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Review

Physiological aspects of fruit ripening: The mitochondrial connection

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ABSTRACT

Fruit ripening is a genetically programmed process which leads to an assortment of physiological and metabolic changes that irreversibly alter its characteristics. Depending on the species, fruit maturation can be either climacteric or non-climacteric. In both cases there is a metabolic shift from normal development conditions toward the fully mature state, but climacteric fruit is characterized by a sharp increase in respiration. In non-climacteric fruit, that generally does not display this feature, respiration changes can be affected by processes related to postharvest storage. This review describes some of the many ways in which mitochondrial metabolism is implicated in this crucial reproductive stage, such as the connection between ethylene production and respiration rate, the involvement of alternative oxidase (AOX) and plant uncoupling mitochondrial protein (PUMP) during the ripening and the common alterations of this organelle in fruits affected by different stress conditions.

Contents

1. Fruit ripening

Fruit ripening is a genetically programmed, highly coordinated process of organ transformation from unripe to ripe stage, to yield an attractive edible fruit with an optimum blend of color, taste, aroma and texture (Brady, 1987). Sourness is generally attributed to proton release from organic molecules, while the anions of each acid such as citric, malic and tartaric, would contribute to a distinctive taste in oranges, apples and grapes, respectively. In general terms, fruits accumulate mainly organic acids during the first period of development, as an energy reserve. Organic acid and amino acid accumulation shifts toward sugar synthesis during the later stage of fruit development (Carrari et al., 2006; Deluc et al., 2007; Fait et al., 2008).

In the case of citrus, for example, sucrose is translocated to the fruits from the leaves throughout fruit development, and constitutes about 50% of the total soluble sugars. During the first half of fruit development, sucrose is hydrolyzed by cytosolic invertases or stored in the acidic vacuoles and hydrolyzed by vacuolar acidic invertases (Echeverria, 1992; Echeverria and Burns, 1990). Meanwhile, citrate begins to accumulate.

Citrate is synthesized in the mitochondrion but accumulates in the vacuole (Martinoia et al., 2007). Export from the mitochondrion is by counter exchange with other carboxylic acids. Therefore, the rate at which TCA intermediates (mainly malate and oxaloacetate, Etienne et al., 2013) are replenished will affect the rate of export of citrate.

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Uptake into the vacuole is ultimately dependent on the tonoplast membrane potential generated by the proton-pumping V-type ATPase but can occur as well by facilitated diffusion through the malate channel (for a review see Etienne et al. (2013)). Accumulation in the vacuole is a function of both the influx and efflux of citrate from this organelle (Shimada et al., 2006) and of subsequent metabolism by the cytosolic isoforms of aconitase and isocitrate dehydrogenase. Thus, the transport of citrate between the different subcellular compartments will influence its rate of accumulation (Shiratake and Martinoia, 2007).

During the second half of citrus fruit development, citrate declines, a trend associated with the activity of CsCit1, a H^+/citrate symporter. Moreover, an increased expression of genes associated with the TCA cycle and genes encoding enzymes mediating sugar accumulation was reported for this stage of development (Deluc et al., 2007). These observations together with the lower activity of the enzymes responsible for sucrose degradation (Katz et al., 2011) suggest that the rapid accumulation of sucrose through the late stages of fruit development is a contribution of the sugar uptake from the tree and *de novo* synthesis of this metabolite in citrus juice sac cells at expense of organic acids deassimilation.

As can be appreciated, metabolic changes along the fruit development involve many mitochondrial enzymatic activities and transporters, which underscores the crucial role of this organelle during fruit ripening. Ripening physiology has been classically defined as either 'climacteric' or 'non-climacteric'. Climacteric fruits show a sudden increase in respiration at the onset of ripening, usually in concert with increased production of the gaseous hormone ethylene. Whereas ethylene is typically necessary for climacteric ripening, non-climacteric fruits do not increase respiration at ripening and often have no requirement for ethylene to complete maturation.

However, these distinctions are not absolute, as closely related melon or Capsicum species can be both climacteric and non-climacteric (Ezura and Owino, 2008). Also, some so-called non-climacteric fruits display enhanced ripening phenotypes in response to exogenous ethylene. Nevertheless, increased ethylene synthesis at the onset of ripening is required for the normal ripening of many fruits (Barry and Giovannoni, 2007).

