Physiologia Plantarum
An International Journal for Plant Biology

ABA and GA_3 increase carbon allocation in different organs of grapevine plants by inducing accumulation of non-structural carbohydrates in leaves, enhancement of phloem area and expression of sugar transporters

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Received 19 June 2015;
revised 3 August 2015

doi:10.1111/ppl.12390

Grape quality for winemaking depends on sugar accumulation and metabolism in berries. Abscisic acid (ABA) and gibberellins (GAs) have been reported to control sugar allocation in economically important crops, although the mechanisms involved are still unknown. The present study tested if ABA and gibberellin A_3 (GA_3) enhance carbon allocation in fruits of grapevines by modifying phloem loading, phloem area and expression of sugar transporters in leaves and berries. Pot-grown *Vitis vinifera* cv. Malbec plants were sprayed with ABA and GA_3 solutions. The amount of soluble sugars in leaves and berries related to photosynthesis were examined at three points of berry growth: pre-veraison, full veraison and post-veraison. Starch levels and amylase activity in leaves, gene expression of sugar transporters in leaves and berries and phloem anatomy were examined at full veraison. Accumulation of glucose and fructose in berries was hastened in ABA-treated plants at the stage of full veraison, which was correlated with enhancement of *Vitis vinifera HEXOSE TRANSPORTER 2* (*VvHT2*) and *Vitis vinifera HEXOSE TRANSPORTER 6* (*VvHT6*) gene expression, increases of phloem area and sucrose content in leaves. On the other hand, GA_3 increased the quantity of photoassimilates delivered to the stem thus increasing xylem growth. In conclusion, stimulation of sugar transport by ABA and GA_3 to berries and stems, respectively, was due to build-up of non-structural carbohydrates in leaves, modifications in phloem tissue and modulation in gene expression of sugar transporters.

Abbreviations – Ψ_o, osmotic pressure; ABA, abscisic acid; asl, above sea level; BSA, bovine serum albumin; cv, cultivar; DAA, days after anthesis; DW, dry weight; FAA, ethanol: acetic acid: formaldehyde: distilled water; GA_3, gibberellin A_3; GAs, gibberellins; GC-FID, gas chromatography-flame ionization detection; g_s, stomatal conductance; IAA, indol-3-acetic acid; IL, average internode length; LA, leaf area; NAA, 1-naphthaleneacetic acid sodium salt; p, phloem; Pn, net CO_2 assimilation rate; SE-CC, sieve elements-companion cell complex; SL, average shoot length; x, xylem.

Introduction

Grape, along with wine industry, is one of the most economically important fruit crops worldwide. The excellence of wine is directly dependent on fruit quality, so carbon allocation in berries to build-up sugar and secondary metabolites with organoleptic properties is a key factor. In grapevine, the carbohydrates produced during photosynthesis are exported from leaves as sucrose and transported via phloem to berries (Conde et al. 2007). Sucrose migrates from mesophyll cells into the sieve elements-companion cell complex (SE-CC) of minor veins, a process known as phloem loading (Sauer 2007, Turgeon and Wolf 2009, Dinant and Lemoine 2010).

Comparative studies indicate that there is more than one plant's strategy for phloem loading (Schulz 2005, Turgeon 2010). Plant species can be grouped into three types according to their plasmodesmatal frequencies in the phloem of minor veins: type 1 has numerous plasmodesmata, type 2 has few, and type 1–2a has an intermediate amount (Gamalei 1989, 1991). Additionally, it has been proposed that type 1 species have passive phloem loading mainly throughout plasmodesmata, while type 2 have active loading via apoplast with involvement of transporters. Type 1 plants are mostly trees and shrubs, like grapevines, while type 2 plants are often herbaceous (Gamalei 1989, Gamalei 1991, van Bel and Gamalei 1992, Slewinski et al. 2013). Some reports suggest that in grapevine phloem loading from cytoplasm of mesophyll cells to SE-CC is mediated by sugar transporters together with passive bulk flow (Afoufa-Bastien et al. 2010, Hayes et al. 2010, Medici et al. 2014, Pastenes et al. 2014). However, there are no reports regarding ABA and GA_3 regulation over phloem loading and unloading in whole plants under field conditions. Three sucrose transporters, *Vitis vinifera SUCROSE CARRIER 11* (*VvSUC11*), *Vitis vinifera SUCROSE CARRIER 12* (*VvSUC12*) and *Vitis vinifera SUCROSE CARRIER 27* (*VvSUC27*) (Ageorges et al. 2000, Manning et al. 2001, Afoufa-Bastien et al. 2010), and 5 hexose transporters named *VvHT1-VvHT5*, have been cloned from various cultivars such as Pinot Noir, Ugni Blanc, Chardonnay, Cabernet Sauvignon and Syrah (Fillion et at. 1999, Vignault et al. 2005, Hayes et al. 2007).

Radial growth of plant stem happens by development of cribro-vascular cambium tissues, which affect the transport of water, minerals and photoassimilates, and ultimately plant height. Several hormonal signals have been shown to be involved in the induction and maintenance of cambium and formation of secondary vascular tissues (Sorce et al. 2013). Stimulation of cell division and maintenance of cell identity in cambium have been found after application of high concentration of indol-3-acetic acid (IAA), whereas with low IAA stimulation of xylem expansion growth and maturation occurred (Tuominen et al. 1997, Milioni et al. 2001, Moyle et al. 2002). Furthermore, xylem vessel density is enhanced by IAA (Aloni 2001). On the other hand, cytokinins combined with IAA stimulate cambium cell division and tracheary elements differentiation (Milioni et al. 2001, Nieminen et al. 2008). It has been shown that gibberellins (GAs) together with IAA stimulate cambial cell proliferation and elongation of xylem fibers (Dayan et al. 2010, Ragni et al. 2011, Dayan et al. 2012). Experiments with ABA show contrasting results. Inhibition of cambium growth through a hypothetical negative interaction with IAA has been suggested (Dumbroff et al. 1979, Little and Wareing 1981), whereas ABA sprayed on maize plants increased phloem and xylem areas in female inflorescences thus enhancing transport of water, nutrients and photoassimilates to grains (Travaglia et al. 2012).

Several studies have demonstrated that partitioning of photoassimilates is a hormonal-regulated process, and plants have the ability to re-direct the resources in response to environmental and developmental changes (Ho 1988, Wardlaw 1990, Geiger and Servaites 1991). ABA enhances the transport of photoassimilates to organs which are economically important as cereal grains (Travaglia et al. 2007) and fruits (Lü et al. 1999, Opaskornkul et al. 1999), although the mechanisms involved are mostly unknown. In *Ilex paraguariensis*, ABA sprayed to the leaves enhanced dry matter accumulation as a result of a better hydric condition of the photosynthetic tissues (Sansberro et al. 2004). In table grapes, application of GAs has been used to increase biomass and sugar content (Fidan et al. 1981, Nakamura and Hori 1985). Bastián et al. (1999) observed that GA_3 promoted carbohydrate accumulation and reduced starch content in *Sorghum bicolor* stems, possibly by stimulating α-amylase activity as it has been demonstrated in germinating seeds (Woodger et al. 2004). It is feasible that GAs facilitate the transport and storage of photoassimilates favoring the discharge from the source (Daie 1987), because it was demonstrated that photosynthesis can be inhibited by end-product accumulation (Sawada et al. 2001).

