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Energy Conservation and Dissipation in Mitochondria Isolated From Developing Tomato Fruit of Ethylene-Defective Mutants Failing Normal Ripening: The Effect of Ethephon, A Chemical Precursor of Ethylene

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Alternative oxidase (AOX) and uncoupling protein (UCP) are present simultaneously in tomato fruit mitochondria. In a previous work, it has been shown that protein expression and activity of these two energy-dissipating systems exhibit large variations during tomato fruit development and ripening on the vine. It has been suggested that AOX and UCP could be responsible for the respiration increase at the end of ripening and that the cytochrome pathway could be implicated in the climacteric respiratory burst before the onset of ripening. In this study, the use of tomato mutants that fail normal ripening because of deficiencies in ethylene perception or production as well as the treatment of one selected mutant with a chemical precursor of ethylene have revealed that the bioenergetics of tomato fruit development and ripening is under the control of this plant hormone. Indeed, the evolution pattern of bioenergetic features changes with the type of mutation and with the introduction of ethylene into an ethylene-synthesis-deficient tomato fruit mutant during its induced ripening.

KEY WORDS: Alternative oxidase; uncoupling protein; mitochondria; respiration; tomato fruit development; ethylene-defective mutants; ethylene precursor treatment.

INTRODUCTION

The plant mitochondrial respiratory chain conserves the redox energy into a proton electrochemical gradient ($\Delta\mu H^+$) built up by the proton-pumping complexes I, III, and IV. $\Delta\mu H^+$ is mainly used for ATP synthesis and ion translocation. Two energy-dissipating systems leading to a decrease in ATP synthesis efficiency exist in plant mitochondria, i.e., an alternative ubiquinol oxidase (AOX) and a plant uncoupling protein (UCP). The cyanide- and antimycin-resistant AOX oxidizes ubiquinol and reduces O_2, dissipating the free redox energy into heat as it is a non-protonmotive enzyme (no $\Delta\mu H^+$ building) (for reviews see Affourtit *et al.*, 2002; Sluse and Jarmuszkiewicz, 1998; Vanlerberghe and McIntosh, 1997). Plant UCP dissipates energy by consuming $\Delta\mu H^+$ built up by the main respiratory chain (Vercesi *et al.*, 1995) as it enables H^+ reentry into the mitochondrial matrix through a free fatty acid (FFA)-activated H^+ cycling process (Borecky *et al.*, 2001; Jezek *et al.*, 1997, 1998). Thus, plant UCP can compete with ATP synthase for the $\Delta\mu H^+$ utilization, uncoupling respiration from phosphorylation (Jarmuszkiewicz *et al.*, 2000).

Key to abbreviations: ACC, 1-aminocyclopropanecarboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; AOX, alternative oxidase; BHAM, benzohydroxamic acid; BSA, bovine serum albumin; chl *a*, chlorophyll *a*; ethephon, 2-chloroethylphosphonic acid; FFA, free fatty acids; LA, linoleic acid; *nor*, "non-ripening" mutant; *Nr*, "never ripening" mutant; *rin*, "ripening inhibitor" mutant; state 4, resting respiration in the absence of added ADP; state 3, phosphorylating respiration in the presence of added ADP; UCP, uncoupling protein; $\Delta\mu H^+$, proton electrochemical gradient.

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The obvious physiological function of UCP and AOX can be recognized in specialized plant and animal thermogenic tissues as heat generation related to an increase in temperature: in spadices of *Araceae* during reproductive processes (AOX activity) (Meeuse, 1975) and in mammal brown adipose tissue (UCP activity) (for review see Ricquier and Bouillaud, 2000). In nonthermogenic plant tissues and some unicellular microorganisms, where AOX and UCP are present together, their role is not fully understood. The two energy-dissipating systems could play a central role in the balance of cell energy metabolism related to the regulation of ATP production, control of the $NADH/NAD^+$ ratio (Sluse and Jarmuszkiewicz, 2000, 2002), and limitation of the level of mitochondrial reactive oxygen species production (Kowaltowski *et al.*, 1998; Maxwell *et al.*, 1999; Popov *et al.*, 1997). To investigate the possible metabolic role of AOX and UCP in the energy balance of the cell in nonthermogenic plant tissues, evolution of the energy-conserving (the cytochrome pathway) and energy-dissipating (AOX and UCP) pathway activities as well as AOX and UCP protein expression have been studied using tomato fruit development and ripening as a model system. The tomato fruit is a climacteric fruit, characterized by a rise in ethylene production just before the onset of ripening accompanying a rise in respiration termed a climacteric burst (Biale and Young, 1981). Thus, climacteric fruits, like tomato, are very useful for defining crucial parameters, which could participate in their development and ripening. Indeed, the evolution of metabolic processes during fruit life (from the growing stage to senescence) is interesting because these processes occur in a relatively short time period during which many changes take place in the cell. Such biological events can thus allow focusing on particular roles of one or both energy-dissipating systems at a precise moment of fruit life.

It must be pointed out that postharvest fruit ripening and ripening on the vine are two physiologically distinct processes (Biale and Young, 1981). Therefore, bioenergetic processes occurring during these two types of ripening could be quite different. Indeed in a previous study, it has been shown that during postharvest tomato fruit ripening, the expression of AOX and the AOX-sustained respiration drop after the mature green stage, whereas the UCP expression and the UCP-activity-sustained respiration decrease weakly from the orange stage (Almeida *et al.*, 1999). These observations suggest that AOX and UCP could work sequentially. More recently, it has been shown that the bioenergetic parameters of tomato fruit development on the plant from the early growing stage to senescence exhibit different evolution patterns compared to postharvest tomato fruit ripening (Almeida *et al.*, 2002). This indicates that the large differences at the level of bioenergetics take place in the physiologically distinct processes peculiar to the two types of ripening. Moreover, comparison of bioenergetic status during tomato development on the vine of wild type fruits and "non-ripening" (*nor*) mutant fruits shows different evolution in the two cultivars that may be attributed to their climacteric/nonclimacteric properties, respectively (Almeida *et al.*, in press). Studies on tomato (Almeida *et al.*, 2002; Holtzapffel *et al.*, 2002) and mango (Considine *et al.*, 2001) fruit ripening on the vine suggest that AOX and UCP could participate in processes occurring at the end of ripening and at senescence, while an overexpression of proteins of the cytochrome pathway complexes could be mainly implicated in the climacteric burst.

