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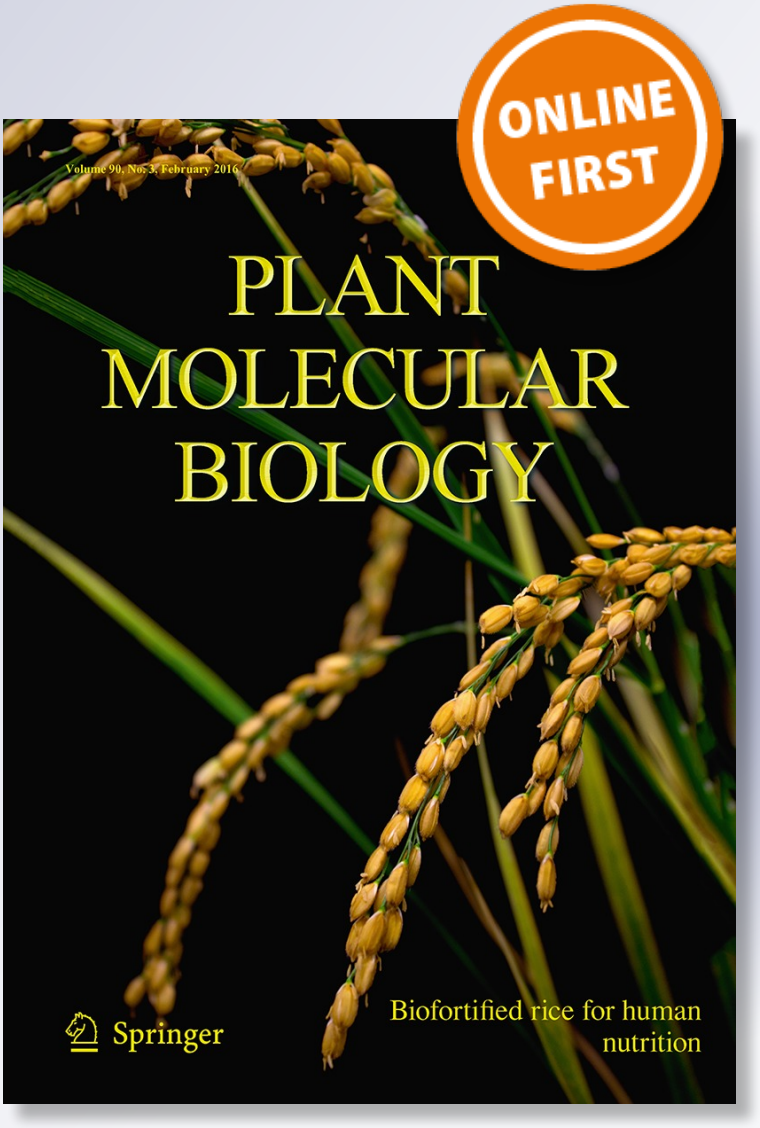

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Expression of grapevine *AINTEGUMENTA*-like genes is associated with variation in ovary and berry size

Constanza Chialva[1] · Estefanía Eichler[1] · Cecilia Grissi[1] · Claudio Muñoz[1] · Sebastian Gomez-Talquenca[2] · José M. Martínez-Zapater[3] · Diego Lijavetzky[1]

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Abstract Fruit size is a highly important trait for most fruit and vegetable crops. This trait has been a main selection target and could be involved in divergent selection processes leading to the differentiation between modern table and wine cultivars. Even though its determination is highly influenced by cultural practices, several regions within the grapevine genome have been identified affecting berry size, either directly or indirectly through their effect on seed content. Using grapevine seeded cultivars, we have analyzed the relationship between ovary cell number and the final size of ovaries and berry fruits. We also performed the characterization of the grapevine *AINTEGUMENTA-LIKE* family, since it is well reported in *Arabidopsis* that *AINTEGUMENTA* (*ANT*) regulates cell proliferation and organ growth in flower organ primordia by maintaining the meristematic competence of cells during organogenesis. Here we show that orthologous grapevine gene expression associate with flower developmental stages suggesting a similar biological role for this gene family in this species. Moreover, we detected a correlation between those organs size and the level of expression of *VviANT1* the grapevine homolog of *AtANT*. This grapevine gene also co-localizes in linkage group 18 with the confidence interval of a previously detected QTL for berry size. Thus our results suggest the involvement of ANT in the regulation of berry size in grapevine.

Keywords Berry size · Cell division · Gene expression · Ovary size · *Vitis vinifera* · AINTEGUMENTA

Introduction

Fruit size is a trait of main importance in most fruit and vegetable species, having undergone selection since the beginning of modern agriculture. While its determination at crop level is strongly influenced by cultural practices (pruning, fertilization, spacing, etc.), several genes controlling the size and shape of fruits have been identified (Tanksley 2004; Giovannoni 2004; Reeves et al. 2012).

In grapevine, the importance of fruit size is associated both to grapes for fresh consumption, where relatively large sizes are wanted, as well as to grapes for wine production, where small sizes are preferably selected (Conde et al. 2007). The genetic control of berry size has been studied in grapevine using quantitative genetic approaches and as in other species it is highly related with the seed content of the fruit (Ledbetter and Burgos 1994). Many genetic analyses involving seedless cultivars have mostly identified QTLs controlling seed content and seed weight and indirectly affecting berry size (Doligez et al. 2002; Cabezas et al. 2006; Mejia et al. 2007; Costantini et al.

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2008). However, some of these reports in table grapes also identified QTLs affecting berry size independently of seed content (Cabezas et al. 2006; Doligez et al. 2013). In addition, a few additional works have analyzed the genetic determinism of berry size in progenies derived from seeded cultivars (Fischer et al. 2004; Doligez et al. 2013). Altogether these reports identified at least seven chromosomal regions on linkage groups (LG) 5, 8, 11, 13, 15, 17 and 18 (Fischer et al. 2004; Cabezas et al. 2006; Doligez et al. 2013). Among them the genomic region on LG 17 could have had an effect in the domestication process of grapevine (Myles et al. 2011; Doligez et al. 2013). As a whole these studies show the complex genetic structure of this trait that also includes genetic and environmental interactions.

Apart from the conclusions of those genetic works identifying the major genetic components responsible for berry size variation, many other genes are likely involved in the process of cell division and expansion that contribute to the final berry sizes. Their genetic redundancy or their involvement in basic or diverse cellular processes exclude them from being identified as major determinants of natural variation for this trait. In this way, several reports have identified genes which mutation can alter berry size or that could have an effect on berry size based on the information obtained from transgenic plants. In this regard, Fernandez et al. (2013) and Nicolas et al. (2013) reported the involvement of genes such as *VvPI* and *VvCEB1*, respectively, in the final berry size. *VvPI*, belongs to the MADS-box transcription factor (TF) family and plays an important role in the flower to fruit transition. A dominant gain of function mutation in this gene originates the *FLESHLESS BERRY* somatic variant, which develops small grape berries lacking the differentiation of flesh cells (Fernandez et al. 2006, 2013). On the other hand, Nicolas et al. (2013) recently reported the identification of *VvCEB1*, a fruit-specific basic helix–loop–helix (bHLH) TF proposed to regulate cell expansion in grape during berry ripening. These studies provide evidence that the processes of cell division around flowering time and cell expansion after anthesis are important components of the variation in the final berry size observed among grape cultivars (Coombe 1992; Gray and Coombe 2009; Houel et al. 2013).

