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The Energy-Conserving and Energy-Dissipating Processes in Mitochondria Isolated From Wild Type and Nonripening Tomato Fruits During Development on the Plant

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Bioenergetics of tomato (*Lycopersicon esculentum*) development on the plant was followed from the early growing stage to senescence in wild type (climacteric) and nonripening mutant (*nor*, nonclimacteric) fruits. Fruit development was expressed in terms of evolution of chlorophyll *a* content allowing the assessment of a continuous time-course in both cultivars. Measured parameters: the cytochrome pathway-dependent respiration, i.e., the ATP synthesis-sustained respiration (energy-conserving), the uncoupling protein (UCP) activity-sustained respiration (energy-dissipating), the alternative oxidase(AOX)-mediated respiration (energy-dissipating), as well as the protein expression of UCP and AOX, and free fatty acid content exhibited different evolution patterns in the wild type and *nor* mutant that can be attributed to their climacteric/nonclimacteric properties, respectively. In the wild type, the climacteric respiratory burst observed in vitro depended totally on an increse in the cytochrome pathway activity sustained by ATP synthesis, while the second respiratory rise during the ripening stage was linked to a strong increase in AOX activity accompanied by an overexpression of AOX protein. In wild type mitochondria, the 10-μM linoleic acid-stimulated UCP-activity-dependent respiration remained constant during the whole fruit development except in senescence where general respiratory decay was observed.

KEY WORDS: Alternative oxidase; uncoupling protein; mitochondria; respiration; tomato fruit ripening; free fatty acids.

INTRODUCTION

The phenomenon of fruit ripening corresponds to a series of biochemical, physiological, and structural changes, involving regulated expression of specific genes. Fruits have been divided into two categories, known as climacteric and nonclimacteric, which differ in their pattern of respiration and ethylene synthesis at the early stage of ripening (Brady, 1987). In climacteric fruits such as tomato, avocado mango, and banana, repening is characterized by a burst in ethylene production concomitant with a burst in respiration (climacteric respiration), which triggers the changes in color, aromas, softening, sugar content, and other biochemical and physiological properties. In tomato fruits, the appearance of the red color is due to lycopene synthesis and chlorophyll degradation.

Key to abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; chl *a*, chlorophyll *a*; FFa, free fatty acids; LA, linoleic acid; state 4, resting respiration in the absence of added ADP; state 3, phosphorylating respiration in the presence of added ADP; UCP, uncoupling protein; *nor*, nonripenig mutant; *wt*, wild type.

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It has been proposed that a cyanid-insensitive respiration plays a role in the climacteric respiration (Kumar *et al.*, 1990; Pirrung and Brauman, 1987; Solomos, 1977). The cyanide- and antimycin-resistant alternative oxidase (AOX) catalyses ubiquinol-oxygen oxido-reduction without H^+ release into cytosol, thus dissipates the redox potential energy instead to promote ATP synthesis as in the cytochrome pathway respiration (Sluse and Jarmuszkiewicz, 1998; Vanlerbergue and McIntosh, 1997; Wagner and Moore, 1997). In plant mitochondria, the activity of AOX is regulated by α-keto acids like pyruvate (activation), by free fatty acids (FFA) (inhibition), and by the redox state of the enzyme that exists in a dimeric structure of around 65 kD. Two distinct states of the dimer have been identified: a less active oxidized form that is cross-linked by a disulfide bridge and a more active reduced form that is maintained by noncovalent interactions and stimulated by pyruvate (Umbach and Siedow, 1993). It has been suggested that the AOX activity is related to thermogenesis in reproductive processes in Aroids (Meeuse, 1975) as well as in the temperature rise at the end of banana and mango repening (Kumar *et al.*, 1990; Kumar and Sinha, 1992). AOX activity can also be induced under stress conditons, such as low temperature, wounding, and pathogen attack (Wagner and Moore, 1997). Some authors have suggested that the respiration in preclimacteric as well as climacteric avocado fruits is mediated by a terminal oxidase with an apparent K_m for oxygen close to that of the cytochrome oxidase and that AOX does not contribute appreciably to the respiration in the climacteric phase of fruit life (Tucker and Laties, 1985). Recently, it has been shown that in mango fruit, the abundance of AOX message and protein peak at the ripe stage (Considine *et al.*, 2001), while proteins of the cytochrome chain (Rieske FeS subunit of complex III and Cox II subunit of cytochrome oxidase) peak at the mature stage of ripening (Considine *et al.*, 2001). This pattern of protein accumulation suggests that AOX could not be implicated in the climacteric burst of respiration, instead the cytochrome pathway could play a role.

In addition to AOX, plant mitochondria contain a second energy-dissipating system, an uncoupling protein (UCP) that dissipates H^+ electrochemical gradient produced by the main respiratory chain (Vercesi *et al.*, 1995). It has been shown that plant UCP works similarly to the uncoupling protein of mammalian brown adipose tissue (Jezek *et al.*, 1996, 1997; Klingenberg and Huang, 1999). It enables H^+ reentry into the mitochondria matrix through a FFA-activated H^+ cycling process, bypassing the ATP sythesis route, and consequently uncoupling respiration from phosphorylation (Jarmuszkiewicz *et al.*, 2000).

Although the two energy-dissipating systems, AOX and UCP, lead to the same final effect (i.e., to a decrease in ATP synthesis per oxygen consumed), they may have different physiological functions. In fact, in isolated tomato fruit mitochondria, they are coregulated by FFA that increase the UCP activity while switch off the AOX activity (Sluse *et al.*, 1998; Sluse and Jarmuszkiewiz, 2000). Consequently, as AOX and UCP seem not to work together, at least at their maximal activities, they could function during different moments of the plant cell life.

In this study we have investigated the changes in AOX and UCP expression and in several respiratory activities during tomato fruit development on the plant. With decreasing chlorophyll *a* (chl *a*) content that is an indicator of the course of fruit development, we have followed the ATP-synthesis-sustained respiration, the cyanide-resistant AOX-mediated respiration, the UCP-activity-sustained respiration, and the H^+-leak-sustained respirtion in isolated tomato mitochondria. In addition, as FFA could modulate in vivo in an opposite way the AOX and UCP activities, we have measured the evolution of composition and content of FFA in the tomato pericarp juice throughout fruit development, In order to reinforce the significance of chages in parameters observed during *wt* development, these measurements have been performed in parallel in wild type (*wt*) tomato and in nonripening (*nor*) tomato mutant. We have showed that the *wt* and *nor* mutant tomato fruits exhibited different evolution patterns of respiratory parameters measured in vitro as well as protein expression and FFA content during their development on the plant.

MATERIAL AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* cv Pearson) seeds of *wt* and *nor* mutant were kindly provided from the Tomato Genetics Resource Center, California Plants were cultivated in a green house of the Botanical Institute under 60 PAR (photosynthetic active radiation), 16-h light/8-h dark at 20°C using standard horticultural practices. The nonripening (*nor*) mutant is an isogenic mutant lineage that possesses *nor* mutation, a recessive mutation on chromosome 10 that results in nonclimacteric fruit (Tigchelaar *et al.*, 1978) as they fail to produce autocatalytic ethylene (Lelievre *et al.*, 1997).

