The mitochondrial elongation factor LeEF-Ts$_{mt}$ is regulated during tomato fruit ripening and upon wounding and ethylene treatment

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Abstract

A gene encoding an elongation factor LeEF-Ts$_{mt}$ that participates in the protein synthesis process in mitochondria shows strong expression in ripening fruit as compared to other organs. It is strongly up-regulated during the first stages of the ripening process in parallel with the climacteric rise in respiration. *LeEF-Ts$_{mt}$* expression is stimulated by ethylene, wounding and high temperature but ethylene-insensitive mutants exhibit normal expression. Transgenic fruit have been generated in which *LeEF-Ts$_{mt}$* has been constitutively up- and down-regulated. Surprisingly, altering the expression of the gene by genetic transformation with antisense and sense *LeEF-Ts$_{mt}$* constructs did not affect the pattern of respiration and ethylene production during ripening and upon wounding. In addition, expression of the alternative oxidase gene which is known to play an important role in respiratory climacteric was not affected. Possible reasons for the absence of effect on respiration of variations of *LeEF-Ts$_{mt}$* gene expression are discussed.

Keywords: Mitochondrial elongation factor; *Lycopersicon esculentum*; Ethylene; Respiration; Transgenic plants; Wounding

1. Introduction

In climacteric fruit, including tomato, ripening is closely associated with an increase in respiration and the plant hormone ethylene stimulates both processes (Abeles et al., 1992). The ethylene-induced respiration has been related to the stimulation of the synthesis of nuclear-encoded proteins that are targeted to the mitochondria such as an alternative oxidase and uncoupling protein (Cruz-Hernandez and Gomez-Lim, 1995; Considine et al., 2001). Data of the screening of genes expressed during tomato ripening using cDNA microarrays (Alba et al., 2005) show that four genes putatively encoding mitochondrial proteins are up-regulated by ethylene: the 7 kDa subunit of a translocase of outer membrane (accession number: SGN-U147420), an NAD aldehyde dehydrogenase (SGN-U149764), a proto-porphyrinogen IX oxidase isozyme (SGN-U147910) and a serine hydroxymethyltransferase (SGN-U148661). Since mitochondria are neither a site for ethylene synthesis (Diolez et al., 1986) nor for ethylene perception (Chen et al., 2002), stimulation of respiration appeared to be related to imported proteins only and not to the stimulation of its endogenous machinery. However, Zegzouti et al. (1999) showed that treating mature green tomato fruit by ethylene resulted in a transient enhanced accumulation of a transcript named ER49 encoded by a nuclear gene later denominated *LeEF-Ts$_{mt}$* and corresponding to a mitochondrial elongation factor involved in protein synthesis in the mitochondria (Benichou et al., 2003). EF-Ts are nucleotide exchange factors that promote the exchange of GDP for GTP with elongation factors of the EF-Tu type (Merrick, 1992). The elongation steps of mitochondrial translation are well documented in mammals and prokaryotes but not in plants. We have recently carried out a study aimed at assessing the function of the LeEF-Ts$_{mt}$ protein. It was demonstrated that the protein was targeted to the mitochondria rather than to the chloroplasts. Biochemical studies with an *E. coli* recombinant protein further

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demonstrated that LeEF-Ts_{mt} was capable of stimulating the poly(U)-directed polymerisation of phenylalanine and to catalyse the nucleotide exchange reaction with EF-Tu (Benichou et al., 2003).

In order to further investigate the role of *LeEF-Ts$_{mt}$*, we have studied the spatio-temporal expression of the gene in different organs of tomato plants with special attention to the ripening fruit and to the effect of ethylene and abiotic stress conditions. Tomato plants in which the *LeEF-Ts$_{mt}$* gene has been over-expressed and down-regulated have been generated. Biochemical and physiological studies have shown that the level of LeEF-Ts_{mt} RNA has no influence on fruit respiratory activity.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum* Mill. cv Micro-Tom and Ailsa Craig) plants were grown in pots in a culture chamber. The conditions were as follows: 14-h day:10-h night cycle, 25 °C day:20 °C night temperature, 70% humidity, 250 μmol m^{-2} s^{-1} light intensity. Seeds were surface-sterilised in 50% bleach solution for 10 min, thoroughly rinsed with sterile distilled water, and were then sown in pots filled with a peat substrate (Klasmann-Deilmann, R.H.P. 15).

