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Biochemical and physiological study of the firmness of table grape berries

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ABSTRACT

Firmness is an essential quality parameter of table grapes (*Vitis vinifera*) for consumers, with grape bunches that contains soft berries less preferred, resulting in a reduction in the market price. The softening of grape berries has been commonly associated with cell walls, especially the disassembly of pectic polysaccharides. However, the process of berry softening is not completely understood. To investigate the softening process of grape berries, we compared the Thompson Seedless variety, which suffers significant economic losses due to fruit softening, and NN107, a new variety with a significantly higher level of berry firmness. The composition of the cell wall during the berry development of these two grape varieties was compared. NN107 berries had a greater amount of calcium and uronic acids in the cell wall material than Thompson Seedless grapes, suggesting a special role for calcium bridge formation in NN107. Additionally, polyacrylamide carbohydrate electrophoresis (PACE) analysis suggested differences between these varieties in pectin structure. Thompson Seedless grapes showed increased pectolyase hydrolysable site dynamics in the cell wall material and higher polygalacturonase activity than NN107. Immunohistochemistry focusing on the pectin structure confirmed the roles of both calcium bridge formation and cell wall integrity as they relate to a firmer grape berry phenotype.

1. Introduction

The firmness of table grapes (*Vitis vinifera*) is an essential quality parameter for the commercialization of table grape berries (Iwatani et al., 2011). Despite this importance, little is known regarding the mechanisms determining grape fruit firmness. Changes in pectins and hemicelluloses in primary cell wall polysaccharides, and alterations in the interactions of these polymers, have been proposed as the primary causes for texture changes that result in a decrease of firmness during the ripening of different fruit (Brummell, 2006; Goulao, 2010). Enzyme activity associated with modifications of the homogalacturonan (HG) fraction has been directly implicated in the softening of several fruit, including grapes (Roe and Bruemmer, 1981; Paull and Chen, 1983; El-Zoghbi, 1994; Cabanne and Donèche, 2001; Quesada et al., 2009). One of these enzymes is polygalacturonase (PG), which catalyzes the hydrolysis of galacturonic acids in the case of exo-PG (EC 3.2.1.67) or (oligo)galacturonate for endo-PG (EC 3.2.1.15). Both activities have been associated with the softening of the berries of *V. vinifera* cv. Sauvignon blanc (Cabanne and Donèche, 2001). Pectin hydrolysis has been associated with the degree of unesterified HG, which is catalyzed by pectin methyl esterase (PME, EC 3.1.1.11) (Bonnin et al., 2002). The activities of PG and PME have been proposed to be interconnected because a decrease in HG methyl-esterification caused by PME generates accessible hydrolysis sites for PG and sites for Ca^{2+} bridge formation (Fischer and Bennett, 1991). Ca^{2+} stabilizes the pectic components of plant cell walls via the formation of ionic bonds between unesterified HG chains in the pectin domain of cell walls, which are referred to as Ca^{2+} bridges (Grant et al., 1973). In the so-called "egg-box" model, HG associates with Ca^{2+} in a specific conformation that resembles an egg-box, with the Ca^{2+} ions acting as eggs and the pectin polymer acting as the box. Many studies have investigated the effects of applying calcium on grapevines (Bonomelli and Ruiz, 2010; Marzouk and Kassem, 2011), but there is no conclusive evidence on the effectiveness or role of calcium applications as they relate to berry firmness.

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Here, we analyze the mechanisms behind grape firmness by an analysis of cellular and biochemical differences between two varieties of table grapes with contrasting berry firmness. The activity of two pectin-/cell wall-modifying enzymes was analyzed, and the amounts of calcium and uronic acids in the cell wall during berry development were quantified. Additionally, the pectin structure was analyzed via immunohistochemistry. We showed a reversal of softening during the final stages of berry ripening in the NN107 variety. A similar reversal of fruit firmness is unusual in grape development and provides an interesting possibility for deciphering the mechanism. This finding assists in the study of the biochemical mechanisms involved in berry firmness, which can be useful for the improvement of table grape quality.

2. Materials and methods

2.1. Plant material and maturity parameters

Two seedless table grape varieties (*V. vinifera*) cv. Thompson Seedless and cv. NN107, were collected at a commercial vineyard located in Llay-Llay (Valparaíso Region, Chile) from December 2011 to March 2012 (Table 1), which is the standard grape growing season in Chile. The grape bunches were analyzed at four different development stages: veraison (P1), two intermediate points (P2 and P3) and harvest (P4). The analyzed parameters were diameter (mm), soluble solids (% w/w of g sucrose per 100 g solution), acidity ($g\,L^{-1}$ of tartaric acid) and firmness ($N\,mm^{-1}$) of five bunches from different plants. The harvest indexes were similar to the requirements commonly used in the industry for each variety (soluble solids between 16.5 and 17.5% and acidity approximately $0.5\,g\,L^{-1}$ for both varieties). Five fruit per bunch were picked and analyzed. The diameter of a total of 25 fruit was measured with a caliper. The soluble solids content of the same fruit was measured with a temperature-compensated digital refractometer (HI 96811, Hanna instruments Inc., Woonsocket, RI, USA). The titratable acidity of the pooled juice of 10 fruit per bunch in three bunches per variety was assayed via titration with 0.1 N NaOH (pH 8.2) and reported as $g\,L^{-1}$ of tartaric acid. The firmness of 25 fruit (five fruit from five bunches picked from five different plants) was measured through the entire fruit (including skin) using a TA-XT plus Texture Analyser equipped with the Volodkevich Bite Jaw probe (Stable Micro Systems Ltd., Surrey, UK). Additionally, a portion of each grape bunch was immediately frozen in liquid nitrogen and stored at −80 °C or fixed with FAA (3.7% formaldehyde; 5% glacial acetic acid; 50% ethanol) for biochemical and microscopic analysis, respectively.