Isolation of Mitochondria

Approximately 0.3 kg of tomato fruits harvested at chosen stage of ripeness (according to fruit color), after

removal of seeds, was sliced and homogenized in a domestic blender. The produced juice was immediately diluted (1:1,v/v) in a medium containing 500 mM sucrose, 0.2 mM EGTA, 4mM cysteine, and 40 mM Hepes, pH 7.8. During homogenization, the pH of the diluted juice was kept between 7.2 and 7.8 by adding 1N KOH. The homogenate was filtered through a layer of polyester and then the crude mitochondria were isolated by a conventional differential centrifugation (500*g* for 10 min followed by 12,300*g* for 10 min) The crude mitochondrial pellet was washed twice in a medium containing 250 mM sucrose, 0.3 mM EGTA, 10 mM Hepes, pH 7.2, and 1% (w/v) bovine serum albumin (BSA).

Mitochondria were purified on a self-generating Percoll gradient according to Jarmuszkiewicz *et al.* (1998). Because of a decrease in the density of the mitochondrial fraction during the ripening of tomato fruits, different concentrations of Percoll in the gradient medium containing 1% BSA (w/v) were used to improve separation: 21% (v/v) for growing, green, and intermediate *wt* tomato mitochondria and for all stages of *nor* tomato mitochondria, and 18% for red *wt* tomoto mitochondria. After centrifugation at 40,000*g* for 30 min, the mitochondrial layer was collected and washed twice in 250 mM sucrose, 0.3 mM EGTA, 10 mM Hepes, pH 7.2, and 1% (w/v) BSA and again twice in the same buffer but without BSA. Protein concentration was determined by the biuret method (Gornall *et al.*, 1949) with correction as described by Szarkowska and Klingenberg (1963). The presence of 1% BSA in isolation media during purification allowed the complete depletion of endogenous FFA from mitochondria.

Quantitative comparison of functional and molecular parameters measured in isolated mitochondria originating from different tomato cultivars (*wt* and *nor* mutant) at different stages of development requires a number of precaution. Therefore, we defined several criteria in order to control the quality of the mitochondrial preparation from each stage of fruit development. Each mitochondrial preparation was submitted to the following assessments: (i) mitochondrial protein yeald per gram of pericarp, after purification with Percoll gradient, had to be at least 20 μg (ii) yield of the cytochrome oxidase activity recovery after purification had to be at least 40% of the activity before the purification step; (iii) factor of purification, determined as a ratio of cytochrome oxidase activity per miligram of mitochondrial protein after purification versus the activity before the purification step, had to be at least 8; (iv) purified mitochondria had to be totally depleted of FFA. Each preparation was checked by measuring respiratory rate in state 4 (in the absence of ADP), followed by the addition of linoleic acid (LA), and then by the addition of BSA/GTP. The first and last rates had to be the same; (v) respiratory control ratio (state 3 respiration versus state 4 respiration) had to be at least 2; (vi) H^+-leak-sustained respiration (state 4 plus oligomycin) had to be less than 30% of state 3 respiration indicating good inner membrane integrity.

Free Fatty Acid Analysis

Total lipids were extracted from 5 mL of tomato fruit pericarp juice in chloroform;methanol (1:1, v/v) (Folch *et al.*, 1957). Lipid and FFA were separated by a thin-layer chromatography using petroleum ether:diethylether:acetic acid (80:20:1, v/v/v) as a solvent system (Deby-Dupont *et al.*, 1983). Ten micrograms of nonadecanoic acid were added to the samples as internal standard for the FFA quantification. The FFa were located on the plates by brief exposure to iodine vapor and identified by a comparison with standards (Sigma). The bands containing FFA were eluted in chloroform and transmethylated as previously described by Schlenk and Gellerman (1960). The methylated fatty acids were analysed by a gas liquid chromatography (Varian, 3400CX gas chromtograph), using a cyanopropyl column (0.2 μm $\times$ 30 m). Peaks were identified by a comparison with standard methyl ester fatty acid mixtures (Supelco).

Pigment Analysis

To evaluate the degree of fruit ripeness and development, the level of major pigments present in tomato fruits, i.e., chlorphyll *a*, β-carotene, and lycopene, were determined spectorphotometriacally in the dried lipid extract obtained from tomato pericarp juice and diluted in chloroform (Bergevin *et al.*, 1993, modified).

Cytochrome Oxidase Activity Measurements

Cytochrome oxidase activity was measured by following the oxidation of reduced cytochrome *c* at 550–540 nm in a DW2000 Aminco-Chance dual-wavelength spectrophotometer (Moller and Palmer, 1982).

Mitochondrial Respiration Measurements

Oxygen consumption was measured using a Clark-type electrode (Hansatech) in 1.3 mL of standard incubation medium (25°C) containing 125 mM sucrose, 65 mM KCL, 2.5 mM KH_2PO_4, 0.33 mM EGTA, 1mM $MgCl_2$,

0.18 mM ATP, and 10 mM Hepes, pH 7.4, with 0.4 mg of mitochondrial protein, in the presence of 10 mM succinate (plus 5 μM rotenone) as the oxidizable substrate. Activation of succinate dehydrogenase was ensured by the presence of ATP. State 3 measurements were performed in the presence of 2 mM (saturating) ADP. To inhibit the AOX and cytochrome pathway activites, 2 mM BHAM and 1.5 mM KCN were used, respectively. The UCP activity was inhibited with 0.5% BSA and 1 mM GTP. To activate AOX, 0.15 mM pyruvate and 1 mM dithiothreitol were supplied. Linoleic acid (10 μM) was supplied to activate UCP.

SDS-PAGE and Immunoblotting of UCP and AOX

Twenty-five and 40 μg of mitochondrial protein, for UCP and AOX detection, respectively, were solubilized in the denaturating sample buffer containing 2% (w/v) SDS, 80 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 30 mM dithiothreitol, 0.5% β-mecaptoethanol, 0.025% (w/v) bromophenol blue, and boiled for 4–5 min. SDS-PAGE was carried using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel, and followed by Western blotting. Prestained low molecular mass markers were used. The protein bands (approximately 34 kD for AOX and 32 kD for UCP) (Almeida *et al.*, 1999) were detected by chemiluminescence (Boehringer system). The antibodies developed against the AOX protein of *Sauromatum guttatum* (generously supplied by Dr. T. E. Elthon, University of Nebraska, Lincoln) were diluted to 1:500. The antibodies developed against the UCP of *Arabidopsis thaliana* (generously supplied by Dr. P. Arruda, Universidade Estadual de Campinas, Brazil) were diluted to 1:1000.