The total cell number of a plant organ is determined by the number of divisions of undifferentiated meristematic cells (Esau 1977). Although most cells in organ primordia are meristematic and proliferate actively, cells lose meristematic competence and withdraw from the cell cycle as organs develop. Thus, the maintenance of meristematic competence of cells is a key mechanism that mediates organ growth and cell proliferation by defining total cell number and thereby the size of plant organs (Mizukami and Fischer 2000). In this sense, the control of the intrinsic organ size has been thoroughly studied in *Arabidopsis* after the analysis of the function of the AINTEGUMENTA (ANT) TF. ANT, together with the AINTEGUMENTA-LIKE proteins (hereinafter, AIL proteins) are members of the APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain family of TFs that are found in a wide group of phylogenetically related plants (Elliott et al. 1996; Klucher et al. 1996; Nole-Wilson et al. 2005; Horstman et al. 2014). *ANT* is required for integument initiation and promotion of growth within developing floral organs (Elliott et al. 1996; Klucher et al. 1996). Loss of function mutations in *ANT* cause a reduction in the number and size of floral organs (Elliott et al. 1996; Klucher et al. 1996), while ectopic expression of *ANT* results in the production of larger floral organs (Krizek 1999; Mizukami and Fischer 2000). The AP2/ERF domain, a DNA binding domain of approximately 60–70 amino acids (Okamuro et al. 1997), was identified initially in the *Arabidopsis* AP2 protein (Jofuku 1994), and later in four tobacco (*Nicotiana tabacum*) ERFs (Ohme-Takagi and Shinshi 1995). The AIL proteins belong to the AP2 family of proteins, which contain two AP2/ERF domains separated by a linker region (Riechmann and Meyerowitz 1998).

Despite individual AIL proteins have been formerly studied in numerous plant species (Licausi et al. 2010; Dash and Malladi 2012; Rigal et al. 2012; Bandupriya et al. 2013; Horstman et al. 2014; Yang et al. 2014) the whole family of a particular plant was just characterized in *Arabidopsis* (Nole-Wilson et al. 2005; Horstman et al. 2014). These reports described and analyzed the eight *AIL* TFs genes within the *Arabidopsis* genome that include *ANT* (Elliott et al. 1996; Klucher et al. 1996), *BABY BOOM* (*BBM*) (Boutilier et al. 2002), and the *PLETHORA* (*PLT*) genes (Aida et al. 2004; Galinha et al. 2007) (Table 1), which are all expressed in young, dividing tissues in the plant. They have overlapping roles in the establishment and maintenance of meristems, as well as organ initiation and growth (Nole-Wilson et al. 2005; Horstman et al. 2014).

While some of the grapevine AIL proteins have been identified in the context of two previous comprehensive searches of the AP2/ERF protein superfamily (Zhuang et al. 2009; Licausi et al. 2010), we present here an exhaustive and specific genome characterization of the whole *AIL* gene family in grapevine. We also identified *VviANT1* as a putative TF associated with the determination of the final berry size through its relationship with cell division during the flower development. *VviANT1* mRNAs accumulates predominantly in the inflorescences, while its expression patterns strongly correlates with number of cells/ovary, the ovary size and the final berry size.

Table 1 Grapevine *ANT* homologous genes

Gene name[a]	Gene ID[b]	Position[b]	Size (aa)	Gene name (Licausi et al. 2010)	*Arabidopsis* ortholog[c]
VviANT1	VIT_18s0001g08610	chr18:7050289..7054311	674	*VvAP2-19*	*ANT*
VviANT2	VIT_07s0151g00440	chr7_random:914799..918602	653	*VvAP2-18*	*ANT*
VviAIL1	VIT_09s0002g01370	chr9:1150521..1153564	561	*VvAP2-10*	*AIL1*
VviAIL2	VIT_04s0023g00950	chr4:17380960..17384755	730	*VvAP2-05*	*AIL2/BBM*
VviAIL8	VIT_06s0004g01800	chr6:2228790..2231310	552	*VvAP2-03*	*AIL3/PLT1*
					AIL4/PLT2
VviAIL5	VIT_11s0052g00840	chr11:18415752..18418607	501	*VvAP2-09*	*AIL5/PLT5*
VviAIL6	VIT_00s0772g00020	chrUn:35248254..35252282	458	*VvAP2-16*	*AIL6/PLT3*
VviAIL7	VIT_00s1291g00010	chrUn:38323287..38328475	516		*AIL7/PLT7*

For every gene, the corresponding gene ID, protein length and *Arabidopsis* ortholog are listed

[a] Gene nomenclature according to Grimplet et al. (2014)

[b] Gene ID according to The CRIBI Biotechnology Center, University of Padua (http://genomes.cribi.unipd.it/grape/)

[c] *Arabidopsis* names according to Horstman et al. (2014)

Materials and methods

Plant material

All grapevine (*Vitis vinifera*) samples used for the different experiments were obtained from the Estación Experimental Agropecuaria of the Instituto Nacional de Tecnología Agropecuaria de Argentina (EEA Mendoza INTA) ampelographic collection. Gene expression analyses throughout the vegetative and reproductive development of the plant were performed on samples collected from cultivar Muscat Hamburg bearing medium sized berries. Developmental stages were classified following the series of Baggiolini (1952) considering the following stages: swelling buds from advanced phenological stage B (B2) bearing inflorescence meristems that are initiating the differentiation of flower meristems; small inflorescences from stage D (D), in which flower meristems are already formed; developing flowers from stage G (G) and early stage H (H1), which corresponds to the development of flower organs, with gynoecium initiated at the latest during stage H; flowers from advanced stage H (flower), just before anthesis; small grapes at fruit set (2 mm); matured grapes (120–140) selected on the basis of berry density as an indicator of accumulated sugar levels. Density was estimated by berry flotation in a range of NaCl solutions, each having a decrease in salinity of 20 g NaCl L^{-1} (from 160 to 100 g NaCl L^{-1}) (Carbonell-Bejerano et al. 2013). Samples were frozen in liquid nitrogen and stored at −80 °C until analysis. Variation for ovary and berry size as well as for gene expression was analyzed in samples of different grapevine seeded cultivars: Cardinal, Alphonse Lavallée, San Geronimo, Fondo de Orza, Afus Ali, Cabernet Sauvignon, Clairette Blanche, Chenin Blanc, Riesling Weiss, Muscat à Petits Grains Blancs and Piccola Nera.

Sequence analysis

All sequence analyses were performed using software MEGA version 6.06-mac (Tamura et al. 2013). Amino acid sequence alignment was done using Muscle (Edgar 2004). The phylogenetic tree was constructed using full-length grapevine, *Arabidopsis*, rice, apple and poplar protein sequences obtained from The National Center for Biotechnology Information (NCBI) and the corresponding genome sequencing initiatives (Sasaki and Burr 2000; The *Arabidopsis* Genome Initiative 2000; Tuskan et al. 2006; Jaillon et al. 2007; Jung et al. 2014). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

RNA isolation and gene expression analyses

Total RNA was extracted from the different tissues according to the procedures described by Reid et al. (2006). Final RNA purification and DNase digestion of contaminating DNA in the RNA samples were performed using the SV Total RNA Isolation System (Promega) following manufacturer's protocols. Reactions for cDNA synthesis and real-time quantitative reverse transcription PCR (qRT-PCR) were performed according to Lijavetzky et al. (2008)

using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies). Non-template controls were included for each primer pair, and each qRT-PCR reaction was completed in triplicate. Expression data were normalized against the grapevine *ACT1* gene (VIT_04s0044g00580). This normalization gene was chosen after the comparison of *ACT1*, *EFa1* (VIT_06s0004g03220) and *UBI* (VIT_16s0098g01190) genes using NormFinder software (Andersen et al. 2004). All three genes were previously tested for grapevine gene expression analysis (Reid et al. 2006). Relative quantification was performed by means of the ΔCt method using StepOne software v2.2.2 (Applied Biosystems, Life Technologies). Gene-specific primers were designed using the Oligo Explorer 1.2 software (Gene Link) and the resulted sequences are described in Online Resource 1. The generation of the heat-map and the cluster analysis of the qRT-PCR expression data were performed with the help of the Genesis v1.7.7 software (Sturn et al. 2002).

