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Research article

Exogenous gibberellic acid application induces the overexpression of key genes for pedicel lignification and an increase in berry drop in table grape

Miguel García-Rojas[a], Marco Meneses[a,c], Kristen Oviedo[a], Carlos Carrasco[a], Bruno Defilippi[a], Mauricio González-Agüero[a], Gabriel León[b], Patricio Hinrichsen[a,*]

[a] Instituto de Investigaciones Agropecuarias, INIA-La Platina, Santiago, Chile
[b] Centro de Biotecnología Vegetal, Facultad de Ciencia Biológicas, Universidad Andrés Bello, Santiago, Chile
[c] Doctorado en Ciencias Silvoagropecuarias y Veterinarias, Campus Sur, Universidad de Chile, Santiago, Chile

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ABSTRACT

Most table grape (*Vitis vinifera* L.) varieties require gibberellic acid (GA_3) applications to obtain an adequate berry size in order to satisfy market requirements. However, GA_3 treatments also produce severe berry drop in some cultivars, which occurs mainly after a cold storage period during post-harvest. Berry drop in bunches treated with GA_3 has been related to the hardening and thickening of the pedicel produced by the over-accumulation of cellulose and its lignification. The main goal of this study was to compare the morphology and gene expression in pedicel samples of genotypes contrasting for berry drop susceptibility. These genotypes are Thompson Seedless, which exhibits a low incidence of berry drop, and a genetic line (Line #23) of INIA's breeding program that is very susceptible to berry drop at harvest and after storage in bunches sprayed with GA_3. The parameters measured to study this phenomenon during fruit growth and post-harvest storage included fruit detachment force (FDF), hardness and thickness of the pedicel and berry drop frequency. Histological analyses of pedicel structures at harvest showed an increase in cell size and deposition of lignin in the cortex zone in both contrasting genotypes treated with GA_3. The expression profile in both genotypes of the key lignin biosynthesis genes *Vv4CL4, VvCCR1L* and *VvCAD1* analyzed by quantitative real time PCR (qPCR) revealed evident changes in response to GA_3 treatments. In particular, gene *VvCAD1* is overexpressed (100X) in pedicels of line #23 treated with GA_3 after 30 and 45 days in cold storage compared to control. Moreover, the frequency of berry drop was higher for Line #23 treated with GA_3 than for the control (23% *vs*. 1%). Our results suggest that gibberellic acid regulates the expression of the biosynthesis of lignin genes, generating changes in cell wall composition and pedicel structure that result in an increase in berry drop.

1. Introduction

Berry drop represents a significant commercial loss for table grape, since it gives a senescent appearance to a fresh product, affecting market sales (Defilippi and Retamales, 2000). Hence it is relevant to predict and control berry drop in table grapes, not only from a scientific perspective, but also because of its commercial importance (Cooper et al., 1993; González-Carranza et al., 1998). For instance, in Chile berry drop is an important post-harvest problem, and is tolerable only if it affects less than 5% of the production (Lizana, 1995). The incidence of berry drop varies considerably from one season to another and among cultivars/genotypes. Kyoho, for example, is very susceptible to berry drop and represents the main problem for grape storage and marketing in China (Wu et al., 1992).

Depending on the cultivar, berry drop can be the result of mishandling of the fruit during harvesting and/or packing. Thus, the incidence of berry drop can be minimized by handling the fruit carefully and maintaining recommended levels of temperature and relative humidity, using shallower packing boxes and packaging bunches individually (Crisosto et al., 1994). Also, the relationship between the use of gibberellins (GAs) for berry growth and shattering has been known for decades. The main roles of GAs are related to the regulation of seed germination, leaf expansion, flowering, fruit development, mediation of environmental signals such as day light and, at cellular level, to stimulate both cell elongation and division (Biemelt et al., 2004; Kende and Zeevaart, 1997).

The most common table grape cultivar produced in Chile is Thompson Seedless; like most commercial table grapes it requires applications of exogenous gibberellic acid (GA_3) to reach a commercial size. These GA_3 applications can have adverse effects, such as increased

* Corresponding author. Santa Rosa 11, 610, Santiago, Chile.
E-mail address: phinrichsen@inia.cl (P. Hinrichsen).

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incidence of berry drop (Crisosto et al., 1994). Other factors such as water loss, in addition to causing browning can also lead to increased incidence of berry drop (Crisosto et al., 2001). Cooper et al. (1993) showed that GA_3 applications on cv. Thompson Seedless produced changes in the length and diameter of the pedicel. Nakamura and Hori (1981) reported similar results in cv. Kyoho, where rachis hardness was increased by GA_3 treatment and often accompanied by an increase in the dimensions of the pedicel and rachis. These changes were associated with cellulose accumulation and lignification of the pedicel, which have been proven to be involved in berry drop (Fidan et al., 1981). Pedicel thickening is produced by an increase in the number of secondary xylem cells and their lignification (Nakamura and Hori, 1985). Likewise, Botti and Cooper (1994) showed that GA_3 generates changes at the cellular level, increasing the number of cells of the cortex, xylem and pith in the pedicel. Retamales and Cooper (1993) proposed the loss of pedicel flexibility as the main factor to explain berry drop in Thompson Seedless in response to GA_3 treatments. In other plant species, changes in gibberellin level affect the expression of certain genes such as lignin biosynthetic genes in tobacco (Biemelt et al., 2004).