RESULTS

Evolution of Energy-Conserving and Energy-Dissipating Respiration in Isolated Mitochondria During *wt* and *nor* Tomato Fruit Development on the Plant

A usual way to define development in fruits is based on their macroscopic properties. For *wt* tomato fruits, four steps can be easily distinguished: (i) growing period, where increase in fruit size is observed; (ii) mature stage, where no more increase in size occurs and green color becomes lighter, (iii) ripening period, where changes in intermediate colors occur successively (green with orange patches, orange with green patches, light red, red) mainly due to an increase in lycopene content; (iv) senescence stage, where fruits are dark red and become softer. In the *nor* mutant, the duration of fruit development was more than twice as long (around 130 days) compared to *wt* fruits. For *nor* tomato fruits, only three steps can be distinguished: (i) growing period and (ii) mature stage that resemble respective stages in *wt* fruits, and (iii) a long senescence period that starts in around 60-day fruits and lasts up to 130 days of development. During senescence, the color of *nor* fruits evolves from light green to yellow with progressive changes in fruit texture (fruits become tough instead of softening) and with progressive dehydration. The procedure based on macroscopic changes cannot allow the comparative description of the whole development of *wt* (four steps) and *nor* (three steps) fruits in a continuous manner. As it is well known that degradation of chlorophyll and accumulation of carotenoids are responsible for color and macroscopic changes in tomato fruits, these parameters were measured continuously during development. In *wt*, chl *a* varied from 45 μmol mL^{-1} of pericarp juice at the growing stage to around 0.4 μmol mL^{-1} at the end of development (Fig. 1). With fruit development, β-carotene decreased by around a factor of 3 while lycopene appeared only at the end of the mature stage (at around 8 μmol mL^{-1} chl *a*) indicating the onset of ripening and reached 90 μmol mL^{-1} in dark red senescent tomato (not shown). In *nor* mutant fruits, chl *a* decreased from 25 to 0.2 μmol mL^{-1} (Fig. 2), β-carotene diminished by around factor of 3, while lycopene did not reveal detectable levels (not shown). As the fruits did not develop at the same rate that results from competition between them in a cluster and among clusters on the plant leads (Beadle, 1937; Lyons and Pratt, 1963), scattering in the pigment content for the same tomato age was observed (not shown). Therefore, taking into account changes in the pigment content and difference in duration of fruit development observed in both cultivars, the course of tomato fruit development is expressed in terms of chl *a* content in μmol per mL of pericarp juice thus in a continuous manner.

Five conditions of mitochondrial respiration with succinate (plus rotenone) as oxidizable substrate (in the presence of ATP to activate succinate dehydrogenase) were followed during tomato fruit development: (i) total state 3 respiration (●) was measured in the presence of ADP, AOX activators (pyruvate and dithiothreitol), and UCP inhibitors (BSA and GTP), and represents the sum of electron fluxes (not necessarily maximal) in phosphorylating conditions through the cytochrome pathway and AOX; (ii) ATP-synthesis-sustained respiration (○) was measured in the presence of ADP and inhibitors of UCP and AOX (benzohydroxamate, BHAM), and represents the electron flux in phosphorylating conditions through the cytochrome pathway only, (iii) AOX-mediated respiration (□) was measured in the presence of

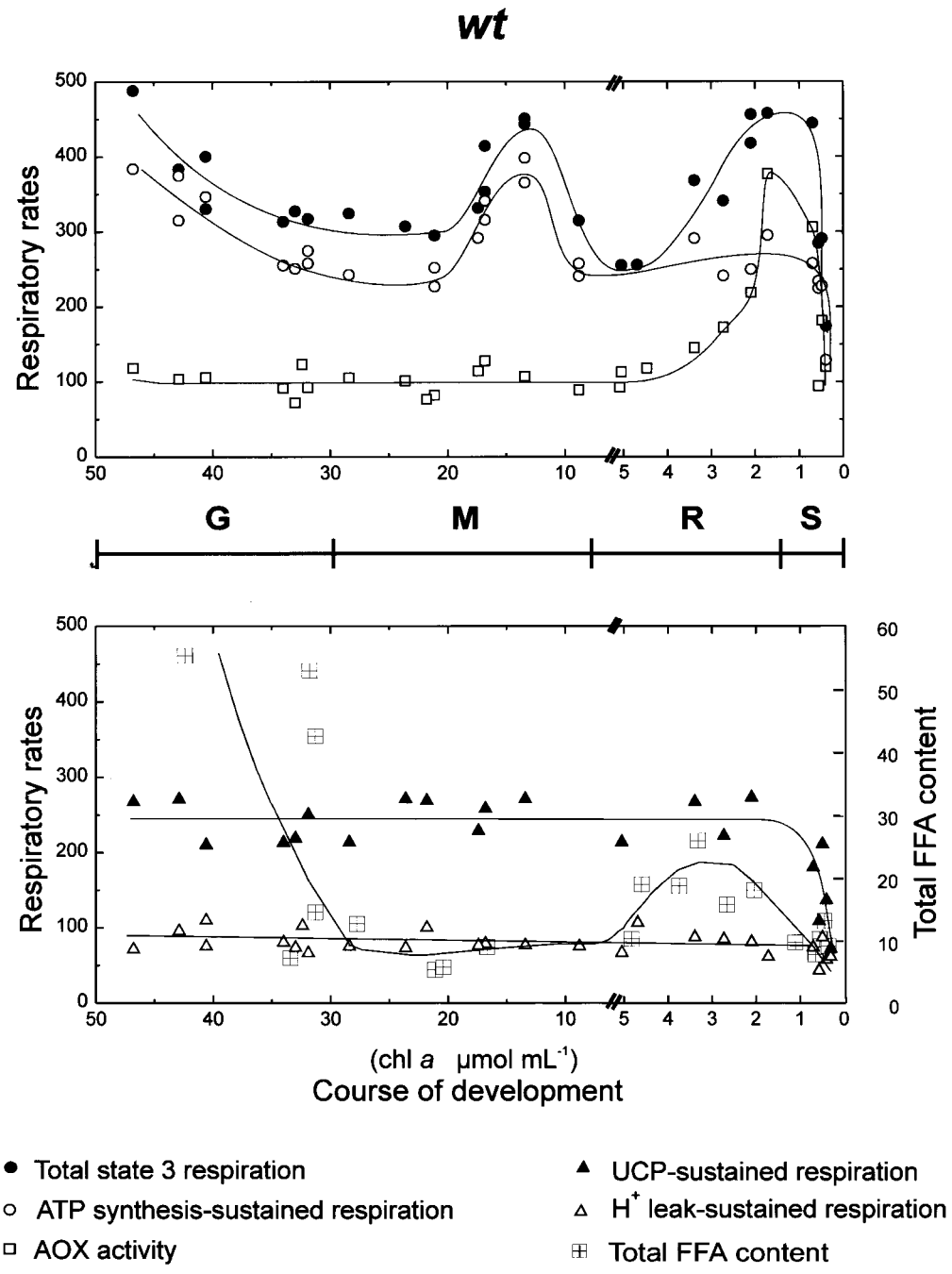

Fig. 1. Evolution of the energy-conserving and energy-dissipating pathways, and total FFA content in *wt* tomato fruit mitochondria during development on the plant. Mitochondria were incubated in a standard reaction conditions as described under "Material and Methods." For total state 3 respiration (●): 2 mM ADP, 0.5% BSA, 1 mM GTP, 0.15 mM pyruvate, and 1 mM dithiothreitol were supplied; for ATP synthesis-sustained respiration (○): 2 mM ADP, 0.5% BSA, 1 mM GTP, and 2 mM BHAM were supplied; for AOX-sustained respiration (□): 0.5% BSA, 1 mM GTP, 0.15 mM pyruvate, 1 mM dithiothreitol, and 1.5 mM KCN were supplied; for LA-activated UCP-sustained respiration (▲): 10 μM LA, 2 mM BHAM, and 2.5 μg mL^{-1} oligomycin were supplied; for H^+ leak-sustained respiration (△): 2 mM BHAM, 2.5 μg mL^{-1} oligomycin, 0.5% BSA, and 1 mM GTP were supplied. Respiratory rates are in nmol 0_2 min^{-1} mg^{-1} protein. Total FFA content (⊞) is expressed in μg mL^{-1} of juce. The course of fruit development is presented in terms of chl *a* content change in μmol mL^{-1}. Expended scale is used for *wt* from 5 to 0 μmol mL^{-1} chl *a*. Different macroscopic stages of fruit development are indicated in parallel with the chl *a* content decrease. G, growing; M, mature; R, ripening; and S, senescence.