On the other hand, patterns of organic acid accumulation and degradation do not always match the classification of species as climacteric or non-climacteric, nor can be attributed to changes in overall respiration rates. For instance, some climacteric fruits, such as tomato, appear to utilize malate during the respiratory burst, while others such as banana, continue to accumulate malate throughout ripening, even at the climacteric stage (Sweetman et al., 2009). In this group of fruits, including mango, strawberry, kiwifruit and others, the conversion of starch into soluble sugars is the most important event during the ripening (Han and Kawabata, 2002; Moing et al., 2001).

Regarding mitochondrial participation in each type of system, the higher respiration rate in climacteric fruits seems to be specifically regulated by ethylene, as will be discussed below. The close functional relationship between increased respiratory activity and intracellular repair has led to the suggestion (Romani, 1974) that the respiratory climacteric may represent an attempt, ultimately futile, to repair and compensate for the effects of incipient senescence. At the climacteric peak, the normal homeostatic reactions or, at least, the respiratory component, will have reached its upper limit and any further injury will not elicit an additional respiratory response.

In this way, rather than being either the metabolic force propelling senescence toward death or simply an unexplained adjunct activity, the respiratory climacteric may represent a final, concerted, homeostatic response.

2. Regulation of the respiratory climacteric

The gaseous plant hormone ethylene has been identified as the major compound that initiates and controls ripening in climacteric fruit, and its biosynthesis in plant tissues has been extensively studied (Argueso et al., 2007; Srivastava and Handa, 2005). The biochemical features of the ethylene biosynthesis pathway in higher plants are well defined and are summarized in Fig. 1. Briefly, ethylene is synthesized from methionine in three steps: (1) conversion of methionine to S-adenosyl-L-methionine (SAM) catalyzed by the enzyme SAM synthetase, (2) formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM via ACC synthase (ACS) activity, and (3) the conversion of ACC to ethylene, which is catalyzed by ACC oxidase (ACO). The formation of ACC also leads to the production of 5′-methylthioadenosine (MTA), which is recycled via the methionine or Yang cycle to yield a new molecule of methionine. Increased respiration provides the ATP required for the methionine cycle and can lead to high rates of ethylene production without high levels of intracellular methionine.

Two systems of ethylene production have been defined in plants. System-1 represents basal ethylene in unripe fruit and vegetative tissues and is regulated in an autoinhibitory manner, whereas system-2 operates during the ripening of climacteric fruit and flower senescence and is autocatalytic (Yokotani et al., 2009).

ACS and ACO are encoded by multigene families in higher plants, and these are transcriptionally regulated along development and ripening. In tomato, for instance, some isoforms are specifically expressed in green fruit that are in a system 1 mode of ethylene synthesis, while others only are present in mature fruits (Barry et al., 1996; Barry et al., 2000). Although numerous transcription factors acting on ethylene synthesis have been identified, the physiologic and molecular pathways that operate to initiate the transition from a system 1 to a system 2 mode of ethylene synthesis remain incompletely defined. Interestingly, it was reported that a high concentration of indole-3-acetic acid (IAA) is required to generate a large amount of ethylene by system 2 in peaches (Tatsuki et al., 2013), suggesting that ethylene biosynthesis transition may be also regulated by auxin (Fig. 2). Small but significant increases in ethylene synthesis and an associated increase in respiration rate have also been detected in non-climacteric fruits like grape berries, citrus fruits and red ripe strawberry fruits (Chervin et al., 2004; Iannetta et al., 2006; Katz et al., 2004). However, the timing of this ripening-related increase in ethylene production is distinct from the patterns of ethylene production typically associated with the ripening of climacteric fruits. In strawberry, for example, the ethylene increase observed was not detected until 24 h after the fruits had developed full red pigmentation. In contrast, while mature citrus fruits do not exhibit an increased ethylene production associated with ripening, harvested immature fruits produce high levels of ethylene that can be further stimulated by ethylene and propylene treatments and inhibited by 1-MCP, indicating the autocatalytic nature of this phenomenon (Katz et al., 2004).