Experiments with *Vitis vinifera* cv. Malbec have shown that ABA and GA_3 enhance sugar content in berries (Moreno et al. 2011). Although these phytohormones regulate the sugar allocation in fruits, the mechanism of this process remained to be elucidated. The hypotheses of this work were that ABA and GAs enhance transport of assimilates in grapevine via: (1) increasing photosynthesis, (2) augmenting sucrose content in source leaves

thus promoting phloem loading, (3) improving phloem growth and (4) stimulating sugar transporters in source leaves and in sink berries. This article reports the effect of sprayed ABA and GA_3 on vascular anatomy, sugar accumulation in berries, physiological parameters and gene expression of sugar transporters in pot-grown plants of *Vitis vinifera* cv. Malbec under field conditions.

Materials and methods

Plant material and experimental conditions

Cuttings of *Vitis vinifera* cv. Malbec were obtained from 1-year-old cane-pruned cv. Malbec shoots collected from an experimental vineyard at INTA-Mendoza (Mendoza, Argentina). The cuttings were embedded 24 h in a solution 0.6 μ*M* NAA (1-naphthaleneacetic acid sodium salt, S. Ando & Cía SA, Buenos Aires, Argentina). Then the bases of the cuttings were maintained at 30°C in a sand/water bed, whereas the tops were exposed to 4°C in a cold room. After 5 weeks, the own-rooted cuttings were planted in 10 l pots containing grape compost as substrate. Plants were watered to field capacity every 2 days. Only one shoot per plant containing one bunch was allowed to grow under field conditions at Instituto de Biología Agrícola de Mendoza (33°0′S, 68°52′W, 940 m asl) during one growing season (2012–2013).

The assay was set in a random design with three treatments, and sampling was performed at three times during berry development (pre-veraison, veraison and post-veraison). Individual plants were used as experimental units. From a set of 12 plants per treatment, 8 replicates were used for pre-veraison and veraison non-destructive measurements, whereas 4 replicates were used in each sampling point (pre-veraison, veraison and post-veraison) for destructive measurements. The treatments consisted in the application of ABA, GA_3 and water (control) solutions with a weekly frequency from fruit set (10 days after anthesis, DAA) until complete berry maturation (130 DAA). The solutions were sprayed with a hand-held sprayer onto the whole plant until runoff in the late afternoon to minimize photodegradation of ABA. The treatment doses were chosen based upon previous work of our group (Quiroga et al. 2009, Berli et al. 2010, Moreno et al. 2011): 250 μg ml^{-1} ABA (±-S-*cis*, *trans* ABA, PROTONE SL, Valent BioSciences, Libertyville, IL), 500 μg ml^{-1} GA_3 (GIBERELINA KA, S. Ando & Cía SA) and control (water). All the solutions were added with 0.05% (v/v) Triton X-100 as surfactant.

The experiment ended at berry maturation but most of the measurements were performed at full veraison (100% colored berries). Full veraison occurred at 70 DAA in control plants, 63 DAA in ABA-treated plants and 75 DAA in GA_3-treated plants. However, pre- and post-veraison samples were taken at the same date independently of the treatment, that is at 30 and 130 DAA, respectively. Determinations of soluble sugars, starch, amylase activity, gene expression as well as anatomical analysis were performed the day after the application of hormones. To avoid circadian effects, either leaves or berries were extracted at 10:00 h.

At full veraison, four replicates per treatment were dissected into leaves, stem, root and bunch. The tissues were dried, weighed and total plant mass calculated. Stems sections (at the level of bunch insertion), leaf discs (taken from the 12th leaf from the shoot apex) and berry pedicels were kept in FAA [ethanol: acetic acid: formaldehyde: distilled water; (50:5:10:35, v/v)] solution for anatomical analysis. Leaves and berries of each phenological stage were kept at −80°C for further analysis.

Growth parameters

Shoot length (SL), number of nodes per shoot and plant leaf area (LA) were measured at 62 DAA (previous to the last application of ABA at full veraison). Internode length (IL) was assessed by dividing SL by the number of nodes per shoot. Plant LA was estimated as follows: 100 leaves per plant from 10 plants picking leaves from the apical, medium and basal zone from the shoot were randomly collected in nylon bags and kept on ice to prevent dehydration. In the laboratory, the length of the main midrib, weight of leaves and weight of two leaf discs (1 cm^2) per leaf were measured. Then, a potential regression model between the area and the midrib length of each leaf was generated. The model, which had a correlation coefficient (r^2) of 0.95, was used to transform the midrib length into LA values.

Anatomical analysis

The material collected was processed according to Travaglia et al. (2012). Micro-sections (10 μm) of leaf midrib and berry pedicels (at the berry proximal zone) were prepared using a rotary microtome, whereas stem cross-sections were prepared by hand-cut. The histological preparations were photographed with a camera (AxioCam HRc camera, Carl Zeiss, Göttingen, Germany) attached to a standard microscope (Model 16, Carl Zeiss). The boundary between xylem and phloem was easily identified because of the differential staining of the cell walls corresponding to sieve and vessel elements. The tissue area was calculated using the software Image Pro-Plus (Media Cybernetics Inc, Rockville, MD).