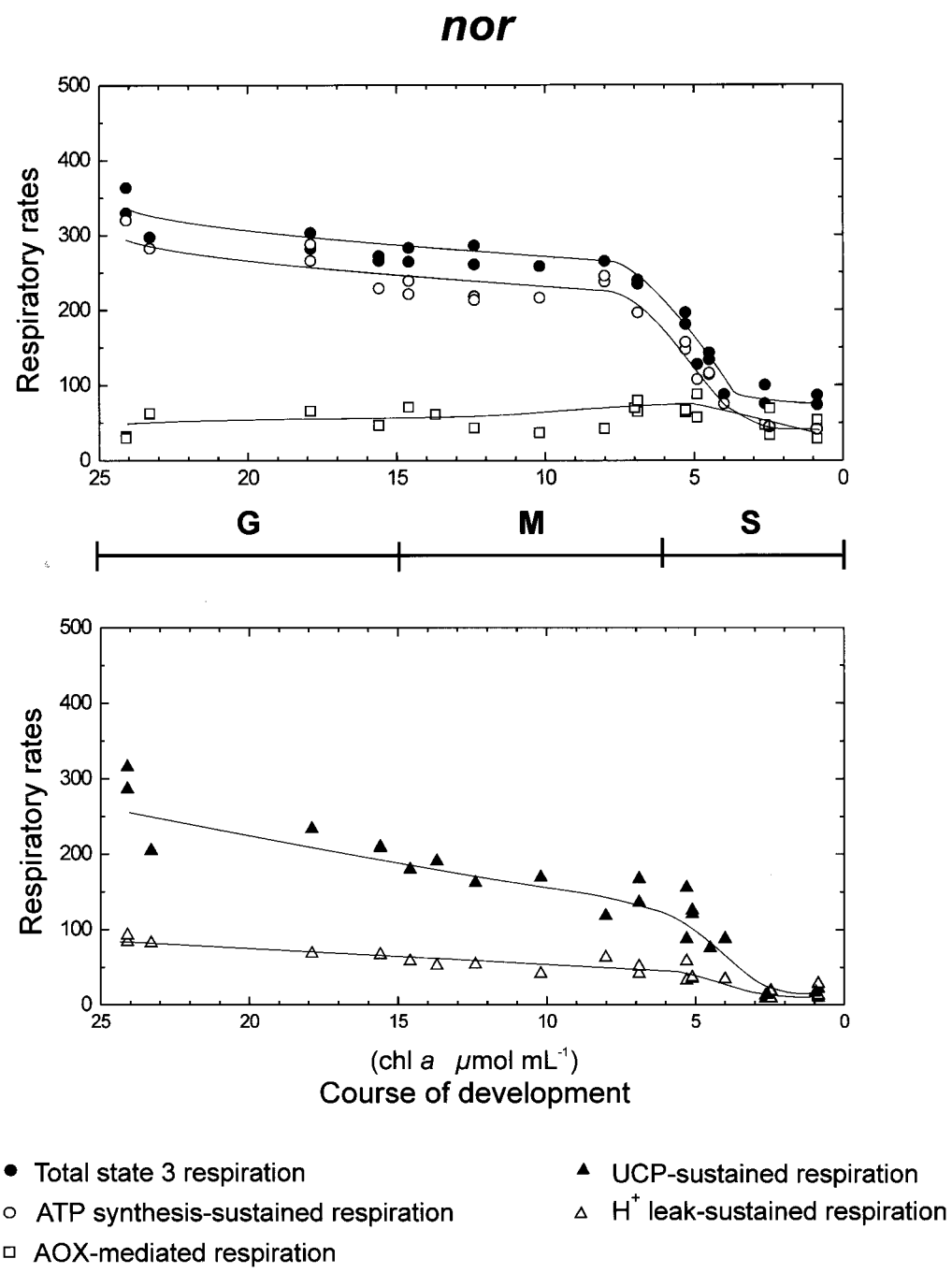

Fig. 2. Evolution of the energy-conserving and energy-dissipating pathways in *nor* (nonripening) mutant tomato fruit mitochondria during development on the plant. Conditions and symbols as in Fig. 1. Data concerning the total FFA content are not shown (see Table I).

AOX activators, cytochrome pathway inhibitor, (cyanide), UCP inhibitors, and ADP; (iv) UCP-sustained respiration activated by 10 μM LA (▲) was measured in state 4 respiration in the presence of oligomycin (an inhibitor of ATP synthase) and BHAM, and represents the electron flux through the cytochrome pathway only, in conditions where UCP is activated by 10 μM LA; (v) proton-leak-sustained respiration (Δ) was measured in state 4 in the presence of oligomycin, BHAM, and BSA/GTP, and represents the electron flux in the cytochrome pathway under the high membrane potential conditions.

However, several points must be stressed: (1) "The UCP-sustained respiration" could reflect not only the sole activity of UCP because a portion of the uncoupling by 10 μM LA could be mediated by the other carriers like ADP/ATP, phosphate, and dicarboxylate carriers. However, the presence of respective translocated substrates or inhibitors of these carriers strongly inhibits their participation in the FFA-induced uncoupling (Andreyev *et al.*, 1989; Sluse and Jarmuszkiewicz, 2002; Więckovski and Wojtczak, 1997; Zackova *et al.*, 2000). Thus, as the UCP-sustained respiration activated by 10 μM LA is measured in the presence of 180 μM ATP, 2 mM inorganic phosphate, and 10 mM succinate, the three translocators that could partly mediated FFA-activated H^+ recycling are substantially excluded. (2) The UCP-sustained respiration in the presence of 10 μM LA represents its standardized assay but not its maximum capacity. The concentration of LA, 10 μM, has been chosen as it is the concentration giving half maximum effect ($S_{0.5}$) for the LA-induced respiration in isolated tomato mitochondria (Sluse *et al.*, 1998). The use of GTP as a UCP-specific inhibitor in order to measure the LA-induced GTP-inhibited UCP-sustained respiration is not possible because GTP is a weak inhibitor of the LA-induced uncoupling in the absence of BSA, in isolated tomato mitochondria (Sluse *et al.*, 1998). (3) "The proton-leak-sustained respiration" measured in state 4 cannot be subtracted from the UCP-sustained respiration because LA induces a drop in membrance potential to a value close to the state 3 membrane potential. Thus in the presence of LA the proton leak is probably negligible (Almeida *et al.*, 1999) compared to the proton-leak-sustaining respiration in state 4 in the presence of oligomycin, BHAM, and BSA/GTP and a under high membrane potential. (4) "The ATP-synthesis-sustained respiration" that represents the phosphorylating respiration through the cytochrome pathway (in the presence of succinate plus rotenone) is equal to the respiration fully uncoupled by the artificial uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) in tomato mitochondria. This observation indicated that maximal electron flux is reached in both state 3 and uncoupled state (Almeida *et al.*, 1999). (5) The difference between "the total state 3 respiration" and "the ATP-synthesis-sustained respiration" is not the contribution of AOX to state 3 respiration, but it is only the BHAM-sensitive respiration in state 3 being underestimation of the AOX-mediated respiration.