Lignin is one of the most important end products in phenylpropanoid metabolism and the main compound implied in secondary cell wall structure, accounting for strength, hydrophobicity and rigidity of vascular and supportive tissues (Bonawitz et al., 2014). Lignin is a polymer derived from the dehydrogenative polymerization of three different monolignols, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Boerjan et al., 2003). To make lignin, all the genes of the lignin biosynthesis pathway need to be coordinately turned on. It has been shown that a common *cis*-element, namely the AC element, is present in the majority of the lignin biosynthetic genes and is required for their expression in lignifying cells (Zhong and Ye, 2009). In addition, different growth regulators such as auxin (IAA) and GA stimulate and control lignin formation in vascular tissues (Aloni et al., 1990; Biemelt et al., 2004).

The aim of the present study was to analyze three of the key genes related to lignin biosynthesis, under the hypothesis that their expression would differentially change after GA_3 treatment, paralleling the increase in berry drop. These genes are 4-coumarate: coenzyme A ligase (4CL) that catalyzes the conversion of cinnamic acid derivatives to their corresponding coenzyme A (CoA) esters, cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD); these latter genes code for the enzymes involved in the last steps of the monolignol pathway. For this purpose, we used pedicel tissue belonging to two genotypes of table grape applied with gibberellic acid which present different susceptibility to berry drop. To our knowledge, this is the first study of gene expression in this relevant tissue, in relation to berry drop incidence in table grape.

2. Materials and methods

2.1. Plant material and maturity parameters

This study was performed in a field located at La Platina Research Center of the Instituto de Investigaciones Agropecuarias (INIA), Santiago-Chile (33°34′20″S; 70°37′32″W; 630 m), during the 2015–2016 growing season. The plant material corresponded to two seedless table grape varieties: Thompson Seedless, which usually exhibits a low incidence of berry drop, and “Line #23” (Ruby Seedless x Centennial Seedless) from INIA's breeding program, which has shown a high incidence of this disorder at harvest and after storage, especially in bunches applied with GA_3. Fruit was harvested when it reached maturity, based on soluble solids content near 18 °Brix. For berry drop evaluations at harvest and post-harvest, 10 bunches per treatment from different plants were stored for 15, 30 and 45 days at 0 °C. The parameters analyzed at harvest were berry and pedicel diameter (mm), soluble solids (% w/w of g sucrose per 100 g solution), acidity (g L^{-1} tartaric acid equivalents) and firmness (N mm^{-1}). For this, 25 healthy and homogenous berries with their cap stems were sampled randomly from each bunch. The diameter was measured with a caliper. The soluble solids content of the same fruit was measured with a refractometer (ATC-1E, Atago, Tokyo, Japan). The firmness of the selected berries (considering skin and flesh) was performed using a firmness tester (Firmtech II, BioWorks, Wamego, KS). The titratable acidity from the pooled juice of 10 berries per bunch per variety was assayed via titration with 0.1 N NaOH (pH 8.2) and reported as g L^{-1} of tartaric acid. Additionally, a pedicel pool (1000 pedicels) was immediately frozen with liquid nitrogen and stored at −80 °C for molecular analysis, and 10 pedicels were fixed in FAA (50% [v/v] ethanol, 5% [v/v] glacial acetic acid, 10% [v/v] formaldehyde) for histological analyses.

2.2. Growth regulator treatment

Bunches of Thompson Seedless and Line #23 were subjected to the following growth regulator treatment: (1) Thompson Seedless bunches were sprayed five times (following industry standards), once with 10 ppm gibberellic acid (GA_3, Pro-Gibb 40% Valent Biosciences Chile S.A., Santiago, Chile) during pre-bloom for bunch elongation, 15 ppm for bunch thinning applied at full bloom and 200 ppm (three doses of 40, 100 and 60 ppm at seven-day intervals) when berries reached 4 or 5 mm for berry growth (Jensen et al., 1994). (2) Line # 23 was sprayed with 120 ppm GA_3 (40 ppm three times at intervals of seven days, starting at a berry diameter of 4 mm) only for berry growth; this dosage was defined in previous assays, looking for a desirable berry enlargement and the lowest possible berry drop (data not shown).

2.3. Fruit detachment force and berry drop

The fruit detachment force (FDF) between pedicel and berry was measured using a TA-XT texture analyzer equipped with a tensile grip probe (Stable Micro Systems Ltd., Surrey, UK) with slight modifications. An individual berry with its stem was put on an acrylic base and firmly clamped with a spring clamp; this clamp was fastened to the load cell fixture. The texture analyzer was programmed so that upward movement was perpendicular to the longitudinal axis of the pedicel-berry system until they were pulled apart. The maximum force was recorded during the tension test. A speed of 2 mm/s was set for test, 2 mm/s for pre-test and 5 mm/s post-test. The triggering force was 5 g; thirty berries with stems from each treatment and genotype were measured. To determine the percentage of berry shattering, 10 bunches were weighed before and after being shaken vertically for 30 s; the weight of the berries that fell was recorded. Fruit drop percentage was calculated as FD% = [(DB + SB)/TB] / 100, where DB represents the weight of berries spontaneously dropped during storage, SB represents the weight of berries dropped after shaking of bunches and TB represents the total weight of the bunch.