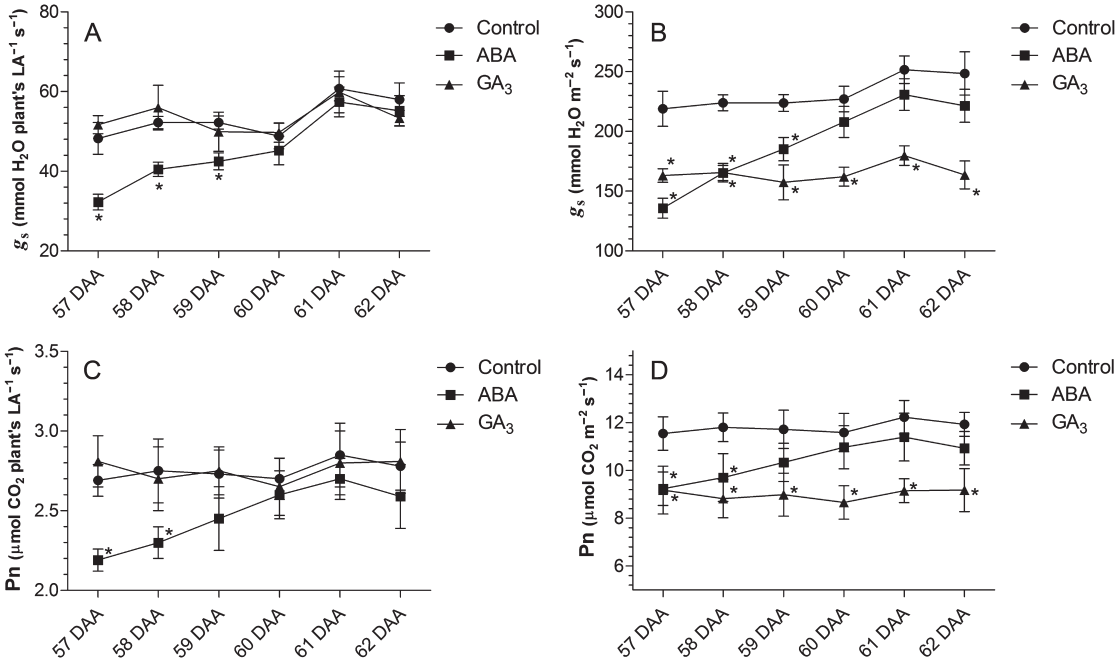

Fig. 1. (A) Plant's stomatal conductance (g_s, mmol H_2O plant's LA^{-1} s^{-1}); (B) stomatal conductance per leaf area basis (g_s, mmol H_2O m^{-2} s^{-1}); (C) plant's net photosynthesis (Pn, µmol CO_2 plant's LA^{-1} s^{-1}); (D) net photosynthesis per leaf area basis (Pn, µmol CO_2 m^{-2} s^{-1}) measured from 57 to 62 DAA. Values are means ± SE, n = 8. Asterisks indicate significant differences ($P < 0.05$).

Stomata density was assessed by taking two imprints of the abaxial surface of a fully expanded leaf (13th from the shoot apex) with transparent nail varnish. Imprints were performed in middle zone between the main midrib and the leaf margin. When imprints dried, they were mounted onto a slide for examination under optical microscope (40×). Three representative photographs per imprint were taken using an AxioCam HRc camera attached to a Zeiss Axiophot (Carl Zeiss) microscope. Stomata density was calculated as the mean value of the number of stomata per square millimeter of six photographs per leaf. The Image Pro-Plus software (Media Cybernetics Inc) was used to analyze the microphotographs.

Physiological measurements

Net CO_2 assimilation rate (Pn, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) per unit of leaf area were measured using an infrared gas analyzer IRGA CIRAS-2 (PP System, Amesbury, MA) from 57 DAA to 62 DAA, that is, the last week before veraison for the ABA-treated plants (Fig. 1). The different photosynthesis variables were set as follows: 380 mg l^{-1} of CO_2 concentration, 2000 µmol m^{-2} s^{-1} of light intensity and 200 ml min^{-1} of cuvette flow. The readings were performed in the morning (09:00 h to 11:00 h) on fully expanded leaves (10th–12th leaf from the shoot apex). To calculate plant's Pn and g_s, Pn and g_s values were multiplied by the plant's LA (calculated at 62 DAA) and expressed as µmol CO_2 plant's LA^{-1} s^{-1} and mmol H_2O plant's LA^{-1} s^{-1}, respectively.

Total chlorophylls (chlorophyll *a* and chlorophyll *b*) were determined at full veraison using a chlorophyll meter (SPAD-502 Plus, KONICA MINOLTA, Osaka, Japan). Measurements of the middle section of five leaves from the medium of the shoot were taken. Then, the mean value of the five SPAD readings per plant was considered for the statistical analysis. The results were expressed in SPAD units.

Soluble sugars and starch determinations

Measurements of soluble sugars were carried out by gas chromatography-flame ionization detection according to Bartolozzi et al. (1997). Ten deseeded berries and 10 discs of 1 cm^2 from adult leaves [collected 1 day after treatment at pre-veraison (30 DAA), full veraison and post-veraison (130 DAA)] per biological replicate were used for the extraction. Two leaf discs (1 cm^2 each) per replicate, collected 1 day after treatment at full veraison were used for starch extraction and quantification according to Moreno et al. (2011).

Protein content and amylase activity

Samples of 500 mg leaf FW collected at full veraison were homogenized using mortar and pestle with 3 ml of buffer (150 m*M* Tris–HCl pH 8.9, 3 m*M* EDTA, 1 m*M* DTT, 5 m*M* ascorbic acid, 10 m*M* $MgCl_2$ and 10% v/v glycerol). Insoluble polyvinylpolypyrrolidone (1% w/v) was then added to the homogenized tissues and centrifuged 20 min at 9000 rpm. All the procedures were done at 0–4°C. Supernatant was collected and protein content was determined at 595 nm according to Bradford (1976) with bovine serum albumin as standard. Amylase activity was assayed according to Hagenimana et al. (1994) with modifications, using 250 µl of 100 m*M* sodium citrate buffer pH 5.6, 50 µl of enzyme extract and 500 µl of 1% w/v of starch solution. The reaction mixtures were incubated 5 min at 40°C and stopped with 1 ml of 0.4 *M* NaOH. The reducing sugars produced were assessed with 3,5-dinitrosalicylic acid reagent according to the method described by Miller (1959). The amylase activity was defined according to the amount of maltose produced per milligram of total proteins and per minute.

Gene expression

RNA from leaves and berries collected at veraison was extracted according to Reid et al. (2006) from 400 mg of a fine frozen powder using liquid N_2 with mortar and pestle. Then, the total RNA was quantified at 260 nm, purified and treated with DNase using RNeasy mini spin columns and the RNase-Free DNase set (QIAGEN, Hilden, Germany) as described by the manufacturer. One microgram of RNA was primed with random hexamers primers and reverse transcribed with RevertAid Reverse Transcriptase (FERMENTAS, Vilnius, Lithuania) according to manufacturer's protocol. Gene expression analysis was carried out by real time PCR system (StepOneTM, Applied Biosytems, Foster City, CA, USA) in a 15 µl mixture containing 3 µl of a fivefold diluted cDNA, 7.5 µl Power SYBR Green PCR Master-Mix (Applied Biosystems) and 0.9 µl of 10 µ*M* of each primer. The PCR cycle was as follows: one cycle of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. A previous standard quantification curve with five serial dilutions of cDNA was constructed for each gene to calculate amplification efficiency. All primer pairs amplified a single product of the expected size, which was confirmed by melt–curve analysis and by agarose gel electrophoresis. Elongation factor 1-α (*VvEF 1-*α) was used for normalization in all experiments. The primers were designed using the software Beacon Designer version 7.70 (Premier Biosoft International, Palo Alto, CA) over the corresponding expressed sequence tags (EST) available at the NCBI GenBank database. The ratio (relative transcription amount) was obtained according to the equation 1 published in Pfaffl (2001). The primer sequences are listed in Appendix S1, Supporting information.

Table 1. Growth parameters of *Vitis vinifera* cv. Malbec plants measured at 62 DAA. Values are means ± SE, n = 8. Different letters indicate significant differences ($P < 0.05$).

Treatment	SL (cm)	IL (cm)	LA ($cm^2 \times 10^3$)
Control	156.40 ± 8.91 *b*	4.15 ± 0.22 *b*	2.33 ± 0.11 *b*
ABA	164.28 ± 11.37 *b*	4.25 ± 0.21 *b*	2.37 ± 0.12 *b*
GA_3	199.11 ± 3.25 *a*	5.06 ± 0.08 *a*	3.06 ± 0.09 *a*

Statistical analysis

One-way ANOVA and Fisher's multiple comparison of means were used to discriminate between the averages by the minimum difference, with a significance level of $P < 0.05$. Regarding relative expression of genes the permutation test was performed. Analysis was done with InfoStat software (http://sites.google.com/site/fgstatistics).