2.2. Ethylene and MCP treatment, wounding and temperature shocks

Ethylene treatments were performed for 15 min and 5 h in 25 l sealed glass boxes. Tomato fruit at the "mature green" (MG) stage were treated with 50 μl l^{-1} ethylene and control fruit were exposed to air alone. For the wounding treatment, fruit were wounded by inserting 30 times a 0.6 mm × 23 mm needle through the fruit pericarp. The ethylene inhibitor 1-MCP was applied to detached MG fruit in 3-l jars at 1 μl l^{-1} during 16 h at room temperature. For the temperature treatments, three sets of fruit at the "breaker" (Br) stage were placed in a dark chamber at 4 °C for 6 days, at 30 °C for 2 h and at 4 °C for 6 days followed by 2 h at 30 °C. After treatments, tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.3. Phylogenetic tree

Phylogenetic analysis of EF-Ts sequences was performed using the neighbour-joining method (Saitou and Nei, 1987) of PHYLIP (Felsenstein, 1992). Bootstrapping was performed by re-sampling from the data 100 times. The tree was drawn by TreeView (Page, 1996). The sequences of the *EF-Ts* genes used in this study were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/) and SGN (http://sgn.cornell.edu) databases. The predicted amino acid sequences were aligned using CLUSTAL X (Jeanmougin et al., 1998) based on the GONNET protein weight matrix.

2.4. RNA isolation, RT-PCR and real-time PCR

Total RNA was extracted as described in Jones et al. (2002). RT-PCR was performed as described previously (Zegzouti et al., 1999). Forward (F) and reverse (R) primers used for RT-PCR amplification of the target genes in each RNA sample are the following: for *LeAOX* (F 5′-AGCTGAAAACGAGAGGATGC-3′ and R 5′-AAGTGGTGCTGGTGAGTCC-3′), and for *LeUbi3* (F 5′-AGAAGAAGACCTACACCAAGCC-3′ and R 5′-TCCCAAGGGTTGTCACATACATC-3′).

For real-time PCR, DNase-treated RNA (2 μg) was reverse transcribed in a total volume of 20 μl using Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA) and then PCR was performed using 2 ng of total RNA in a 10 μl reaction volume using SYBR GREEN PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence-detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers: for *LeEF-Ts$_{mt}$* (F 5′-CAGCCGAGATTTCCTCTTCTGA-3′, R 5′-TGCTAGTGACAAGAGCAGCTTTG-3′ and 3′UTR F 5′-TGTCAGGATCGTGAGAGAAACTACCT-3′ and R 5′ CACGTTCAGAAGTGCCCACCA for AS RNA) and for *LeActin* (F 5′-TGTCCCTATTTACGAGGGTTATGC-3′ and R 5′-CAGTTAAATCACGACCAGCAAGAT-3′). Optimal primer concentration was 50 nM. RT-PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and one cycle 95 °C for 15 s and 60° for 15 s. Samples were run in triplicate on a 384-well plate. For each sample, a C_t (threshold cycle) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the β-actin as an internal standard.

2.5. CO_2 and ethylene production measurements

Individual fruit of the Micro-Tom variety were placed in sealed 120 ml-flasks for 1 h (entire fruit) or 20 min (wounded fruit) at 25 °C. Gas samples (1 ml) were withdrawn from the headspace for evaluation of $C0_2$ by infrared gas analyzer (LI-COR model LI 820, Lincoln, Nebraska) and ethylene by gas chromatography (Intersmat IGC 120 FB). Following measurements, flasks containing the fruit were fully ventilated. Results were expressed as mmoles CO_2 kg^{-1} h^{-1} and μmoles ethylene kg^{-1} h^{-1}.

2.6. Generation of sense and antisense LeEF-Ts$_{mt}$ plants

The coding region of the *LeEF-Ts$_{mt}$* gene was obtained by PCR using Vent polymerase (New England Biolabs) and the following primers: forward 5′-CCTCCTGATTAGCATCA-CAGATC-3′ and reverse 5′-ATCAAGCAGCATTAGCC-

AAAGG-3′. This fragment was then cloned into pGA643 binary vector either in antisense or sense orientation under the transcriptional control of the cauliflower mosaic virus *35S* promoter and the *Nos* terminator. Transgenic plants were generated by *Agrobacterium*-mediated transformation according to Jones et al. (2002) and transformed lines were first selected on kanamycin (70 mg l^{-1}) and then analyzed by PCR to check the presence of the T-DNA insertions. Homozygous plants of the R2 and further generations were used for subsequent physiological experiments.

