′42″O and 616 m altitude). The cultivars were grafted on Paulsen 1103 rootstock, planted on pergola trellising system and managed with conventional annual pruning and control of pests and diseases. Cultivars assayed were the pirenic ‘Prosecco’, ‘Alvarinho, “Chardonnay”, ‘Italia’, ‘Moscato giallo’, ‘Pinot Noir’, ‘Ruby’, ‘Trebbiano’ and apirenic ‘Clara, “Linda” and ‘Sultanine’ (Table 1). Grapevine bunches chosen for treatment with plant plasmids were at the phenological scales D, G and H1 (Supplementary Fig. 1), according to Baggiolini [30]. Plants from the same cultivars were also kept under the same conditions and used as controls plants, in which no treatment was performed.

2.2. Vector construction and preparation

VviAGL11 DNA fragments were amplified via PCR from a previous cloned ‘Chardonnay’ seed cDNA [12]. Primers *VviAGL11CDSF* 5′CACC ATGGGGAGAGGAAAGATCG3′ and *VviAGL11CDSR* 5′TTACCCGAGAT GGAGGACCTTCTTATC3′ were defined, allowing the amplification of a 676 bp fragment that was subsequently cloned into the overexpression plasmid pH7WG2D ([31]; Ghent University, Ghent). Primers

VviAGL11F 5′CACCATTGTTCATCTGGGCATTTCG3′, and *VviAGL11R* 5′ GGAGATGAAGTTGGCGGATA-3′ were designed to amplify the initial portion of *VviAGL11* gene. with. The resulting 110 bp amplicon was cloned into the silencing plasmid pH7WIWG2D ([31]; Ghent University, Ghent). All PCR amplifications reported in this study were performed with 10–25 ng of grapevine genomic DNA using Platinum® Pfx DNA polymerase (Applied Biosystems, Foster City) and reaction conditions recommended by the manufacturer. The 35S CaMV-*VviAGL11* cassette and the *VviAGL11* hairpin were respectively transferred from pH7WG2D and pH7WIWG2D to the plant plasmid pIR (Morflora) using the restriction enzymes combinations HindIII/XbaI and NsiI/BglII. Final constructs were confirmed by sequencing in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using primers *pIR*F1 5′TTGCTCACATG TAATG3′ and *pIR*R1 5′ AGTTTTTTGTCTTGCA3′). All primers were synthesized by Integrated DNA Technologies (IDT). The final piIR-derived vectors were named piIR-*VviAGL11*-OX (overexpression) and piIR-*VviAGL11*-RNAi (silencing). Both plasmids were prepared in large amounts and purified using the PureLink® HiPure Plasmid Filter Maxiprep Kit (Thermo Fischer Scientific) following manufacturer's instructions. Solutions combining the p1470 helper plasmid with either piIR-*VviAGL11*-OX or piIR-*VviAGL11*-RNAi were prepared to a final concentration of 20 ng/μL of each plasmid

2.3. Grapevines bunches treatment with plant plasmids

Plant plasmids administration was performed at the base of the grapevine bunch peduncle with a 1 mL hypodermic syringe armed with a needle. A 1:1 proportion solution with 2 μg of the helper plasmid (p1470 [27]) plus 2 μg of the *VviAGL11* overexpression vector (pIRVviAGL11OX) or of the *VviAGL11* silencing vector (pIRVviAGL11RNAi) in was injected in each bunch. The treatment in the peduncle was performed at early D or G stages of development as shown in Supplementary video 1 and Supplementary Fig. 1. The treatments with the plant plasmids were repeated after three days, when bunch development was at the H1 stage, to guarantee maximum plasmid distribution in the growing bunches. Grapevine cultivars, combination of plasmids, number of bunches injected and analyzed are presented in Table 1. A total number of 133 bunches were treated with plasmids solutions, and more than 9.000 berries were analyzed molecular and phenotypically. At six weeks of fruit development, three bunches of each cultivar were sampled. Thereafter, all berries from the middle section of the bunch were picked and mixed. Thirty randomly sampled berries were pooled to constitute three representative samples (10 berries each). The replicates were stored into 50 mL Falcon tubes in −80 °C for later RNA extraction and RT-qPCR reactions. At berry maturation, all bunches available for each cultivar were harvested. Posteriorly, all the berries from the bunches were collected and mixed. Three replicates of 100 berries each were used for phenotypic analysis (300 berries per cultivar/per treatment, divided in three replicates). For anatomical purposes, seeds representing distinct phenotype variants were used as described later on this section.

2.4. Reporter genes analysis

GFP: Leaves from grapevine plants (1 m high) were treated with a pIRGFP + p1470 combination in a concentration of 200 ng/μL. The visualization of the GFP fluorescence was obtained in a stereo microscope equipped with a GPF filter (Leica 165FC).

GUS: Bunches from adult grapevines were treated with a pIRGUS + p1470 combination in a concentration of 1 μg/uL. GUS histochemical assays were performed in different organs of the grapevine plants (berry, seed, leaf, stem, pollen). The tissues were incubated in 1 mM X-Gluc, 100 mM phosphate buffer (pH 7.0), 2 mM KH2Fe, and 0.5% Triton X-100. The samples were incubated for 16 h at 37 °C. Thereafter, the tissues were incubated in 70% ethanol for chlorophyll removal.