Results

Sprayed ABA and GA_3 modify the plant's phenology and physiology

ABA shortened the time to full veraison by 7 days (63 DAA), whereas GA_3-treated plants showed a delay of 5 days (75 DAA) compared with the control (70 DAA). As expected, applications with GA_3 increased shoot length as a consequence of higher internode length (Table 1). Also, LA was enhanced by GA_3, whereas ABA did not modify any growth parameter. ABA diminished plant's g_s, and hence plant's Pn during 3 and 2 days post applications, respectively. Then, the values of g_s and Pn increased reaching the control ones (Fig. 1). The same pattern was observed when the results were expressed per unit of LA. On the other hand, applications with GA_3 reduced g_s and Pn per unit of LA, whereas when those variables were expressed per plant basis, no differences were observed between GA_3 and control (Fig. 1). Furthermore, stomatal density and chlorophylls content were decreased by GA_3 applications (Figs. 2 and 3).

ABA hastens berry ripening allowing sugar accumulation in berries

Fig. 4A–C show sucrose, glucose and fructose leaf contents expressed in µg cm^{-2} at the three phenological stages. All the treatments presented the same pattern

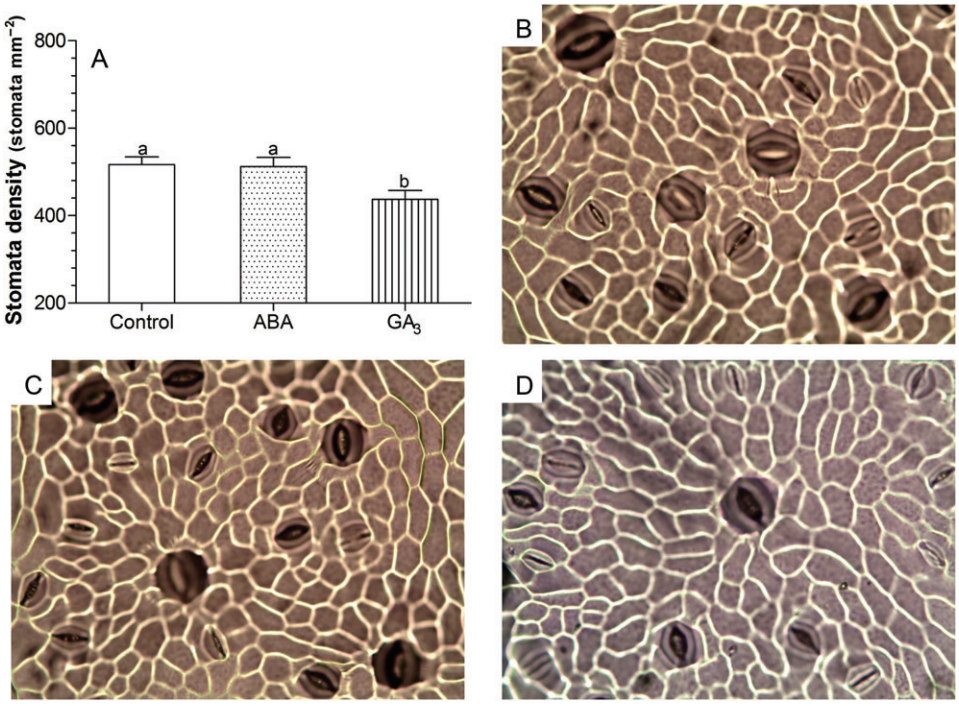

Fig. 2. (A) Stomata density (stomata mm^{-2}) in leaves of *Vitis vinifera* cv. Malbec plants at full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Values are means ± SE, n = 8. Different letters indicate significant differences ($P < 0.05$). Microphotographs of leaves imprints; (B) control; (C) ABA; (D) GA_3.

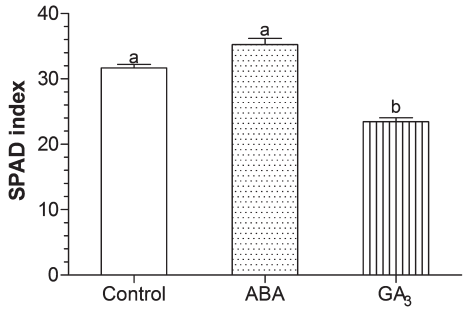

Fig. 3. Photosynthetic pigments (SPAD index) in leaves of *Vitis vinifera* cv. Malbec plants at full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants).Values are means ± SE, n = 8. Different letters indicate significant differences ($P < 0.05$).

in leaf soluble sugars accumulated along berry maturation. The sucrose content decreased from pre-veraison to full veraison and then increased at post-veraison. Glucose and fructose contents decreased constantly from pre- to post-veraison, and their levels were lower compared with control. However, ABA- and GA_3-treated plants showed almost constant leaf sucrose concentration throughout berry ripening.

ABA-treated berries accumulated the same amount of glucose and fructose as the control during berry ripening (Fig. 5A and B), but in a shorter time. While control berries reached full veraison in 70 DAA, ABA berries did it in 63 DAA, and GA_3 berries in 75 DAA with the higher concentration of hexoses at post-veraison (Fig. 5A and B). The pattern of sucrose accumulation during berry ripening was similar between control and GA_3 berries, increasing from pre-veraison until full veraison and then decreased in post-veraison. Whereas ABA-treated berries showed a lower sucrose concentration at full veraison and a higher concentration at post-veraison as compared with the other treatments (Fig. 5C).

Fig. 6A and B shows starch content and amylase activity in leaves at full veraison. Applications with ABA and GA_3 reduced the leaf starch content as compared with control, having the GA_3-treated plants the lower values (Fig. 6A). Amylase activity was promoted by GA_3, whereas ABA treatment remained similar to control (Fig. 6B). Even though the plant's DW showed no differences among treatments (data not shown), an increase of the relative berries DW was observed in ABA-treated plants (18%) as compared with GA_3 and control (9 and 12% respectively, Fig. 7). GA_3-sprayed plants showed the major proportion of DW in the stem (65 vs 43% of control, Fig. 7) in detriment to root, leaves and berries DW.