3. Results

3.1. Tomato LeEF-Ts$_{mt}$ is encoded by a single copy gene

Lycopersicon esculentum unigenes BLASTn similarity search (http://sgn.cornell.edu) of the mitochondrial LeEF-Ts$_{mt}$ nucleotidic sequence gave no other similar sequence than LeEF-Ts$_{mt}$ itself. In addition, tBLASTn showed that the chloroplastic EF-Ts$_{cp}$ was also a unigene. *Arabidopsis thaliana* genome analysis and database search also identified AtEF-Ts$_{mt}$ and AtEF-Ts$_{cp}$ as single-copy genes. These results show that nucleotidic sequences of mitochondrial and chloroplastic EF-Ts are highly divergent and strongly suggest that as for *A. thaliana*, the corresponding genes of tomato are unique moreover, the phylogenetic relationship of EF-Ts predicted amino acid sequences from prokaryotic and eukaryotic organelles indicates that mitochondrial EF-Ts are more related to bacterial genomes (Fig. 1) and are highly divergent from their chloroplastic homologs.

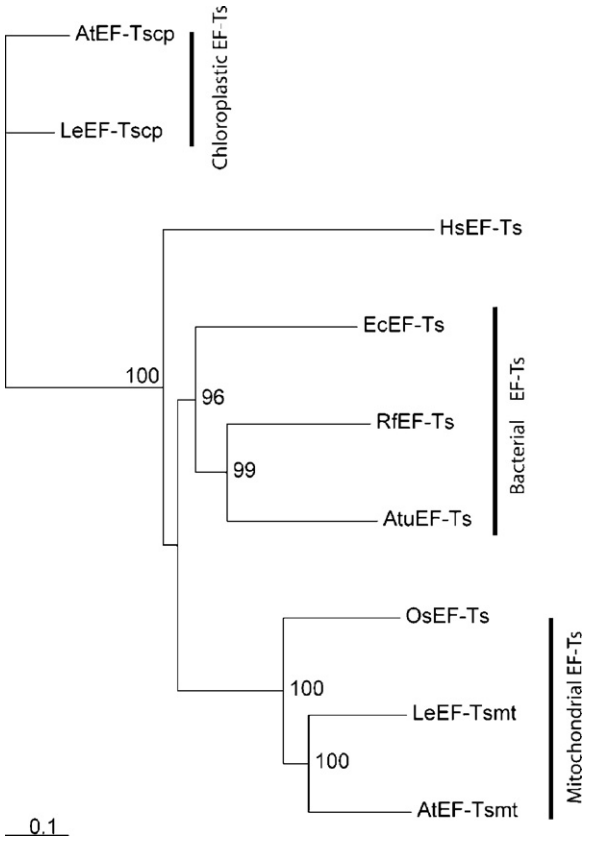

Fig. 1. Consensus neighbour-joining tree based on EF-Ts sequences (bootstrapped 100 times) showing the relationship between various prokaryotic and eukaryotic organelles homologs. Accession numbers of sequences and corresponding databases are: *Lycopersicum esculentum* LeEF-Ts$_{mt}$ (GenBank, AF096247_1) and LeEF-Ts$_{cp}$ (SGN, SGN-U218510); *Arabidopsis thaliana* AtEF-Ts$_{mt}$ (GenBank, NP_192850) and chloroplastic AtEF-Ts$_{cp}$ (GenBank, NP_567820); *Oryza sativa* mitochondrial OsEF-Ts (GenBank, XP_482256.1); *Rickettsia felis* RfEF-Ts (GenBank, AAY60921.1); *Agrobacterium tumefaciens* AtuEF-Ts (GenBank, AAK87167.1); *Escherichia coli* EcEF-Ts (GenBank, BAB96746.1) and *Homo sapiens* mitochondrial HsEF-Ts (GenBank, NP_005717.2).