In this study, the evolution of several bioenergetic parameters has been investigated during the course of tomato fruit development in ethylene-defective mutant. In isolated mitochondria, the evolution of ATP-synthesis-sustained respiration, AOX-mediated respiration, and UCP-activity-sustained respiration, as well as AOX and UCP protein expression have been followed. Moreover, the total FFA content in fruit pericarp juice has been measured. In order to determine how the evolution of the bioenergetic status during tomato fruit development is under the control of ethylene, two different mutants have been studied: "never ripening" (*Nr*) (partially nonclimacteric) mutant deficient in exogenous ethylene perception and "ripening inhibitor" (*rin*) (nonclimacteric) mutant deficient in the ethylene biosynthesis pathway. Moreover, in order to mimic the surge of ethylene production before the onset of wild type ripening, the *rin* mutant fruits at the green mature stage have been treated with ethephon, a chemical precursor of ethylene.

The results described in this paper show that (i) the *Nr* and *rin* mutants differ in the evolution of the activity and expression of AOX and UCP during fruit development, and (ii) treatment with ethephon of *rin* fruits, deficient in ethylene synthesis pathway, modifies the evolution pattern of their bioenergetic parameters. The whole set of observations made with wild type tomato and three ethylene-defective mutants (*rin*, *Nr*, *nor*) indicates clearly for the first time that the bioenergetics of tomato fruit development and ripening is under the control of the plant gaseous hormone, ethylene.

MATERIAL AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* cv Pearson) seeds of *Nr* and *rin* were provided from the Tomato Genetics

Resource Center, California. Plants were grown in a greenhouse of the Botanical Institute under 60 PAR (photosynthetic active radiation) at 20°C with a photoperiod of 16 h using standard horticultural practices. The *Nr* mutant possesses a dominant mutation in the NR ethylene receptor gene located on chromosome 9 (Rick and Yoder, 1988) that results in a lower sensitivity to ethylene (Lanahan *et al.*, 1994). The *rin* mutant possesses the recessive *rin* mutation located on chromosome 5 (Rick and Yoder, 1988) that results in the incapacity to increase ethylene production at the onset of ripening (Lincoln and Fischer, 1988). As a result of these mutations, *Nr* and *rin* fruits fail to undergo normal ripening.

Ethephon Treatment

Ethephon (2-chloroethylphosphonic acid), a chemical precursor of ethylene, was used as an exogenous source of ethylene. Tomato fruits were dipped for 1 min in ethephon solution. Diluted solutions (from 70 to 5 mM) of commercial concentrated solution of ethephon (PROTEX) were used. Aqueous solutions of ethephon are stable below pH 4. Above this pH value, as is the case when entering plant tissues, ethephon decomposes to ethylene, phosphate, and chloride ion.

Pigment Content Analysis

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

Isolation of Mitochondria

Mitochondria of *Nr* and *rin* tomato fruits were isolated and purified on a selfgenerating Percoll gradient as described by Almeida *et al.* (in press). Because of variation in the density of the mitochondrial fraction during development of the mutant fruits, different concentrations of Percoll in the gradient medium were used to improve separation: for *Nr* mutant: 25% (v/v) for growing, 21% for mature and partially ripe, and 18% for senescent fruits; for *rin* mutant: 25% for growing, 21% for mature and ageing, and 28% for senescent fruits. The gradient was generated during centrifugation at 40,000 g for 30 min. The mitochondrial fraction was collected and washed twice in 250 mM sucrose, 0.3 mM EGTA, 10 mM Hepes, pH 7.2, and 1% (w/v) bovine serum albumin (BSA) and finally twice in the same buffer without BSA. The presence of 1% BSA in the medium during purification allowed the complete depletion of endogenous FFA from mitochondria. Protein concentration was determined by the biuret method (Gornall *et al.*, 1949).

To control the quality of mitochondrial preparation from each stage of fruit development of both cultivars, we applied the same selection criteria as described by Almeida *et al.* (in press).

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 KCI, 2.5 mM KH_2PO_4, 0.33 mM EGTA, 1 mM $MgCl_2$, 0.18 mM ATP (to ensure the activation of succinate dehydrogenase), 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.

Five respiratory activities were measured during tomato fruit development: (i) Total state 3 respiration was measured in the presence of 2 mM ADP, AOX activators (0.15 mM pyruvate and 1 mM dithiothreitol), and UCP inhibitors (0.5% BSA and 1 mM GTP). This respiration represents the sum of electron fluxes (not necessarily maximal) in phosphorylating conditions through the cytochrome pathway and AOX. (ii) The ATP-synthesis-sustained respiration was measured in the presence of 2 mM ADP and in the presence of inhibitors of AOX and UCP [2 mM benzohydroxamic acid (BHAM) and 0.5% BSA plus 1 mM GTP, respectively]. It represents the electron flux in phosphorylating conditions solely through the cytochrome pathway. (iii) AOX-mediated respiration was measured in state 3 (plus 2 mM ADP) in the presence of AOX activators and respective inhibitors of the cytochrome pathway (1.5 mM KCN) and UCP. (iv) The UCP-sustained respiration activated by 10 μM Linoleic acid (LA) was measured in state 4 respiration with 2.5 μg mL^{-1} oligomycin in order to inhibit ATP synthase and with 1.5 mM BHAM to inhibit AOX. This respiration represents the electron flux only through the cytochrome pathway, in conditions where UCP is activated by 10 μM LA. It reflects UCP activity (plus a H^+ endogenous leak) (Almeida *et al.*, 1999) because other carriers like the ADP/ATP, phosphate, and dicarboxylate carriers are excluded from the uncoupling process by the presence of 180 μM ATP, 2 mM inorganic phosphate, and 10 mM succinate in the incubation medium (Andreyev

et al., 1989; Sluse and Jarmuszkiewicz, 2002; Wieçkowski and Wojtczak, 1997; Zackova *et al.*, 2000). (v) The proton leak-sustained respiration was measured in state 4 respiration in the presence of oligomycin, BHAM, and BSA/GTP, and represents the electron flux through the cytochrome pathway under the high membrane potential conditions. Additional restrictions and justifications of the use of these respiratory measurements are given by Almeida *et al.* (2002).

SDS-PAGE and Immunodetection of AOX and UCP

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

FFA Analysis

Total lipids were extracted from 5 mL of tomato fruit pericarp juice in chloroform: methanol (1:1, v/v), according to Folch *et al.* (1957). Lipids and FFA were separated and analyzed as described in Almeida *et al.* (in press).