Figures 1 and 2 show changes of the five respiratory rates during *wt* and *nor* mutant tomato development. The course of fruit development (abscissa) is expressed in terms of chl *a* evolution in parallel with indication of the different macroscopic stages. In *wt*, the total state 3 respiration changed during the four stages of fruit development in the following way (Fig. 1): (i) in the growing stage (chl *a* higher than 30 μmol mL^{-1}) it decreased from 500 to 300 nmol O_2 min^{-1} mg^{-1} protein; (ii) in the mature stage (for chl *a* between 30 and 8 μmol mL^{-1}), it peaked at 450 nmol O_2 min^{-1} mg^{-1} protein that corresponds to the climacteric respiratory rise occurring before the beginning of ripening (before lycopene appearance); (iii) in the ripening stage (for chl *a* between 8 and 1.5 μmol mL^{-1}), it reincreased to 450 nmol O_2 min^{-1} mg^{-1} protein; (iv) in the senescence stage (chl *a* below 1.5 μmol mL^{-1}), it exhibited a sharp decrease. In *nor* mutant (Fig. 2), the total state 3 respiration evolved in a different way compared to *wt* fruits. Indeed, during the growing stage (till 15 μmol mL^{-1} chl *a*) and the mature stage (between 15 and 6 μmol mL^{-1} chl *a*), there was a slow decrease in the total state 3 respiration from 350 to 250 nmol O_2 min^{-1} mg^{-1} protein. Afterwards, at the end of ripening stage and at the early senescence, an abrupt decrease occurred in the total state 3 respiration to around 80 nmol O_2 min^{-1} mg^{-1} protein.

In *wt*, the ATP-synthesis-sustained respiration (phosphorylating respiration) paralleled the total state 3 respiration until the end of the mature stage (Fig. 1). Starting at 400 nmol O_2 min^{-1} mg^{-1} protein, it decreased to 250 nmol O_2 min^{-1} mg^{-1} protein at the end of the growing stage, then peaked at 400 nmol O_2 min^{-1} mg^{-1} protein (climacteric burst) and dropped to 250 nmol O_2 min^{-1} mg^{-1} protein at the end of the mature stage. However, it remained roughly constant (250 nmol O_2 min^{-1} mg^{-1} protein) during the ripening stage and finally dropped at senescence. On the contrary, in *nor* mutant, the ATP-synthesis-sustained respiration paralleled the total state 3 respiration during the whole fruit development (Fig. 2).

In mitochondria of *wt* fruits, the AOX-mediated respiration remained constant (around 100 nmol O_2 min^{-1} mg^{-1} protein) during the growing stage and the mature stage, increased strongly during ripening (to 400 nmol O_2 min^{-1} mg^{-1} protein) and dropped during senescence (Fig. 1). In *nor* mutant mitochondria, a slight increase (from 50 to 100 nmol O_2 min^{-1} mg^{-1} protein) in the AOX-mediated respiration occurred until the beginning of senescence, then it decreased to around 50 nmol O_2 min^{-1} mg^{-1} protein (Fig. 2). It must be pointed out that the sum of the ATP-synthesis-sustained respiration and the AOX-mediated respiration is always higher than the total state 3 respiration.

In *wt*, UCP-activity-sustained respiration induced by 10 μM LA remained constant (around 250 nmol O_2 min^{-1} mg^{-1} protein) until the end of ripening and dropped during senescence (Fig. 1). In *nor* mutant, the

UCP-sustained respiration decreased progressively from 300 nmol O_2 min^{-1} mg^{-1} protein until the end of the mature stage (down to 150 nmol O_2 min^{-1} mg^{-1} protein), then dropped during senescence (Fig. 2).

The proton-leak-sustained respiration was almost constant in *wt* (around 80 nmol O_2 min^{-1} mg^{-1} protein) and decreased progressively (starting from the same rate) in *nor* mutant.

Differences in the mitochondrial respiratory pattern between *wt* and *nor* mutant are significant and can be attributed to their climacteric/nonclimacteric properties. It seems that the climacteric burst observed in vitro for *wt* fruits depends totally on the cytochrome pathway activity, which is responsible for the ATP-synthesis-sustained respiration. This profile was not observed in *nor* mutant, as expected if the respiratory burst is a climacteric characteristic. The second respiratory rise during the ripening stage in *wt* is mainly linked to a strong increase in the AOX activity. This rise, not observed in *nor* mutant, seems to be related to the ripening processes. The UCP-sustained respiration induced by a fixed (10 μM) concentration of LA did not change during the full development of *wt* fruits except a drop during the senescence stage. On the contrary, in *nor* mutant, the UCP-sustained respiration, starting as in *wt* at 250–300 nmol O_2 min^{-1} mg^{-1} protein, paralleled roughly the total state 3 respiration and ATP-synthesis-sustained respiration profiles. In *wt* and *nor* mutant, the absence of increase in the H^+-leak-sustained respiration (that was around 80 nmol O_2 min^{-1} mg^{-1} protein in the growing stage for both cultivars) proves that all mitochondrial preparations had an intact inner membrane. The decrease that occurred during the senescence stages was concomitant with the general decrease in respiration.

Immunological Analysis of UCP and AOX

In order to assess the role of protein expression in variations of the UCP-sustained respiration and the AOX-mediated respiration, the evolution of UCP and AOX protein levels during tomato fruit development of *wt* and *nor* mutant was determined by an immunological analysis of total mitochondrial proteins. Figure 3 (upper part) shows that a protein band of approximately 34 kD, that corresponds to a monomeric form of tomato AOX (as samples were treated with dithiothreitol and β-mercaptoethanol), was present in all stages of fruit development in both *wt* and *nor* mutant. In *wt* fruits, the AOX protein level remained almost constant at 41, 18, and 8.8 μmol mL^{-1} chl *a* (i.e., till the end of the mature stage), accumulated three times at the end of the ripening stage (at 1.8 μmol mL^{-1} chl *a*), then decreased during senescence (for less than 0.8 μmol mL^{-1} chl *a*) (Fig. 3(A)). This profile of protein expression fits the activity profile observed in vitro in isolated mitochondria (Fig. 1) In *nor* mutant, the AOX