Determination of natural variation for ovary and berry size

Healthy flowers and berries were collected in 2013 from the central part of 10 clusters belonging to five plants per cultivar according to Organisation Internationale de la Vigne et du Vin (OIV, http://www.oiv.int/) indications. Thirty-five berries per cultivar were measured and the perimeter was determined using an electronic digital caliper (Digimess, Argentina).

Preparation of ovary cross section

Collected flowers were processed essentially as described by Auge et al. (2012) with slight modifications. Ovaries were cut in transversal thin microsections of 10 μm with a sliding microtome and the microsections were mounted on slides. Slides were stained by immersion in Cresyl Brilliant Blue solution (0.05 %) for 2 min, washed with distilled water and dried at 25 °C for 24 h. Samples were dewaxed with xilene 100 %. Finally, ovary sections were mounted with synthetic Canada Balsam and were observed at 10× and 40× magnification under a Nikon Eclipse E200 optical microscope (Japan) and photographed with a TV Lens 0.55× DS Nikon (Japan). The ovary external epidermal cells were counted in the equatorial microsection. The number of cells/ovary and the cell perimeter were evaluated in five to eight ovaries per cultivar. Cell counting and perimeter measures were performed by analyzing the digital images with the help of the Image Tool software. (http://compdent.uthscsa.edu/dig/itdesc.html).

Statistical analysis of the data

Student's *t* test or ANOVA followed by LSD Fisher post-test was performed when appropriate, in order to asses the differences between means with a $P < 0.05$ significance level using INFOSTAT software (Di Rienzo et al. 2011). Linear regression analysis was also performed by means of INFOSTAT software.

Results

Identification and annotation of grapevine ANT and AIL homologous genes

Using the *Arabidopsis* information regarding the different *AINTEGUMENTA* (*ANT*) and *AINTEGUMENTA*-like (*AIL*) sequences (hereinafter *AIL* genes) available in public databases we performed a BLAST search at a grapevine specific database (The CRIBI Biotechnology Center, University of Padua; http://genomes.cribi.unipd.it/grape/) and at The National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). A total of eight *AIL* genes were identified in the grapevine genome and are listed in Table 1. We named the *AIL* genes based on their homology to previous related genes annotated in *Arabidopsis* (Nole-Wilson et al. 2005; Horstman et al. 2014) and following the nomenclature recommendations proposed for grapevine (Grimplet et al. 2014). In Table 1 we detailed the assigned gene names, gene IDs according to the 12× V2 genome annotation, the physical location, the predicted protein size and the corresponding *Arabidopsis* orthologs. The eight *AIL* genes were found spread on at least six of the 19 grapevine chromosomes. In contrast to what was observed in *Arabidopsis*, two duplicated *ANT* genes were identified in grapevine. The most similar to *AtANT* was named *VviANT1*, mapping on chromosome 18, while the other (*VviANT2*) was located on chromosome 7. On the other hand, the grapevine genome bears only one of the two highly similar and redundant *PLETHORA* (*PLT*) genes (*PLT1/AIL3* and *PLT2/AIL4*; (Nole-Wilson et al. 2005; Litt 2007) that was named as *VviAIL8* (Table 1). It's worth to mention that two automatically annotated sequences are mapped at the same chromosomal position (chr06: 02228790..02231310) as a putative grapevine *PLT*, possibly corresponding to two alternative splicing variants of the same transcript. *AIL* sequences previously identified by Licausi et al. (2010) were also referred to in Table 1.

The close relationship between grapevine *AIL* genes became evident after conducting their alignment analysis by the MUSCLE algorithm (Edgar 2004). The alignment of the fragment of around 170 amino acids containing the two AP2-domain repeats and the linker region of AtANT and

the eight grapevine AIL proteins is shown in Fig. 1. Within the AP2 repeat region, these proteins share 86 % of sequence similarity. These values increased to 97 % for the pair-wise comparison VviANT1/VviANT2 and to 100 % for VviAIL6/VviAIL7. Moreover, VviANT1 and VviANT2 displayed >91 % similarity with AtANT in this region (Online Resource 2a). The 19 amino acids within the two AP2-domain repeats and the linker region identified in AtANT as essential for the DNA binding activity (Krizek 2003) were conserved in VviANT1 but not in VviANT2 (Fig. 1). VviANT2 shared 16 out of the 19 mentioned amino acids, particularly lacking the "VYL" motif, described as a critical sequence for transcription activation (Masaki et al. 2005; Ma et al. 2013). In fact, this motif was present only in two out of the eight grapevine AIL proteins (VviANT1, and VviAIL5) (Fig. 1). Additionally, upstream of the AP2 repeat region, VviANT1 and VviANT2 presented a basic motif (TKKR) similar to the nuclear localization signal (KKKR) present in AtANT (Online Resource 3) (Krizek and Sulli 2006). While the only region shared by all members of the grapevine AIL family is the AP2 repeat region, pairs of proteins also shared high similarity throughout their sequence. That is, VviANT1 and VviANT2 are 79 % similar throughout their entire sequence, while VviAIL6 and VviAIL7 are 84 % similar throughout their sequence (Online Resource 2b).

Phylogenetic analysis of AIL proteins

To examine the phylogenetic relationships among grapevine AIL proteins and cluster them within their putative orthologous groups, we constructed a phylogenetic tree (Fig. 2) from the alignment of full-length grapevine, *Arabidopsis*, rice, poplar and apple protein sequences obtained from NCBI and the corresponding genome sequencing initiatives (Sasaki and Burr 2000; The *Arabidopsis* Genome Initiative 2000; Tuskan et al. 2006; Jaillon et al. 2007; Vitulo et al. 2014; Jung et al. 2014). The phylogenetic tree revealed three major clades grouping the six orthologous groups. One clade clustered the AIL1 group, while another included the, AIL2, AIL3-4, AIL5, AIL6 and AIL7 groups. The ANT proteins from the five species grouped in the third clade (Fig. 2). All grapevine AIL proteins were grouped with corresponding *Arabidopsis*, rice, poplar and apple counterparts, with high bootstrap support. Remarkably, in most cases, two poplar genes were found for every homolog in grapevine or *Arabidopsis*, with the exception of the AIL6-AIL7 group, where no putative ortholog was