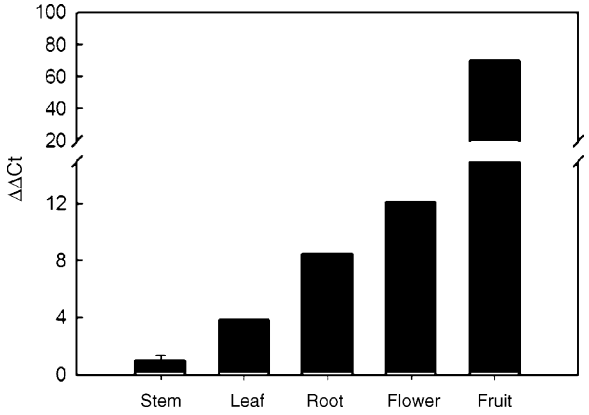

Fig. 2. Levels of *LeEF-Ts$_{mt}$* transcripts in various organs of tomato of the Ailsa Craig variety assessed by real-time PCR. The *X*-axis represents various organs of tomato stem, leaf, root, flower and fruit at Tu (turning stage). $\Delta\Delta C_t$ in the *Y*-axis refers to the fold difference in *LeEF-Ts$_{mt}$* expression relative to stems. Values are the mean of three replicates ±S.E.

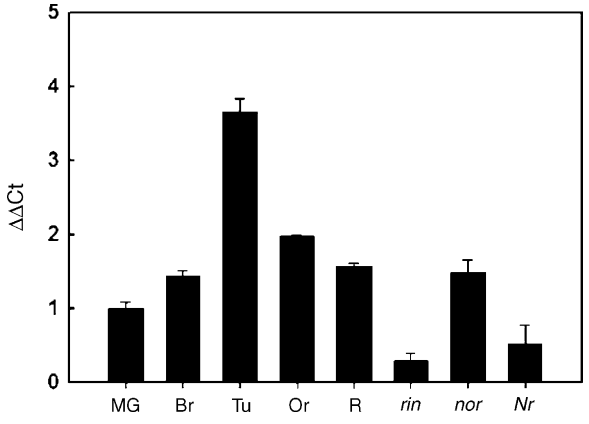

Fig. 3. Levels of *LeEF-Ts$_{mt}$* transcripts during ripening of Ailsa Craig tomatoes and in ripening mutants (*rin*, *nor* and *Nr*) assessed by real-time PCR. The *X*-axis represents various stages of fruit ripening (MG, mature-green; Br, breaker; Tu, turning; Or, orange and R, red) and ripening mutants collected 70 days after anthesis. $\Delta\Delta C_t$ in the *Y*-axis refers to the fold difference in *LeEF-Ts$_{mt}$* expression relative to MG stage. Values are the mean of three replicates ±S.E.

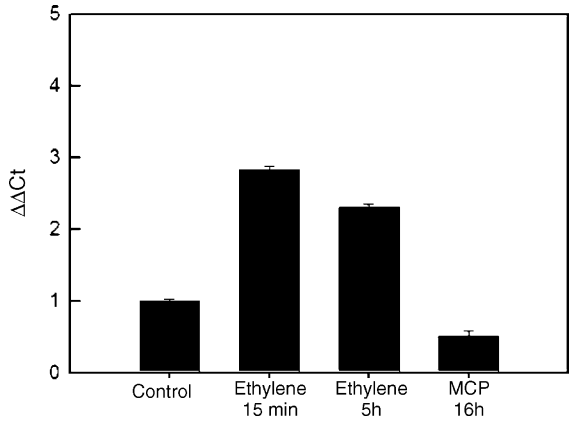

Fig. 4. Effects of treating Ailsa Craig MG fruit with ethylene and 1-MCP on the levels of *LeEF-Ts$_{mt}$* transcripts assessed by real-time PCR. The *X*-axis represents control fruit, fruit treated for 15 min and 5 h with 50 $\mu l l^{-1}$ ethylene and fruit treated with 1 $\mu l l^{-1}$ 1-MCP for 16 h. $\Delta\Delta C_t$ in the *Y*-axis refers to the fold difference in *LeEF-Ts$_{mt}$* expression relative to untreated control fruit. Values are the mean of three replicates ±S.E.