RESULTS

Impact of *Nr* and *rin* Mutations on the Tomato Fruit Development

According to macroscopic properties, the growing period (defined by increase in fruit size) and the mature stage (when fruits reach their final size and the green color becomes lighter) were similar for *Nr* and *rin* mutant fruits compared to wild type and *nor* mutant fruits (Almeida *et al.*, 2002). After the mature green stage, *Nr* and *rin* fruits underwent different changes. The color of *Nr* fruits evolved from green with orange patches to fully orange, then ligneous-looking cracks appeared from the level of the peduncle. The period from the initiation of the orange color to the fully orange fruits (between 43 and 65 days after fruit bud appearance) corresponds to a partial ripening process, as fruits did not become red and soft. This partial ripening with a lower intensity of pigmentation and softening compared to wild type fruits can be explained by the fact that although *Nr* mutant fruits are defective in the NR ethylene receptor because of a single amino acid change located in the first hydropobic domain of the protein (Bleecker and Schaller, 1996), they still possess enough other isoforms of ethylene receptor to respond partially to exogenous ethylene (Hua and Meyerowitz, 1998; Lashbrook *et al.*, 1998; Tieman and Klee, 1999; Tieman *et al.*, 2000). Thus, the transcriptional cascade under the control of exogenous ethylene (Giovannoni, 2001; Gray *et al.*, 1994; Streptanova and Ecker, 2000), leading to the expression of isoforms (inducible by exogenous ethylene) of two key enzymes [1-aminocyclopropanecarboxylic acid synthase (ACS) and 1-aminocyclopropanecarboxylic acid oxidase (ACO)] (Lelièvre *et al.*, 1997; Yang and Hoffman, 1984) responsible for the increase of intracellular ethylene synthesis at the onset of ripening, occurs in *Nr* fruits but at a level of 50% of the wild type fruits (Tigchelaar *et al.*, 1978). Carotenoid synthesis seems also to be altered in *Nr* fruits (Tighchelaar *et al.*, 1978) as they did not become red but only reached a fully orange stage mainly because of chlorophyll degradation, which allows β-carotene pigment to be visible (Piechulla *et al.*, 1987). The moment when cracks occur (around 70–80 days after fruit bud appearance) corresponds to the beginning of senescence, defined as the process that follows physiological maturity (fruits are ready for seed dispersion) and leads to death of tissue (Brady, 1987).

From the end of the mature green stage, the color of *rin* fruits evolved slowly to a light yellow-greenish (about 45–65 days after fruit bud appearance), then turned to a light yellow stage where fruits (older than 70 days) had many brown vessels. There was no ripening at all in the *rin* fruits. The period when they underwent the color change to reach a yellow-greenish color was rather an aging process followed by senescence, when brown vessels became visible. This results from the nature of *rin* mutation. The *rin* mutant seems to be blocked at the level of induction of transcription factors responsible for the expression of additional ACS isoforms (Giovannoni, 2001) implicated in the overproduction of ethylene precursor, 1-aminocyclopropanecarboxylic acid (ACC) (Yang and Hoffman, 1984). As a result, *rin* fruits can only produce

ethylene at a basal level without any rise responsible for ripening that occurs in wild type. Thus, concerning ethylene production, the *rin* mutant fruits behave like nonclimacteric fruits (Herner and Sink, 1973; Lincoln and Fischer, 1988) but *rin* fruits do not undergo ripening. Although *rin* mutant fruits are defective in ethylene overproduction, they are able to respond to ethylene as it has been shown that when exposed to exogenous ethylene they could express some genes inducible by ethylene (Gray *et al.*, 1994).

Observation only of macroscopic changes would not allow a comparative description of the whole fruit development in a continuous manner or a comparative description of the evolution of bioenergetic parameters in wild type and mutant fruits. The tomato fruit age was also not a good criterion because fruits did not develop on the plant at the same rate, resulting from competition between them in a cluster and among clusters on the plant (Beadle, 1937; Lyons and Pratt, 1963). Thus, fruits of the same age could be at different physiological stages. In order to express the course of *Nr* and *rin* tomato development, we measured the content of chlorophyll and carotenoids throughout the whole development. Changes in pigment content are biochemical data that reflect the true physiological stage of each cultivar. Therefore, taking into account the fact that fruits of both mutants evolved in a different way after the macroscopic mature green stage, the course of their development is expressed in terms of chlorophyll *a* (chl *a*) content in micromoles per milliliter of pericarp juice. All the measured parameters in this work are expressed according to this biochemical scale, thus in a continuous manner.

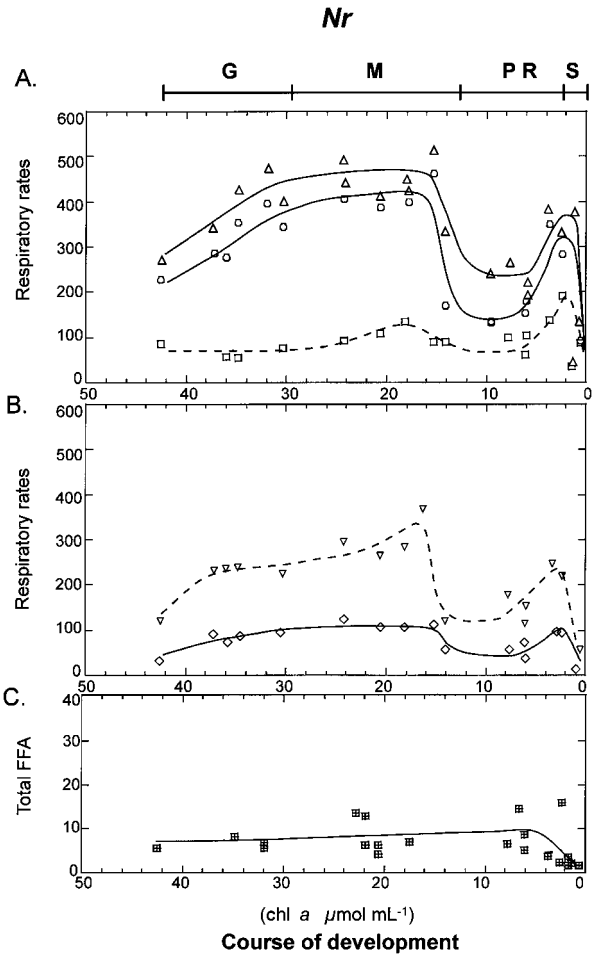

Fig. 1. Evolution of respiratory activities and FFA content in *Nr* tomato fruit mitochondria during development on the vine. Mitochondria were incubated in a standard incubation medium as described under "Material and Methods." The course of fruit development is expressed in terms of chl a content in micromol per milliliter of pericarp juice in parallel with the macroscopic stages: G, growing; M, mature green; PR, partial ripening; and S, senescence. Respiratory rates are expressed in nmol O_2 min^{-1} mg^{-1} protein. FFA content is expressed in μg mL^{-1} of juice. (A) (△), total state 3 respiration in the presence of 2 mM ADP, 0.5% BSA, 1 mM GTP, 0.15 mM pyruvate, and 1 mM dithiothreitol; (○), ATP-synthesis-sustained respiration with 2 mM ADP, 0.5% BSA, 1 mM GTP, and 2 mM BHAM; (□, dotted line), AOX-mediated respiration with 2 mM ADP, 0.5% BSA, 1 mM GTP, 0.15 mM pyruvate, and 1 mM dithiothreitol. (B) (▽, dotted line), UCP-activity-sustained respiration measured in the presence of 10 μMLA, 2 mM BHAM, 2.5 μg mL^{-1} oligomycin, 0.5% BSA, and 1 mM GTP; (◇), H^+ leak-sustained respiration with 2 mM BHAM, 2.5 μg mL^{-1} oligomycin, 0.5% BSA, and 1 mM GTP (C) (⊞), total FFA content.