2.5. Plant DNA/RNA purification

Genomic DNA was extracted from leaves and fruits at six weeks of development after fruit establishment (fruit set), and from seeds after dissection from fruit pulp following the protocol of Lefort and Douglas [32]. RNA extraction from the same organs was performed by LiCl precipitation using the Zeng and Yang [33] protocol scaled to 2 mL micro centrifuge tubes. Each sample extraction was performed in triplicate and final volumes were pooled before the LiCl precipitation step. Genomic DNA was removed using the TURBO DNA-free Kit (Ambion, Foster City) according to the manufacturer's protocol. RNA integrity and quantity were monitored by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

2.6. RT-qPCR analysis

For *VviAGL11* expression analysis complementary DNAs were synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City) according to manufacturer's instructions. We used biological for each cultivar, and also technical quadruplicates for the RT-qPCR evaluation. The gene-specific primers *VviAGL11F* 5′CACTTAAT GGGTGATTCCTTGGC3′ and *VviAGL11R* 5′AGCAACTCATGCTTCTTCG ACC3′ were designed with the Oligo Analyzer 3.1 tool (IDT, http://www.idtdna.com) and synthesized by IDT, with the standards settings of 0.2 μM of oligo concentration, 1.5 mM of $MgCl_2$ and 0.2 mM of dNTP. The evaluated tissues were leaves and fruits at six weeks of development after fruit establishment (fruit set). Seeds were dissected from the pulp in seeded cultivars samples and the whole berry was evaluated in seedless cultivars. For the plasmid quantification on berries and seeds, we used a serial standard curve produced by pIRVviAGL1OX (at initial concentration of $1{,}2.10^{-9}$g), diluted in five magnitude orders: p1 (10^{-9}), p2 (10^{-10}), p3 (10^{-11}), p4 (10^{-12}) and p5 (10^{-13}). 5 ng of total DNA from each sample tested were used for the quantitation assay. All RT-qPCR were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City). SYBR Green (Invitrogen, Carlsbad city) was used to monitor dsDNA synthesis. 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 expected size in 1% ethidium bromide-stained agarose gel electrophoresis and sequencing of the amplicons. Primer efficiency was calculated by LinRegPCR (version 11.0 [34]). Mean relative gene expression was calculated by the Pfaffl [35] method employing *ACTIN* (GenBank EC969944), *EF1-α* (GenBank EC959059), *GAPDH* (GenBank CB973647) and *α-Tubulin* (GenBank EC930869) as reference gene [36]. Statistical analysis was performed using Prism 6.1 with a Parametric paired T test.

2.7. Characterization of VviAGL11 among grapevine cultivars

Genomic DNA was extracted from leaves following the protocol from Lefort & Douglas [32]. Grapevines cultivars were genotyped by PCR and sequencing with the employment of primers *VviAGL11 F10* 5′GCAAAGCAGTTATTGAAAGC*3′* and *VviAGL11 R15* 5′GATGGGAGAA AATATCCGCC*3′* for PCR. Amplification products were purified and sequenced with primers *VviAGL11 F14* 5′*CGTATATCTCCGAACCAAG3′* and *VviAGL11 R14* 5′*GCTTAATGAGACATTTCAAGCC3′*. Primer design and synthesis were described previously. This strategy enabled us to observe *VviAGL11* SNPs in the CDS region for the determination of *VviAGL11* allele's composition. The products were sequenced in an ABI Prism® 310 Genetic Analyser (Applied Biosystems, Foster City) using standard sequencing protocols described in Falavigna et al. [37]. Sequence analysis was carried with DNA Sequencing Analysis Software v5 (Applied Biosystems, Foster City) and MEGA7 software (http://www.