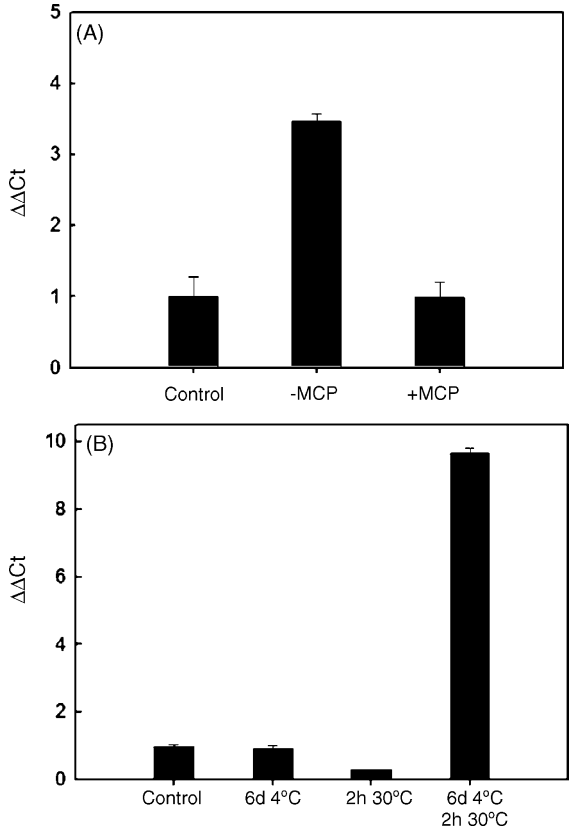

Fig. 5. Effects of wounding in the presence or absence of 1-MCP (A) and temperature treatments (B) on the levels of *LeEF-Ts$_{mt}$* transcripts assessed by real-time PCR in Micro-Tom tomato fruit at the breaker stage. Wounding, 1-MCP and temperature treatments were performed as described in Section 2. $\Delta\Delta C_t$ in the *Y*-axis refers to the fold difference in *LeEF-Ts$_{mt}$* expression relative to untreated control fruit. Values are the mean of three replicates ±S.E.

3.2. LeEF-Ts$_{mt}$ gene is expressed at higher levels in fruit and is regulated during fruit ripening

RNA extracted from stem, leaf, flower, root and fruit tissues of the Ailsa Craig variety were used to assess the accumulation of tomato LeEF-Ts$_{mt}$ transcripts by real-time PCR. Fig. 2 shows that transcript were present in all tissues tested. Accumulation in flowers and roots was about 12 and 8 times higher respectively than in stems. However the most striking observation is that accumulation in ripening fruit was about 80 times higher than in stems. This prompted us to study in more detail the pattern of *LeEF-Ts$_{mt}$* gene expression at different stages of ripening and in non-ripening tomato mutants. Fig. 3 shows that *LeEF-Ts$_{mt}$* gene undergoes a ripening-associated regulation with increasing expression from mature-green to turning and decreasing thereafter. Ripening impaired mutants *rin*, *nor* and *Nr* taken at stages equivalent to red, show substantial expression. The comparison of data of Figs. 2 and 3 shows that the level of *LeEF-Ts$_{mt}$* transcripts in the *rin* and *Nr* fruit is similar to that in leaves and roots, while expression in *nor* is higher and similar to that in breaker fruit. Since these mutants are affected for ethylene response, it can be concluded that the

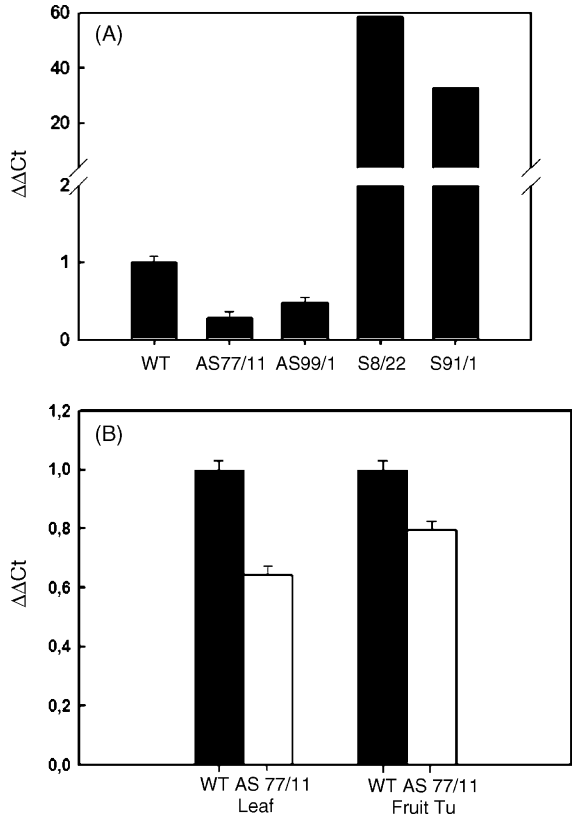

Fig. 6. Expression of the *LeEF-Ts$_{mt}$* gene in wild type (WT), sense (S) and antisense (AS) in leaves (A) and leaves and fruit at the turning stage (B) of Micro-Tom tomato plants. The level of *LeEF-Ts$_{mt}$* transcripts were assessed by real-time PCR. $\Delta\Delta C_t$ in the *Y*-axis refers to the fold difference in *LeEF-Ts$_{mt}$* expression relative to WT leaves (A) and WT leaves or fruit (B). Values are the mean of three replicates ±S.E.

regulation of *LeEF-Ts$_{mt}$* gene (at least for *rin* and *Nr*) has a major developmental component independent of ethylene action corresponding to expression in vegetative tissues.