Nr, the "Never Ripening" Mutant: Evolution of Energetic Status

The evolution of energy-conserving and energy-dissipating respirations in isolated mitochondria during *Nr* fruit development on the vine is shown in Fig. 1(A) and (B). Total state 3 respiration (Δ) increased during the growing period (chl *a* higher than 30 μmol mL^{-1}) and then remained roughly constant (around 450 nmol O_2 min^{-1} mg^{-1} protein) till the end of the mature stage (around 16 μmol mL^{-1} chl *a*) where it drastically decreased to around 200 nmol O_2 min^{-1} mg^{-1} protein. It remained at this low level for the most part of partial ripening, and then reincreased to reach almost 350–400 nmol O_2 min^{-1} mg^{-1} protein at the end of the partial ripening process (between 6 and 2 μmol mL^{-1} chl *a*). In the senescence stage (below 2 μmol mL^{-1} chl *a*), the total state 3 respiration exhibited a sharp decrease as previously reported for wild type tomato fruits (Almeida *et al.*, in press). Evolution of the ATP-synthesis-sustained respiration (○) closely paralleled (but 75–100 nmol O_2 min^{-1} mg^{-1} protein below) the total state 3 respiration during the whole *Nr* fruit development (Fig. 1(A)). The AOX-mediated respiration

(□) remained constant during the growing stage (around 75 nmol O_2 min^{-1} mg^{-1} protein), then started to increase to reach almost a peak value twice as high (for chl *a* around 20 μmol mL^{-1}), and then decreased to around 75 nmol O_2 min^{-1} mg^{-1} protein at the end of the mature stage. Afterwards at the end of partial ripening, the AOX-mediated respiration reincreased, reaching its maximum at 200 nmol O_2 min^{-1} mg^{-1} (for chl *a* between 4 and 2 μmol mL^{-1}) and finally dropped during senescence (Fig. 1(A)).

The UCP-activity-sustained respiration (∇) induced by 10 μM LA (Fig. 1(B)) increased (from 125 to 250 nmol O_2 min^{-1} mg^{-1} protein) during the *Nr* fruit growing stage, remained quite constant during most of the mature stage, except at the end (for chl *a* between 20 and 15 μmol mL^{-1}) where it reached more than 300 nmol O_2 min^{-1} mg^{-1} protein just before dropping to around 120 nmol O_2 min^{-1} mg^{-1} protein. Then at the end of partial ripening, it reincreased to around 250 nmol O_2 min^{-1} mg^{-1} protein and finally dropped at senescence like all measured respiratory activities. The proton leak-sustained respiration (◇) increased slightly during the growing stage (from 50 to less than 100 nmol O_2 min^{-1} mg^{-1} protein), was almost constant throughout the whole mature stage, and paralleled the evolution of the other respiratory activities during partial ripening and senescence (Fig. 1(B)).

The evolution of the AOX and UCP protein expression levels during *Nr* tomato fruit development on the vine was determined by an immunological analysis of total mitochondrial proteins (Fig. 2). Monoclonal antibodies developed against *S. guttatum* AOX revealed in all samples a single band of approximately 34 kD corresponding to the monomeric form of AOX, as all samples were treated with dithiothreitol and β-mercaptoethanol. The immunodetected level of AOX increased during the growing period (for chl *a* between 42.5 and 32 μmol mL^{-1}), reached a peak at around the middle of the mature stage (for 20.6 μmol mL^{-1} chl *a*), decreased at the end of the mature stage (for 15 μmol mL^{-1} chl *a*), and then reincreased strongly during the end of partial ripening and beginning of senescence (for 3.7 and 2.4 μmol mL^{-1} chl *a*). This profile of changes of AOX protein expression fits perfectly the evolution of the AOX-mediated respiration measured in vitro in isolated mitochondria (Fig. 1(A)).

A single band of around 32 kD was detected in all samples with antibodies developed against *A. thaliana* UCP, indicating that UCP protein was present at every stage of *Nr* fruit life. The UCP expression level remained constant till the middle of the mature stage, and then increased (at the end of the mature stage, for 15 μmol mL^{-1} chl *a*) and remained at the higher level during partial ripening until the senescence stage. The increase observed at the end of the mature stage corresponds to the peak in the UCP-sustained respiration measured in isolated mitochondria (Fig. 1(B)). contrarily, the high level of UCP expression during partial ripening and senescence seems to contradict the low UCP-sustained respiration during partial ripening. In fact, from the end of the mature stage to senescence, the UCP activity (around 100 nmol O_2 min^{-1} mg^{-1} protein) may be limited by the cytochrome pathway activity (around 100 nmol O_2 min^{-1} mg^{-1} protein) rather than by the UCP protein expression.

As has been shown for LA in isolated mitochondria of green mature tomato fruits (Sluse *et al.*, 1998), FFA can play a connecting role between the activities of

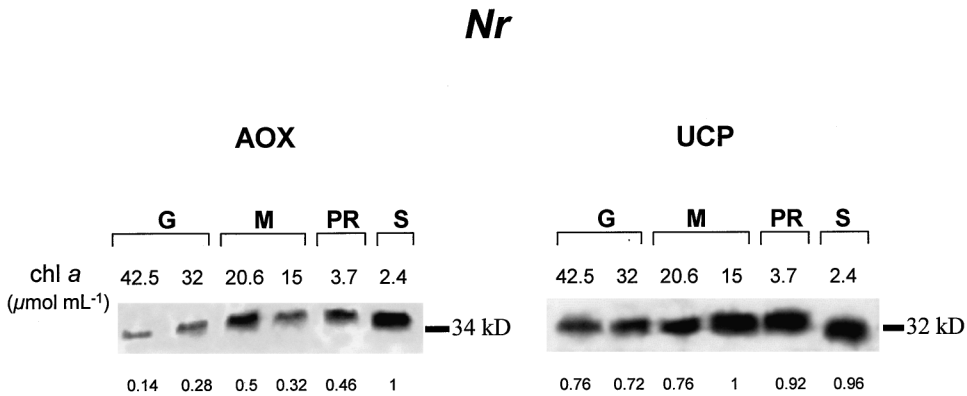

Fig. 2. Immunodetection of AOX and UCP during *Nr* tomato fruit development on the vine. Stage of fruit development is given as macroscopic stage (G, growing stage; M, mature green stage; PR, partial ripening; S, senescence) in parallel with chl *a* content. The protein bands were visualized by chemiluminescence. Densitometry of bands was made digitally using the *Scion Image* program. The highest intensity band of the blot was set to 1 and others estimated relative to that value. The relative density is given below each band.