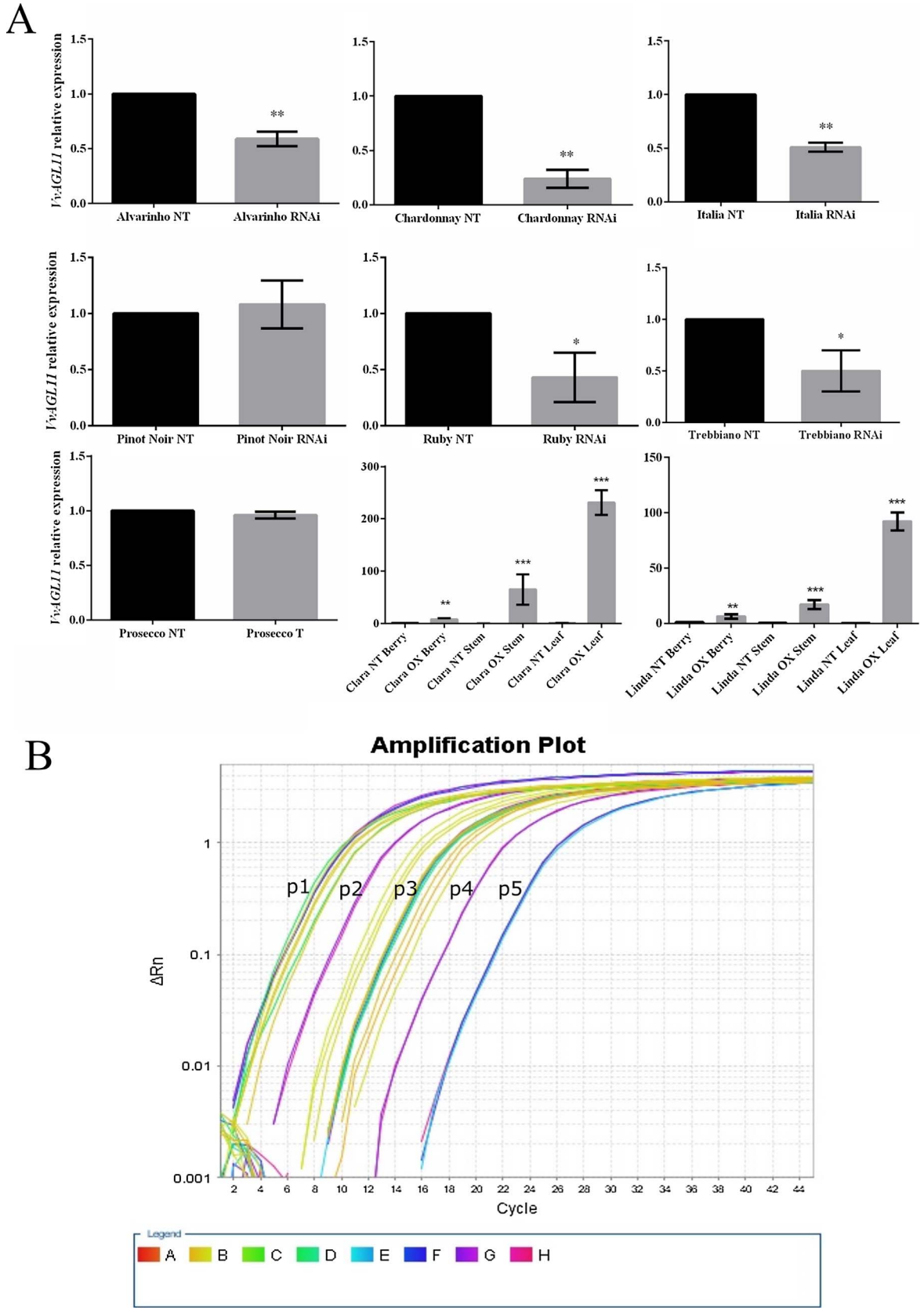

Fig. 1. *VviAGL11* relative expression and vector detection after treatment with plant plasmids. (A) The silenced cultivars, 'Italia' and 'Ruby', demonstrated a decrease in *VviAGL11* expression in seeds of six weeks of development. Linda cultivar demonstrated an accumulation of *VviAGL11* transcripts in berries, stem and leaves after six weeks of fruit development. Prosecco was used as a control. Bars show standard deviation. *α-Tubulin*, *GAPDH* and *EF1-α* were used as reference genes. OX = Overexpression, RNAi = RNA interference, NT = non-treated and T = treated. Parametric paired T test = * p < ,05; ** p < ,001; *** p < ,00001. (B) Quantitative evaluation of pIRVviAGL11 vectors by RT-qPCR. The serial standard curve presents five points: p1 (10^{-9}), p2 (10^{-10}), p3 (10^{-11}), p4 (10^{-12}) and p5 (10^{-13}). The curves from berries and seeds from the treated grapevines shows the high amount of plant plasmids in the samples.

megasoftware.net/home). Sequences were compared with the grapevine reference ('Pinot Noir' PN40024) genome.

2.8. Seed morphological analysis

After grapevine bunches reaches maturity, quantitative and qualitative evaluations of seeds were carried out. The maturation stage of the bunches was determined by Brix degree (°Brix) evaluation in a digital refractometer (Abbe) model 1421 with ten berries from each sampled bunch. The measurements represent an estimate of the total soluble solids on the liquid (mostly sugar), which indicates the sugar content and the phenological maturation of the berries [38]. The total number of seeds and seed traces was estimated from an average of 300 berries from each treated and non-treated cultivar. After determining seed fresh weight, seeds were dried at 50 °C for three days and dry weight was measured, both in a Shimadzu AUX22 analytical balance. We followed the OIV standard descriptor 243 in which the fruit seedlessness classification refers to the seed/seed trace dry weight content, for the classification of resulting fruits of pirenic or apirenic. Statistical analysis was performed using the Prism 6.1 with a Parametric paired T test and $\chi 2$ test.

2.9. Seed morpho-anatomical assay

Fresh seeds and seed traces were transferred to a fixation solution under vacuum [39]. Thereafter, samples were dehydrated in an increasing gradient of ethanol [40] and embedded in 2-hydroxyethyl methacrylate resin [41]. The 8 µm sections were obtained in a Leica RM 2255 microtome. The metachromatic reagent Toluidine Blue O [42] was used to determine seed structure. Slides with sections were submerged in the reagent for 1 min, washed with water and dried on plate at 45 °C. The photographs were obtained with the support of ZEN microscope software from ZEISS.

3. Results

3.1. Reporter gene experiments (GFP and GUS)

Prior to the experiments with adult grapevines, we evaluated the capacity of these vectors to multiply themselves in grapevine leaves and express reporter genes. Our pilot test showed that the leaves were expressing the Green Fluorescent Protein (GFP) after one month of the treatment with pIRGFP + p1470 (Supplementary Fig. 2). Furthermore, we tested the expression of the Beta-Glucuronidase protein (GUS) by injecting a combination of pIR/GUS + p1470 vectors on bunches. After a month we were able to observe the blue staining in seed tissues (Supplementary Fig. 2). Nevertheless, we could not see a GUS activity in other organs evaluated, such as leaves, stem and pollen. We believe that these results are due to the place of injection since the treatments were made in the bases of the bunch peduncle and not in leaves. The absence of GUS activity in pollen samples is in agreement with the plant plasmids technology that states that its vector was not reported in the progeny of treated plants [28].