3.3. LeEF-Ts$_{mt}$ gene expression is stimulated in fruit by ethylene, wounding and temperature shocks

Since ethylene is known to control respiratory activity, the ethylene responsiveness of the gene was further assessed upon ethylene and 1-MCP treatment of fruit and upon wounding and temperature shock. Fig. 4 indicates that ethylene treatment of mature-green of Ailsa Craig tomato fruit results in almost a three-fold increase in transcript accumulation after 15 min. Accumulation was somewhat lower after 5 h. Fruit treated with 1-MCP showed reduced expression (about half of the control) indicating that ethylene plays an important role in the stimulation of *LeEF-Ts$_{mt}$* expression. Wounding, which is known to stimulate both ethylene production and respiration, resulted in a 3.5-fold stimulation of *LeEF-Ts$_{mt}$* gene expression after 72 h in mature-green Micro-Tom tomato fruit (Fig. 5A). Stimulation was prevented by treating wound fruit tissues with 1-MCP indicating that the bulk of wound-induced stimulation of *LeEF-Ts$_{mt}$* gene expression is mediated by ethylene. Since the *LeEF-Ts$_{mt}$* gene has been isolated by mRNA differential display on the basis of ethylene response (Zegzouti et al., 1999), these data represent a new demonstration of the role of ethylene in regulating *LeEF-Ts$_{mt}$* gene expression.

In considering the role of ethylene in abiotic stresses, an assessment was made of the effect of temperature shocks on Micro-Tom fruit. Fig. 5B indicates that continuous application of low (4 °C) or high (30 °C) temperatures had no effect on LeEF-Ts$_{mt}$ RNA accumulation. However, fruit that were first stored at 4 °C for 6 days and then transferred to 30 °C for 2 h showed strong stimulation of gene expression.

3.4. Physiological characterisation of sense and antisense LeEF-Ts$_{mt}$ plants

In order to address the function of the *LeEF-Ts$_{mt}$* gene *in planta*, transgenic tomato plants of the Micro-Tom genotype were generated and brought to homozygocity by successive self-pollinations. Among the various lines generated, two sense lines showed strong over-expression (up to 60 times in line S8/22) in the leaves, while two antisense lines showed moderate reduction of gene expression, specially the AS 77/11 line (Fig. 6A). The reduction in the levels of transcripts was similar in AS leaves and fruit at the turning stage with about 40% and 30% reduction, respectively (Fig. 6B).

Respiratory activity of untransformed (Wild Type, WT) and up- (S) and down-regulated (AS) Micro-Tom tomato fruit was evaluated after harvesting fruit at the mature-green