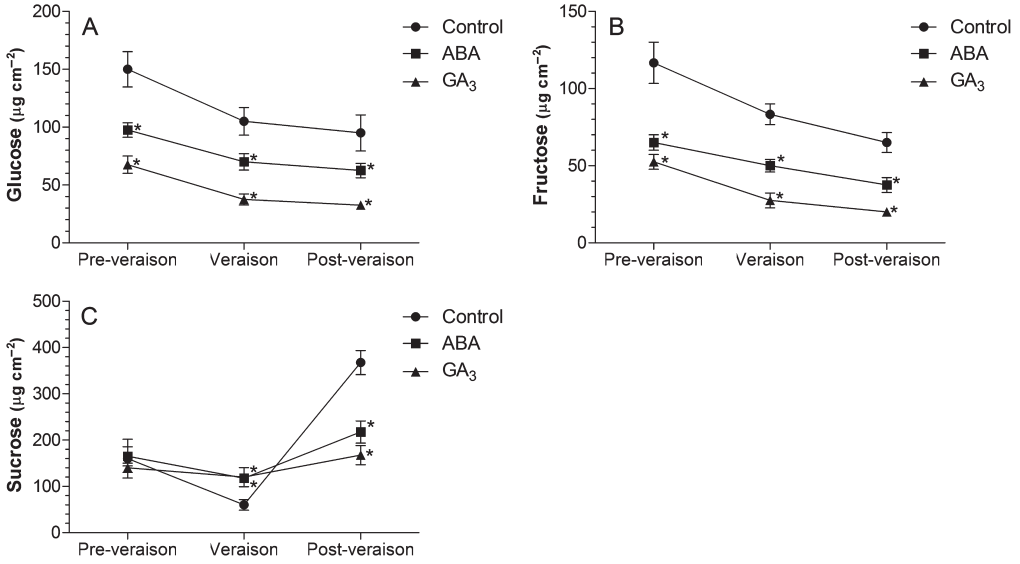

Fig. 4. Effect of ABA and GA_3 on leaf sugars content (µg cm^{-2}). Measurements were performed at pre-veraison (30 DAA), full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants) and post-veraison (130 DAA). Content of (A) glucose; (B) fructose; (C) sucrose. Values are means ± SE, n = 4. Asterisks indicate significant differences ($P < 0.05$).

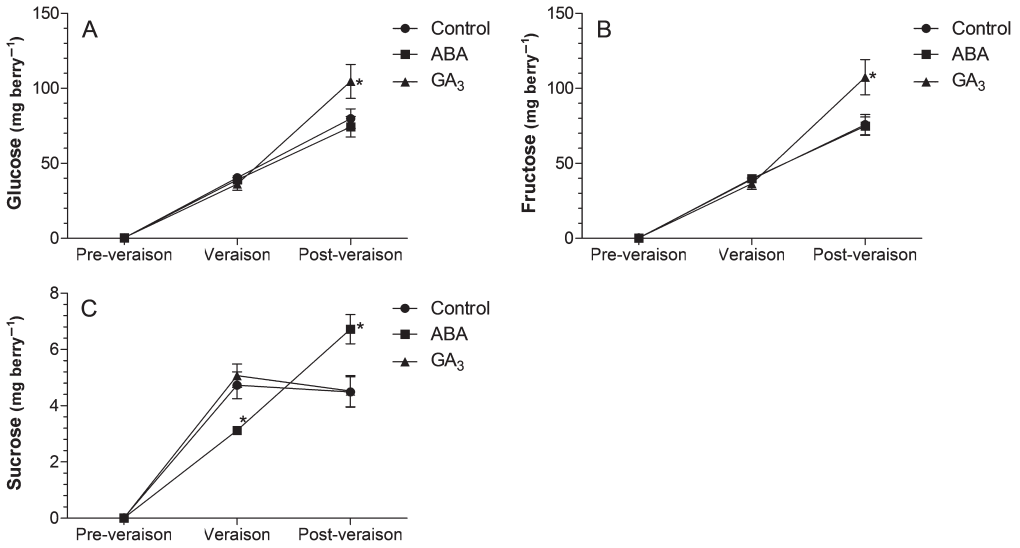

Fig. 5. Effect of ABA and GA_3 on berry sugars content (mg $berry^{-1}$). Measurements were performed at pre-veraison (30 DAA), full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants) and post-veraison (130 DAA). Content of (A), glucose; (B), fructose; (C), sucrose. Values are means ± SE, n = 4. Asterisks indicate significant differences ($P < 0.05$).

ABA and GA_3 enhance phloem and xylem tissue areas

Leaf midrib and berry pedicel phloem areas were enhanced by ABA and GA_3 in a similar way as compared with the control (Table 2 and Fig. 8A–F). In addition, the stems of ABA-treated plants presented the highest phloem area (Table 2 and Fig. 8G–I). ABA and GA_3 significantly increased cross-section area of midrib, berry pedicel and stem xylem, although GA_3 presented the higher values (Table 3 and Fig. 8).

ABA and GA_3 modify the expression of sugar transporters genes in leaves and fruits

The expression of seven genes purportedly involved in sugar transport (*VvHT1*, *VvHT3*, *VvHT5*, *VvHT6*, *VvSUC12* and *VvSUC27*) and metabolism (*Vitis vinifera*

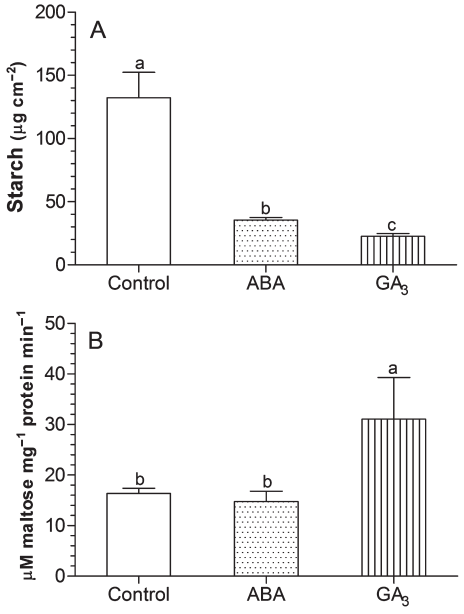

Fig. 6. Effect of ABA and GA_3 on (A) starch content of leaves (μg cm^{-2}) and (B) amylase activity (μmol maltose mg^{-1} protein min^{-1}). Measurements were performed at full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Values are means ± SE, n = 4. Different letters indicate significant differences ($P < 0.05$).

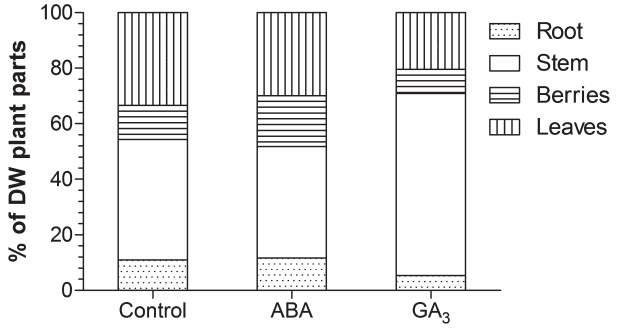

Fig. 7. DW distribution (%) in the different plant parts of *Vitis vinifera* cv. Malbec at the stage of full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Values are means ± SE, n = 4. Different letters indicate significant differences ($P < 0.05$).

VACUOLAR INVERTASE 1, *VvGIN1*) were measured in leaves at full veraison (Fig. 9). ABA upregulated *VvHT1* and *VvGIN1* by threefold and sixfold respectively, while the hexose transporter genes *VvHT3*, *VvHT5* and *VvHT6* were downregulated twofold, threefold and 1.6-fold, respectively as compared with control. Furthermore, the sucrose transporter genes *VvSUC12* and *VvSUC27* were downregulated 1.6-fold in relation to the control. Applications of GA_3 downregulated the hexose transporter genes, *VvHT3* and *VvHT5*, by threefold and 1.6-fold compared with control, and also downregulated

Table 2. Phloem area of leaf midrib, berry pedicel and stem cross-sections of *Vitis vinifera* cv. Malbec measured at the stage of full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Values are means ± SE, n = 4. Different letters indicate significant differences ($P < 0.05$).