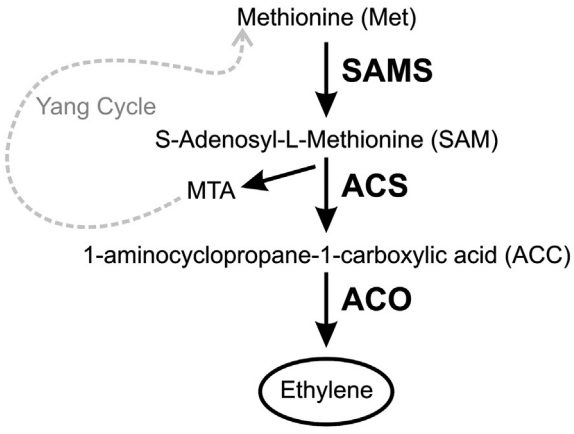

Fig. 1. Representation of ethylene biosynthesis from methionine. The main enzymes are: SAMS, S-Adenosyl-L-methionine synthetase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase, ACO: 1-aminocyclopropane-1-carboxylic acid oxidase. MTA is 5′-methylthioadenosine, another product of the ACS reaction from which methionine is regenerated by the Yang cycle.

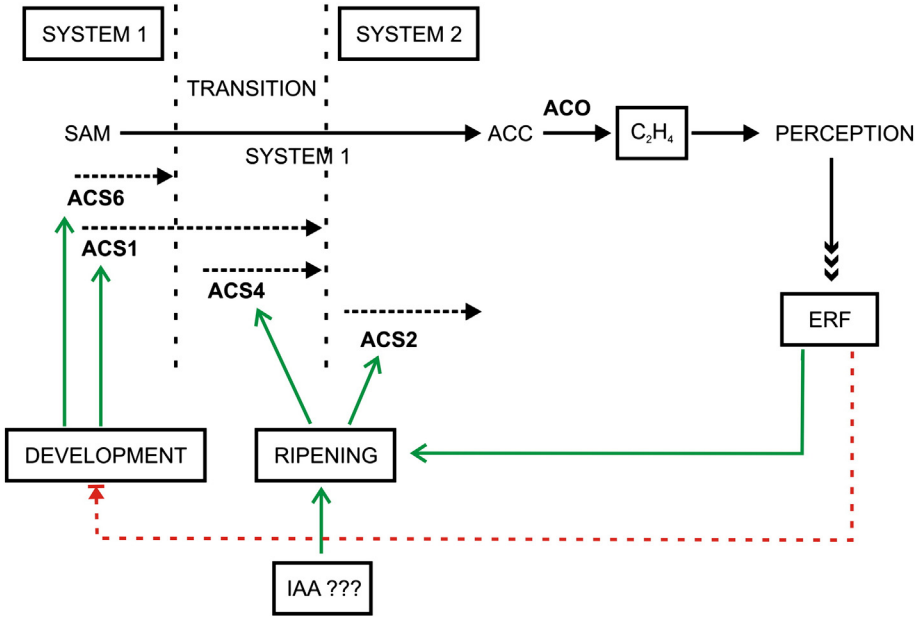

Fig. 2. Regulation of ACS gene expression during the transition from system-1 to system-2 ethylene synthesis. Ethylene response factors (ERF) acting on ethylene synthesis to initiate the transition from a system 1 to a system 2 mode of ethylene synthesis regulate the expression of the different ACS genes. This transition may be also regulated by high concentrations of indole-3-acetic acid (IAA). Positive regulation is indicated by green arrows and negative regulation is represented in red.
Adapted from model proposed by Barry et al. (2000).

Therefore, further investigations are necessary to understand the physiological role of the increased ethylene synthesis and the concomitant rise in respiration rate in so different scenarios. Since ethylene regulation can occur not only at the level of synthesis but also at the level of hormone perception or signal transduction, these aspects should be taken into account to understand the global regulation of this pathway. Tomato ripening is regulated independently and cooperatively by ethylene and transcription factors, including nonripening (NOR) and ripening inhibitor (RIN). Mutations of NOR, RIN, and the ethylene receptor Never-ripe (Nr), which block ethylene perception and inhibit ripening, have proven to be great tools for advancing our understanding of the developmental programs regulating ripening (Osorio et al., 2011).

Ethylene is perceived by a family of integral membrane receptors with similarity to two-component His kinases and function as negative regulators. Six different receptors have been identified in the tomato genome, all of which are differentially expressed in various tissues (Lashbrook et al., 1998). The *CTR1* gene is a negative regulator of the pathway acting downstream of the receptors and encodes a protein that belongs to the Raf family of Ser/Thr protein kinases (Kieber et al., 1993). *CTR1* may interact directly with the receptors and would be the head of a protein kinase cascade (Chang and Shockey, 1999). The signaling events from CTR1 to the nucleus are unclear but appear to involve EIN2, an integral membrane protein, and EIN3, a transcriptional regulator, which acts directly upon ethylene response factors to activate ethylene-inducible gene expression (Chao et al., 1997; Solano et al., 1998).