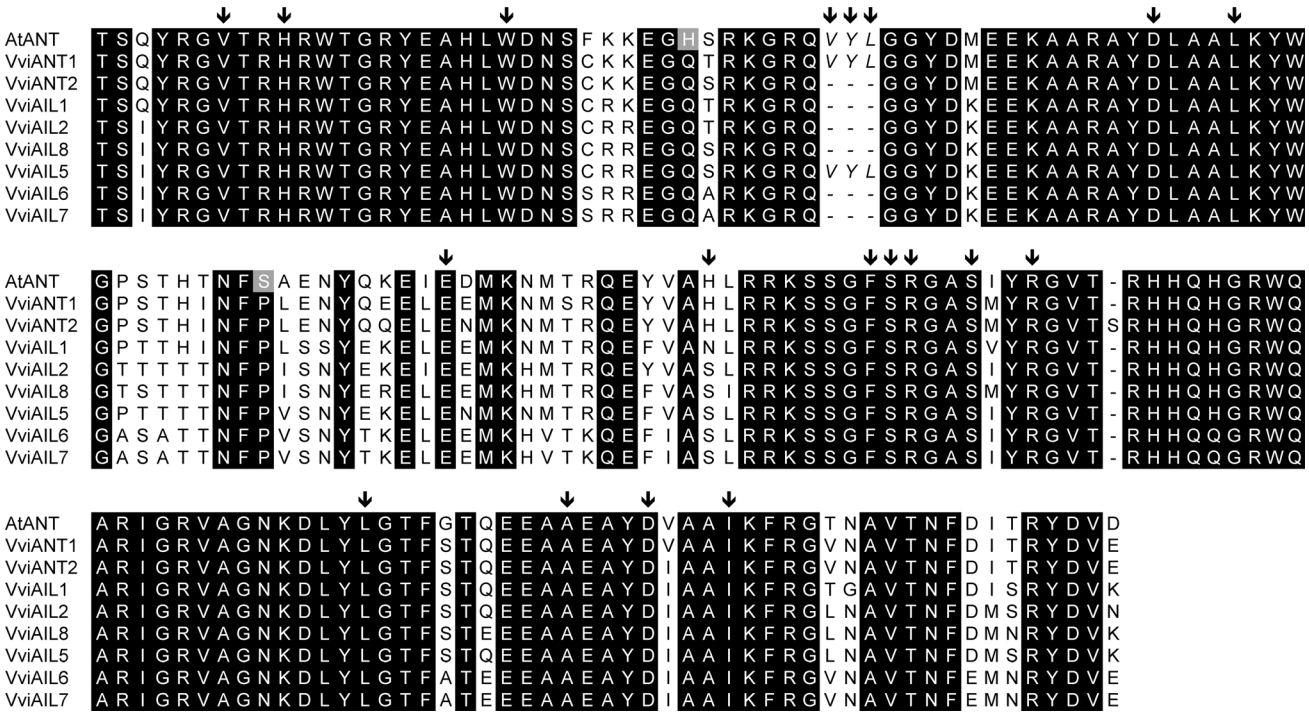

Fig. 1 Comparison of predicted amino acid sequences within the DNA binding domain of grapevine AIL proteins. The predicted amino acid sequence of the two AP2 domains and the linker region are shown for eight grapevine AIL proteins and AtANT. Amino acids that are identical in all sequences are shaded in *black*. Additional amino acids that are identical in all AILs proteins but differing with AtANT are indicated in *grey*. *Arrows* denote the 19 amino acids within the two AP2-domain repeats and the linker region identified in AtANT as essential for the DNA binding activity (Krizek 2003)

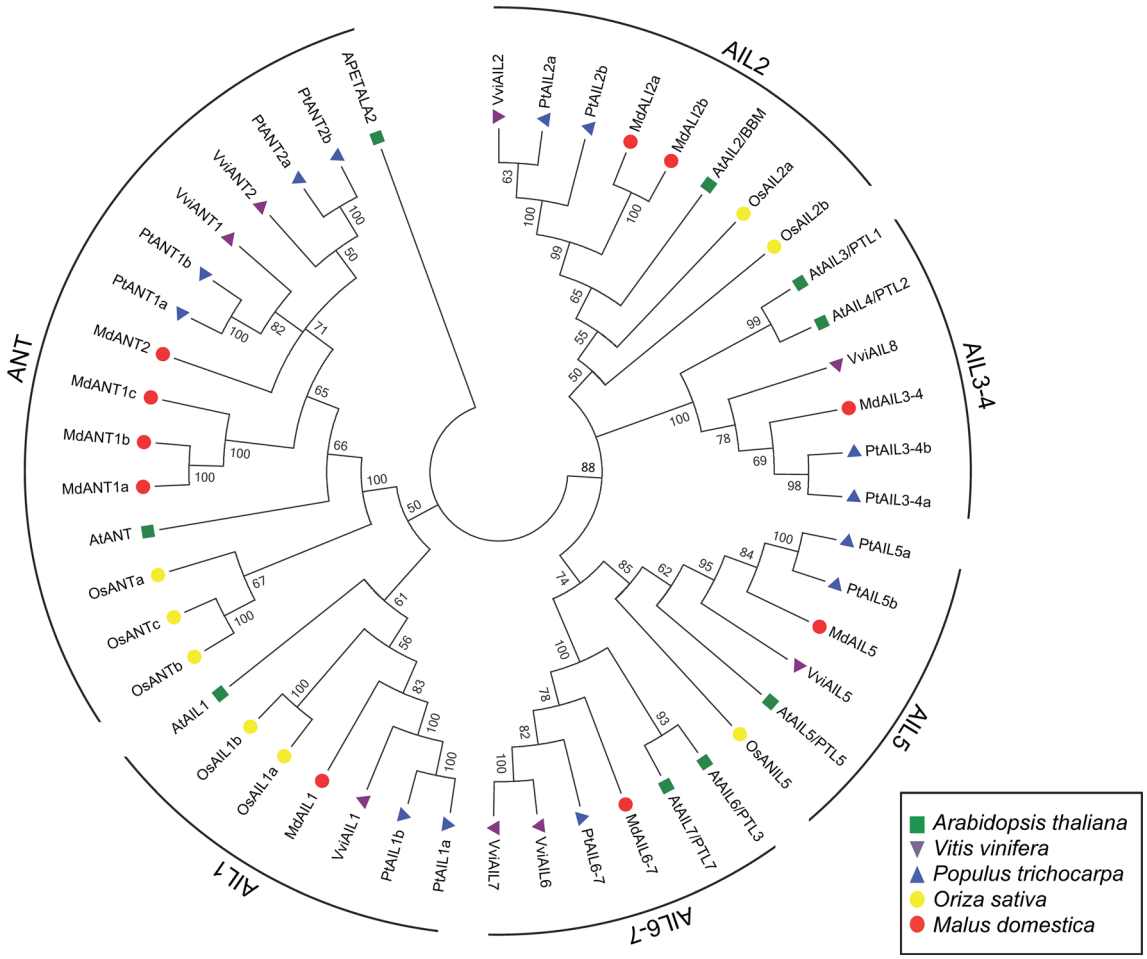

Fig. 2 Phylogenetic relationships among grapevine AIL proteins and their putative orthologous groups from other sequenced genomes. The tree was constructed from the alignment of full-length *Arabidopsis* (At), rice (Os), poplar (Pt), apple (Md) and grapevine (Vvi) proteins. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013) using the Neighbor-Joining (NJ) model (bootstrap = 1000). The AP2 protein (GenBank: AAC13770.1) was used as outgroup

found for AIL7. On the other hand, the apple ANT protein seems to be widely duplicated.

AIL genes are mainly expressed during flower development

AIL genes have mainly been involved in the specification of meristematic or division-competent cellular states (Nole-Wilson et al. 2005). In order to further associate their biological function in grapevine with specific developmental processes, we analyzed their expression in seven representative vegetative and reproductive organs of the plant using real-time quantitative reverse transcription PCR (qRT-PCR). Figure 3 displays the tissue-specific mRNA accumulation of these genes clustered according to their expression patterns. Two clusters were differentiated by means of the Genesis software (Sturn et al. 2002). In the first group (*VviAIL1*, *VviAIL2* and *VviAIL8*) which higher expression was observed in inflorescences from stage "H1", while in the second (*VviANT1*, *VviANT2*, *VviAIL5*, *VviAIL6* and *VviAIL7*), the maximum expression was detected in inflorescences from stage "G". *VviANT1*, *VviANT2* and *VviAIL5* also displayed strong expression in stage H1. As a general rule, the expression patterns of the *AIL* genes increased from the bud (stage B2) until the corresponding peaks, declining later until fruit maturation. Just three genes presented significant expression during other additional stages: *AIL1* (stage B2), *AIL2* (fruit set = 2 mm) and *AIL7* (stages B2 and fruit set). At ripening all the genes showed very low expression that was almost undetectable for most of them (Fig. 3). Globally, the clustering of the expression patterns of the *AIL* genes agreed with their phylogenetic relationships (Figs. 2, 3).