3.2. Plasmid DNA is systemically translocated and retained in grapevine

Seeded grapevine cultivars Alvarinho, Chardonnay, Italia, Moscato giallo, Pinot Noir, Ruby and Trebbiano, were treated with the *VviAGL11* silencing construction (pIRVviAGL11RNAi + p1470) whereas seedless cultivars Clara, Linda and Sultanine, were treated with *VviAGL11* overexpression construction (pIRVviAGL11OX + p1470). After a month of plant plasmid inoculation, leaves proximal to injected bunch peduncles were sampled for plasmid DNA detection. All treated cultivars resulted PCR-positive. 'Prosecco', randomly selected as the control cultivar and injected with the empty plasmid, was also PCR-positive for vectors presence after treatment with pIR + p1470 (Supplementary Fig. 3). We were able to confirm that all treatments combining different versions of pIR and the helper p1470 plasmid were fully efficient, demonstrating that plasmid DNA was translocated through plant tissues, demonstrating a sectorized branch systemic translocation in treated plants.

3.3. The introduced genes remained stable and functional in grapevine reproductive and vegetative tissues

The expression of *VviAGL11* in Clara and Linda apirenic cultivars was evaluated by RT-qPCR in leaves, stems, and berries. Compared to field plants of the same cultivars, treated 'Clara' and 'Linda' overexpressed *VviAGL11* in all evaluated organs. *VviAGL11* overexpression in 'Linda' ('Linda' OX) berries presented six times more *VviAGL11* transcripts compared to non-treated 'Linda' ('Linda' NT) berries. It was also possible to observe a 17-fold increase in *VviAGL11* relative expression in stems and, in leaves, the expression was up to 90-fold higher than non-treated samples (Fig. 1A). 'Clara' OX also showed a significant increase in *VviAGL11* relative expression. Compared to non-treated plants, the levels of *VviAGL11* mRNA in berries was eight times higher, whereas stems presented a 65-fold increase and leaves demonstrated a remarkable mean of a 231-fold increase in *VviAGL11* relative expression (Fig. 1A). These results show that the plant plasmid harboring the *VviAGL11* overexpression construct was effective in conferring high levels of *VviAGL11* expression in apirenic grapevines. We were able to observe that the introduced gene remained stable and functional after three months of plant treatments.

It was previously demonstrated that *VviAGL11* transcripts accumulate at higher levels in seeds of two, four and six weeks after fruit set [12,19]. We, therefore, evaluated the expression levels of *VviAGL11* in seeds of pirenic cultivars Alvarinho, Chardonnay, Italia, Pinot Noir, Ruby, and Trebbiano treated with the silencing *VviAGL11* construct version (pIRVviAGL11RNAi + p1470) by RT-qPCR. The level of transcripts in plants silenced for *VviAGL11* was statistically lower than those observed in control plants, most of them presenting half or even lower levels of the *VviAGL11* normal expression (Fig. 1A). 'Chardonnay' showed the higher differences between the treated and the control plants. Moreover, 'Pinot Noir' was the only genotype that presented similar levels of *VviAGL11* expression relative to the control plants. The Prosecco plants, treated with an empty vector as control, showed similar levels of *VviAGL11* expression between seeds from treated and non-treated plants (Fig. 1A).

Furthermore, we also quantified the amount of plant plasmids in berries and seeds of the treated cultivar (Fig. 2B). When we compare the amount of plant plasmids in the total DNA extracted from the samples, it is clear that there is a correlation between vector quantity and gene expression. The samples from the cultivars Linda, Clara, Italia and Ruby presents the highest amounts of plant plasmids, almost 1/80 of the total DNA (Supplementary Table 1). These are some of the cultivars that had the most drastic phenotype modifications on their seed content: Linda presenting miniseeds after treatment; and Italia and Ruby presenting significate amounts of seed traces and less total seed dry weight. For the others cultivars, including Prosecco, the rate of plant plasmids versus total DNA varied between 1/600 −1/800.

3.4. Manipulation of VviAGL11 expression affects seed morphogenesis in several grape cultivars

Our study aimed to assess the phenotypic effects of altered *VviAGL11* expression on seed characteristics of each grapevine cultivar. Therefore, we analyzed the mature bunches of treated cultivars and their respective non-treated controls. The phenological maturation stage of berries was estimated by the determination of the °Brix from each bunch sample. Because berries from all cultivars exhibited °Brix degrees above 13, they were all characterized as belonging to the mature stage for final seed size evaluation (Supplementary Fig. 4).