Using the tomato system as a model of climacteric fruit ripening, it was possible to identify putative conserved orthologous ripening-related genes in many other species, including climacteric and non-climacteric ones (Aharoni and O'Connell, 2002; Costa et al., 2010; Lee et al., 2010). The conservation of ripening mechanisms is surprising considering the significant differences in the morphological, physiological, and biochemical characteristics of fruits.

Ethylene does not act to regulate ripening in isolation, but rather acts in concert with other plant hormones like IAA and abscisic acid (ABA), as has been recently reviewed by Seymour et al. (2013).

3. Role of AOX and PUMP during fruit ripening

It is well known that the branched electron transport chain of plant mitochondria contains a CN- and antimycin-resistant AOX (Vanlerberghe and McIntosh, 1997; Wagner and Moore, 1997) that catalyzes the reduction of oxygen to water with electrons derived directly from ubiquinol, bypassing the energy-conserving sites (i.e. proton-translocating complexes III and IV) of the Cyt pathway. Since no protonmotive force is generated during this reaction, electron flow through AOX appears to dissipate energy (thermogenesis), decreasing ATP synthesis.

Changes in the expression and/or activity of the alternative oxidase have been observed during fruit ripening. Furthermore, the alternative oxidase has been implicated to play a role during conditions such as oxidative stress (Cramer et al., 2007; Maxwell et al., 1999).

Several studies have revealed that AOX may play an important role in the climacteric or post-climacteric senescent processes during fruit ripening. However, the precise role of AOX in respiratory climacteric is still unknown. Thus, for example, the activity of AOX in mango peaks after climacteric respiration, which contributed to fruit senescence, rather than the respiratory climacteric (Considine et al., 2001). Conversely, studies in apple fruit demonstrated that climacteric increases in respiration during fruit ripening were linked to increased AOX capacity and that AOX was induced at the climacteric during post-harvest storage (Duque and Arrabaça, 1999).

Interestingly, a recent study suggests that AOX would affect system-2 ethylene synthesis during tomato ripening (Xu et al., 2012). It has been proposed that AOX activity in vivo may be regulated in a feed-forward fashion by upstream respiratory carbon metabolism. Accordingly, intramitochondrial pyruvate was proved to stimulate the AOX capacity (Pastore et al., 2001). Interestingly, the main source for pyruvate, the glycolytic pathway, is stimulated at the climacteric peak. Thus, increase levels of pyruvate will lead to an accumulation of intramitochondrial reducing power. Under this scenario it is to be expected that AOX activity will be enhanced.

In summary, AOX permits carbon flow through glycolysis and the citric acid cycle by removing excess carbohydrates and avoiding the over-reduction of the electron transport chain as well. In consequence, AOX would be indirectly allowing the production of the large amount of ATP that is needed for system-2 ethylene synthesis and a series of ethylene-regulated ripening processes.

Since AOX activity can control the level of potential mitochondrial signaling molecules such as superoxide, nitric oxide and important redox couples, AOX also provides a degree of *signaling homeostasis* to the mitochondria, able to influence processes such as nuclear gene expression (Vanlerberghe, 2013).

Similar to the alternative oxidase, the plant uncoupling mitochondrial protein (PUMP), another energy-dissipating system, has also been suggested to prevent generation of reactive oxygen species by the respiratory chain (Kowaltowski et al., 1998) and to be involved during fruit ripening. Changes in the amounts of immunodetected PUMP were coherent with the decrease in potential PUMP activity observed during tomato ripening (Almeida et al., 1999). Nevertheless, the three respiratory pathways (ATP synthesis, PUMP-, and AOX-sustained respirations) in isolated mitochondria decreased progressively throughout the period of ripening, working apparently in a concert way. Further studies are necessary to determine the functional connection between these two energy-dissipating systems, PUMP and AOX, in plant mitochondria, particularly during fruit ripening.