AOX and UCP. Therefore, the total FFA content (⊞) in juice of *Nr* fruits was determined during their development (Fig. 1(C)). The amount of FFA was low compared to that observed in wild type fruits (Almeida *et al.*, in press) and did not change significantly (remaining around 10 μg mL^{-1}) throughout the whole *Nr* fruit development except the drop at senescence. This may lead to a prediction that the evolution pattern of AOX and UCP activities in vivo may be quite similar to the evolution pattern measured in vitro.

Rin, the "Ripening Inhibitor" Mutant: Evolution of Energetic Status

Figure 3 shows the evolution of energy-conserving and energy-dissipating pathways during *rin* fruit development on the vine. Total state 3 respiration (Δ) remained almost constant (around 300 nmol O_2 min^{-1} mg^{-1} protein) throughout the growing and mature stages (from 21.5 to 10 μmol mL^{-1} chl *a*), increased smoothly (till 400 nmol O_2 min^{-1} mg^{-1} protein) during the aging stage (from 9.5 to 4 μmol mL^{-1} chl *a*), and then dropped abruptly at senescence (for chl *a* below 4 μmol mL^{-1}) (Fig. 3(A)). The ATP-synthesis-sustained respiration (○) starting at around 250 nmol O_2 min^{-1} mg^{-1} protein during the growing stage decreased to around 200 nmol O_2 min^{-1} mg^{-1} protein during the mature period and then paralleled the total state 3 respiration during fruit aging and senescence (i.e., slightly increasing till around 350 nmol O_2 min^{-1} mg^{-1} protein during aging then dropping at senescence). The AOX-mediated respiration (□) increased progressively from the growing stage (around 50 nmol O_2 min^{-1} mg^{-1} protein) to the end of the aging stage, when it reached its maximum at around 200 nmol O_2 min^{-1} mg^{-1} protein, and then it decreased at senescence.

The UCP-activity-sustained respiration induced by 10 μM LA (∇, Fig. 3(B)) paralleled the ATP-synthesis-sustained respiration during the whole *rin* fruit development and seems to be limited by the cytochrome pathway activity except at the end of the aging period (when remained below 300 nmol O_2 min^{-1} mg^{-1} protein). The proton leak-sustained respiration (◇) was almost constant (around 75 nmol O_2 min^{-1} mg^{-1} protein).

Figure 4 (left part) shows an immunological analysis of the evolution of AOX and UCP protein levels during *rin* fruit development on the vine. The level of AOX protein increased progressively to peak at the end of the aging process (for 4.3 μmol mL^{-1} chl *a*) and then decreased during *rin* fruit senescence. This evolution pattern of AOX protein expression fits the AOX activity determined in isolated mitochondria (Fig. 3A). The level of UCP protein was also maximum at the end of the aging stage (for 4.3 μmol

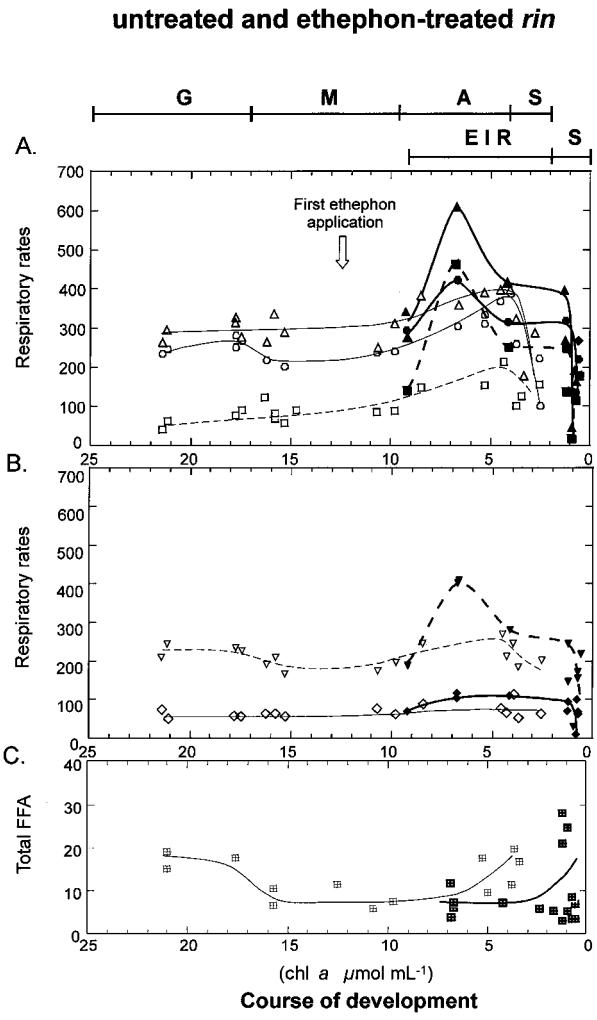

Fig. 3. Evolution of respiratory activities and FFA content in *rin* tomato fruit mitochondria during development on the vine. Effect of ethephon treatment on the evolution of these parameters. The course of fruit development is expressed in terms of chl *a* content in micromoles per milliliter of pericarp juice in parallel with the macroscopic stages: G, growing; M, mature green; A, ageing; EIR, ethylene-induced ripening; S, senescence. Respiratory rates are expressed in nmol O_2 min^{-1} mg^{-1} protein. FFA content is expressed in μg mL^{-1}. Conditions and symbols as in Fig. 1. Bold symbols and lines deal with ethephone-treated *rin*. (A) (△, ▲), total state 3 respiration; (○, ●), ATP synthesis-sustained respiration; (□, ■ dotted lines), AOX-mediated respiration. (B) (∇, ▼, dotted lines), UCP-activity-sustained respiration; (◇, ◆), H^+ leak-sustained respiration. (C) (⊞), total FFA content.

mL^{-1} chl *a*), then decreased a little in senescent fruits (Fig. 4, left part). In *rin* fruit mitochondria, the profile of UCP-expression evolution fits perfectly the profile of UCP- activity-sustained respiration evolution observed in vitro (Fig. 3(B)).