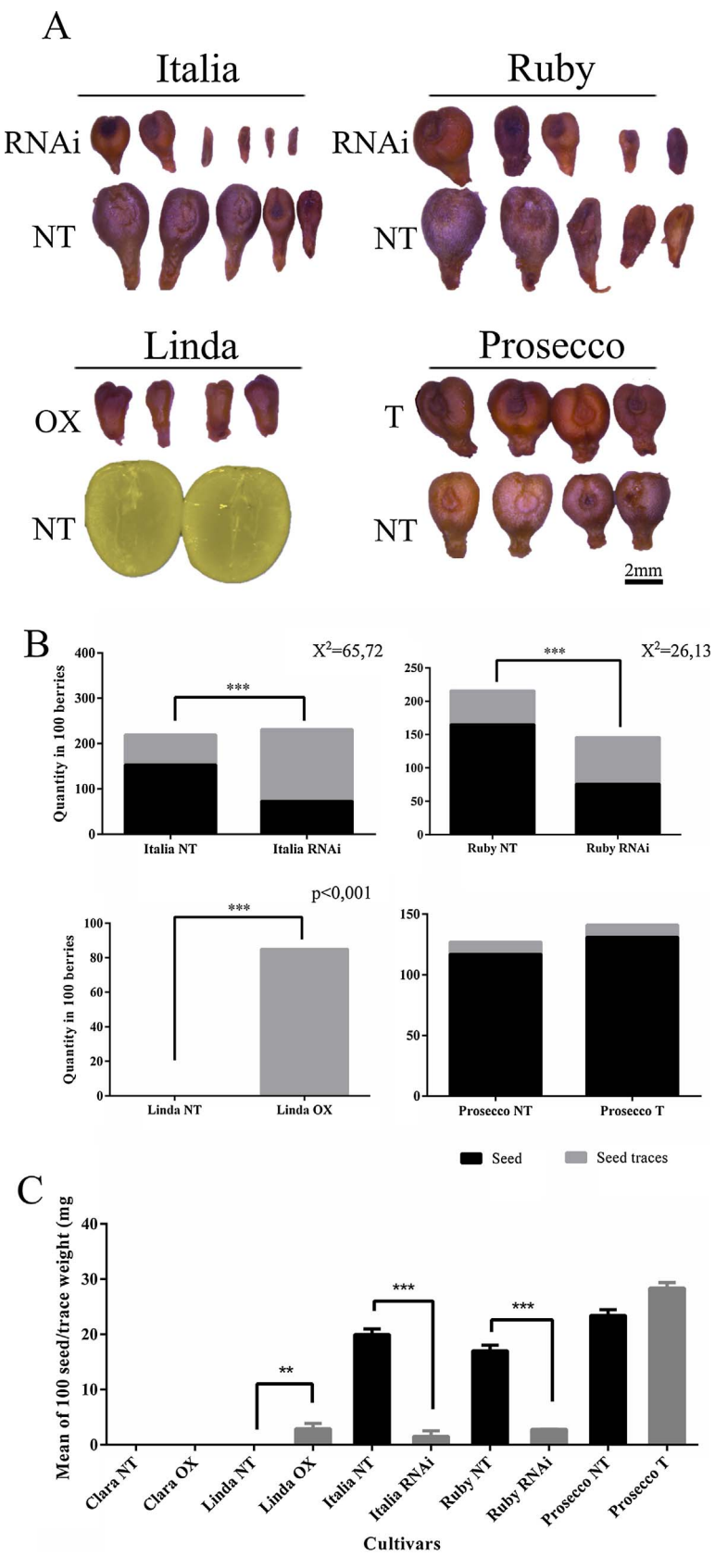

Fig. 2. Seeds and seed traces quantitative evaluation. (A) Seeds and seed traces morphological analysis. 'Linda' OX presents miniseeds that do not exist in 'Linda' NT. (B) The number of seed and seed traces was counted of 100 berries per cultivar. 'Italia' RNAi and 'Ruby' RNAi present an increased number of seed traces and fewer seeds, while 'Linda' OX presents small seeds (counted as seed traces) that 'Linda' NT does not present. 'Italia', 'Ruby' and 'Prosecco' $\chi 2$ test * p < ,05; ** p < ,001; *** p < ,00001. (C) Dry seed/traces weight evaluation. Italia RNAi and Ruby RNAi had a significant decrease in dry seed weight. 'Linda' OX presents a dry seed weight that was nonexistent in 'Linda' NT. Prosecco was used as a control. OX = Overexpression, RNAi = RNA interference, NT = non-treated and T = treated. Linda Parametric paired T test = * p < ,05; ** p < ,001; *** p < ,00001.

Subsequently, mature bunches were evaluated for quantitative measurements including berry weight, seed/seed-trace number, fresh seed/seed-trace weight, and dry seed/seed-trace weight. The quantitative evaluation of seeds and seed traces showed that cultivars Alvarinho, Chardonnay, Italia, Linda, Pinot Noir, Moscato giallo and Ruby treated with the silencing version of the *VviAGL11*-harboring plasmid presented a significant reduction in all parameters analyzed between treated and non-treated plants (Fig. 2B and Supplementary Fig. 5).