	Phloem area		
Treatment	Midrib (μm^2 10^3)	Pedicel (μm^2 10^3)	Stem (mm^2)
Control	34.73 ± 1.08 *b*	13.63 ± 1.21 *b*	10.16 ± 0.55 *b*
ABA	48.81 ± 1.91 *a*	19.15 ± 0.48 *a*	12.49 ± 0.53 *a*
GA_3	48.76 ± 6.44 *a*	19.28 ± 0.79 *a*	9.75 ± 0.41 *b*

the sucrose transporter gene *VvSUC27* by 10-fold. Contrariwise, the vacuolar invertase gene *VvGIN1* was upregulated almost 30-fold in relation to the control, whereas the genes *VvHT1*, *VvHT6* and *VvSUC12* remained unaltered as compared with control.

Fig. 10 shows expression of the genes involved in sugar transport and metabolism of berries at full veraison. ABA upregulated *VvHT2*, *VvHT6* by 2.5-fold and *VvGIN1* by sevenfold while the gene *VvSUC12* remained unaltered compared with control. In addition, GA_3 downregulated approximately twofold the genes *VvHT2*, *VvHT6*, whereas the genes *VvSUC12* and *VvGIN1* remained unaltered as compared with control.

Discussion

The grapevine berries are fruits that from veraison (50% of colored berries in red grapes) store considerable amounts of sugars in vacuoles of the mesocarp cells. Although the main long-distance transported sugar is sucrose, sugars are stored as hexoses, specifically glucose and fructose (Conde et al. 2007). In this study, we observed that ABA modifies the berry phenology reaching veraison 7 days earlier with respect to control, confirming thus the role of ABA on the regulation of the timing of ripening (Wheeler et al. 2009).

Like in most fruits, sugar accumulation in grape berries does not only depends on the amount of carbon fixed by photosynthesis but also on the sink strength. In this work, ABA-treated plants showed a transient decrease in Pn caused by g_s temporal depletion because of partial stomatal closure. In the case of GA_3-treated plants, a clear correlation was found between g_s and Pn related to stomatal density and chlorophylls content, suggesting that photosynthesis was affected by leaf expansion (dilution effect). In fact, when g_s and Pn were expressed per plant basis no differences were observed between GA_3 and control.

It is known that the driving force which moves the assimilate flux throughout the phloem is the difference of turgor pressure ($\Delta P = \Delta \Psi_p$) between two zones of the sieve element (Taiz and Zeiger 1998). The ΔP is given by

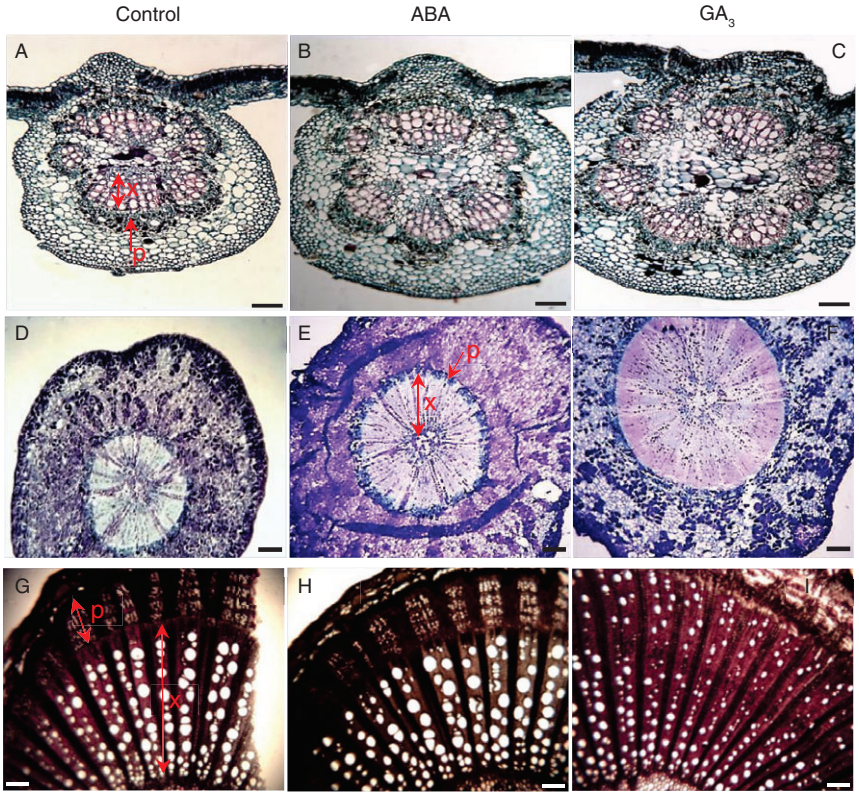

Fig. 8. Microphotographs of cross-sections corresponding to (A–C) leaf midrib; (D–F) berry pedicel and (G–I) stem of *Vitis vinifera* cv. Malbec plants at full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). (A), (D), (G), control; (B), (E), (H), ABA-treated; (C), (F), (I), GA_3-treated. Scale bar 100 μm, except for berry pedicel cross-sections which is 50 μm. x: xylem; p:phloem.

two components P_{source} and P_{sink}, thus $\Delta P = P_{source} - P_{sink}$ (Keller 2010). So, the higher the ΔP, the higher will be the sink strength of a certain organ. It has been postulated that woody plants, like grapevines, are mainly passive phloem loaders (Slewinski et al. 2013). This means that sucrose must freely diffuse, via plasmodesmata, from the cytoplasm of mesophyll cells to the SE-CC augmenting P_{source}. Thus, the concentration of sucrose must be higher in the cytoplasm of mesophyll cells to sustain the transport. Pastenes et al. (2014) have shown that at the time of strong sugar accumulation in berries, the sucrose: starch ratio was elevated in leaves. According to this, the higher sucrose content measured in ABA- and GA_3-treated leaves at the stage of full veraison might be related with an improvement in phloem loading (higher P_{source}). However, it seems that the minor leaf starch content observed in ABA-treated plants was a consequence of the lower photosynthetic rate per leaf area basis rather than a major rate of starch: sucrose conversion mediated by amylases (see Fig. 6). In addition, GA_3-treated leaves showed the minor starch amount as a consequence of a minor Pn per LA basis and greater amylases activity. Furthermore, the low content of hexoses in leaves observed in ABA- and GA_3-treated plants, likely by sucrose hydrolysis, was observed only in samples at full veraison. Altogether, the results suggest that the starch: sucrose: monosaccharaides conversion is not lineal and there might be other biochemical processes that regulate the concentration of each compound in grapevine leaves.

Table 3. Xylem area of leaf midrib, berry pedicel and stem of cross-sections of *Vitis vinifera* cv. Malbec plants measured at full veraison (70 DAA in control, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Values are means ± SE, n = 4. Different letters indicate significant differences ($P < 0.05$).