Large berry size is associated to large ovary size and higher cell number

Several reports showed that ANT is involved in the control of organ growth during *Arabidopsis* flower development affecting the final size of flower organs (Elliott et al. 1996;

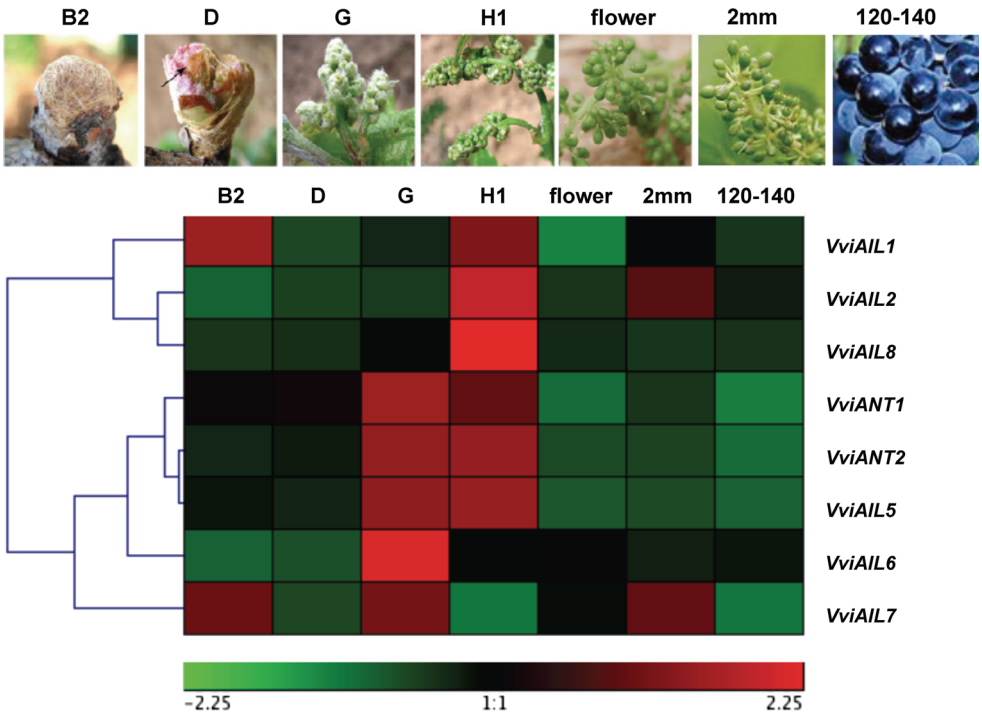

Fig. 3 Expression profiles of grapevine *AIL* genes during flower development and berry ripening. Expression analyses were performed by qRT-PCR, and relative gene expression data were gene-wise normalized. *Color scale*, representing signal values, is shown at *bottom*. At *top*, photographs from the different developmental stages are shown. Developmental stages correspond to buds from advanced stage B (B2), inflorescences of stage D (D), and flowers from inflorescences at stage G (G), early stage H (H1), fruit set (2 mm) and ripe berries (120–140)

Klucher et al. 1996; Krizek 1999). In order to evaluate the association between the expression of *VvAIL* genes and final berry size, we first analyzed the variation in ovary and berry size, focusing on the relationship between those two traits. We selected a set of 11 seeded grapevine cultivars from the EEA Mendoza INTA Grapevine Collection known to present differences in berry size and we measured the size of their ovary and berries (Fig. 4a, b). We used seeded cultivars with the aim of avoiding the effects of seed content on berry size. Despite the berry size of the chosen cultivars did not correspond to extreme examples within the collection (Alercia et al. 2009) they presented contrasting measurements. As expected, large-berry cultivars ("LBC": 'Cardinal', 'Alphonse Lavallée', 'San Geronimo', 'Fondo de Orza' and 'Afus Ali') presented larger berries than small-berry cultivars ("SBC": 'Cabernet Sauvignon', 'Clairette Blanche', 'Chenin Blanc', 'Riesling Weiss', 'Muscat à Petits Grains Blancs' and 'Piccola Nera'), with an average size (equatorial perimeter) of 66.0 ± 1.54 mm for the LBC and 42.8 ± 1.41 mm for the SBC (Fig. 4a and Online Resource 4a). Interestingly, LBC cultivars also presented larger ovaries than SBC, with an average pistil perimeter of 5.0 ± 0.22 mm for the LBC and 3.35 ± 0.20 mm for the SBC (Fig. 4b and Online Resource 4b). That close relationship was confirmed by linear regression analysis ($R^2 = 0.84$ and P value <0.0001; Fig. 4c). Additionally, LBC and SBC showed similar average seed number/berry (2.17 ± 0.15 vs. 2.24 ± 0.14, respectively) suggesting that this trait is no playing a key role in determining the final berry size (Online Resource 4c).

We wonder whether larger ovaries and berries were related to the presence of larger cells or to the presence of a higher number of cells. With this purpose we analyzed transversal pistil sections from the 11 contrasting cultivars at anthesis (Fig. 6) and measured the number of cells/ovary. As shown Online Resource 4d, LBC cultivars presented ovaries with significantly higher numbers of cells per ovary than SBC cultivars, with an average number of 488.46 ± 29.63 for the LBC and 331.28 ± 27.05 for the SBC. Linear regression analysis showed that the number of cells/ovary and the berry size are significantly correlated (Fig. 7b; $R^2 = 0.75$; P value = 0.0004). As expected, the correlation between the number of cells/ovary and the ovary size was even higher (Fig. 7c; $R^2 = 0.92$; P value <0.0001). As a verification, we calculated the number of cells/mm (cells/ovary perimeter) for what no significant differences were observed between LBC and SBC, with an average cells/mm of 98.83 ± 2.74 for the LBC and 97.20 ± 3.00 for the SBC (Online Resource 6), meaning that cells from both groups of cultivars had a similar size.

VviANT1 expression is associated with a large number of cells per ovary

It has been proposed that ANT regulates cell proliferation and organ growth by maintaining the meristematic competence of cells during organogenesis, showing additionally that ANT ectopic expression enlarges embryonic and all shoot organs by increasing cell number in both *Arabidopsis* and tobacco plants (Mizukami and Fischer 2000). Once we verified that differences in ovary size were related to differences in cell number