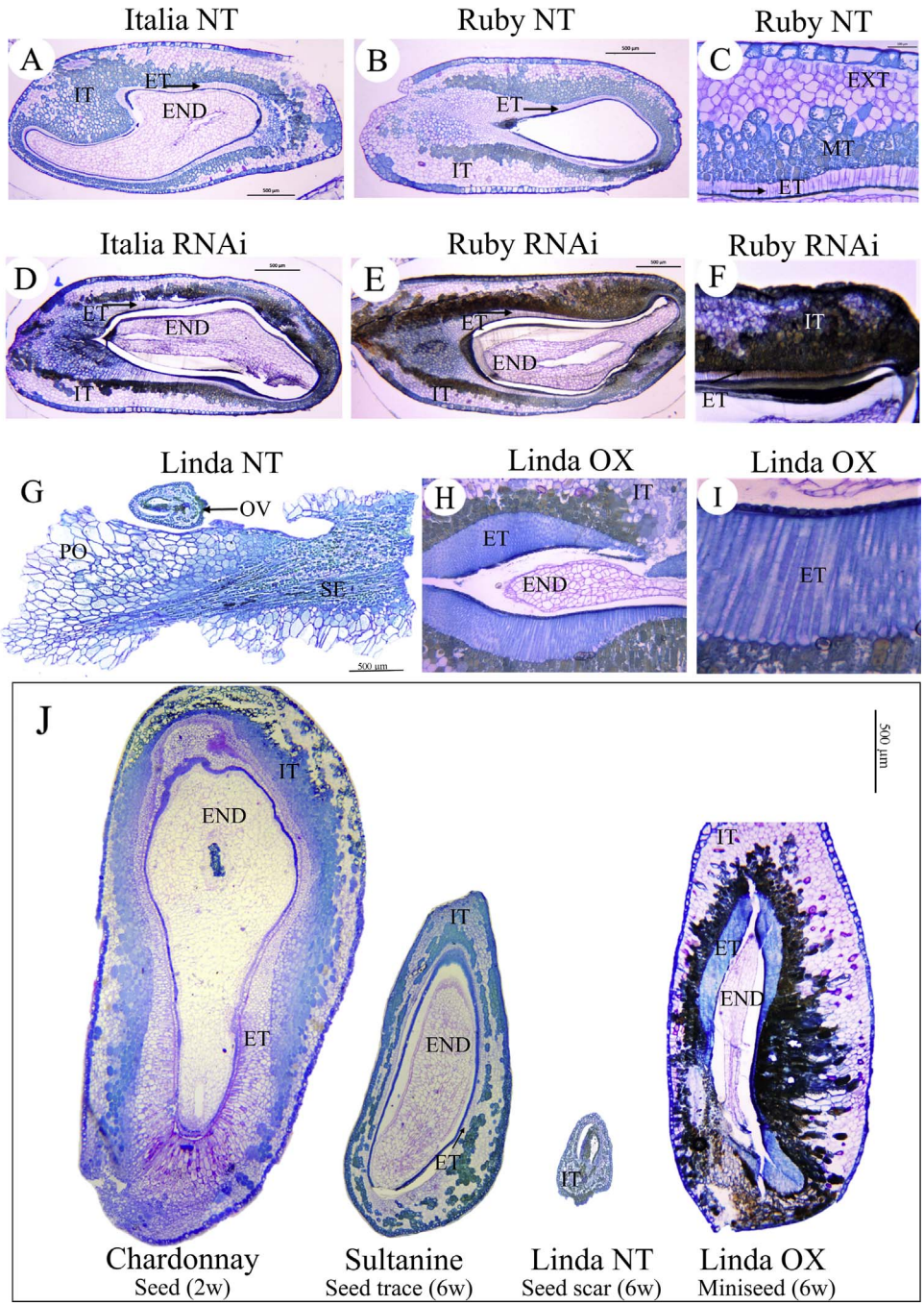

Fig. 3. Comparison of grapevine seed and seed trace morpho-anatomical structure. Seeds were sampled six weeks after fruit set. Light micrographs of grape seed cross sections were stained with toluidine blue O. (A) 'Italia' NT; (B) 'Ruby' NT; (C) 'Italia' RNAi; (D) 'Italia' RNAi; (E) 'Ruby "RNAi"; (F) 'Ruby' RNAi (G-I) 'Linda' OX. (J) Comparison between a normal seed of 'Chardonnay' with two weeks of development, a 'Sultanine' seed trace of six weeks after fruit set, the 'Linda' NT seed trace scar and the 'Linda' OX that presents a miniseed. Endosperm (END), endotesta (ET), mesotesta (MT), exotesta (EXT), integuments (IT), ovule (OV), polp (PO), septum (SE). Scale bar: 500 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Silencing of *VviAGL11* in the seeded cultivars Italia and Ruby rendered a visible and drastic decrease in their total seed dry weight with a reduced number of seeds and a concomitant increase in the number of seed traces (Fig. 2A). Consequently, the reduction in seed size and in number may allow 'Italia' RNAi and 'Ruby' RNAi to change their classification for the OIV-243 description, being considered seedless (apirenic) cultivars (Fig. 2A–C).

Overexpression of *VviAGL11* in the seedless Linda cultivar (Linda OX) resulted in the formation of miniseeds that were not present in the 'Linda' non-treated bunches. Its control, Linda NT, typically presents only seed traces that are almost unobservable to the naked eye (Fig. 2A). The diminutive seeds from 'Linda' OX berries were bigger in size than a seed trace, but they did not achieve a normal mature seed size. Despite the miniseed development in the 'Linda' OX berry samples, its total dry weight was still classified as seedless following the OIV-243 descriptor (Fig. 2C). This indicates that *VviAGL11* overexpression by plant plasmid delivery did affect seed size, increasing it, but it was not sufficient to restore the complete seed development in the Linda genotype background although it can partially restore the formation of the seed coat (Fig. 3G–J).

Finally, the quantitative evaluation of seeds and of seed traces in berries from cultivars Clara (OX), Trebbiano (RNAi) and Prosecco (control) showed no statistically significant differences in the number of seeds or traits comparing treated and untreated plants (Supplementary Fig. 5).

3.5. Seed traces from VviAGL11RNAi-treated plants are anatomically different from non-treated control plants

The seeds from 'Pinot Noir' and 'Chardonnay' were characterized in

previous studies with a particular pear triangular shape and composed of a cuticle, an epidermis, three layers of outer integument (exotesta, mesotesta and endotesta), two inner integument layers, endosperm and embryo [12,43]. The structure of the seed traces from 'Sultanine' was characterized by the undifferentiation of the seed coat inner layer – endotesta (ET), as a consequence of the absence of *VviAGL11* expression, that leads to endosperm and embryo abortion, forming a seed trace [12]. We conducted the morpho-anatomical characterization of seed traces resulting from the treatments of pirenic cultivars Italia and Ruby (VviAGL11RNAi treatment). These two cultivars showed the most significant differences in final seed size and dry weight after plasmid treatments for *VviAGL11* silencing.