	Xylem area		
Treatment	Midrib (μm² 10³)	Pedicel (μm² 10³)	Stem (mm²)
Control	67.04 ± 1.69 *c*	39.64 ± 1.79 *c*	19.88 ± 1.12 *c*
ABA	83.87 ± 2.52 *b*	63.17 ± 2.73 *b*	23.30 ± 1.03 *b*
GA_3	108.85 ± 1.11 *a*	113.13 ± 4.18 *a*	32.21 ± 1.69 *a*

Some reports suggest that in grapevine an active phloem loading mediated by sugar transporters might be acting together with the passive bulk flow from

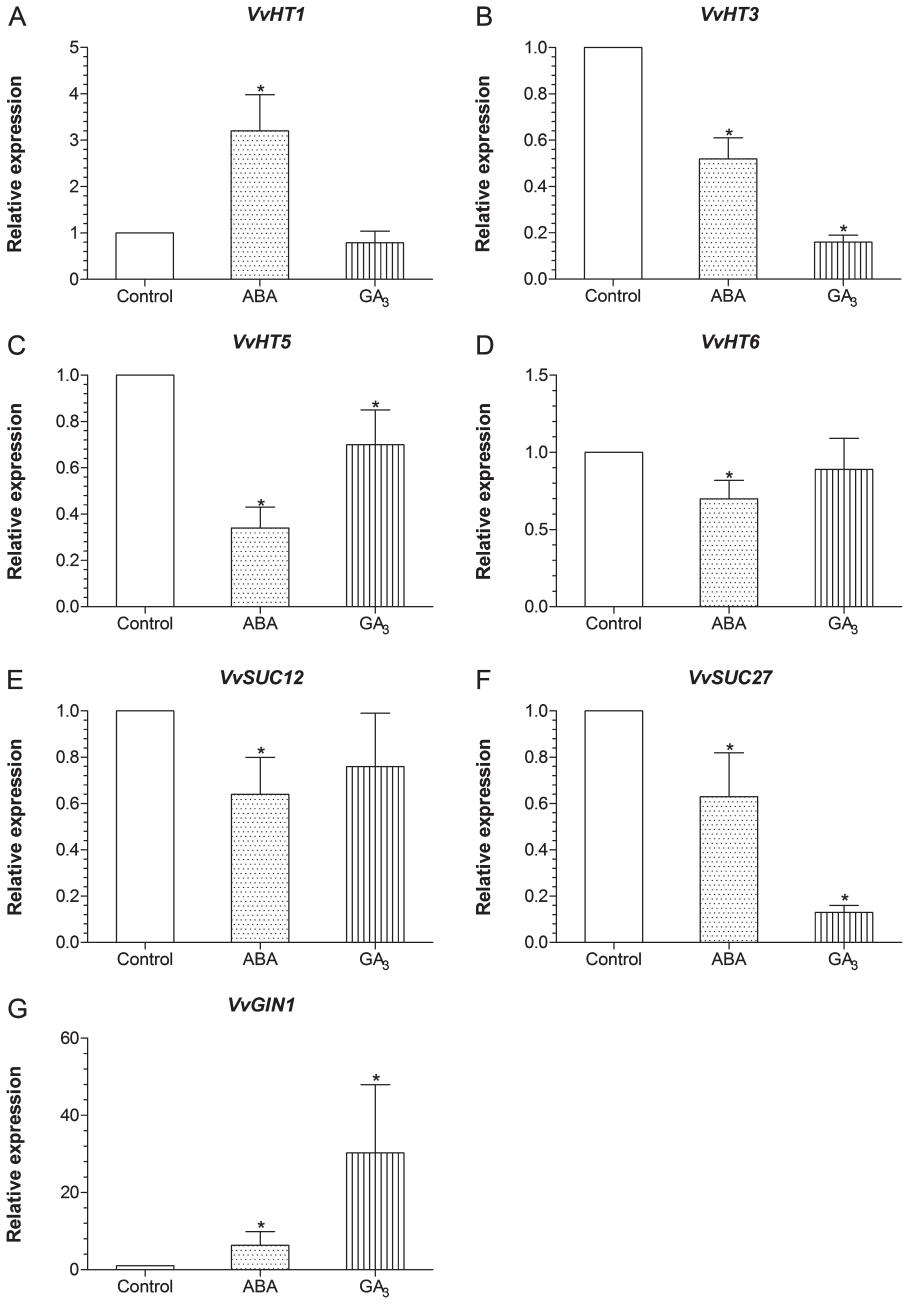

Fig. 9. Effect of ABA and GA_3 on gene expression of sugar transporters and vacuolar invertase measured in leaves at full veraison (70 DAA in control plants, 63 DAA in ABA- and 75 DAA in GA_3-treated plants). Relative expression of (A) *VvHT1*; (B) *VvHT3*; (C) *VvHT5*; (D) *VvHT6*; (E) *VvSUC12*; (F) *VvSUC27*; (G) *VvGIN1*. Values are means ± SE, n = 4. Asterisks indicate statistically significant differences by the permutation test ($P < 0.05$). All values were normalized to the expression of *VvEF 1-α*.

cytoplasm of mesophyll cells to SE-CC (Afoufa-Bastien et al. 2010, Hayes et al. 2010, Medici et al. 2014, Pastenes et al. 2014). The results presented here give new insights about the regulation of sugar transporters by ABA and GA_3 in whole plants growing under field conditions. Our results show a general downregulation of either hexose or sucrose transporters with the applications of the phytohormones. The genes *VvHT3*, *VvHT5* that encode for two plasmatic membrane hexose transporters (Hayes et al. 2007) and the gene *VvHT6* that encodes for a purportedly tonoplast hexose transporter (Afoufa-Bastien et al. 2010, Çakir and Giachino 2012),

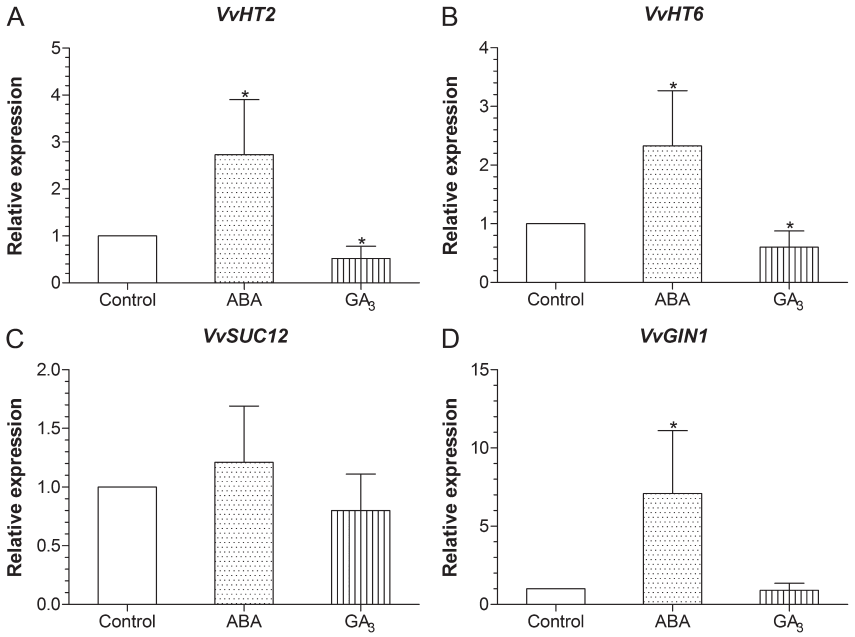

Fig. 10. Effect of ABA and GA_3 on genes expression of sugar transporters and vacuolar invertase measured in berries at full veraison (70 DAA in control plants, 63 DAA in ABA plants and 75 DAA in GA_3 plants). Relative expression of (A) *VvHT2*; (B) *VvHT6*; (C) *VvSUC12*; (D) *VvGIN1*. Values are means ± SE, n = 4. Asterisks indicate statistically significant differences by the permutation test ($P < 0.05$). All values were normalized to the expression of *VvEF 1-α*.

