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Functional uncoupling of the tonoplast proton pump and its effect on the flesh gelling physiological disorder in papaya fruit

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ABSTRACT

A physiological pre-harvest disorder known as flesh gelling has been verified in the 'Golden' papaya culture in the main papaya-producing regions of Brazil. While this disorder has been described in the literature, its cause has not been identified yet. There is evidence in other works which associated papaya gelling with cell plasmolysis in fruit tissue. The primary system of ion and sugar transport by proton pump enzyme activity of papaya fruit was investigated in this work and may help identify the cause of this disorder. A reduction in the P-ATPase activity of flesh gelling pulp was verified, which was more evident in the activity of ATP hydrolysis than in the H^+ transport. On the other hand, the V-ATPase exhibited a strong increase in its ATP hydrolysis activity, but completely uncoupled to the H^+ pumping capacity, which markedly decreased compromising the establishment of the ATP-dependent proton gradient in tonoplasts of flesh gelling fruits. Our results indicated a drop in cellular capacity for sugar and ion compartmentalization and water retention in cells, which could be the cause of cell plasmolysis and the soaked tissue appearance, among other alterations observed in the flesh gelling disorder.

1. Introduction

Brazil is one of the world's most important producers of papaya, and its role is particularly important in the production and exportation of the cv. Golden. This cultivar was originated from a natural mutation that occurred in cv. Sunrise Solo, and is characterized by a reduced susceptibility to a specific physiological disorder that affects the skin of fruits (Oliveira and Vitoria, 2011) exhibiting a more uniform yellowing of the fruit surface, resulting in a product with a better appearance when compared with cv. Sunrise Solo (Costa and Pacova, 2003). However, the quality of the 'Golden' papaya has been compromised due to the occurrence of another physiological disorder—pulp gelling (Oliveira et al., 2010; Oliveira and Vitoria, 2011).

Pulp gelling has a sporadic occurrence, with higher rates of incidence during periods of low temperatures (e.g., from May to July in Southeastern Brazil), and can cause significant losses in production (Oliveira et al., 2010; Oliveira and Vitoria, 2011). Fruit with flesh gelling does not differ from unaffected samples in outward appearance, as the disorder is characterized by a gelatin-like feature of the pulp developed from the endocarp outward to the exocarp. Another symptom of this disorder is the conspicuous presence of a partially fluid-filled central cavity of the fruit, making gelled fruits denser than the healthy ones (Oliveira et al., 2010). In addition, in the orchard, both normal and gelled fruits can be encountered on the same papaya tree (Oliveira, 2005).

Transmission electron microscopy analysis previously performed by Campos et al. (2004) and Oliveira et al. (2010) revealed that the pulp tissues in gelled papayas exhibited cells with intense plasmolysis and unbroken cell walls. The irregular contours of gelled tissue cells may be the result of excessive water loss from these cells to the apoplastic environment, which could account for the soaked appearance of the gelled mesocarp as suggested by Oliveira et al. (2010).

In a previous study we described a possible relationship between flesh gelling and a reduced capacity to accumulate K^+ ions and water in the interior of mesocarp cells (Oliveira et al., 2010). Oliveira et al. (2010) also verified that gelled papaya tissues presented a lower level of Mg^{++} and of K^+, but without significant differences in Ca^{++} levels between gelled and healthy fruits. Based

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on these early results, we have investigated the ionic transport system in cell membranes of healthy and gelled tissues of papaya fruit.

The primary system of ion transport has the participation of proton pump enzymes present in all plant cell membranes. By converting the chemical energy of ATP or pyrophosphate (PPi) to the proton motive force, the proton pumps play a central role in the function of the cell membranes by generating and maintaining an electrochemical gradient in these membranes that energizes the secondary transport of many ions like Mg^{++}, K^{+}, and Ca^{++}, and also of sugars, organic acids, and other metabolites (see, e.g., Milner et al., 1995; Morsomme and Boutry, 2000; Zeng et al., 2012; Etienne et al., 2013). The principal enzymes that make up these systems in the plasma membrane and in the tonoplast are, respectively, the P-type H^{+}-transporting ATPase (P-ATPase; EC 3.6.1.35), and the V-type H^{+}-transporting ATPase (V-ATPase; EC 3.6.1.34). In the tonoplast of plant cells there is also a H^{+}-transporting inorganic pyrophosphatase (V-PPase; EC 3.6.1.1), which has been described as a backup system for the V-ATPase under ATP-limiting conditions like anoxia and cold stress (Maeshima, 2000), salt tolerance and drought resistance (Gaxiola et al., 2007), and fruit development (Gaxiola et al., 2007; Shiratake and Martinoia, 2007). These enzymes are able to couple the chemical energy of the hydrolysis of ATP or PPi to proton translocation from the cytoplasm to the apoplast or into the vacuole.

The transport and maintenance of ions and solutes into the cell compartments, such as the cytoplasm and vacuole, are coupled to the proton pump activity of membranes which determine the hydric potential balance between the apoplast and cytoplasm, and between the latter and the vacuole favoring the entrance of water into the cell and the vacuole with resulting cell turgor (Palmgren and Nissen, 2011; Schulz et al., 2011) and cell expansion during fruit development (Shiratake and Martinoia, 2007). Furthermore, the transport of sugars, organic acids, and secondary metabolites into the cell compartments is very important for fruit quality because these compounds are responsible for the typical taste of fruits. The failure in the transport of these osmotic components into the cell results in alterations in the hydric potential gradient resulting in water leaving the cell and the consequent plasmolysis which alters cell morphology. On the other hand, the accumulation of liquid in intercellular spaces has consequences on cell metabolism due to the reduction of O_2 diffusion to the cell (Licausi, 2010; Wang and Wu, 2010).