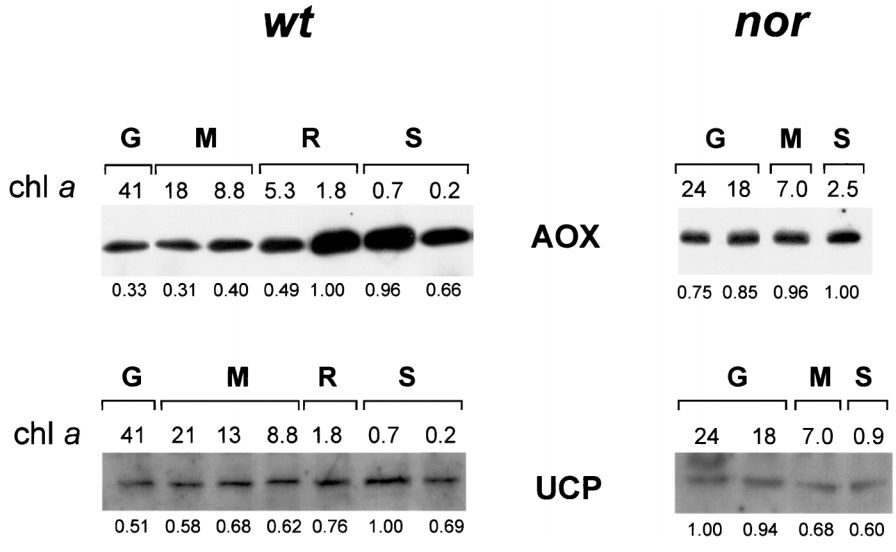

Fig. 3. Immunoblots of total mitochondrial proteins from *wt* and *nor* tomato at different stages of fruit development using antibodies raised against AOX and UCP. The total mitochondrial protein load was 25 μg for UCP detection and 40 μg for AOX detection. The protein bands were visualized by chemiluminescence and quantitated digitally. The highest intensity band of the profile was set to 1 and others calculated relative to that value. Relative intensity is given below each band. Different stages of fruit development are given as described under "Results" (macroscopic stages and chl *a* content). G, growing; M, mature; R, ripening; and S, senescence.

protein level increased very slightly with the decrease in chl *a* content until the senescence stage (Fig. 3(B)). This protein evolution fits with activity evolution.

An approximately 32-kD UCP protein can be detected immunologically in tomato fruit mitochondria (Almeida *et al.*, 1999). During development of *wt* fruits on the plant, the level of UCP protein slightly rose with the decrease in chl *a* content until the beginning of senescence (0.7 μmol mL^{-1} chl *a*), then decreased (Fig. 3(A), lower part). These slight changes in the UCP protein level do not influence significantly the UCP-sustained respiration induced by 10 μM LA measured in vitro as it is constant (Fig. 1). In *nor* mutant, the UCP protein level was a little lower in the mature and senescence stages compared to the growing stage (Fig. 3(B), lower part). Thus, this could account for the progressive decrease in UCP-sustained respiration with decrease in chl *a* content but not for the drop in this respiration in senescent fruits. Differences in the UCP and AOX expressions between *wt* and *nor* mutant can also be attributed to their climacteric/nonclimacteric properties.

Free Fatty Acid Content Analysis

FFA were extracted from the juice obtained by homogenization of tomato fruit pericarp. Concentrations given in Table I were calculated taking into account the total volume of juice. Therefore, they represent overall concentrations (i.e., including the aqueous phase and lipid phase concentrations) and indicate the relative amounts of FFA at different stages of fruit development.

As shown for LA in isolated mitochondria from green mature tomato fruits (Sluse *et al.*, 1998), FFA can play a connecting role between the activities of UCP and AOX. Therefore, level and composition of FFA in vivo during fruit development have to be taken into account together with the AOX and UCP expression and with AOX-mediated respiration and UCP-sustained respiration measured in vitro, if extrapolation of changes of these two respiratory activities to in vivo conditions is attempted.

In *wt* and *nor* fruits, the content of long chain FFA (longer than C-18) was low and did not change significantly throughout the tomato fruit development (between 5 and 9% of total FFA in *wt* and between 12 and 15% in *nor*, not shown). Table I shows variations in the content of saturated, unsaturated, and total FFA (excluding long chain FFA, i.e., from C-12 till C-18) during development in both types of fruits. In *wt* fruits, the total concentration of FFA was around four times higher in the growing stage compared to the mature stage. Between the mature stage and the ripening stage, the total FFA concentration increased 2.4 times as the content of saturated FFA rose around 3.7 times while that of unsaturated FFA did not change. Between the ripening and senescence stages, the content of saturated FFA decreased a little (around 1.5 time) while the content of unsaturated FFA dropped more strongly (2.5 times). Thus, during development of *wt* fruits, the saturation ratio of FFA varied as well as total FFA concentration. In Fig. 1, for *wt* fruits, the total FFA content (excluding long chain FFA) is plotted versus course of fruit development. A very abrupt decrease in the total FFA level (from 55 to around 15 μg mL^{-1}) was observed at the end of growing stage. During the mature stage, the total FFA level remained stable (around 15 μg mL^{-1}) until the beginning of ripening stage where it peaked reaching around 27 μg mL^{-1}. At the end of ripening stage and at the early senescence, the total FFA level decreased to around 10 μg mL^{-1}. In *nor* mutant fruits, no significant change was observed during development and the overall content of FFA was much lower compared to *wt* (around eight times in the growing stage). Comparison of FFA content in *wt* and *nor* mutant fruits indicates that their overall steady-state concentration are probably related to their climacteric/nonclimacteric properties.

Table I also reports changes in the content of nine individual FFA (from C 12:0 to C 18:3) expressed as changes in concentration and percentage of total FFA (excluding long chain FFA) during development of *wt* and *nor* fruits. In *wt*, the predominant free fatty acid in all stages of development was palmitic acid, followed by lauric acid in the growing stage, by α-linolenic acid in the mature stage, by myristic and lauric acids in the ripening stage, and by lauric acid in the senescence stage. In particular, the percentage of lauric acid decreased almost three times between the growing and mature stages, the percentage of myristic acid decreased by 4 between the growing and mature stages as well as between the ripening and senescence stages, the percentage of palmitic acid increased from 26 to 45 during the whole development, the percentage of stearic, oleic, and γ-linolenic acids did not change much, percentage of palmitoleic acid decreased 2.5 times between the growing and mature stages, the percentage of linolenic and α-linolenic acid peaked at the mature stage. In *nor* mutant, lauric acid and palmitic acid were predominant. In particular, the level of lauric acid decreased almost twice between the mature and senescence stages, the level of myristic and oleic acids increased around twice between the growing and mature stages and the level of other acids was quite stable. Thus, the evolution pattern of individual FFA during tomato fruit development was very different in *wt* and *nor* and seemed to be also related to their climacteric/nonclimacteric properties.

Table I. Comparison of the Evolution of the FFA Level and Composition in *wt* and *nor* Tomato Pericarp During Fruit Development

	wt								*nor*					
	G		M		R		S		G		M		S	
Free fatty acids	μM	%	μM	%	μM	%	μM	%	μM	%	μM	%	μM	%
Lauric (12:0)	22.82	21	1.95	7	9.65	14	9.66	25	3.51	28	3.81	24	1.87	14
Myristic (14:0)	13.44	13	0.70	3	10.8	16	1.60	4	0.74	6	1.35	9	1.42	11
Palmitic (16:0)	27.95	26	8.82	32	23.2	35	17.70	45	2.64	21	3.31	21	2.36	18
Palmitoleic (16:1)	12.13	11	0.66	2	7.84	12	1.36	4	1.10	9	1.65	10	1.42	11
Stearic (18:0)	5.48	5	1.24	5	4.84	7	2.87	7	1.19	10	1.10	7	1.31	10
Oleic (18:1n9)	9.47	9	0.72	3	3.43	5	1.29	3	0.41	3	1.07	7	1.23	10
Linoleic (18:2n6)	5.76	5	3.85	14	2.97	5	3.04	8	0.79	7	0.71	4	0.43	3
α-inolenic (18:3n3)	6.73	6	8.28	31	2.10	3	1.31	3	0.92	7	1.15	7	0.92	7
γ-linolenic (18:3n6)	4.40	4	0.79	3	1.99	3	0.27	1	1.16	9	1.74	11	2.03	16
Total	108.2	100	27	100	66.8	100	39.1	100	12.50	100	15.90	100	12.99	100
Saturated	69.7	64	12.7	47	48.5	72	31.8	81	8.08	65	9.57	61	6.96	53
Unsaturated	38.5	36	14.3	53	18.3	28	7.27	19	4.38	35	6.32	39	6.03	47