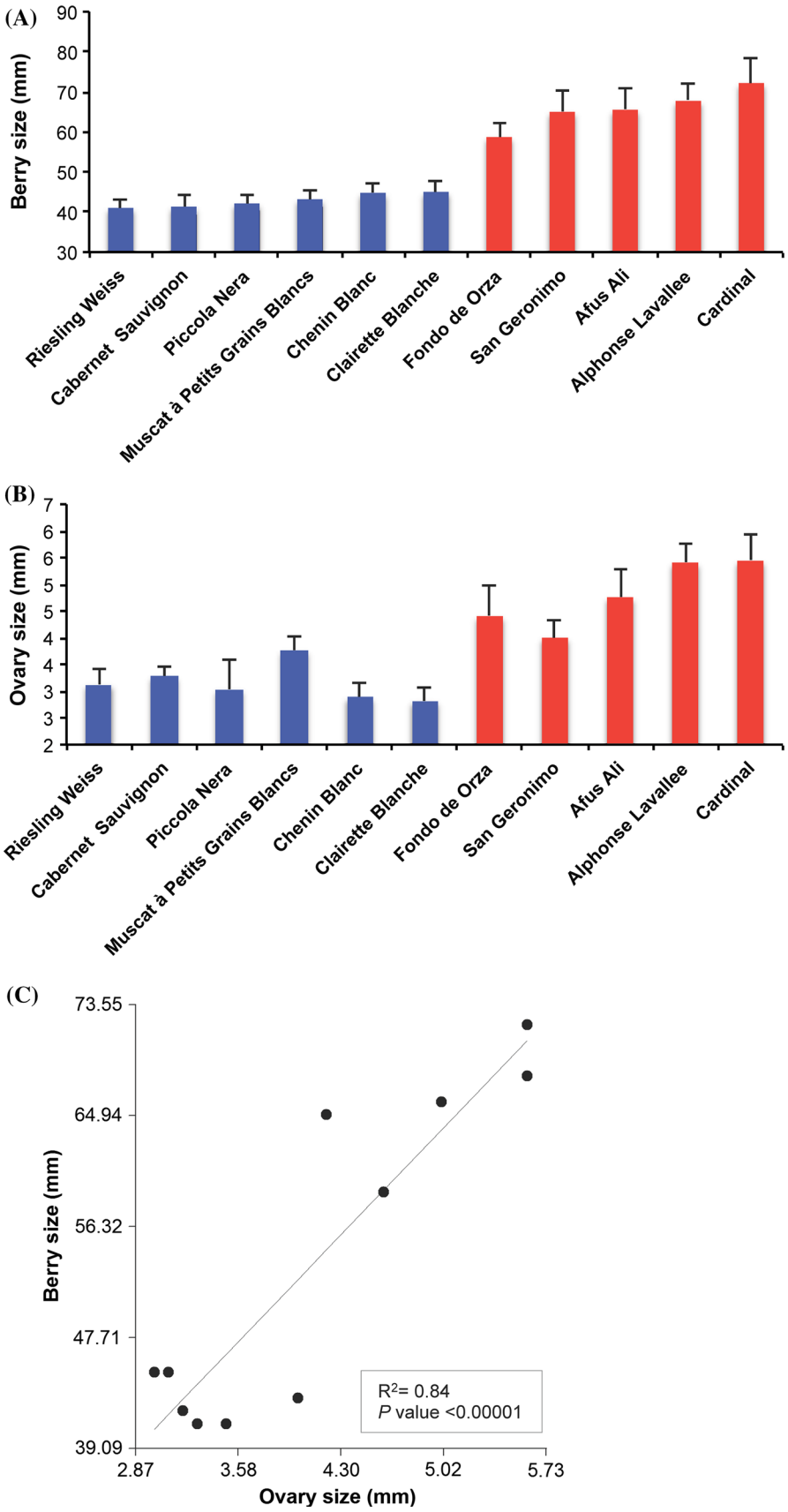

Fig. 4 Analysis of natural variation for ovary and berry size. Determination of the size (perimeter) of the berries (**a**) and ovaries (**b**) in a set of 11 grapevine cultivars with contrasting berry size. Large-berry cultivars (LBC): Cardinal, Alphonse Lavallée, San Geronimo, Fondo de Orza and Afus Al). Small-berry cultivars (SBC): Cabernet Sauvignon, Clairette Blanche, Chenin Blanc, Riesling Weiss, Muscat à Petits Grains Blancs and Piccola Nera. (**c**) Linear regression analysis between berry and ovary size

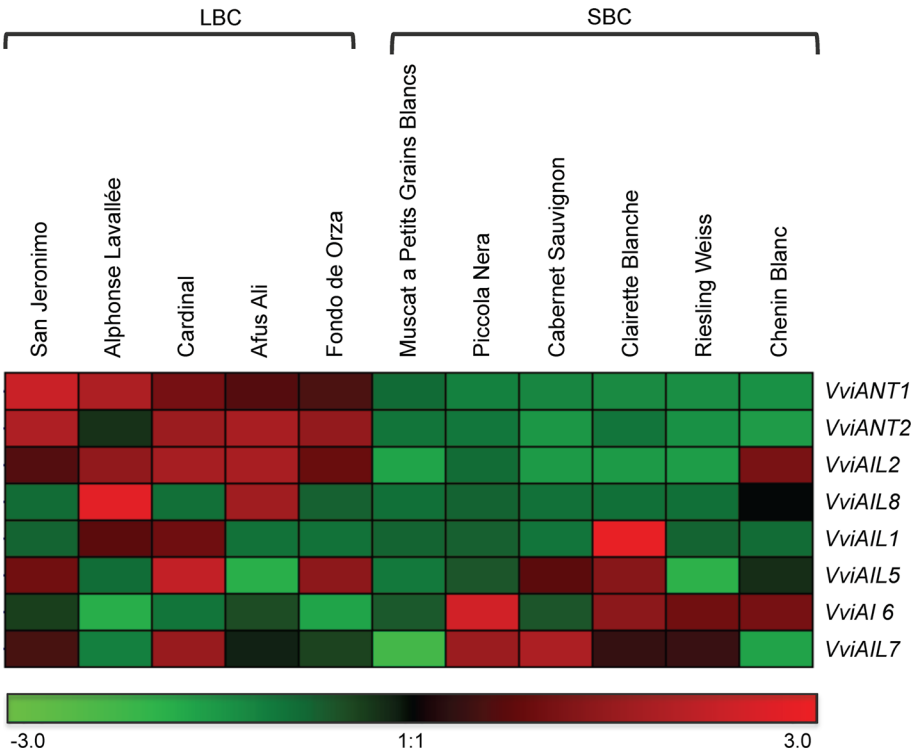

Fig. 5 *VviAIL* genes expression on grapevine cultivars with contrasting berry size. Large-berry cultivars (LBC): Cardinal, Alphonse Lavallée, San Geronimo, Fondo de Orza and Afus Ali. Small-berry cultivars: (SBC) Cabernet Sauvignon, Clairette, Chenin Blanc, Riesling Weiss, Muscat à Petits Grains Blancs and Piccola Nera. Expression was determined on stage "G" samples. The normalization of data and the construction of the heat-map were performed with the help of the Genesis software (Sturn et al. 2002)

and not in cell size, and that ovary size was directly related to final berry size, we investigated whether ovaries with higher cell number displayed an increased expression of *VviAIL* genes. With this goal we performed a qRT-PCR expression analysis of the eight *VviAIL* genes on the flowers of the 11 cultivars under analysis. Flower samples were collected in stage "G" since most *VviAIL* genes displayed their higher expression at this stage (Fig. 3). As shown in Fig. 5, expression of *VviANT1* was significantly higher in LBC than in SBC, presenting an average fold-change (FC) expression (LBC/SBC) of 7.0 with a *P* value of 0.0296 (Fig. 5 and Online Resource 4e). Moreover, the linear regression analysis of berry size and *VviANT1* expression (Online Resource 5a) yielded a R^2 of 0.75 (P value = 0.0005). Additionally, *VviANT2* and *VviAIL2* also showed a similar expression pattern than *VviANT1* but with particular differences: *VviANT2* displayed low expression for a LBC (Alphonse Lavallee) while *VvAIL2* displayed high expression for a SBC (Chenin Blanc) (Fig. 5). As expected, both genes showed a lower average FC expression than *VviANT1*, whit a FC of 3.99 ($P = 0.0017$) for *VviANT2* and 2.87 ($P = 0.0339$) for *VviAIL2* (Online Resource 4f and 4g).