The total content of FFA in *rin* fruit juice during development (⊞, Fig. 3(C)) was at a level of around

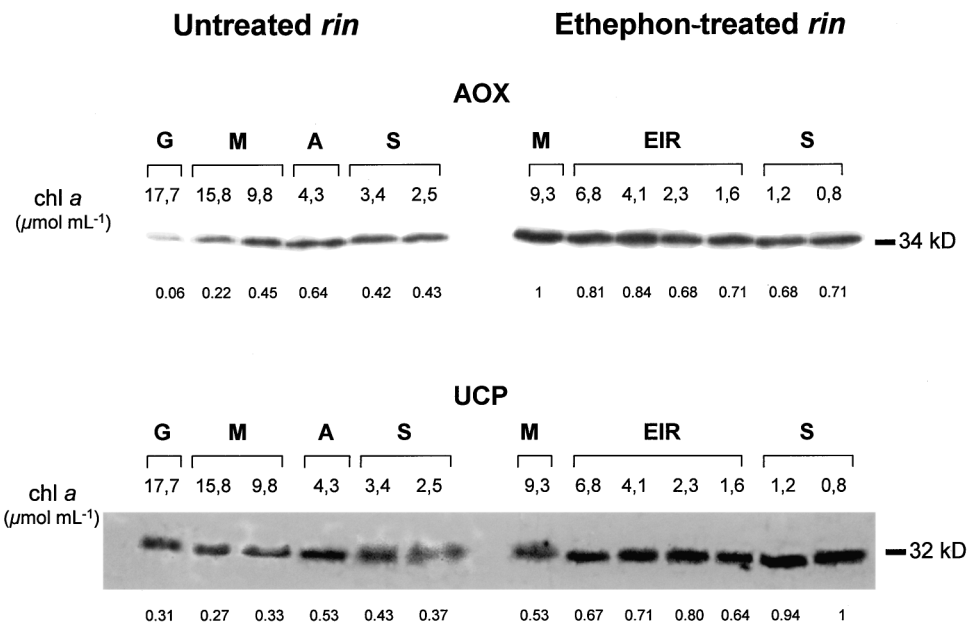

Fig. 4. Immunodetection of AOX and UCP during untreated and ethephon-treated *rin* tomato fruit development on the vine. Stage of fruit development is given as macroscopic stage (G, growing stage; M, mature green stage; A, partial ripening; S, senescence; EIR, ethephon-induced ripening) in parallel with chl *a* content. Untreated and ethephon-treated *rin* samples were electrotransferred on a single membrane to allow comparison between them. The highest intensity band of the blot was set to 1 and others estimated relative to that value.

20 μg mL^{-1} during the growing period, decreased to less than 10 μg mL^{-1} for the mature stage and half of the aging stage (from 16 to 8 μmol mL^{-1} chl *a*), and then rose to reach its initial value at the end of *rin* fruit life. These variations in the FFA content could influence the in vivo activities of two energy-dissipating systems, UCP and AOX.

The Effect of Ethephon on Evolution of Bioenergetic Parameters in *rin* Mutant Fruits

As described above, during fruit development on the vine, the evolution of bioenergetic parameters was different for *Nr* and *rin* mutants, as well as for wild type and *nor* mutant as described previously (Almeida *et al.*, 2002). The tested mutant cultivars are defective in ethylene perception (*Nr*) or in the climacteric ethylene overproduction (*rin* and *nor*). They were used as tools to focus on a possible implication of ethylene in controlling the evolution of bioenergetic parameters during tomato fruit development. The results described so far seem to indicate that the gaseous plant hormone, ethylene, may play a key role by controlling some of the bioenergetic parameters.

To further investigate and confirm this role of ethylene, we studied the effect of ethephon, a chemical precursor of ethylene, on the evolution of bioenergetic parameters during development of *rin* tomato fruits. In this way, it could be possible to observe if treatment with ethephon brings the *rin* mutant nonclimacteric fruits closer to wild type climacteric fruits. The *rin* mutant was the most interesting cultivar to use because (i) *rin* fruits presented the most abnormal development as they exhibited less macroscopic changes after the green mature stage compared to *Nr* or *nor* mutants; (ii) *rin* mutants are defective in the ethylene precursor overproduction; (iii) it has been reported previously that treatment of *rin* fruits with ethephon induces ripening (Buescher, 1977; Mizrahi *et al.*, 1975).

To optimize the protocol of treatment of *rin* fruits with ethephon, we checked four conditions differing in concentration of ethephon and frequency of its application (Table I). In all conditions, the first ethephon application was made in the middle of the mature stage that corresponds to the onset of climacteric respiratory burst in wild type tomato (Almeida *et al.*, 2002). Treatment n° 1 with 70 mM ethephon was not successful in inducing full ripening of *rin* fruits contrarily to Mizrahi *et al.* (1975). The maximal obtained macroscopic stage of fruits was orange not red. Thus, even if some lycopene content was measured (around 2.1 μmol mL^{-1}) this treatment did not lead to a wild-type-like ripening. Moreover, the applied concentration of ethephon (70 mM) seems to be lethal for

Table I. Different Treatments of *rin* Fruits with Ethephon Tested to Optimize the Treatment Protocol

Treatment N°	Number of ethephon applications	Ethephon concentration (mM)	Fruit age at the beginning of the treatment and frequency of applications
1	1	70	Started with 25-day-old fruits. Application only once.
2	4	50	Started with 20-day-old fruits. Application every 2 days, stopped when fruits were 26 days old.
3	2	50	Started with 24-day-old fruits. Application every 2 days, last application (5 mM) when fruits were 32 days old.
	2	10	
	1	5	
4	till 10	50	Started with 25-day-old fruits. Application every 2 days.

Treatment n° 1 was based on Mizrahi *et al.* protocol (1975), the other on Buescher (1977) protocol.

fruits and the entire plant, as burning-like spots appeared. Treatments n° 2 and n° 3 (details in Table I) appeared worse than the first one. They did not induce a strong macroscopic change of *rin* fruits (only a yellow-orange color was reached) and no lycopene was detected. However, 50 mM ethephon did not induce damage in fruits and whole plants like 70 mM solution. Fruits submitted to treatment n° 4 underwent an induced ripening leading to light red fruits without any damage due to the applied ethephon concentration (50 mM) or frequency of ethephon applications. Lycopene concentration in these fruits reached a concentration of 11.3 μmol mL^{-1}, that remained around four times less than in wild type red fruits. Nevertheless, treatment n° 4 gave the best results in the induction of *rin* fruit ripening by ethephon. Therefore, all measurements of bioenergetic parameters were performed on *rin* fruits treated according to this treatment and the course of development of these fruits was expressed in terms of chl *a* content degradation in parallel with the description of the macroscopic stage.