4. ROS in ripening and senescence

Ripe fruit, climacteric or not, decays with time in a process known as senescence. Although widely different among plant species, this naturally programmed degeneration process is a fundamental factor in the postharvest management of any kind of fruit. The postharvest lifetime of fruits can vary from several months to a few days, and is dependent on a multiplicity of internal and external factors (Tian et al., 2013). During decay, fruit quality and resistance to pathogen attack are progressively lost. In recent years, research has shown that reactive oxygen species (ROS) play a key role in the establishment and progress of senescence (Jimenez et al., 2002; Lacan and Baccou, 1998; Qin et al., 2009; Tian et al., 2013).

The production of ROS in cells and organelles was a direct consequence of the evolutionary process of acquisition of aerobic metabolic pathways. Partial reduction of oxygen generates intermediates such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH); while O_2 physical excitation can lead to the production of oxygen singlet ($^1O_2$) (Fig. 3). ROS in plants are originated in organelles: chloroplasts, peroxisomes and mitochondria in photosynthetic tissues, and mostly mitochondria in non-photosynthetic cells. ROS-mediated mitochondrial function impairment has been indicated as one of the major factors associated with senescence in fruits (Tian et al., 2013).

As the different ROS types all have in common the power to inflict different degrees of oxidative damage to cellular components, a concomitant evolution was that of different detoxification mechanisms in plants. All organisms, plants in particular, are subject to changing environmental conditions that, by altering general metabolism, can cause an imbalance in between the synthesis and degradation of ROS. Under this condition, an oxidative burst, characterized by a fast increase in some or all ROS can take place. Biotic (e.g. pathogen attack) or abiotic factors (temperature, water or nutrient stress) are responsible for triggering an oxidative burst (Apel and Hirt, 2004).

Plant mitochondria are essential in non-photosynthetic tissues where ATP demands are solely met by this organelle. Respiration leads to oxygen being reduced to H_2O, but during the process a reactive intermediate molecule, the superoxide anion O_2^-, may arise by incomplete reduction, as was described above. Complex I and complex III are the main sites of O_2^- production. Most of it is rapidly converted to H_2O_2 by a manganese-containing superoxide dismutase (MnSOD) (Navrot et al., 2007). Purposely, it was showed that this is one of the enzymes

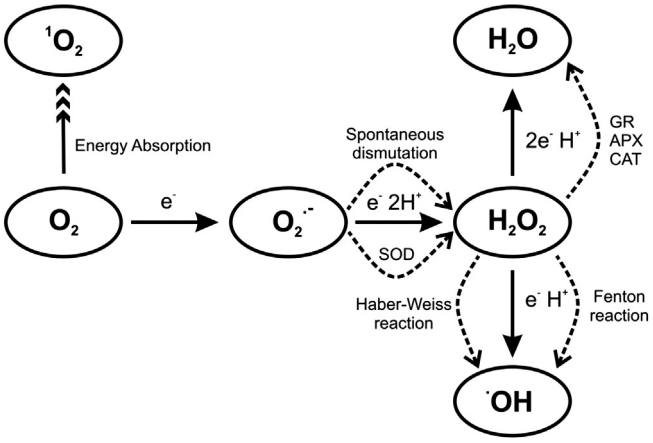

Fig. 3. Schematic representation of generation of reactive oxygen species (ROS) in plants. Stepwise monovalent reduction of O_2 leads to formation of $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$, whereas energy transfer to O_2 leads to formation of $^1O_2$. $O_2^{\bullet -}$ is easily dismutated to H_2O_2 either nonenzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX).

affected by oxidative damage during fruit senescence, together with other mitochondrial proteins such as an outer membrane transporter and some tricarboxylic acid cycle enzymes (malate dehydrogenase and aconitase) (Qin et al., 2009).

Different mitochondrial macromolecules are vulnerable to oxidative damage because this organelle is a primary generator of endogenous ROS. In particular, mitochondrial DNA is more sensitive to oxidative damage than nuclear DNA because of the absence of chromatin organization and lower DNA repair activities (Yakes and Van Houten, 1997). Another factor that makes mitochondrial DNA more susceptible is the localization because it is close to the inner mitochondrial membrane, where many ROS are generated.

ROS are not only involved in oxidative damage; they also regulate plant development, stress responses, and programmed cell death. Previous studies have shown that they can act as messengers to trigger protein de/activation (Desikan et al., 2001). Moreover, it has been demonstrated that the transcription of specific sets of genes in plant cells is induced by superoxide ions, H_2O_2 and singlet oxygen (Gadjev et al., 2006). This signaling system also interacts with other pathways, like phosphorylation cascades by kinases or Ca^{2+} signaling, and is a part of the regulation network that connects the cell to its environment (Navrot et al., 2007).