Note. Concentration (μM) and relative amount (%) of particular FFA are given. Long chain fatty acids are omitted, also in the total FFA content presented in the Table. Fatty acids are abbreviated with the first figure showing the number of carbon atoms and the second indicating the number of double bonds. The positions of double bonds are also given. Macroscopic stages of fruit development are indicated. For *wt* fruits: G, growing (till 30 μmol mL^{-1} chl *a*); M, mature (30-8 μmol mL^{-1} chl *a*); R, ripening (8–1.5 μmol mL^{-1} chl *a*); and S, senescence (1.5–0.4 μmol mL^{-1} chl *a*). For *nor* fruits: G, growing (till 15 μmol mL^{-1} chl *a*); M, mature (15-6 μmol mL^{-1} chl *a*); and S, senescence (6–0.8 μmol mL^{-1} chl *a*). Values in μM are means of 4–6 determinations where SEM was from 5 to 15%.

DISCUSSION

In the present work, we have described changes in energy-conserving and energy-dissipating respiration, AOX and UCP protein expression, and FFA content during the development on the plant of *wt* tomato, a climacteric fruit, and its isogenic nonripening (*nor*) mutant that behaves as a nonclimacteric fruit. In order to keep track of fruit development uninterruptedly, the determination of chl *a* degradation was performed as content of this pigment depends on fruit development in both *wt* and *nor* mutant.

The wild type and *nor* mutant presented very different developmental profiles for respiratory activities in vitro, protein expression, and FFA concentration in vivo. This general observation indicates that these parameters can be related to the climateric/nonclimacteric properties of *wt* and *nor* mutant, respectively. The respiratory pattern in vitro shows that in *wt* fruits, the total state 3 respiration, i.e., total electron flux from succinate through the cytochrome pathway and AOX (that is not the sum of maximal fluxes through both pathways, their "capacities"), exhibits an expected decrease during the growing period and two rises in the following stages. The first peak occurs during the mature stage and corresponds to the climacteric burst. It is result of a transient increase in the cytochrome pathway activity as shown by the concomitant peak in the ATP-synthesis-sustained respiration (maximal phosphorylating respiratory rate). The second peak observed in total state 3 respiration, beginning in the ripening stage (rise) and ending in the senescence stage (drop), is due to a large increase in AOX activity. The senescence stage is accompanied by a drop in total respiration due to simultaneous decrease in the AOX activity and in the ATP-systhesis-sustained respiration. The UCP-sustained respiration (plus BHAM), measured in vitro with 10 μM LA, showed no change until the senescence stage and its rate (around 250 nmol O_2 min^{-1} mg^{-1} protein) was equal to the rate of phosphorylating respiration (plus BHAM) during the "preclimacteric" mature stage and during the ripening stage but lower during the growing stage and the climacteric burst. The equality between the UCP-sustained respiration and the ADP-stimulated respiration, in the presence of BHAM, has already been described in green-mature tomato fruits (Jarmuszkiewicz *et al.*, 1998). This was believed to be the result of saturation of the respiratory chain in both conditions, with a limiting step likely being the succinate dehydrogenase. On the contrary, the results presented in the present study suggest that the electron flux in the cytochrome pathway itself could be the limiting step outside the growing period and the climacteric period as the ADP-stimulated respiration is higher than the UCP-sustained respiration in these two periods. Moreover, possibly because of this limitation, the increase in UCP protein expression during ripening is not reflected in the UCP-sustained respiration measurements in vitro.

On the other hand, during the growing period, when phosphorylating respiration (thereby the cytochrome pathway activity) was much higher compared to the ripening stage, the UCP-sustained respiration was not higher. This indicates that limitation of the UCP-sustained respiration is linked to the lower amount of UCP protein. The immunologically detected increase in the AOX protein level during ripening was perfectly reflected as an increase in the AOX-dependent repiration measured in vitro. Accordingly, neither phosphorylating respiration nor AOX-mediated respiration was limited by the succinate dehydrogenase side of respiratory chain at least until the senescence stage but by the cytochrome pathway and AOX proteins, respectively.

Knowledge of FFA concentration and composition in vivo can help to predict from in vitro measurements how the AOX and UCP activities as well as the associated respiration change in vivo. There are very few data about the specificity of inhibition of AOX by FFA except in a fungi-type AOX (Minagawa *et al.*, 1992). Namely, oleic, linoleic, and α-linolenic acids are the most efficient tested FFA in inhibiting AOX activity in *Hansenula anomala* mitochondria. Concerning UCP, much more is known about its FFA-dependent activation. Free fatty acids must have at least a C-10 chain and a sufficient hydrophobicity (Klingenberg and Huang, 1999). Their efficiency increases with carbon chain length up to C 14:0 (myristic acid) and decreases for palmitic acid (C 16:0) and more strongly for stearic acid (C 18:0) because of a decrease in water solubility. Oleic, linoleic, and linolenic acids that are unsaturated fatty acids of the C-18 group are as good activators as myristic acid (Klingenberg and Echtay, 2001). On this basis, it appears that FFA specificity could be similar for AOX and UCP, i.e., efficient inhibitors of AOX could at the same time be efficient activators of UCP. Our results indicate that the content of unsaturated FFA decreased during *wt* fruit development between the growing and mature stages and again between the ripening and sensecence stages. Moreover, a sum of overall concentrations of the known most efficient FFA (lauric 12:0, myristic 14:0, oleic 18:1, linoleic 18:2, and α-linolenic 18:3n3) (calculated from data in Table I) was 58 μM in the growing stage, 16 μM in the mature stage, 29 μM in the ripening stage, and 17 μM in the senescence stage. Taking these concentrations as indicators of the overall concentration of efficient FFA at different stages of *wt* fruit development, it can be predicted that AOX-mediated respiration in vivo could increase (being less inhibited) between the growing and mature stages contrary to the in vitro results obtained with a fixed 10 μM LA. Indeed, higher FFA concentration could inhibit AOX in vivo during the growing stage, whereas lower FFA concentration could allow increased activity of AOX in vivo in the mature stage. During the ripening stage, presence of FFA at an intermediate concentration concomitant with a high protein expression could nevertheless allow high AOX activity in vivo. Thus, it can be predicted that the AOX activity in vivo could peak twice, at climacteric (even if the protein expression is unchanged) and at the end of the ripening stage (due to a large increase in the AOX expression level, even if FFA level increases). Following the same analysis, the UCP-sustained respiration could be predicted from in vitro information (respiratory rate measurements) and from variations in FFA overall concentration in vivo. Indeed in vivo, the UCP-sustained respiration could be minimum during the mature stage and higher during the growing stage (even if less UCP protein is expressed) and during the ripening stage (as FFA level and protein expression increase). Thus, the UCP-sustained respiration in vivo could display a minimum at climacteric and peak during the ripening stage.