The evolution profiles of respiratory activities in mitochondria isolated from *rin* fruits treated with 50 mM ethephon are shown in Fig. 3 (bold symbols and lines). In *rin* fruits, during the ethephon-induced ripening (for chl *a* between 9 and 1.5 μmol mL^{-1}), the total state 3 respiration (▲) peaked at 600 nmol O_2 min^{-1} mg^{-1} protein at 7 μmol mL^{-1} chl *a* (instead of at 400 nmol O_2 min^{-1} mg^{-1} protein at 4 μmol mL^{-1} chl *a* in untreated *rin* fruits) and dropped at senescence at chl *a* concentration below 1.5 μmol mL^{-1} (instead of at 4 μmol mL^{-1} chl *a* in untreated fruits). The ATP-synthesis-sustained respiration (●) peaked at 400 nmol O_2 min^{-1} mg^{-1} protein at 7 μmol mL^{-1} chl *a* (instead of at 350 nmol O_2 min^{-1} mg^{-1} protein at 4 μmol mL^{-1} chl *a* in untreated fruits) and also dropped for chl *a* concentration below 1.5 μmol mL^{-1}. The AOX-mediated respiration (■) peaked at around 450 nmol O_2 min^{-1} mg^{-1} protein at 7 μmol mL^{-1} chl *a* (instead of at 200 nmol O_2 min^{-1} mg^{-1} protein at 4 μmol mL^{-1} chl *a* in untreated fruits). Therefore, increased activity of AOX seems to be the main reason of increase in total state 3 respiration observed during ethephon-induced ripening. Evolution of the UCP-sustained respiration (▼) paralleled the evolution of the ATP-synthesis-sustained respiration. The proton leak-sustained respiration (♦) was roughly constant during ethephon-induced ripening. Therefore, evolution profiles of the measured bioenergetic parameters were different for mitochondria isolated from untreated and ethephon-treated *rin* fruits (Fig. 3), indicating that intracellular ethylene is an important regulator of the bioenergetic status during tomato fruit ripening.

As shown in Fig. 4 (right part), 2 days after the first ethephon application at the end of the mature stage (for 9.3 μmol mL^{-1} chl *a*), the immunodetected level of AOX protein was twice higher than that observed for nontreated *rin* fruits at a similar chl *a* concentration (9.8 μmol mL^{-1}). This means that the AOX protein expression was quickly stimulated by the ethephon treatment. Then, the amount of AOX protein decreased a little but remained higher when compared to the nontreated *rin* fruits during the whole ethphon-induced ripening period and senescence. Similarly, expression of the UCP protein was also rapidly stimulated by ethephon, as it increased by almost 1.5 times after 2 days of ethephon treatment (for 9.3 μmol mL^{-1} chl *a*). Moreover, the UCP expression remained upregulated throughout ethephon-induced ripening and senescence, reaching the highest level at the end of fruit life. Comparison of the expression level of the AOX and UCP proteins in untreated and ethephon-treated *rin* fruits reinforces the proposal of a regulatory role of intracellular ethylene at the bioenergetic level of ripening fruit mitochondria.

The fast upregulation of AOX expression after ethephon treatment clearly fits the large increase of the AOX-mediated respiration at the beginning of ethephon-induced ripening (Figs. 3 and 4). However, the peak of overexpression looks to precede the peak of activity. A slow decrease in both, AOX activity and protein level, was observed during the ethephon-induced ripening. The immediate and progressive overexpression of UCP during the whole ethephon treatment did not lead to a corresponding in vitro increase in the UCP-sustained respiration. The UCP-sustained respiration increased during

ethephon-induced ripening but it was limited by the cytochrome pathway activity, i.e., the ATP-synthesis-sustained respiration activity.

Different evolution of FFA content during the wild type and *nor* mutant fruit development on the vine observed previously seems to be linked to the climacteric/nonclimacteric properties of these cultivars (Almeida *et al.*, in press). Treating nonclimacteric *rin* fruits with ethephon, it could be expected to observe an FFA profile closely related to that of wild type fruits, i.e., an increase in FFA content during ripening process. However, as shown in Fig. 3(C), the FFA content evolution measured in *rin* fruits after ethephon treatment was not significantly different from that of nontreated fruits but with a shift to the right in the chl *a* scale (to lower content of the pigment) as it was also observed for every respiration in vitro (Fig. 3(A) and (B)).

DISCUSSION

In this study we analyzed the impact of ethylene deficiency and the effect of treatment with an exogenous ethylene source (ethephon) on the bioenergetic parameter evolution during development of tomato fruits of ethylene-defective mutants. For this purpose, in isolated mitochondria we have followed the respiration sustained by ATP synthesis, AOX, cytochrome pathway, and UCP, as well as the protein expression of UCP and AOX. Changes in total concentration of FFA (activators of UCP and inhibitors of AOX) in pericarp juice throughout the fruit development have also been measured. Studies with two, *Nr* and *rin*, tomato mutants were designed to enlighten the relationship between the ethylene deficiency (deficiency in hormone perception or synthesis, respectively) and the measured bioenergetic parameters, on one side, and the possible role of ethylene as a regulator of protein expression and activity of energy-dissipating and energy-conserving systems in mitochondria, on the other side.

As described previously (Almeida *et al.*, in press), the development of wild type tomato fruits is characterized by (i) a strong increase in the AOX activity and protein expression during ripening with a peak at the end of this process, followed by a drop in senescence; (ii) a strong increase in the ATP-synthesis-sustained respiration (cytochrome-pathway-dependent) corresponding to the climacteric burst (at the second half of the mature green stage) and linked to the intracellular ethylene synthesis rise under the control of exogenous ethylene binding on its receptors; (iii) a constancy of the UCP activity in vitro even if its expression increases from the end of ripening till the early senescence stage; (iv) large variations in FFA content, high during growing (up to 50 μg mL^{-1}), very low during the mature stage (10 μg mL^{-1}), and re-increasing during ripening (20 μg mL^{-1}).