In 'Italia' RNAi (Fig. 3D) and 'Ruby' RNAi (Fig. 3E), seed traces presented the same characteristics when compared to 'Italia' NT (Fig. 3A) and 'Ruby' NT (Fig. 3B). However, the internal layer of the seed coat in seeds from *VviAGL11*RNAi-treated plants exhibited an accumulation of phenolic compounds that were probably involved in the beginning of the lignification process (maximization in Fig. 3F). These cells are stained brown because of their polyphenols content. They are also present in the outer integument, both in the exotesta and in the endotesta layers (maximization in Fig. 3F). This concentration of phenolic compounds did not occur in non-treated seed traces from 'Italia' and 'Ruby' (Fig. 3C). Also, the endosperm and the embryo were not developed properly, as expected for a normal seed trace. We were able to observe that the ET layer neither elongated nor duplicated in most of the seed traces from 'Italia' RNAi and 'Ruby' RNAi (Fig. 3D and E). This suggests that *VviAGL11* induces modifications in the seed coat, initiating the lignification of the ET layer and, therefore, producing a high effect on seed development and maturation even without the normal formation of endosperm and embryo.

3.6. *VviAGL11 overexpression induces the development of the endotesta layer in 'Linda' miniseeds*

In order to evaluate the effect of *VviAGL11* overexpression in the 'Linda' seedless cultivar, we first performed an anatomical evaluation of the diminutive seed traces scars present in non-treated plants. These structures present minimal conducting vessels that were identified as septum cells surrounded by parenchyma cells derived from the pulp (Fig. 3G). Additionally, a structure composed of maternal tissues was identified as being the ovule. Within this structure, we were able to observe inner and outer integuments that never developed (Fig. 3G), and that could only be identified after microscopic examination.

'Linda' OX miniseeds presented the most interesting morpho-anatomical results when compared to the seed traces scars from 'Linda' NT. 'Linda' OX miniseeds presented the typical characteristics of seed trace concerning size and death of reproductive tissues, such as endosperm and embryo. However, the seed coat layers were found to be completely different from standard seed traces observed in 'Sultanine' [12]. The ET layer was duplicated and elongated (Fig. 3H and maximization on Fig. 3I). It was possible to observe the accumulation of phenolic compounds on the exotesta layer as well (Fig. 3H and I). The miniseeds presented a vulvar form due to ET layer overdevelopment (Fig. 3J). As a result, OX treated seedless plants showed a seed trace that was intermediary in terms of seed size and lignification profile, being bigger than a typical stenospermocarpic seed trace but, nevertheless, not achieving the size nor the shape of a wild-type like seed.

4. Discussion

Since the beginning of grapevine domestication, first by artificial selection and later by intercrossing and vegetative propagation, the main desirable traits selected were the ones involving fruit taste, size, and reduced seed size and number [44]. Unluckily, this was not the easiest trait combination to be achieved due to the seed effects on fruit size in grapevine [45]. Consequently, its improvement was and still is a laborious and time-consuming work. Furthermore, the molecular mechanisms underlying seed development in grapevine were not fully understood. Previous reports merely stated that the anatomical and morphological changes during normal seed development, from fertilization to maturity, involve three phases (i) rapid cell division; (ii) reserve accumulation and cell expansion due to water uptake; and (iii) slowing down growth and arrest of reserve accumulation [46].

By the time *VviAGL11* was confirmed as a master controlling gene of the seed development in grapevine [12], we then envisaged to use its sequence in a biotechnological approach. In the present study, we constructed vectors for *VviAGL11* ectopic overexpression and silencing through RNA interference. Overall, we treated more than 130 bunches and evaluated more than 9.000 berries from 11 grapevine cultivars. By detecting the plasmid DNA in different plant tissues and organs after months of the original injection, it was possible to confirm that 100% of the plants were multiplying the IL-60 plasmids systemically. Through the evaluation of the *VviAGL11* relative expression, 90% of the cultivars showed a direct correlation between gene expression and plasmid presence. 'Pinot Noir' plants treated with the overexpression construction was the exception, in which no differences in the expression of *VviAGL11* were observed comparing treated and non-treated seeds. This might be due to the phenological stage of the 'Pinot Noir' plants when treatments were performed. 'Pinot Noir' plants presented an early bud break and, at the time of treatment, bunches were at the H1 stage instead of D or G stages like the other cultivars. Therefore, the bunches were more developed. There are no studies suggesting the best combination of time and place of plasmid injection in order to optimize its movement to other tissues in grapevine. For the purpose of our study, plasmids had to reach the ovule integuments in time to allow the overexpression or the silencing of the *VviAGL11* gene during seed formation. It seemed to us that the best stage of bunch development for treatment was the first week after bunch appearance, during grapevine floral development and before ovule fertilization. This stage is typically achieved in a couple of weeks after bud break [30]. Nevertheless, there is room for optimizing the protocol in the DNA delivery strategy, assuring that plasmid constructs will reach the target tissues at the appropriate time.