2.4. Quantification of pedicel hardness/stiffness

In order to approach a representative value for pedicel hardness/stiffness, a compression test was performed on the middle zone of the pedicel. For this we used a TA-XT plus texture analyzer (Stable Micro Systems) equipped with a cylindrical probe (2 mm diameter) with a 30 Kg load cell. Thirty samples were compressed to 50% deformation at a speed of 1 mm/s, 1 mm/s for pre-test, 10 mm/s post-test; the trigger force was 5 g. The texture parameter determined was hardness/stiffness, measured in Newtons (N), and defined as the maximum force necessary to compress the sample. The data were analyzed by software provided with the texturometer (Exponent Lite, version 5.0.9.0; Stable Micro Systems).

2.5. Histological analyses

Six pedicel samples of each genotype and treatment were analyzed as follows, using blind samples to avoid sample bias. Middle sections of each pedicel were longitudinally cut into 1–2 mm thick sections and immediately placed in FAA (50% [v/v] ethanol, 5% [v/v] glacial acetic acid, 10% [v/v] formaldehyde). Samples were then dehydrated in serial ethanol solutions with increasing concentrations (10, 30, 50, 70, 85 and 95%), followed by 100% xylol and saturated with paraffin at 65 °C for two days. Then 6–9 µm thick sections were cut using a microtome (Leica RM 2125 RT, Leica Biosystem, Nussloch, Germany) and mounted on microscope slides. After paraffin removal and rehydration, the samples were stained by immersion in a solution of Safranin-Fast Green for 12 h; a red-purple color indicates a positive signal for lignin. The slides were analyzed using a Leica microscope (DM500) and images were captured using the built-in camera (Leica ICC50W).

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated from 1 to 2 g of frozen tissue using the modified hot borate method (Gudenschwager et al., 2013). The quantity and quality of the RNA were assessed with a Qubit® 2.0 fluorometer (Invitrogen™ by Life Technologies, Singapore) by measuring the $A_{260}/_{280}$ ratio and by electrophoresis on a 1.2% formaldehyde-agarose gel. The first strands of cDNA were obtained by reverse transcription reactions with 2 µg of total RNA as the template, using MMLV-RT reverse transcriptase (Promega, Madison, WI) and oligo dT primers according to standard procedures. The concentration of cDNA was assessed by measuring the absorbance at 260 nm. Each cDNA sample was diluted to 50 ng μl^{-1} prior to use in the real-time quantitative PCR (qPCR) assays.

2.7. Real-time qPCR assays

The genes analyzed in this study were the following: *VvCCR1L* (cinnamoyl-CoA reductase 1-like, GenBank XM 002285332); *Vv4CL4* (4-coumarate-CoA ligase 4; GenBank XM 002272746); *VvCAD1* (cinnamyl alcohol dehydrogenase, GenBank XM 002285370) and *VvTCPB* (T-complex protein 1 subunit beta, GenBank XM 002285876). The transcript abundance was analyzed via qPCR with the LightCycler® 96 system from Roche (Roche Diagnostics, Mannheim, Germany), using SYBR Green to measure the amplified RNA-derived DNA products, as described by García-Rojas et al. (2012). Gene-specific primers (Table 1) were designed using the Primer Premier 5.0 software package (Premier Biosoft International, Palo Alto, CA) and were synthesized by IDT (Integrated DNA Technologies, Coralville, IO). The qPCR assays were performed on three samples (biological replicates) using technical duplicates for each sample. The gene expression values were normalized to the *VvTCPB* expression according to González-Agüero et al. (2013).

2.8. Experimental design and statistical analysis

A fully randomized experimental design was used; 10 bunches and 25 berries per bunch were used to evaluate physiological parameters in each treatment and genotype (biological replicates). Respect to cDNA, 10 bunches were used to collect a pool of 1000 pedicels which were homogenized and processed to obtain three samples of RNA (biological replicates). The cDNA obtained from each RNA sample was used in real time PCR assays, twice each (technical replicates). The data set was subjected to analysis of variance, and the means were separated by a least significant difference (LSD) test at 5% significance using the Statgraphics Centurion Plus 5 software package (Manugistics Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Fruit detachment force and berry drop