The partially nonclimacteric *Nr* mutant fruits, described in this study, partially lost their perception of exogenous ethylene and exhibited an abortive ripening. The following bioenergetic features were observed: (i) two rises of AOX activity and protein expression, the first one (not present in wild type) at about the level of the wild type climacteric burst (second half of the mature stage) and the second rise at the end of partial ripening process, corresponding to the peak at the end of wild type ripening; (ii) two periods of high ATP synthesis-sustained respiration (separated by a drastic decrease) at the mature stage and at the end of partial ripening, with the first plateau covering widely the wild type climacteric period; (iii) two peaks in the UCP activity (instead of constancy observed in wild type), first at the end of the mature green stage and second at the end of partial ripening. As the UCP expression remained constant after reaching the highest level at the end of the mature stage, variation of the UCP activity after this stage looks to be strictly limited by the cytochrome pathway activity; (iv) a low and quite constant total FFA content, contrarily to the large variations observed in wild type. Thus, partial loss of exogenous ethylene sensibility, which normally triggers off the intracellular ethylene synthesis, had dramatic effects on the expression and activity of proteins implicated in energy conservation and energy dissipation during the tomato fruit development run. The pleiotropic effects of the *Nr* mutation also seem to alter fatty acid metabolism, mostly during early fruit development, as the FFA level was low during growing in *Nr* fruits compared to wild type.

The nonclimacteric *rin* mutant, which is defective in the ethylene biosynthesis pathway, did not ripen at all and presented the following peculiar bioenergetic properties: (i) AOX activity and protein expression increased progressively and culminated in aging stage. This culmination could correspond to a high expression and activity of AOX in wild type at the end of ripening and at early senescence. (ii) The ATP-synthesis-sustained respiration appears to be depressed during the mature stage and higher during the aging process, contrarily to wild type where it revealed the respiratory burst during the mature stage. (iii) The UCP expression culminated at the end of the aging period as in UCP-sustained respiration, which nevertheless could be limited by the cytochrome pathway activity before aging. The increase in UCP expression in *rin* resembles the increase observed in wild type till senescence stage. On the contrary, the wild type UCP activity was constant. (iv) The total FFA content was twice as low in the mature stage compared to the growing, aging, and

senescence stages. This mimics the evolution of FFA content in wild type except that the level of FFA during the growing stage was much higher in wild type. Thus in *rin* mutant, the loss of ability to overproduce ethylene precursor and thereby ethylene which triggers off the onset of ripening has also important effects on the evolution of expression and activity of proteins implicated in energy transduction during fruit development.

Although the *rin* mutant fruits must be considered, like the *nor* mutant fruits, as nonclimacteric because they only produce ethylene at a basal level during the whole fruit life, the behavior of both cultivars at the level of mitochondrial bioenergetics is different. Indeed, the *nor* mutant, which is defective in the ethylene biosynthesis pathway at the level of ACC oxidase isoforms inducible by exogenous ethylene and responsible for conversion of the overproduced ethylene precursor into ethylene, exhibits more drastic differences than *rin* mutants when compared to wild type (Almeida *et al.*, in press). The evolution of bioenergetic features during *nor* mutant fruit development is characterized by constant AOX activity, smooth decline of all other respirations, almost no change in the AOX expression, a little decline in the UCP expression, and low and constant FFA level. As in the *Nr* mutant, the pleiotropic effects of *rin* as well as *nor* mutations seem to deeply disturb the fatty acid metabolism especially during the early fruit development leading to a low FFA level. The low FFA content could have in vivo an important effect on the AOX and UCP activities, leading to low or no inhibition of AOX and low activation of UCP. Moreover, analysis of these results indicates that the loss (*rin* and *nor*) or the decrease (*Nr*) of intracellular ethylene overproduction ability, a common feature of the three mutants, has important but various effects on the expression and activity of AOX, UCP, and cytochrome pathway.

The use of ethephon to treat *rin* fruits at the mature green stage during tomato development on the vine allows an increase in intracellular ethylene level, thereby mimicking the "autocatalytic" intracellular ethylene overproduction occurring in wild type. This is an ultimate tool to demonstrate the key role of ethylene in the control of energetic status during tomato fruit development. Rin fruits treated with ethephon showed important and fast upregulations. An extensive rise in the AOX-mediated respiration during the ethylene-induced ripening fitting the rapid change in the AOX protein expression was observed. The AOX activity was multiplied at least by 2 and reached the same value as the peak value observed during wild type ripening (Almeida *et al.*, in press). Moreover, the ATP-synthesis-sustained respiration increased by 15% during the ethephon-induced ripening and reached the rates observed during wild type climacteric period (around 400 nmol O_2 min^{-1} mg^{-1} protein). As a consequence, the total state 3 respiration (around 600 nmol O_2 min^{-1} mg^{-1} protein) exceeded during ethephon-induced ripening the highest value reached in wild type during the climacteric burst and ripening process (450 nmol O_2 min^{-1} mg^{-1} protein). Both UCP activity and expression increased during ethephon-induced ripening. The UCP-sustained respiration being limited by the cytochrome pathway activity paralleled the ATP-synthesis-sustained respiration, and the UCP expression exceeded the highest level observed for untreated fruits and peaked at senescence. Compared to wild type fruits, during ethephon-induced development, the UCP-sustained respiration exceeded the stable value observed throughout wild type development which was limited by the cytochrome pathway activity (Almeida *et al.*, in press). The FFA content did not increase during the ethephon-induced ripening, in contrast to the FFA increase observed during wild type ripening. For ethephon-treated *rin* fruits, the general profile of changes of FFA content and the relative values were similar to those of untreated fruits. Only a shift of the curve to the lower chl *a* concentrations was observed because the senescence process was delayed compared to untreated *rin* fruits because of the induction of the partial ripening process.

Careful analysis of the events during ethephon-induced *rin* ripening reveals that the partial ripening observed in treated fruits was accompanied by mixed simultaneous changes in bioenergetic parameters: (i) an increase in AOX activity and expression characteristic of wild type ripening and an increase in the cytochrome pathway activity characteristic of wild type climacteric burst; (ii) an increase in the UCP expression (as in wild type ripening) which was translated in an increase in the UCP-sustained respiration (not observed in wild type) owing to increase in the cytochrome pathway activity; (iii) no change in the FFA level in contrast with wild type ripening. Therefore, it can be concluded that ethephon treatment of *rin* fruits is responsible for an overlapping of events characteristic of wild type climacteric burst and ripening, leading to excesses in some activities (in the total state 3 respiration and in the UCP-sustained respiration) and protein expression (UCP, AOX). This response to ethylene, introduced into fruits by ethephon treatment, could be due to the fact that at the moment of ethephon application, mature green *rin* fruits were different from those of wild type because of the pleiotropic nature of the mutation. Finally, the overall analysis of the results demonstrates nonambiguously for the first time that ethylene, the gaseous plant hormone, controls the bioenergetics of tomato fruit during its development.

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