In the nonclimacteric *nor* mutant, the respiratory pattern in vitro showed a significant decrease in the UCP-sustained respiration during the growing and mature stages, which can be related to the decrease in UCP protein expression. AOX-mediated respiration evolution (slight increase) can also be related to AOX protein expression. The other respiratory activities (i.e., the total state 3 respiration, the ATP-synthesis-sustained respiration, and the H^+-leak-sustained respiration) decreased progressively until the senescence stage where they dropped in unison. In *nor* mutant, the overall concentration of the most efficient FFA was 6 μM in the growing stage, 8 μM in the mature stage, and 6 μM in the senescence stage. Thus, concentration of these FFA was low and change was negligible during fruit development. Then, for *nor* mutant it can be predicted that the evolution profile of the in vivo AOX-mediated respiration and the in vivo UCP-sustained respiration are similar to the in vitro measurements.

The results of respiratory activities measured with isolated mitochondria and their evolution during tomato fruit ripening on the plant described in the present study differ from the results obtained with mitochondria isolated from tomato fruits during postharvest ripening (Almeida *et al.*, 1999) In the previous study, the ATP-synthesis-sustained, UCP-sustained, and AOX-mediated respiration as well as the immunologically detectable UCP and AOX protein levels decreased with ripening and no respiratory burst was observed. These differences may be due to the altered process of ripening in harvested fruits, which depends on preharvest factors (Ferguson *et al.*, 1999, Woolf *et al.*, 2000). Level of UCP, AOX, and cytochrome pathway proteins have been described in mango fruits during ripening on the tree (Considine *et al.*, 2001). These molecular results, suggesting that the cytochrome pathway is implicated in the climacteric burst and that AOX

is implicated in postclimacteric ripening, are functionally demonstrated in this study.

In conclusion, the energy-conserving and energy-dissipating processes play an important role in the progress of tomato fruit development, ripening, and sensence. Sequential up- and down-regulations of expression of implicated proteins and their activities can account for the successive steps from growing to senescence including the climacteric period. This is demonstrated by the absence of such regulations in the nonripening (*nor*) mutant.

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REFERENCES

Almeida, A. M., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A. E., and Sluse, F. E. (1999). *Plant Physiol.* **119**, 1323–1329.

Andreyev, A. Y., Bondareva, T. O., Dedukhova, V. I., Mokhova, E. N., Skulachev, V. P., Tsofina, L. M., Volkov, N. L., and Vygodina, T. V. (1989), *Eur. J. Biochem.* **182**, 585–592.

Beadle, N. C. W. (1937). *Aust. J. Exp. Biol. Med. Sci.* **15**, 173–189.

Bergevin, M., L'Heureux, G. P., Thompson, J. E., and Willemot, C. (1993). *Physiol. Plant.* **87**, 522–527.

Brady. C. J. (1987). *Annu. Rev. Plant Physiol.* **38**, 155–178.

Considine, M., Daley, D., and Whelan, J. (2001). *Plant Physiol.* **126**, 1619–1629.

Deby-Dupont, G., Ducarne, H., de Lanndsheere, C., Ancion, J. C., Noel, F. X., Dadoux, L., and Deby, C. (1983). *Biomed. Pharmacother.* **37**, 386–391.

Ferguson, I., Volz, R., and Woolf, A. (1999). *Postharv. Biol. Tech.* **15**, 255–262.

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957). *J. Biol. Chem.* **226**, 497–509.

Gornall, A. G., Bardawill, C. J., and Dawid, M. M. (1949). *J. Biol. Chem.* **177**, 751–757.

Jarmuszkiewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (1998). *J. Biol. Chem.* **273**, 34882–34886.

Jarmuszkiewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (2000). *J. Biol. Chem.* **275**, 13315–13320.

Jezek, P., Costa, A. D. T., and Vercesi, A. E. (1996). *J. Biol. Chem.* **271**, 32743–32748.

Jezek, P., Costa, A. D. T., and Vercesi, A. E. (1997). *J. Biol. Chem.* **272**, 24272–24278.

Klingenberg, M., and Echtay, K. S. (2001). *Biochim. Biophys. Acta* **1504**, 128–143.

Klingenberg, M., and Huang, S. G. (1999). *Biochim. Biophys. Acta* **1415**, 271–296.

Kumar, S., and Sinha, S. H. (1992). *J. Exp. Bot.* **43**, 1639–1642.

Kumar, S., Patil, B. C., and Sinha, S. K. (1990). *Biochem. Biophys. Res. Commun.* **168**, 818–822.

Lelievre, J.-M., Latche, A., Jones, B., Bouzayen, M., and Pech, J.-C. (1997). *Physiol. Plant.* **101**, 727–739.

Lyons, J., and Pratt, H. K. (1963). *Am. Soc. Hort. Sci.* **84**, 491–500.

Meeuse, B. J. D. (1975). *Annu. Rev. Plant Physiol.* **26**, 117–126.

Minagawa, N., Sakajo, S., and Yoshimoto, A. (1992). *Biosci. Biotech. Biochem.* **56**, 1342–1343.

Moller, I. M., and Palmer, J. M. (1982). *Physiol. Plant.* **54**, 267–274.

Pirrung, M. C., and Brauman, J. I. (1987). *Plant. Physiol. Biochem.* **25**, 55–61.

Schlenk, H. L., and Gellerman, J. L. (1960), *Anal. Chem.* **32**, 1412–1414.

Sluse, F. E., Almeida, A. M., Jarmuszkiewicz, W., and Vercesi, A. E. (1998). *FEBS Lett.* **433**, 237–240.

Sluse, F. E., and Jarmuszkiewicz, W. (1998). *Braz. J. Med. Biol. Res.* **32**, 733–747.

Sluse, F. E., and Jarmuszkiewicz, W. (2000). *Braz. J. Med. Biol. Res.* **33**, 259–268.

Sluse, F. E., and Jarmuszkiewicz, W. (2002). *FEBS Lett.* **510**, 117–120.

Solomos, T (1977). *Annu. Rev. Plant Physiol.* **28**, 279–297.

Szarkowska, L., and Klingenberg, M. (1963). *Biochem. Z.* **338**, 674–697.

Tigchelaar, E. C., McGlasson, W. B., and Buescher, R. W. (1978). *Hort. Sci.* **13**, 508–513.

Tucker, M. L., and Laties, G. G. (1985). *Plant Cell. Environ.* **8**, 117–127.

Umbach, A. L., and Siedow, J. N. (1993). *Plant Physiol.* **103**, 845–854.

Vanlerbergue, G. C., and McIntosh, L. (1997). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 703–734.

Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cucovia, I. M., and Chaimovich, H. (1995). *Nature* **375**, 24.

Wagner, A. M., and Moore, A. L. (1997). *Biosci. Rep.* **17**, 319–333.

Więckowski, M., and Wojtczak, L. (1997). *Biochem. Biophys. Res. Commun.* **232**, 414–417.

Woolf, A. B., Wexler, A., Prusky, D. N., Kobiler, E., and Lurie, S. (2000). *J. Am. Soc. Hort. Sci.* **125**, 370–376.

Zackova, M., Kramer, R., and Jezek, P. (2000). *Int. J. Biochem. Cell Biol.* **32**, 499–508.