Fruits were harvested at 18° Brix with a firmness of 249 g/mm for Thompson Seedless and 236 g/mm for Line #23. Both genotypes presented higher FDF in samples treated with GA_3 compared to the control at every stage evaluated (Fig. 1). Similar results were reported by Retamales and Cooper (1993), where the force required to separate the berry from the pedicel, when applied longitudinally, increased in GA_3-treated samples. Thompson Seedless did not show a direct relationship between FDF and berry drop, despite the GA_3 treatment (Fig. 1A). The FDF decreased steadily in samples of Line #23 treated with GA_3 through the storage period, from 7.66 N at harvest to 4.80 N after 45 days in cold storage at 0 °C, while berry drop increased gradually from 9% to 21% (Fig. 1B). Deng et al. (2007) observed a close relationship between fruit detachment force and berry drop in Kyoho table grapes, whereas berry drop increased gradually concomitantly with reduction in the FDF. The decrease in FDF may be explained by changes in the abscission zone cells generated by forming intercellular cavities between berry and pedicel (Deng et al., 2007). Nakamura and Hori (1981) observed that the percentage of berry drop was much higher in GA_3-treated seedless berries than in seeded and seedless berries of control, non-treated clusters. Zoffoli et al. (2009) showed that pedicel thickness increased after several treatments with GA_3 (eight applications) or CPPU (GA_3 + CPPU). These treatments produced an increase in the shattering incidence on different varieties, including Thompson Seedless, Red Globe and Ruby Seedless. Other researchers have proposed that the angle of insertion between the pedicel and berry determines (to some extent) its susceptibility to berry shattering, being lower with a more obtuse insertion angle (Sarig et al., 1998). We hypothesize that one of the main factors producing higher susceptibility to berry drop is the increase in stiffness/hardness of the pedicel produced by GA_3 treatment, which in turn would not allow the berries to accommodate and change their position as they are manipulated during harvest and post-harvest, generating breakage at the pedicel-berry union.

3.2. Pedicel hardness/stiffness in contrasting phenotypes

To measure pedicel hardness/stiffness, a texture analyzer was used to determine the force required for a 50% deformation of this tissue. The control samples did not present differences in hardness between the genotypes at harvest and post-harvest stages (Fig. 2). However, bunches treated with GA_3 showed an increase in pedicel hardness after cold

Table 1
List of primers designed for qPCR considered in this study.

Gene abbreviation	Enzyme	GenBank	Primer Sequence (5′-3′)	Product size (bp)	Tm (°C)
VvCCR1L	Cinnamoyl-CoA reductase 1 like	XM_002285332	F: AGCAATGGTGATTGGTCCTCTT R: ATGTGCCTCAGCTACATCTTTA	139	60
VvCAD1L	Cinnamyl alcohol dehydrogenase 1 like	XM_002283320	F: CTGGAAGTTTCATTGGAAGCAT R: CACCACAAACCGATACCTGAC	158	60
Vv4CL4	4-coumarate-CoA ligase 4	XM_002272746	F: ACCGAGTTCGTGTTCGCATTTC R: GGAGTAGCAAGCCTGGGTGATT	144	64

F: forward primer. R: reverse primer. bp: base pairs. Tm: melting temperature given in °C.

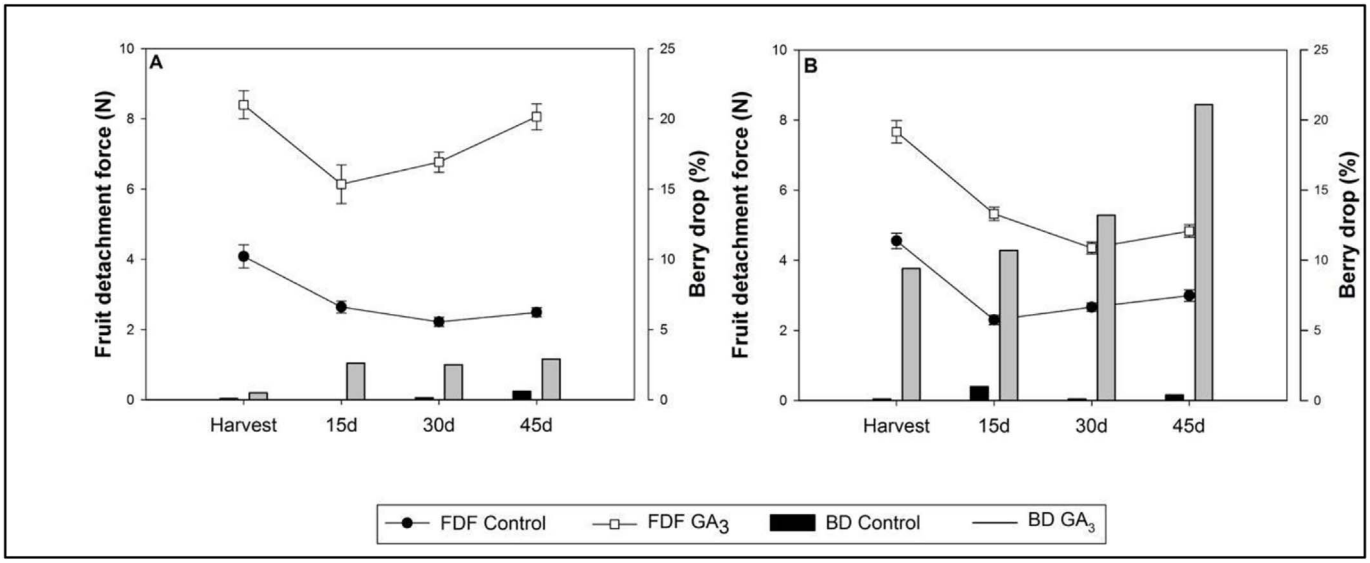

Fig. 1. Changes in the fruit detachment force (FDF) and berry drop during cold storage in contrasting phenotypes treated with gibberellic acid (GA_3). Fruits were stored for 15, 30 and 45 days at 0 °C. Data of FDF are mean of 30 berries ± standard deviation. Berry drop percentage values correspond to means of 10 bunches. **A**) Thompson Seedless harvested at 16,5° Brix. **B**) Line #23 harvested at 17° Brix. FDF: fruit detachment force; N: Newton; BD: berry drop.