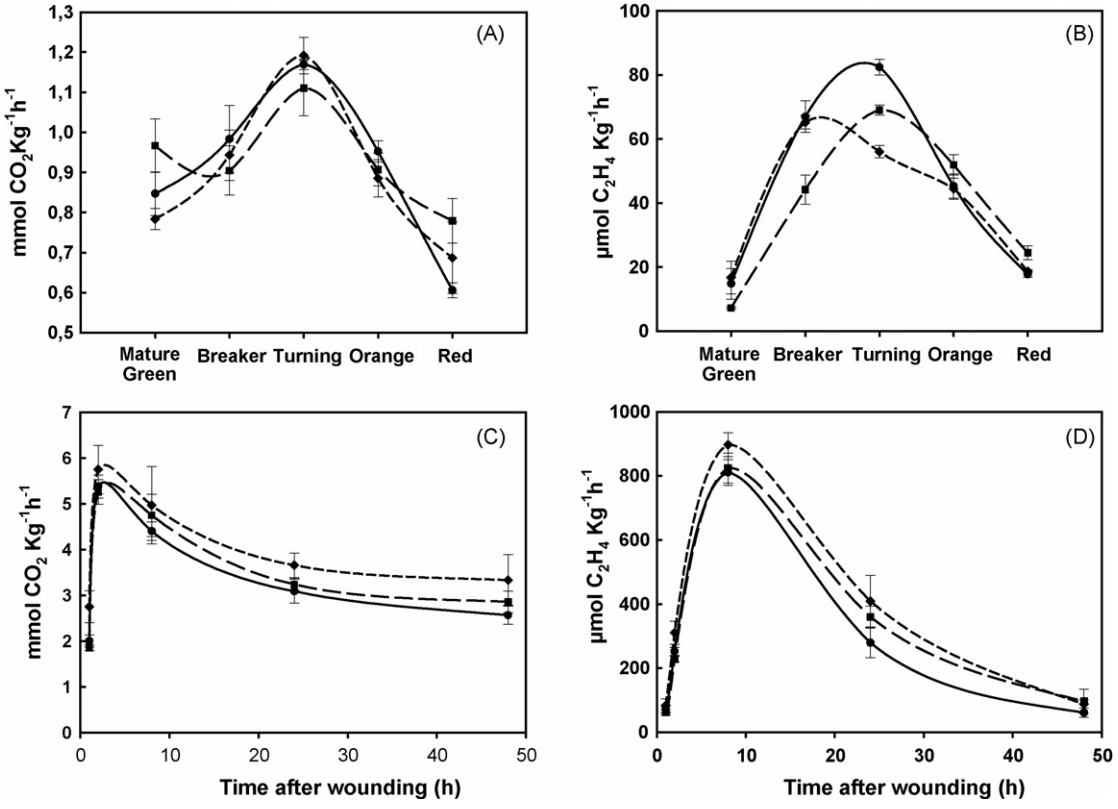

Fig. 7. Respiration and ethylene production of WT Micro-Tom fruit (♦) and transgenic fruit in which the *LeEF-Ts$_{mt}$* gene has been over (●) or down-regulated (■). CO_2 production of whole (A) and wounded fruit (C). Ethylene production of whole (B) and wounded (D) fruit. Each data point represents the mean of five replicates ±S.E.

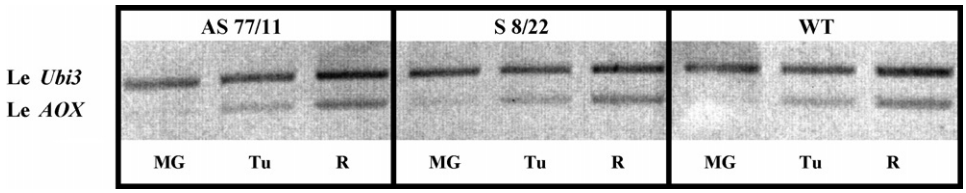

Fig. 8. RT-PCR analysis of LeAOX transcript accumulation in wild type (WT), sense (S) and antisense (AS) Micro-Tom tomato fruit at the MG, Tu and R stages. In each sample, the internal reference *LeUbi3* was co-amplified with the cDNA as described in Section 2.

stage and ripening at 25 °C. Fig. 7A shows a smooth climacteric respiratory rise in all type of fruit without any statistical difference between WT, S and AS fruit. In addition, the climacteric rise in ethylene production was not significantly affected by the genetic transformation in both intensity and timing (Fig. 7B), indicating that the overall ripening process remained unchanged. An attempt was made to strongly stimulate respiratory activity by wounding of mature-green fruit tissues. Although wounding resulted in about 3-fold stimulation of respiration (Fig. 7C) and 20-fold stimulation of ethylene production (Fig. 7D), no difference could be observed between WT fruit and S and AS fruit.

It has been proposed that cyanide-insensitive respiration plays a major role in the climacteric respiration (Solomos, 1977; Pirrung and Brauman, 1987; Kumar et al., 1990). Therefore, the expression of the alternative oxidase (*AOX*) gene was evaluated in WT, S and AS ripening fruit. Fig. 8 clearly indicates that the amount of AOX mRNA is increasing similarly in WT, AS and S fruit during ripening.