Discussion

In this work we have used a new annotation of the grapevine genome (Grimplet et al. 2014) to characterize the members of the grapevine AIL family of putative transcription factors that belong to the AP2/ERF superfamily (Elliott et al. 1996; Klucher et al. 1996; Nole-Wilson et al. 2005; Zhuang et al. 2009; Licausi et al. 2010; Horstman et al. 2014). In this way, we were able to update and complete the previous exhaustive work performed by Licausi et al. (2010). Based on de v0 version of the 8× grapevine genome these authors reported the characterization of the AP2/ERF superfamily in grapevine. Within this superfamily they identified 10 AIL related sequences. From those, seven (*VvAP2-19*, *VvAP2-18*, *VvAP2-10*, *VvAP2-05*, *VvAP2-03*, *VvAP2-09* and *VvAP2-16*) correspond respectively to *VviANT1*, *VviANT2*, *VviAIL1*, *VviAIL2*, *VviAIL8*, *VviAIL5* and *VviAIL6* described in the present work (Table 1) for which slight differences related to the annotation update were observed despite the general agreement in the protein sequences. The other three sequences described by Licausi et al. (2010) (*VvAP2-04*, *VvAP2-07* and *VvAP2-17*) correspond to truncated and/or wrongly annotated sequences. Additionally, our *VviAIL7* corresponds to a new gene not previously identified by Licausi et al. (2010). All the mentioned disagreements can be explained by the different version of the grapevine genome utilized. While Licausi et al. (2010) worked with 8× (v0), we utilized the last predictions (v2) from the 12× version (Jaillon et al. 2007; Vitulo et al. 2014).

The phylogenetic analysis of the grapevine AIL proteins together with those identified in *Arabidopsis*, rice, poplar and apple (Fig. 2) showed that the corresponding putative orthologs from each species cluster in good agreement with

the well characterized *Arabidopsis* AIL proteins (Nole-Wilson et al. 2005; Horstman et al. 2014). A remarkable feature of this analysis is the presence of two poplar sequences for every grapevine or *Arabidopsis* ANT-AIL proteins (Fig. 2). Similar observations were reported during the study of the grapevine MIKCC-Type MADS-Box (Diaz-Riquelme et al. 2009) and bZIP (Liu et al. 2014) transcription factors and may be explained by an additional poliploidization event taking place during poplar evolution (Jaillon et al. 2007). On the other hand, the apple genome displayed a particular amplification of the *ANT* orthologous sequences (Fig. 2). While the presence of two *ANT* genes (*MdANT1* and *MdANT2*) was previously reported (Dash and Malladi 2012) our own search revealed the existence of three isoforms for MdANT proteins (Jung et al. 2014). In general, a the good concordance between the clustering of the grapevine AIL proteins based on both the phylogenetic and their gene expression analyses was observed (Figs. 2, 3). Interestingly, *VviAIL6* and *VviAIL7*, having the highest pairwise similarity values (Online Resource 2a and 2b), show a differential expression pattern, with *VviAIL7* displaying two additional expression peaks besides the shared expression at the "G" stage (Fig. 3). Comparable observations were reported in *Arabidopsis*, for its closely related genes *AtAIL6* and *AtAIL7*, they are 74 % similar throughout their protein sequence but display distinct expression patterns (Nole-Wilson et al. 2005).

The sequence of VviANT1 characterized in grapevine is consistent with its putative role as a transcription factor (TF). The deduced grapevine protein contains a particular motif (TKKR), also present in the other VviANT isoform (Online Resource 3). As reported for the apple ANT proteins (Dash and Malladi 2012), the major part of the TKKR element is conserved and aligned to the "KKKR" motif from AtANT, a determinant for the nuclear localization of the protein (Krizek and Sulli 2006). Moreover, this conserved motif was detected in all the ANT proteins from grapevine, rice, poplar and apple (Online Resource 3). Nole-Wilson and Krizek (Nole-Wilson and Krizek 2000) showed that the *Arabidopsis* ANT protein binds to the DNA through two AP2 domains and a conserved linker region. VviANT1 and VviANT2 shared more than 91 % sequence identity with AtANT within these regions, a slightly greater value than the 88 % reported for the apple ANT proteins (Dash and Malladi 2012). Moreover, similarly to that observed in apple (Dash and Malladi 2012), all the 19 residues identified as essential for the DNA binding activity of AtANT (Krizek 2003) are conserved in VviANT1. The main difference observed between VviANT1 and VviANT2 within the AP2 DNA binding domain is the absence of the "VYL" motif (Fig. 1 and Online Resource 3). In AtANT, this 9 bp micro-exon (Okamuro et al. 1997) may contact DNA, and mutations in any of the "VYL" residues affects the DNA binding activity of the protein (Krizek 2003). Additionally, this element was also reported as essential for the function of WRINKLED1 (Masaki et al. 2005; Ma et al. 2013), another member of the AP2 family of transcription factors (Cernac and Benning 2004). Whether this means that VviANT2 and other VviAIL proteins lacking this motif are altered in their binding activities remains to be shown.

Previous work has pointed out that cell division before anthesis and cell expansion after anthesis are likely major determinants for the variation in the final berry size among grape cultivars (Coombe 1992; Gray and Coombe 2009; Houel et al. 2013). Still, part of the variation in the final berry size can also be attributed to differences in cell division after fruit set and particularly to differences in cell expansion after veraison as has been pointed out by Houel et al. (2013). In agreement with those results, large berry cultivars (LBC) contained significant greater number of cells per ovary than small berry cultivars (SBC), and there was a strong correlation ($R^2 = 0.92$) between berry size and ovary size (Fig. 7c). This correlation was lower ($R^2 = 0.76$) but still highly significant between berry size and number of cells/ovary ($R^2 = 0.76$) in agreement with

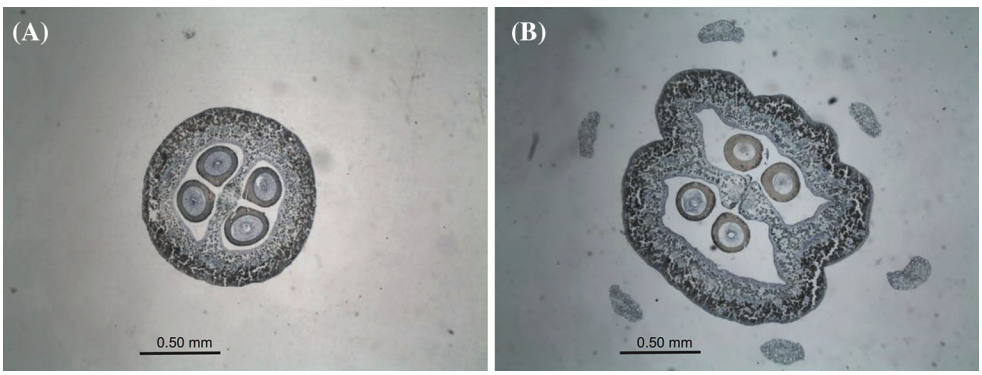

Fig. 6 Variation of ovary size in the analyzed cultivars. Transversal sections (×10) of pistils at anthesis from **a** a small-berry cultivar (Cabernet Sauvignon) and **b** from a large-berry cultivar (Afus Ali)