storage independently of the genotype (Fig. 2). This increase in the force after cold storage may be associated with water loss in the pedicel during post-harvest, which may also lead to stem browning and berry shattering during commercialization (Crisosto et al., 2001). These results confirm the quantitative effect of GA_3 in increasing the rigidity of the pedicels, determining a reduction in rachis flexibility. Nakamura and Hori (1981) demonstrated that rachis hardness is associated with post-harvest berry drop and that it is hastened by GA_3 treatment.

3.3. Anatomical changes in the pedicel

Since the pedicel plays a major role in terms of berry drop, the next step was to study the morphological changes that the pedicel undergoes with GA_3 treatment, knowing that it results in thicker and larger pedicels for both genotypes compared to untreated controls, similar to what happens with berry width and length (Table 2). The effects of GA_3 on increase in berry size and pedicel thickening have been reported in many studies (Venkataratnam, 1964; Weaver, 1972; Singh and Weaver, 1978; Cooper et al., 1993; Pérez and Gomez, 1998). In this case, we focused on the changes observed in the hardness/stiffness of the pedicel for both genotypes treated with GA_3 (Fig. 2). To look into a possible physiological mechanism underlying these changes, longitudinal sections from pedicels at harvest were analyzed using safranin (purple-red color) for lignin identification. Thompson Seedless showed a larger cortex cell size and greater accumulation of lignin only in the samples treated with GA_3 (Fig. 3A–B), while samples from Line #23 did not present differences in the accumulation of lignin between control and treated, but showed larger cortex cells in pedicels treated with GA_3 (Fig. 3C–D). Interestingly, Fidan et al. (1981) observed that cellulose accumulation and lignification of the pedicel are associated with berry drop. Nakamura and Hori (1984) showed that the hardening of the rachis and pedicel are related to pedicel thickening, which in turn derives from an increase in the number of secondary xylem cells and their lignification. A limitation of this study is the lack of quantitative data regarding the accumulation of lignin in GA_3-treated pedicels of both genotypes, in order to validate the histological observations (Fig. 2). Indeed, we did preliminary quantifications of lignin content

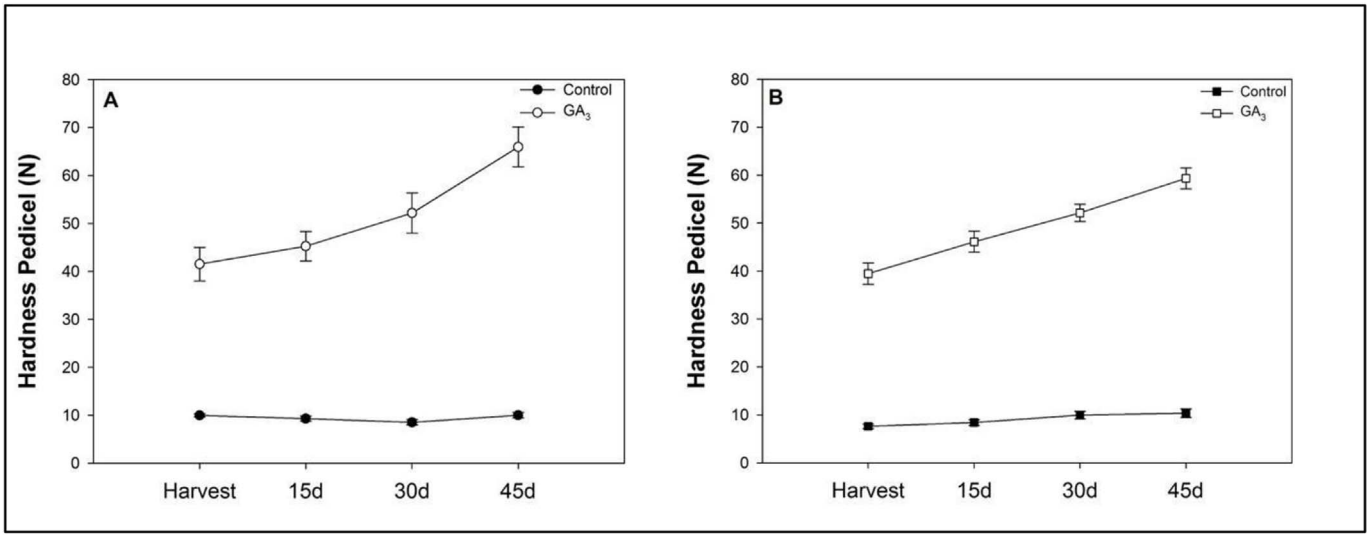

Fig. 2. Hardness of pedicels during harvest and post-harvest in contrasting phenotypes for berry drop treated with gibberellic acid (GA_3). Values correspond to the mean from 30 berries ± standard deviation. Texture analyzer was used to quantify the maximal force needed to deform 50% of the pedicel. **A**) Thompson Seedless **B**) Line #23. Fruits were stored at 0 °C for 15, 30 and 45 days. N: Newton.