4. Discussion and conclusion

The up-regulation of the *LeEF-Ts$_{mt}$* gene during fruit ripening suggest that the protein synthesis machinery of the mitochondria is stimulated. A stimulation of the translation machinery for nuclear encoded proteins is well documented during fruit ripening (Brady, 1987). To our knowledge, changes in the translation machinery in the mitochondria has never been evaluated. Cytosolic elongation factors of the EF-Tu type are generally considered as constitutively expressed, including during fruit ripening of tomato (Bartley and Ishida, 2003) and strawberry (Wilkinson et al., 1995) but nothing is known on the mitochondrial EF-Tu in plants. No information is available on elongation factors of the EF-Ts type. LeEF-Ts$_{mt}$ is the first mitochondrial elongation factor characterised in plants (Benichou et al., 2003). The *LeEF-Ts$_{mt}$* gene has been isolated on the basis of the response to the plant hormone ethylene (Zegzouti et al., 1999). It encodes a functional elongation factor involved in providing energy for the synthesis of proteins in the mitochondria (Benichou et al., 2003). Knowing the essential role of ethylene in the stimulation of the respiration, it was hypothesised that *LeEF-Ts$_{mt}$* could play a role in the respiratory rise accompanying climacteric fruit. This hypothesis is supported by the high expression of *LeEF-Ts$_{mt}$* in fruit as compared to other plant tissues and by its up-regulation during the first steps of the ripening process. Ethylene stimulates and 1-MCP partially inhibits *LeEF-Ts$_{mt}$* expression in fruit. However non-ripening mutants exhibit significant expression indicating that the gene has a basal constitutive expression which is ethylene independent. Wounding of fruit tissues, which is known to greatly enhance respiration (McGlasson and Pratt, 1964) also stimulates *LeEF-Ts$_{mt}$* gene expression in a partial ethylene-dependent manner as well as temperature shocks. The stimulation of the translation mitochondrial machinery via the up-regulation of the *LeEF-Ts$_{mt}$* gene could correspond to an adaptation of the mitochondrial protein complexes to the hosting of increased levels of imported proteins that are know to be stimulated during ripening and upon ethylene action such as the alternate oxidase and uncoupler protein. The peculiar expression pattern of *LeEF-Ts$_{mt}$* gene prompted us to generate transgenic tomatoes in which the *LeEF-Ts$_{mt}$* gene was up- or down-regulated. Despite a strong difference in *LeEF-Ts$_{mt}$* gene expression between transgenic mutants and control fruit, the respiratory activity of either intact or wounded fruit remained unaffected. Since the gene is present in single copy in the tomato genome, the absence of effect on respiration cannot be attributed to a compensation by an homolog gene. A possible explanation is that the down-regulation of the gene was not sufficient to render the LeEF-Ts$_{mt}$ protein limiting of the protein synthesis process. Transgenic lines with complete inhibition of the gene may not be viable and may have been deleted during the transformation procedure. This hypothesis is supported by the fact that all *Arabidopsis* plants mutated for this gene are mutated in introns and never in the open-reading frame of the *AtEF-TS$_{mt}$* gene (The Arabidopsis Information Resource, http://arabidopsis.org/), indicating that the absence of transcription of the protein is probably lethal. The absence of effect on respiration could also be related to the presence of regulatory processes that would prevent the alteration of such an important function in plants. Some chaperon proteins of the heat-shock (Hs) type are known to be involved in the transport of nuclear-encoded proteins from the cytosol to the mitochondria (Kang et al., 1990) and in stabilizing the target protein under stress conditions (Jinn et al., 1995). Interestingly, a gene encoding an Hsp70 type of proteins, named *ER21*, is ethylene-regulated and exhibits a pattern of expression which is similar to *LeEF-Ts$_{mt}$* (Zegzouti et al., 1999). It can be speculated that these proteins regulate the transport and/or the stability of the LeEF-Ts$_{mt}$ protein so as to modulate the turn-over of the protein and thus extend the energy

supplying function for protein synthesis under stress conditions or when the level of LeEF-Ts_{mt} protein is decreasing. However, we have so far no information on variation of the Le-EF-Ts_{mt} protein under stress conditions in WT and AS plants. In any case, these data show that, despite a strong effect of ethylene, the respiratory activity of mitochondria appears to be unaffected by variations of the expression of the *LeEF-Ts_{mt}* gene. The regulation of respiration would rather depend upon a signal transmitted to the nucleus for enhancing the expression of genes such as the alternative oxidase or the uncoupling protein (Almeida et al., 1999, 2002). Further work is needed (i) to determine whether changes in the expression of *LeEF-Ts_{mt}* result in alterations of the synthesis of proteins in the mitochondrion, (ii) to understand the role of ethylene in stimulating *LeEF-Ts_{mt}* expression and (iii) to know whether post-translation regulatory processes exist that prevent the synthesis of proteins in mitochondrion from alteration.

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