In this work the activities of the proton pumps present in the cell membrane and tonoplast of the 'Golden' papaya cells were investigated to verify the involvement of these primary transport systems in the flesh gelling of papaya fruit. The consequence of the loss of cellular capacity of ion transport into and accumulation in the papaya flesh vacuole is highly consistent with the symptoms observed in the gelled flesh.

2. Materials and methods

2.1. Plant material

Papaya fruit (*Carica papaya* cv. Golden) originating from fields in the Linhares region (19°15′ S, 39°51′70″ W) of Espírito Santo, Brazil were provided by Caliman S/A (www.calimanpapaya.com). The fruits employed in all experiments were at the full ripe stage (with at least 75% yellow skin). The number of replications was at least three healthy and three gelled fruits for biochemistry analysis and ten replications for physico-chemical analysis. The fruits were transported to the laboratory in a refrigerated (15 °C) container. The elapsed time between sample collection and laboratory analysis was approximately 7 h. The time period between fruit arrival in the laboratory and analysis was 1 h, while the fruit were kept at room temperature (24 ± 2 °C and 75 ± 5% RH).

Fruits had been selected in the reception tank in the packing house. After being placed in the water tank, fruits were separated according to their tendency to sink or float in the sanitizing solution, due to differing densities. The fruits that had dropped to the bottom of the tank were cut in half to confirm the occurrence of the gelling disorder. Healthy fruits were encountered in the same lot of fruits from which gelled fruits were selected, and in the same full ripe stage.

2.2. Physic-chemical analysis

2.2.1. Pulp firmness

Pulp firmness was determined by means of penetration resistance measurements according to Azevedo et al. (2008). Initially, each fruit was longitudinally sectioned in two halves. By using a bench penetrometer model 53205 (Fruit Pressure Tester, Italy) with an 8 mm × 8 mm (height × diameter) probe, the firmness was determined at three equidistant points of each face, 5 mm inwards from the exocarp. The results were expressed in Newton (N) units.

2.2.2. Titratable acidity and pH

Two samples (total of 30 g) were collected from the equatorial region of the fruit mesocarp in order to measure titratable acidity (TA) essentially following standard procedures (AOAC, 1975). Samples were homogenized using a mortar and pestle with 50 mL distilled water and the titratable acidity (TA) determined by titration with 0.1 mol L^{-1} NaOH solution to an end point of pH 8.1. The results of TA were converted to percent of citric acid and expressed as $[H^{+}]$ in mol L^{-1}. The pH of the juice was determined with pHmeter (ORION, Model 410 A) before TA determinations.

2.3. Biochemical analysis

2.3.1. Vacuolar and plasma membrane-enriched vesicles

Vacuolar and plasma membrane vesicles were isolated from papaya mesocarp by using differential centrifugation essentially as described by Giannini and Briskin (1987) with modifications (Azevedo et al., 2008). One hundred grams, fresh weight, of mesocarp were homogenized using a mortar and pestle in 2 L/kg fresh weight of ice-cold buffer containing 500 mmol L^{-1} sucrose, 30% (v/v) glycerol, 0.3% (v/v) PVP (PVP-40, 40 kDa), 15 mmol L^{-1} EDTA, 0.4% (w/v) BSA, and 210 mmol L^{-1} Tris–HCl buffer, pH 8.0. Just prior to use, 10 mmol L^{-1} DTT and 2 mmol L^{-1} PMSF were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 1500 × *g* for 10 min. The supernatant was recovered and centrifuged at 100,000 × *g* for 40 min. The microsomal pellet was resuspended in a small volume of ice-cold buffer containing 10 mmol L^{-1} Tris–HCl, pH 7.6, 15% (v/v) glycerol, 1 mmol L^{-1} DTT, 1 mmol L^{-1} PMSF, and 1 mmol L^{-1} EDTA. The microsomal fraction was layered over a 30%/46% (w/w) discontinuous sucrose gradient containing 10 mmol L^{-1} Tris–HCl buffer, pH 7.6, 1 mmol L^{-1} DTT, and 1 mmol L^{-1} EDTA. After centrifugation at 100,000 × *g* for 90 min in a swinging bucket rotor, vesicles that sedimented at the interface between 30% and 46% sucrose were collected, diluted with 50 mL of ice-cold buffer containing 10 mmol L^{-1} Tris–HCl, pH 7.6, 10% (v/v) glycerol, 1 mmol L^{-1} DTT, and 1 mmol L^{-1} EDTA, and centrifuged at 100,000 × *g* for 40 min. The pellet containing the plasma membrane vesicles was resuspended in the same buffer, and the vesicles were either used immediately or frozen under liquid N_2 and stored at −70 °C until use.

The cell fractionation procedure was validated by measuring the sensitivity of ATPase activity to specific inhibitors of the P- and V-ATPases used as classical marker enzymes for membranes