Table 2
Morphological parameters for berry and pedicel during development, harvest and post-harvest.

Genotypes	Stage	Berry growth (mm)				Pedicel growth (mm)			
		Width		Length		Thickness		Length	
		Control	GA $_3$[a]	Control	GA $_3$	Control	GA $_3$	Control	GA $_3$
Thompson Seedless	*Véraison*	13.5 ± 0.17	14.7 ± 0.23	20.1 ± 0.25	23.1 ± 0.34	3.1 ± 0.06	4.8 ± 0.12	8.3 ± 0.24	8.5 ± 0.21
	Pre-Harvest	14.7 ± 0.20	17.0 ± 0.35	20.7 ± 0.25	22.9 ± 0.58	2.9 ± 0.05	4.6 ± 0.14	7.7 ± 0.21	9.0 ± 0.23
	Harvest	15.2 ± 0.23	17.2 ± 0.20	20.4 ± 0.67	25.5 ± 0.30	2.9 ± 0.06	4.8 ± 0.13	6.7 ± 0.17	8.6 ± 0.20
	15 d	13.6 ± 0.20	17.5 ± 0.27	19.5 ± 0.34	24.9 ± 0.40	2.6 ± 0.06	4.2 ± 0.12	7.0 ± 0.18	8.5 ± 0.30
	30 d	14.2 ± 0.20	17.9 ± 0.22	18.8 ± 0.59	27.2 ± 0.31	2.2 ± 0.07	4.0 ± 0.13	7.5 ± 0.25	8.8 ± 0.25
	45 d	14.1 ± 0.40	18.3 ± 0.26	20.1 ± 0.33	18.3 ± 0.25	2.7 ± 0.09	4.6 ± 0.12	6.3 ± 0.21	9.0 ± 0.25
Line # 23	*Véraison*	14.6 ± 0.11	15.6 ± 0.12	19.5 ± 0.21	21.5 ± 0.17	3.1 ± 0.10	3.9 ± 0.16	8.9 ± 0.35	9.7 ± 0.22
	Pre-Harvest	15.6 ± 0.24	17.7 ± 0.18	20.9 ± 0.31	24.5 ± 0.86	3.2 ± 0.09	3.9 ± 0.08	9.4 ± 0.32	10.4 ± 0.29
	Harvest	14.9 ± 0.52	17.3 ± 0.69	20.2 ± 0.77	25.3 ± 0.49	3.1 ± 0.05	4.3 ± 0.09	8.5 ± 0.20	9.4 ± 0.19
	15 d	15.4 ± 0.14	18.3 ± 0.38	21.8 ± 0.30	26.5 ± 0.34	2.2 ± 0.07	3.6 ± 0.09	8.3 ± 0.16	10.8 ± 0.22
	30 d	15.8 ± 0.19	17.5 ± 0.18	20.7 ± 0.34	24.8 ± 0.30	2.4 ± 0.09	3.3 ± 0.09	7.0 ± 0.22	9.3 ± 0.21
	45 d	16.1 ± 0.22	17.3 ± 0.21	22.4 ± 0.40	24.7 ± 0.22	2.2 ± 0.07	3.3 ± 0.09	8.8 ± 0.18	10.7 ± 0.28

ˆGenotypes with low berry drop treated with 200 ppm of gibberellic acid.
+Genotypes with high berry drop treated with 120 ppm of gibberellic acid.
[a] Gibberellic acid: GA_3.

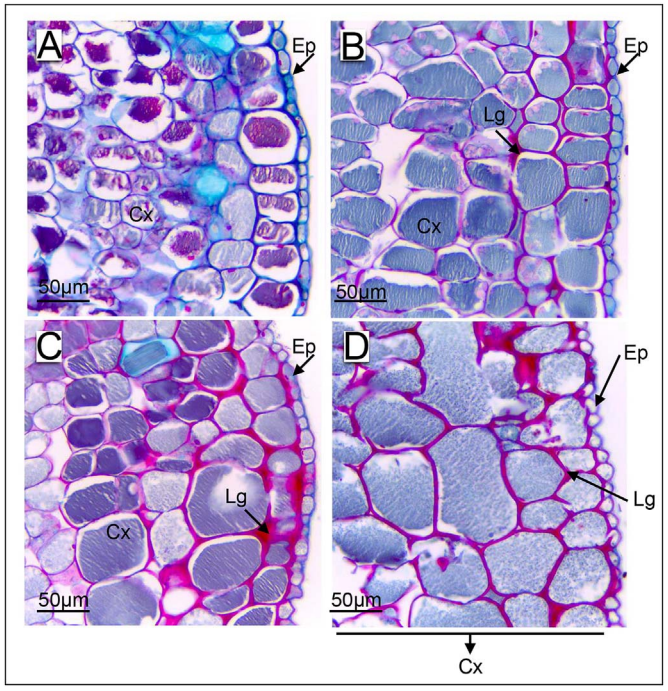

Fig. 3. Changes in the accumulation of lignin and in the size of cortex cells in contrasting phenotypes for berry drop at harvest time. Light microscopy of longitudinal section of pedicels in Thompson seedless **A**) non-treated or **B**) treated with GA3, and Line # 23 **C**) non-treated or **D**) treated with GA3. Scale bar = 50 µm. Ep: Epidermis; Lg: Lignin; Cx: Cortex cells.