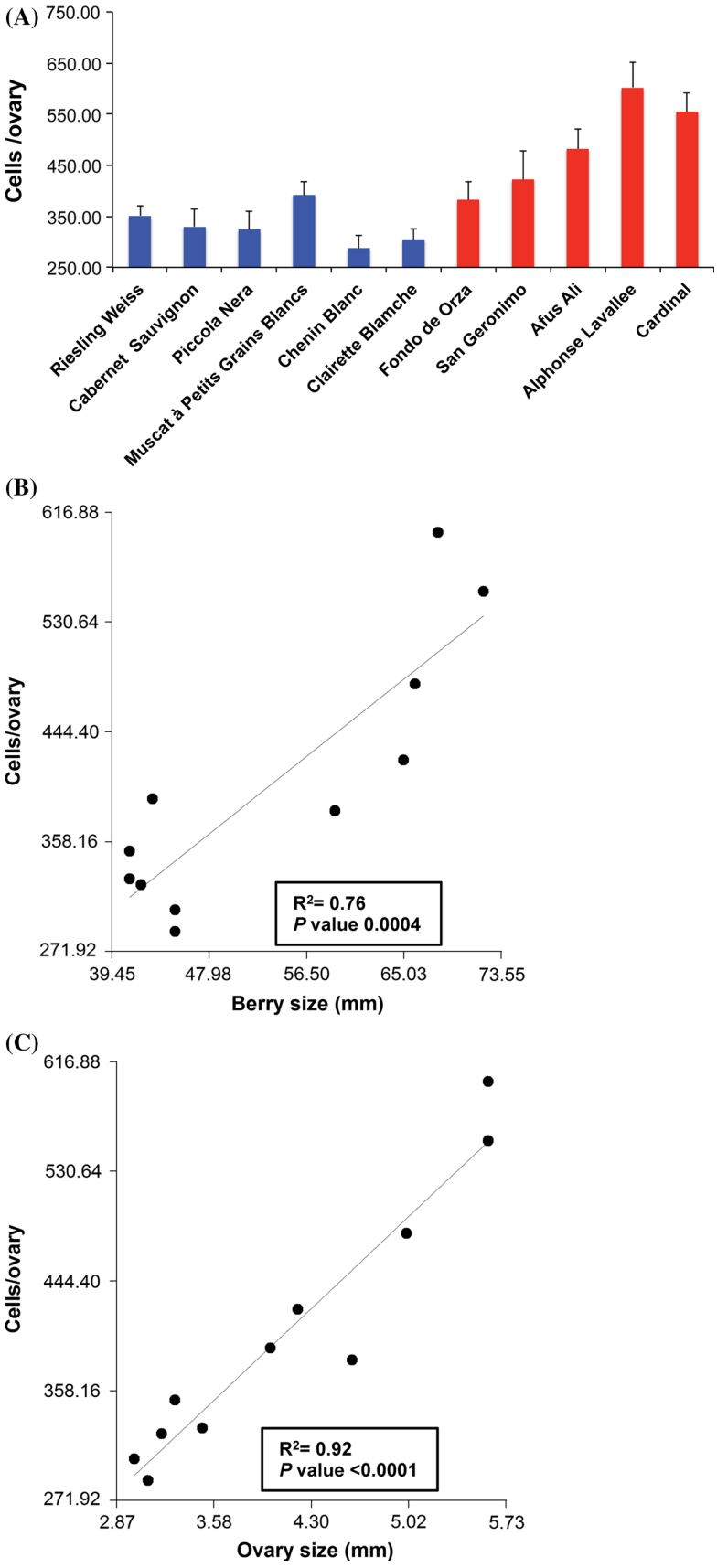

Fig. 7 Variation in number of cells/ovary in the analyzed cultivars. **a** Cell number in the 11 contrasting berry-size cultivars. **b** Linear regression analysis between number of cells/ovary and berry perimeter (mm); **c** Linear regression analysis between number of cells/ovary and ovary perimeter (mm)

the role of other factors, mostly related to cell expansion, in the determination of the final berry size (Nicolas et al. 2013; Houel et al. 2013; Fernandez et al. 2013). Thus, the number of cells/ovary and not the size of those cells is the key factor in determining the size of this flower organ (Figs. 6, 7a and Online Resource 4e) and directly affects the final berry size.

Expression of *VvANT1* and *VviANT2* and other *VviAIL* genes was preferentially detected in inflorescences (Fig. 3), markedly increasing throughout the stages corresponding to flower development till anthesis and becoming undetectable after anthesis. These results are in agreement with those described in the atlas of gene expression developed for cultivar Corvina (Fasoli et al. 2012). Considering that Coombe (1973) reported up to seventeen doublings in cell number during ovary development before anthesis and only two doublings afterwards, our expression results would be consistent with a role of *VviANT* and *VviAIL* genes in the regulation of cell division during flower development in grapevine as has been shown in other species like *Arabidopsis* and tobacco (Krizek 1999; Mizukami and Fischer 2000). Other grapevine genes such as *VvCEB1* encoding a fruit-specific bHLH TF was found to be predominantly expressed during berry ripening after veraison and has been suggested to play a key role in the regulation of cell expansion through ripening (Nicolas et al. 2013).

In agreement with a putative role of *VviANT* and *VviAIL* related genes in the positive regulation of ovary cell division, several of them seem to be more highly expressed in the flowers of cultivars with higher number of ovary cells and larger fruits than in the flowers of small berry size cultivars. This relationship was particularly clear for *VviANT1* expression. Two additional genes (i.e. *VviANT2* and *VviAIL2*) also displayed a similar behavior, although with a weaker relationship. While these results suggest this small gene family and particularly *VviANT1* to be related with the process of cell division that take place during ovary development and contribute to determine berry size, they are not enough to support a major role of *VviANT1* as responsible for part or the genetic variation in berry size. Association genetic analyses within a large sample of cultivars representing the existing variation for berry size and considering their genetic relationships will be required to proof this role.

The quantitative genetic analyses of berry size in grapevine has been addressed in several reports involving table and/or wine grape cultivars and QTL for this trait have so far been detected in ten out of the nineteen linkage groups (LG) defined (Doligez et al. 2002; Fischer et al. 2004; Cabezas et al. 2006; Costantini et al. 2008; Doligez et al. 2013). Many of these works use segregating populations derived from crosses involving seedless cultivars that carry QTL for seed number and seed size indirectly affecting berry size. Additionally, using a seeded segregating population, Houel et al. (2015) recently reported a major QTL for berry weight also co-localizing with QTL for seed number. When we only considered reported berry size QTL that are independent of seed size and/or seed number, *VviANT* or *VviAIL* genes co-located in two linkage groups with stable berry size QTL. In this way, *VviAIL5* co-localizes in LG11 with a stable QTL MBW6.16.1 (Doligez et al. 2013) although the physical positions for the gene sequence (18.415.752) and a linked QTL marker (*VVS2* at 3.909.894) likely indicate that this gene would not be a good candidate for such a QTL. Additionally, *VviANT1* co-localizes in LG18 with a stable QTL detected in both table grape (BW18b, Cabezas et al. 2006) and wine grape segregating progenies (MBW6.598.0, Doligez et al. 2013) and linked to marker *VVIN83*. In this case the physical positions of *VviANT1* gene (7.050.289) and the *VVIN83* linked marker (10.665.387) are close enough as supporting the possibility of *VviANT1* as candidate gene to underlie the effect of the BW18b/MBW6.598.0 QTL. This locus, although located in the same linkage group as *SDI* (*Seed Development Inhibitor*), which is responsible for seedlessness in table grape and indirectly affects berry size, affects berry size independently of seed content (Cabezas et al. 2006; Doligez et al. 2013). Further co-segregation analyses and genetic and molecular proofs will be required to confirm this possibility.

In conclusion, this work showed a relationship between berry and ovary size, with ovary cell number being an important determinant of the ovary size. Analyses of *VviANT1* and *VviAIL* grapevine genes also suggest their possible role in the regulation of cell division during ovary development and open the possibility that *VviANT1* could contribute to part of the natural variation in berry size observed in grapevine. Further analyses will be required to confirm those possibilities.

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