were downregulated by ABA and GA_3. Hayes et al. (2010) showed that *VvHT5* was positively regulated by ABA in leaves of Chardonnay plants deprived of bunches, but in this case it is possible that the source: sink relationship had been highly modified as compared with our experiments. In this experiment, it is likely that the expression of *VvHT3*, *VvHT5* and *VvHT6*, at least in leaves, may have been regulated by hexoses content rather than by ABA and GA_3 because the change observed in carbohydrates concentration in the phytohormones-treated plants (see Fig. 4). Santi et al. (2013) working with laser micro-dissection of leaf grapevine phloem showed that *VvSUC27* was expressed in phloem cells while *VvSUC11* and *VvSUC12* were expressed mostly in mesophyll cells. So far, the subcellular localization of those proteins remains unexplored. The high sucrose content and the downregulation of *VvSUC12* and *VvSUC27* in phytohormone-treated leaves, suggest a sucrose negative regulation at full veraison. On the other hand, there was no correlation between the high expression of the vacuolar invertase *VvGIN1* in ABA and GA_3 treatments and the content of sucrose and hexoses. That is, it seems that sucrose was not accumulated in vacuole because of the depletion in carbon fixation (Pn) per LA basis and the loading of the sieve element as a result of ABA and GA_3 signaling.

During veraison grape berries switch their phloem unloading mechanism from symplastic, mediated by plasmodesmata, to apoplastic, mediated by sugar transporters (Zhang et al. 2006). It has been reported that at this phenological stage, the sucrose unloaded in the apoplast, is mainly hydrolyzed by cell wall invertases (cwINVs) producing glucose and fructose (Zhang et al. 2006) but a little fraction gets into the mesocarp cells mediated by sucrose transporters. Once there, the sucrose is hydrolyzed by vacuolar invertases (GINs). In our results, it can be observed that ABA upregulated *VvGIN1* whereas *VvSUC12*, the main sucrose transporter in berries at veraison stage (Ageorges et al. 2000, Manning et al. 2001, Afoufa-Bastien et al. 2010), remained unaltered as compared with control. This was in concordance with results of Pan et al. (2005) who found that ABA enhances VvGINs activity in developing berries. The results suggest that the minor sucrose content in berries of ABA-treated plants (see Fig. 5) might be because of overexpression of *VvGIN1*.

After sucrose hydrolysis by cwINVs, hexose transporters take up glucose and fructose into the mesocarp cells reducing the osmotic pressure (Ψ_o) in the apoplast. As a consequence, the sieve turgor pressure at berry level (P_{sink}) diminishes, because it has been stated that a drop in Ψ_o leads to a fall in P_{sink} (Keller 2010). Genes *VvHT1*, *VvHT2*, *VvHT3* and *VvHT6* are highly expressed at all

stages of berry development (Afoufa-Bastien et al. 2010). However, only *VvHT2* and *VvHT6* transcripts are highly accumulated at veraison (Afoufa-Bastien et al. 2010). When the relative expression of *VvHT2* and *VvHT6* were measured in ABA treatment, it was observed an increment of their transcripts, so suggesting an improvement of sugar phloem unloading. Assuming a correlation between the phloem area and the number of sieve tubes (Canny 1973), the upregulation of those genes in addition to a major phloem area at berry pedicels, leaf midrib and stems may lead to a major flux of assimilates and in consequence an advance of ripening, as it was observed after ABA application. The increment on phloem area in ABA-treated plants is supported by the results of Travaglia et al. (2012) who found out in maize that phloem connections toward the growing ovaries were enhanced by ABA applications. Conversely, other researchers have found negative effects of ABA regarding vascular development. Popko et al. (2010) stated that ABA might operate by downregulating aquaporin genes, reducing auxin concentration in the stem or interfering with auxin signal transduction. ABA is known to repress several genes involved in the hydrolysis of various cell wall polymers; in this way, the hormone hinders cell wall loosening, which is a prerequisite for cell expansion (Gimeno-Gilles et al. 2009).

A different scenario can be visualized regarding GA_3-treated plants. Applications with GA_3 showed high leaf midrib, stem and berry pedicel phloem area, so improving the structural capacity for the conduction of assimilates. However, GA_3 stimulated C allocation in stems instead of berries. Thereby, it seems that GA_3 may prevent the flow of C to bunches, diminishing the expression of hexose transporters in berries. In this sense, GA_3 delayed veraison (5 days later respect to control), so the GA_3-treated berries have similar fructose and glucose content than the control ones but in different times. Furthermore, unlike ABA treatment, the plants applied with GA_3 showed similar amounts of sucrose which is explained by the same transcription pattern of *VvSUC12* and *VvGIN1*.

Conclusions

To our knowledge, this is the first report showing that ABA and GA_3 regulate the timing of ripening in grapevine by modifying non-structural carbohydrates concentration in leaves, expression of sugar transporter genes and phloem area. ABA-and GA_3-enhanced source (leaves) passive phloem loading keeping high sucrose concentration at the time of strong sugar accumulation. In addition, ABA-enhanced sink (berries) strength due to upregulation of *VvHT2* and *VvHT6* genes coding for hexose transporters. Applications of phytohormones, at physiological doses, promoted phloem and xylem area growth in mature leaves, berry pedicels and stems, leading to a purportedly better transport of assimilates from leaves to sink. In addition, even though GA_3 showed the same phloem development pattern as ABA, the major quantity of carbohydrates was delivered to the stem xylem tissue due to a downregulation of *VvHT2* and *VvHT6* in berries.

Authors' contributions

G. M. designed the experiments, performed the physiological, transcriptional and biochemical experiments, analyzed data and wrote the manuscript. M. P. participated in the physiological and biochemical experiments and assisted in writing the manuscript. H. R. performed the anatomical analysis. R. B. and G. B. performed the soluble sugars determinations. S. G. T. participated in primers design and assisted in transcriptional experiments. R. B. and P. N. P. assisted in analysis of data and in writing the manuscript. All authors have read and approved the final manuscript.

Acknowledgements – The authors are grateful to D. Moreno (IBAM, CONICET-UNCuyo) for her excellent assistance in the starch content assay. This work was financially supported by CONICET (PIP 2009 to P. N. P.), FONCYT (PAE-PICT2007-02190 to P. N. P., PICT2008-1666 and PAE-PID2007-00149 to R. B.) and SeCTyP-UNCuyo to P. N. P. and R. B.).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Primer sequences used in qRT-PCR experiments.

Edited by C. Foyer