on both genotypes, treated or not with GA_3, by using the acetyl bromide method described by Moreira-Vilar et al. (2014). However, the results were not very convincing, just revealing a slight but significant difference in lignin concentration in pedicels of Line #23 with or without GA_3 treatment, contrasting to Thompson seedless, in which case there were no significant differences (data not shown). Next seasons we expect to repeat these analyses in order to the measure "total content" of lignin, that has not been precisely quantified yet. The presence of different types of the polymer (lignin variants) in response to the application of GA_3, as well as the possible changes of the solubility of the lignin monomers from each genotype and condition, would be evaluated using this same or other techniques to quantify lignin content in pedicel tissue.

3.4. Expression pattern of genes involved in lignin biosynthesis

Lignin provides rigidity and hydrophobic properties to woody tissues, favoring resistance to mechanical damage, water transport, and defense against pathogen attack (Boerjan et al., 2003; Boudet et al., 2003). Lignification of cell walls in plants involves monolignol formation, transportation and polymerization, each of which is the result of the coordinated action of many enzymes (Anterola and Lewis, 2002; Baucher et al., 2003) which belong to different gene families (Xu et al., 2009). Here we studied the expression pattern of three key genes of the lignin biosynthesis pathway in pedicels collected from both contrasting genotypes. These genes are 4-hydroxycinnamoyl CoA ligase (*Vv4CL4*), cinnamoyl CoA reductase (*VvCCR1L*) and cinnamyl alcohol dehydrogenase (*VvCAD1*). In the case of Thompson Seedless not treated with the hormone, the three genes presented the same relative expression pattern in all stages studied, in contrast to Line #23, in which the expression of *Vv4CL4, VvCCR1L* and *VvCAD1* did not show significant differences during fruit development and harvest, but decreased significantly after cold storage for 30 and 45 days (Fig. 4). Also, the changes in expression in response to GA_3 treatment had different performance between the genotypes. In this case, the expression pattern for the three genes in Thompson Seedless was similar, exhibiting a maximal expression level at *véraison*, which decreased significantly during fruit development and harvest, without significant changes in post-harvest (Fig. 4). In pedicel samples of Line #23 treated with GA_3 there were different expression patterns, depending on the gene. *Vv4CL4*, for instance, did not show significant differences in expression during development, harvest and post-harvest, however *VvCCR1L* and *VvCAD1* had an expression peak during *véraison* which decreased during development, harvest and post-harvest (Fig. 4). When treated with GA_3, both genotypes showed at *véraison* an up-regulation of 4–5 times for the *Vv4CL4*, *VvCCR1L* and *VvCAD1* genes in comparison to the untreated control samples (Fig. 4). This response in the pedicels treated with gibberellic acid suggests that the expression of the lignin biosynthetic genes is up-regulated since before *véraison*. It has been described that gibberellic acid participates in the regulation of lignin biosynthesis (Aloni et al., 1990). Nakamura and Hori (1984) studied the effect of GA in the lignification of the rachis; they showed differences in lignin accumulation and phenylalanine ammonia-lyase (PAL) activity after applications of GA_3 which were genotype-dependent. Particularly in the rachis of cultivar Kyoho, an increased lignin content at three and 34 days after GA_3 treatment was determined; a similar behavior was observed for PAL activity. By contrast, in control rachis the increase of lignin content began 11 days later than in GA-treated rachis and the lignin content only reached one fourth (Nakamura and Hori, 1984). The expression of *VvCAD1* in the