2.2. Protein extraction

The grape berries (5 g) were ground to a fine powder in liquid nitrogen. Proteins were extracted at 4 °C in 5 mL of extraction buffer (0.1 M Tris–HCl, pH 7; 13 mM EDTA; 20 mM β-mercaptoethanol; 1 M NaCl; 1% polyvinylpyrrolidone (w/v); 20% glycerol (v/v); 1% Triton X-100). The mixture was centrifuged at 9000 × *g* for 20 min at 4 °C. The supernatant was transferred to a new tube and mixed with five volumes of chloroform:methanol (1:4) and three volumes of distilled water. The mixture was centrifuged at 9000 × *g* for 10 min. The supernatant was transferred to a new tube and mixed with three volumes of methanol and centrifuged at 9000 × *g* for 10 min; the supernatant was then discarded, and the pellet was suspended in 5 mL of 1.0 M Tris–HCl, pH 7.5, to solubilize the crude extract. The protein concentration was determined using the Bradford method (Bradford, 1976) with a bovine serum albumin as a standard.

2.3. Pectin methylesterase (PME) activity

The PME activity of three different protein extractions was measured from the berries of different bunches (Hagerman and Austin, 1986). In a 1.5 mL glass cuvette, 1 mL pectin solution (0.5% pectin from citrus peel [Sigma–Aldrich, St. Louis, MO, USA] in water adjusted to pH 7.5 with NaOH), 0.075 mL bromothymol blue solution (0.01%, w/v in 3 mM phosphate potassium buffer, pH 7.5) and 0.425 mL protein extract were mixed. The absorbance at 620 nm was measured immediately and after 30 s. One unit (U) is defined as the amount of enzyme required to produce 1 μmol of GalA per second.

2.4. Polygalacturonase (PG) activity

The PG activity of three different protein extractions from berries of different bunches was measured (Lohani et al., 2004). The reaction mixture of 0.3 mL of 200 mM NaCl + 200 mM sodium acetate, pH 4.5, 0.3 mL of 1% polygalacturonic acid (1%, w/v, Sigma–Aldrich, St. Louis, MO, USA) and 0.1 mL of protein extract was incubated at 37 °C for 15 min and terminated via heating at 100 °C for 5 min. The activity was determined via colorimetric assay using 100 μL of 3,5-dinitrosalicylic acid (Sigma–Aldrich, St. Louis, MO, USA) with 100 μL of reaction mixture via heating for 15 min at 100 °C. After incubation, 1 mL of distilled water was added to the reaction mixture. The formation of reducing groups was quantified against a standard curve of glucose (US Biological, Swampscott, MA, USA) measured at 540 nm. One unit (U) of enzyme was defined as the amount of enzyme required to produce 1 $\mu g\,mL^{-1}$ of reducing sugar per min.

2.5. Alcohol-insoluble residue (AIR) preparation

AIR preparation was performed (Lefever et al., 2004). A total of 10 g of frozen grapes was ground with a mortar and pestle with 50 mL of 80% ethanol. After grinding, 50 mL of 80% ethanol was added, and the mixture was boiled at 80 °C for 20 min. Then the mixture was cooled to room temperature (RT). The solid residue was filtered over Miracloth (CalBiochem, San Diego, CA, USA) and suspended in 25 mL ethanol 80% by stirring for 30 min. This process was repeated three times. The solid residue was resuspended in 25 mL of 95% ethanol and mixed via vortex for five min. The solution was filtered through Miracloth and resuspended in 25 mL of 100% acetone. The solution was mixed via vortex for five min and was filtered again. The filtered material (AIR) was dried overnight at RT.

2.6. Uronic acid analysis

The uronic acid extractions were performed via three different AIR preparations from berries of different bunches (Jones and Albersheim, 1972). Two mg of AIR was hydrolyzed for 3 h with 250 μL 2 M trifluoroacetic acid (TFA, Merck, Darmstadt, Germany) at 100 °C. TFA was evaporated at 60 °C in a hood, and the samples were resuspended in 300 μL of isopropanol. The samples were then evaporated at 40 °C in a hood. This process was repeated three times. The hydrolyzed cell wall material was suspended in 0.2 mL ultrapure water and sonicated for 10 min using an ultrasonic cleaning machine (VWR International, Radnor, PA, USA). The suspension was centrifuged at 9000 × *g* for 5 min at RT, and the supernatant was transferred to a new tube and used for analysis. Then 0.01 mL of 4 M sulfamic acid, pH 1.6, was added to the reaction mixture and heated to 100 °C for 5 min to remove the interfering neutral sugars. The uronic acids content was measured using a spectrophotometric method (Blumenkrantz and Asboe-Hansen, 1973).