Treatments with the *VviAGL11*-harboring plasmids led to quantitative and qualitative changes in seeds and/or traits in 78% of the cultivars. Three cultivars showed more evident modifications in the number and characteristics of seeds and/or seed traits. The seeded cultivars 'Italia' and 'Ruby' are two of the main commercialized table grapes in South America, contributing to the economic success of table grape varieties, but still lacking the desirable seedless trait. In the present work, 'Italia' and 'Ruby' were treated for *VviAGL11* silencing. The suppression of *VviAGL11* expression caused a reduction in their seed quantity in a way that their total dry weight decreased to a level acceptable as seedless. To access this information, the standard dry matter data of 100 seeds randomly sampled is used, classified in the following order: class 1 – very low ($\leq$10 mg/seed); class 3 – low (21–29 mg/seed); class 5 – average (36–44 mg/seed); class 7 – high (51–59 mg/seed) and class 9 – very high ($>$ 65 mg/seed) [47]. The varieties that fit in classes 1 and 3 are the most desired for table grape seedless selection. Both cultivars, after treatment, turned out to be considered as elite seedless because their dry weight from 100 seeds is less than 10 milligrams. Nevertheless, the small seeds could be sensed by chewing during berry consumption because of their unique seed coat development. These results show that the decrease of *VviAGL11* expression on the integuments was sufficient to interrupt normal seed development, and many seeds became seed traces. Furthermore, the analysis of control plants from 'Prosecco' allowed us to demonstrate that the empty pIR +1470 plasmids alone did not promote changes in treated plants, confirming that the modifications were caused directly by the overexpression or silencing of *VviAGL11*.

'Linda' (BRS Linda) is a seedless grape developed by the Embrapa Uva e Vinho breeding program. Its berry is elliptical, greenish, with a

tiny seed trace, practically invisible. Exploring the extreme seedless phenotype from this cultivar, it was possible to observe that ectopic *VviAGL11* overexpression during berry development partially restored seed formation, determining elongation and duplication of the ET layer in 'Linda' OX berry samples, leading to the development of miniseeds. Notwithstanding, the novel seeds from *VviAGL11*-overexpressed 'Linda' samples did not achieve a normal final size. Nevertheless, the morpho-anatomical examination of the miniseeds samples allowed to show the critical role of this gene in seed formation and growth, being able to 'open space' for endosperm growth and, consequently, embryo development. These results are in agreement with the findings reported by Garcia et al. [48], showing that integuments are able to control and determine the final seed size, due to the elongation of their layers, producing a strong maternal effect on seed growth and development.

Even though we detected the pIR DNA and also *VviAGL11* transcripts level modifications in seeds, only the ET layer was influenced by *VviAGL11* overexpression; and, as a maternal tissue, this layer does not remain in the progeny. This provides an important biosafety barrier for future field applications in grapevines and other plants. Peretz et al. [28] showed that when the progenies (up to F3) of treated tomato plants were evaluated, the pIR plasmid and p1470 were not present. As it was observed for the ASLV vectors [49], the TYLCV-derived plasmids are not heritable, and the progeny will be devoid of plasmid DNA.

VviAGL11, as a MADS-box protein class D, is able to form the so-called floral quartets protein complexes. In this case, MADS-box proteins will bind other proteins from the same family but different classes (A, B, C and E), which afterwards can bind to two *cis*-elements called CArG-boxes and activate gene transcription [50,51]. The formation of transcription factor dimers and multimeric complexes offers a mechanism to increase the diversity of possible DNA-binding proteins enormously [52]. The fact that only the ET layer of grapevine seeds and seed traces presented a contrasting development after the treatments and that *VviAGL11* is the single class D gene in grapevine [12,18] brings into light how important is the arrangement of the MADS-box proteins complexes in a certain temporal and spatial site. Nevertheless, another point to focus is the amount of VviAGL11 protein in the ET layer needed for its proper elongation and duplication. For normal seed formation, the expression of the *VviAGL11* gene should be around 15–25-fold higher relatively comparing to seedless cultivars in which this gene is expressed at a very low rate [12,19]. Regrettably, there is still no clear clue about the reasons for the low expression of *VviAGL11* in seedless cultivars. However, in our study, it was possible to see that the ET layer is actively responsive to the expression levels of this gene. With its overexpression provided by plant plasmids, a higher level of *VviAGL11* transcripts was present in 'Linda' OX samples, leading to the formation of miniseeds in a higher number and more developed than seed traces. This probably induced the overdevelopment of 'Linda' OX seed structure causing the duplication and elongation of the ET layer in a faster way and, consequently, initiating the lignification process. Because the timing of the expression of this key gene was not regulated by our approach, the lignification process was rapid and, consequently, stalled seed growth. These unusual processes formed a final structure that is no longer a seed trace and not even a normal seed, and therefore was named 'miniseed' (Fig. 3J).

The significant differences observed in *VviAGL11* transcript levels combined with the phenotypic seed modifications observed both in 'Italia' RNAi and 'Rubi' RNAi samples, and also the restored seed structure in 'Linda' OX, confirm the previous study of *VviAGL11* complementation of *stk* (*AtAGL11* mutant) that was also able to restore seed number and seed size in the Arabidopsis heterologous background [12]. In addition, it seems clear that the *VviAGL11* transcript levels can be directly correlated to the rate of plant plasmids versus total DNA. The present work contributes with key functional data about *VviAGL11* in grapevine itself, showing, *in situ,* that this gene is a major regulator of seed morphogenesis in grapevine. Furthermore, these results demonstrated the scientific applicability of the IL-60-derived plasmids to manipulate the *VviAGL11* gene expression. However, from the results observed, it was possible to verify that the efficiency of this tool is dependent on the context of its application. Nevertheless, this work represents the follow up characterization step of the *VviAGL11* locus as a target for manipulation of seed number and size in grapevine.

Authors contribution

L.F.R and J.M. conceived original screenings, research plans, designed experiments and analyzed resulting data; L.F.R, V.B., F.S.M, M.M.P, G.P. and J.E.A.M. supervised experiments and writing; J.M. and V.B. performed most experiments; F.S.M and J.E.A.M. provided technical assistance. J.M. wrote the article with contributions from all authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2018.01.013.

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