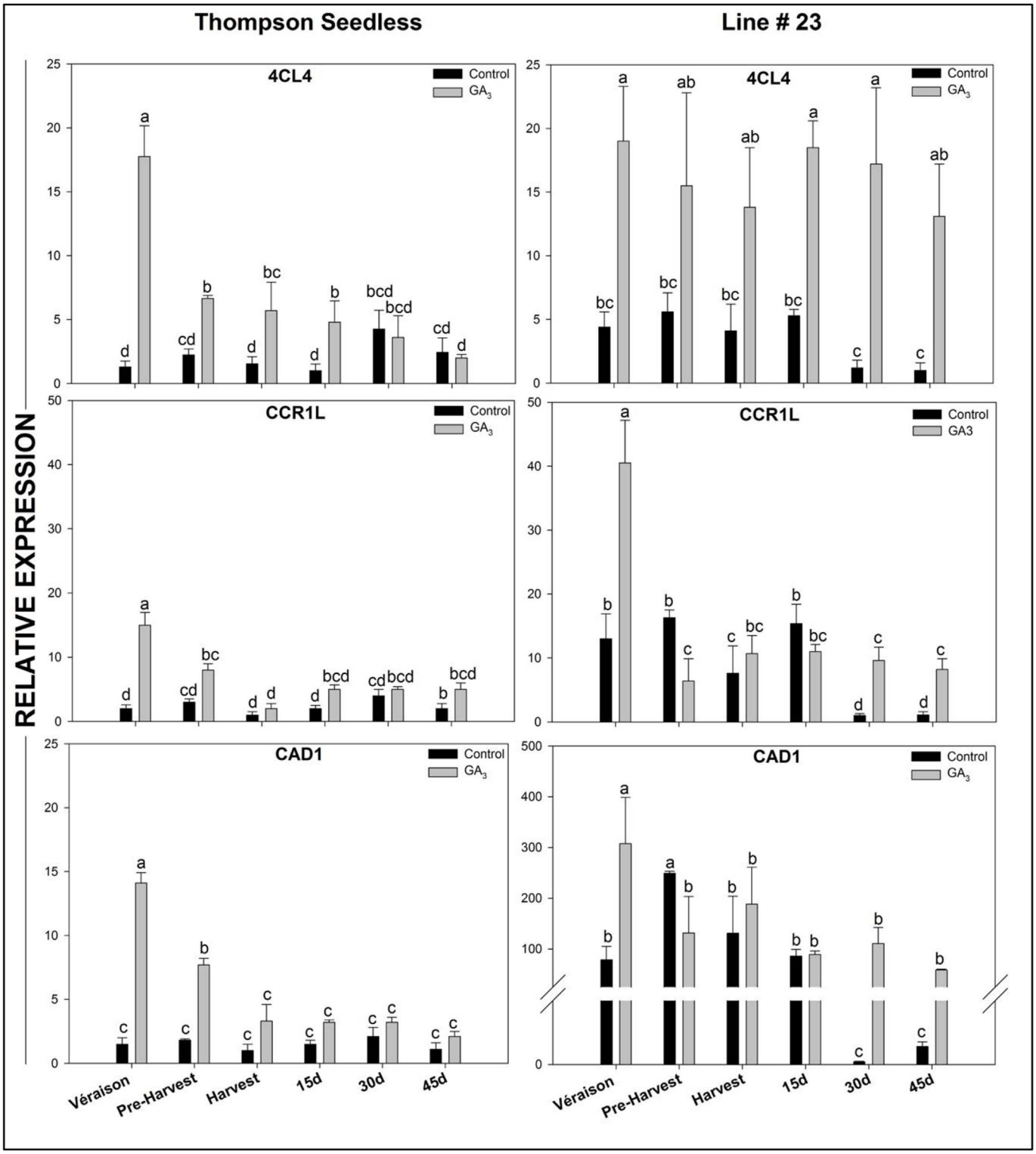

Fig. 4. Effect of gibberellic acid (GA_3) treatment on the expression of genes of the lignin biosynthesis pathway in cv. Thompson Seedless and Line #23. qPCR relative expression levels for 4-hydroxycinnamoyl CoA ligase (4CL4), cinnamoyl CoA reductase (CCR1L), cinnamyl alcohol dehydrogenase (CAD1) in pedicel during pre-and post-harvest, without (black) and with GA_3 treatment (grey). The relative abundance of each mRNA was normalized to the Vv*TCPB* gene. The results are presented as the relative expression against the transcription level of the lowest expression gene. Bars in the graphs correspond to standard error from three biological replicates, assayed in duplicate. Different letters represent significant differences at $P < 0.05$ by the LSD test. Pre-harvest: one week before harvest. Bunches sampled at post-harvest storage at 0 °C is indicated (15, 30 and 45 days).

samples of Line #23 treated with GA_3 showed an overexpression of 100 times after 30 and 45 days of cold storage compared to the control, a change that could be related to an increase in berry drop during the same stage (Fig. 1B).

4. Conclusions

This is the first report of gene expression in grapevine pedicels, a key structure for the support of the berry and for the flow of nutrients and water from the plant to the fruit. It was observed that the increase

in both the hardness/stiffness and the diameter of pedicel could be associated with an increment in cortex cell size and in lignin accumulation. This process may be related to an overexpression of any of the three genes analyzed, *Vv4CL4*, *VvCCR1L* or *VvCAD1*, resulting in a substantial increase in berry drop at post-harvest in the susceptible Line #23 treated with GA_3. This is the first study linking differences in gene expression and susceptibility to berry drop, setting the basis for future research dealing with the identification of marker tools for the breeding and management of this trait. All these results are reinforcing the importance of doing careful evaluations of the dosage of GA_3 used by the table grape growers when adopting a new variety, in order to obtain the proper balance between the largest berry size and the minimal berry drop.

Author contributions

All the authors contributed to the study supporting this manuscript. The design of the experiments and discussion of the results were performed by MG, GL, MG-A, BGD and PH. MG performed the experiments and analyzed the data. MM, CC and KO performed RNA extractions, cDNA preparations and gene expression analyses. MM prepared the first draft of the paper and all authors proofread the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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