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Involvement of AOX and UCP pathways in the post-harvest ripening of papaya fruits

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ABSTRACT

Enhanced respiration during ripening in climacteric fruits is sometimes associated with an uncoupling between the ATP synthesis and the mitochondrial electron transport chain. While the participation of two energy-dissipating systems, one of which is mediated by the alternative oxidase (AOX) and the other mediated by the uncoupling protein (UCP), has been linked to fruit ripening, the relation between the activation of both mitochondrial uncoupling systems with the transient increase of ethylene synthesis (ethylene peak) remains unclear. To elucidate this question, ethylene emission and the two uncoupling (AOX and UCP) pathways were monitored in harvested papaya fruit during the ripening, from green to fully yellow skin. The results confirmed the typical climacteric behavior for papaya fruit: an initial increase in endogenous ethylene emission which reaches a maximum (peak) in the intermediate ripening stage, before finally declining to a basal level in ripe fruit. Respiration of intact fruit also increased and achieved higher levels at the end of ripening. On the other hand, in purified mitochondria extracted from fruit pulp the total respiration and respiratory control decrease while an increase in the participation of AOX and UCP pathways was markedly evident during papaya ripening. There was an increase in the AOX capacity during the transition from green fruit to the intermediate stage that accompanied the transient ethylene peak, while the O_2 consumption triggered by UCP activation increased by 80% from the beginning to end stage of fruit ripening. Expression analyses of *AOX* (*AOX1* and *2*) and *UCP* (*UCP1–5*) genes revealed that the increases in the AOX and UCP capacities were linked to a higher expression of *AOX1* and *UCP* (mainly *UCP1*) genes, respectively. In silico promoter analyses of both genes showed the presence of ethylene-responsive cis-elements in *UCP1 and UCP2* genes. Overall, the data suggest a differential activation of AOX and UCP pathways in regulation related to the ethylene peak and induction of specific genes such as *AOX1* and *UCP1*.

1. Introduction

Ripening is one of the phases of fruit development that dramatically changes the characteristics of fleshy fruits. Fruit can be classified according to respiratory pattern into two physiological groups, i.e., climacteric and non-climacteric. The presence of an ethylene peak due to auto-catalytic ethylene biosynthesis and respiratory burst during ripening are characteristics of the climacteric group (Osorio et al., 2012; Seymour et al., 2013; Klie et al., 2014). Ethylene acts to initiate and coordinate ripening in these fruits. Changes in texture, color, sugar accumulation, and increased respiration during fruit ripening are attributed to the action of ethylene and are under genetic regulation and/or in response to environmental perturbations (Hiwasa et al., 2003; Krongyut et al., 2011; Perotti et al., 2014); however, the physiological roles of the respiratory and ethylene peaks are not well understood (Seymour et al., 2013; Perotti et al., 2014).

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These changes in climacteric respiration are likely associated with a very rapid reconfiguration of mitochondrial energy-dissipating systems, which leads to decreased oxidative phosphorylation and higher heat production. Plant mitochondria possess two energy-dissipating systems, the alternative oxidase (AOX) and the uncoupling protein (UCP, also termed PUMP in plants), which are independent and modulated in different ways (Borecký et al., 2006; Vercesi et al., 2006).

The AOX pathway directly couples the oxidation of ubiquinol to the reduction of O_2–H_2O, preventing the oxidation of ubiquinol through complex III, cytochrome c, and finally cytochrome c oxidase (complex IV). Therefore, the electron flow via AOX introduces a branch in the mitochondrial electron transport chain (mETC) which bypasses two important energy conservation sites (complex III and IV) and reduces the generation of membrane potential and ATP synthesis (Zhang et al., 2012; Vanlerberghe, 2013). Thus, the AOX pathway by electron flow through parallel oxygen reduction to water increases the rate of electron transport into mETC, dissipating energy as heat. Also AOX activity dampens the generation of superoxide anion, O_2^-, which in turn will reduce its conversion to other reactive oxygen species (ROS) such as H_2O_2 and hydroxyl radical (Vanlerberghe, 2013).

The pumping of protons from the matrix out to the intermembrane space generates an electrochemical proton potential ($\Delta\mu H^+$) across the inner mitochondrial membrane. This proton potential is the driving force used by the ATP synthase to phosphorylate ADP (Borecký and Vercesi, 2005). The UCP pathway is attributed to the proton leak by allowing the reentry of protons from the intermembrane space to the matrix, bypassing the ATP synthase complex (Vercesi et al., 1995). Thus, UCP protein uncouples ATP synthesis from respiration in plant mitochondria (Jezek et al., 1997; Laloi et al., 1997; Vercesi et al., 2006), while both AOX and UCP pathways increase the respiratory rate (Calegario et al., 2003).