5. Mitochondrial alterations in fruit exposed to different types of stress

5.1. Biotic stress

The first barrier that a pathogen must confront in fruit is the cellular wall and the adjacent plasma membrane which is involved in reception and transduction of signals. Increased membrane permeability, accumulation of free radicals and enhanced lipid peroxidation are common host cell membrane responses under infection stress (de Wit et al., 1997). Alterations in lipid compositions appear when mitochondria are damaged and result in an unfavorable energy status.

Treatments with ATP on litchi fruit revealed lower activities of phospholipase D, acid phosphatase and lipoxygenase involved in membrane lipid peroxidation and hydrolysis. Thus, higher energy levels allow us to maintain membrane integrity of the harvested litchi fruit at the early stage of storage (Yi et al., 2008).

It is known that several types of stress effectively increase mitochondrial activity, oxidative phosphorylation rates and ATP synthesis. This behavior seems to have two goals: the maintenance of metabolic

homeostasis and stimulating the accumulation of natural defense compounds, including phytoalexins and pathogenesis-related proteins.

However, not all the host strategies are successful; many pathogens have developed different mechanisms to overcome the host-defenses in what is called suppression response. An example is the infection of citrus fruit by *Penicillium digitatum*. It has been demonstrated that *P. digitatum* suppresses a defense-related hydrogen peroxide (H_2O_2) burst in the host tissue. In contrast, the nonhost pathogen, *Penicillium expansum*, triggers production of a significant amount of H_2O_2 in citrus fruit exocarp. Additionally, pathogenicity of both *P. digitatum* and *P. expansum* on citrus fruit was significantly enhanced by the H_2O_2-scavenging enzyme catalase (Macarisin et al., 2007). Similarly, higher catalase transcript and protein levels were observed in the pathogen *Blumeria graminis* when it was incubated on barley leaves (Zhang et al., 2004), demonstrating the implication of catalase in pathogenesis.

5.2. Abiotic stress

Abiotic stresses usually cause protein dysfunction. Molecular chaperones are responsible for protein folding, assembly, translocation and degradation in a broad array of normal cellular processes. They are crucial in the stabilization of proteins and membranes, especially under stress conditions.

NADH:ubiquinone oxidoreductase (complex I) is the most thermolabile protein complex of oxidative phosphorylation. During heat stress conditions, complex I breaks down to a lower molecular mass and exhibits lower activity. Therefore, oxidative phosphorylation rate decreases at high temperatures, being complex I the limiting element (Zhang et al., 2004). It has been demonstrated that in *Pyrus pumila* fruits the mitochondrial small heat-shock protein (lmw Hsp) is an important determinant of the thermotolerance of oxidative phosphorylation, since this protein specifically protects complex I during heat stress (Downs and Heckathorn, 1998). In the same way, mitochondrial small Hsps were shown to improved mitochondrial electron transport during salt stress in maize, mainly by protection of the complex I (Katz et al., 2011).

Orange fruit affected by natural frosts have been shown to possess less MnSOD and glutathione reductase but higher levels of catalase, ascorbate peroxidase and peroxidase than non-exposed fruit (Falcone Ferreyra et al., 2006). In this tissue, mitochondrial integrity seems to be severely compromised, as indicated by lesser protein content, the reduced respiration rate and the lower activities of enzymes such as malate dehydrogenase and fumarase and a reduced capacity for exogenous cytochrome *c* reduction (Falcone Ferreyra et al., 2006; Perotti and Podestá, unpublished results). A thwarted respiratory capacity of damaged fruit could be one of the major causes of the interruption of the ripening process by frost in citrus fruit.

Similarly to the ROS signaling system, generation of reactive nitrogen species (RNS) such as nitric oxide (NO) has been linked to plant mitochondria. However, the mechanism of generation of RNS by mitochondria is not yet well understood. In recent years, there is growing evidence that ROS and RNS specifically generated by mitochondria influence plant responses to stress, suggesting that they act as signaling molecules for stress acclimation (Guptaa et al., 2011; He et al., 2012).

6. Concluding remarks

Mitochondrial activity is one of the most important players in the process of fruit ripening and senescence. As a site of production of ROS, but also as the main energy provider of the fruit cell, its role in this very essential stage of plant reproduction makes it a significant target in the assessment of the biochemical changes surrounding the last stages of fruit life.

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