Compared with the AOX pathway, the role of the UCP pathway in the energy-dissipation mechanisms during fruit ripening is less understood. Both systems are simultaneously present in the mitochondria of green tomato fruit (Almeida et al., 1999; 2002; Sluse and Jarmuszkiewicz, 2000) and have also been suggested to prevent generation of ROS by the respiratory chain (Calegari et al., 2003; Pinheiro et al., 2004; Perotti et al., 2014) and working sequentially to be involved in fruit ripening (Vercesi et al., 2006). Protein accumulation and/or capacity increases in these two energy-dissipating systems during fruit ripening on the plant suggests that AOX and UCP could participate in processes occurring at the end of ripening and at senescence in tomato (Almeida et al., 2002; Holtzapffel et al., 2002) and in mango (Considine et al., 2001). The protein accumulation in mango was linked to specific transcripts of *AOX* (*AOX1a* and *1b*) and *UCP* (*UCP1*) genes, which were mainly up-regulated at turning and ripe stages (Considine et al., 2001). In postharvest studies the stage of fruit ripening significantly influences fruit respiration and, certainly, the AOX and UCP activities (Souza et al., 2014; Silva et al., 2015a). Therefore, in contrast to tomato (Almeida et al., 1999), in which protein levels and AOX and UCP capacities decreased during ripening, in mango (Cruz-Hernandez and Gomez-Lim, 1995) and apple (Duque and Arrabaça, 1999) the AOX protein increased at the climacteric stage. UCP, however, was not studied by Cruz-Hernandez and Gómez-Lim (1995) or Duque and Arrabaça (1999). Thus, while there is little data available to date for these proteins in postharvest ripening, no studies have related AOX and UCP capacities with the climacteric burst. It is not clear whether these differences are due to the ripening stage or are fruit speciedependent.

Navet et al. (2003) showed that ethylene controls the bioenergetics (including AOX and UCP) of tomato fruit development, including ripening. More recently, Xu et al. (2012) showed that AOX is involved in climacteric respiration in tomato fruit controlling, interestingly, the ethylene production and ripening-associated genes. Although UCP was not studied by Xu et al. (2012), this report indicates a crucial role of AOX in ripening whereby events take place earlier than has been stated, i.e., at the end of ripening and at senescence (Considine et al., 2001; Almeida et al., 2002; Holtzapffel et al., 2002).

Papaya, the model chosen in this study, is characterized as a fruit in which the ripening-related changes take place very quickly when compared to some climacteric fruits (Fabi et al., 2007; Oliveira and Victoria, 2011). This feature makes it a good model for studying the relation between mitochondrial uncoupling and ethylene during ripening. Thus, in this paper the AOX and UCP capacities as well as gene expression during ripening in harvested papaya fruit were studied. Concomitantly, ethylene emission was followed in order to gain insight into the regulation of both energy-dissipation systems during papaya ripening.

2. Material and methods

2.1. Plant material and fruit ripening

'Golden' papaya fruit were furnished by the Caliman Agrícola S.A. Company, located in Espírito Santo State, Southeastern Brazil. Mature fruit with totally green skin (maturity stage 0) were harvested, washed, carefully selected, and transported in a refrigerated truck at 15 °C during 4 h to the laboratory before performing assays. Afterwards, fruit were allowed to fully ripen for up to nine days in chambers with controlled temperature (25 °C ± 1 °C) and relative humidity (85% ± 5%). The skin color and flesh firmness were monitored daily as described below in Section 2.2.

2.2. Assessment of fruit ripening

Fruit were classified into five ripening stages (E1–E5), defined according to color development and softening (see criteria for fruit classification in Supplementary material I). Changes in skin color and pulp firmness were used to assess the stage of ripening. The skin color was measured using a portable colorimeter (Chroma Meter, model CR-300, Minolta, Japan). Pulp firmness was obtained by penetration resistance, using a digital fruit firmness tester (Fruit Pressure Tester, model 53205, Italy) with a probe adapter of 8.0 mm × 8.0 mm (height × diameter). The soluble solids (SS) content was determined with a portable refractometer (Atago N1, France).

2.3. Measurements of CO_2 and C_2H_4 emissions

Ethylene emission and respiration were measured in selected fruit with different ripening stages as described above. To monitor the emissions of CO_2 and C_2H_4 from individual intact fruit, a laser-driven photoacoustic spectrometer coupled with an infrared gas analyzer was used. In this sensitive-system, measurements can be taken simultaneously in real-time without the need for preincubating fruit in a hermetically sealed recipient for hours to detect ethylene (Corrêa et al., 2011; Souza et al., 2014).

2.4. Isolation and purification of mitochondria

To get novel insights about this typical respiration pattern and its relation to ripening, we assessed the respiratory activity (measured as O_2 consumption) in mitochondria purified from the same fruit used to assess the degree of ripening, after monitoring of CO_2 and C_2H_4 emissions as mentioned above.

Mitochondria were isolated from the same fruit used to assess the stage of ripening in three ripening stages (E1, E3, and E5) representing the green, intermediate, and ripe papaya fruit. Three