Tomato EF-Ts$_{mt}$, a functional mitochondrial translation elongation factor from higher plants

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Abstract

Ethylene-induced ripening in tomato (*Lycopersicon esculentum)* resulted in the accumulation of a transcript designated *LeEF-Ts*$_{mt}$ that encodes a protein with significant homology to bacterial Ts translational elongation factor (EF-Ts). Transient expression in tobacco and sunflower protoplasts of full-length and truncated *LeEF-Ts*$_{mt}$-*GFP* fusion constructs and confocal microscopy observations clearly demonstrated the targeting of LeEF-Ts$_{mt}$ to mitochondria and not to chloroplasts and the requirement for a signal peptide for the proper sorting of the protein. *Escherichia coli* recombinant LeEF-Ts$_{mt}$ co-eluted from Ni-NTA resins with a protein corresponding to the molecular weight of the elongation factor EF-Tu of *E. coli*, indicating an interaction with bacterial EF-Tu. Increasing the GDP concentration in the extraction buffer reduced the amount of EF-Tu in the purified LeEF-Ts$_{mt}$ fraction. The purified LeEF-Ts$_{mt}$ stimulated the poly(U)-directed polymerization of phenylalanine 10-fold in the presence of EF-Tu. Furthermore, LeEF-Ts$_{mt}$ was capable of catalysing the nucleotide exchange reaction with *E. coli* EF-Tu. Altogether, these data demonstrate that LeEF-Ts$_{mt}$ encodes a functional mitochondrial EF-Ts. LeEF-Ts$_{mt}$ represents the first mitochondrial elongation factor to be isolated and functionally characterized in higher plants.

Introduction

During fruit ripening ethylene induces the expression of a number of gene products (Zegzouti *et al*., 1999). In a screening for ethylene-responsive genes in tomato (*Lycopersicon esculentum*) fruit, we have isolated a differential display mRNA, initially called ER49, which showed sequence homology to both prokaryotic and mitochondrial elongation factor Ts (EF-Ts). EF-Ts is the nucleotide exchange factor that promotes the exchange of GDP for GTP with elongation factor Tu (EF-Tu). In *Escherichia coli*, EF-Tu facilitates the binding of aminoacyl-tRNA (aa-tRNA) to the ribosome during the elongation cycle of protein biosynthesis (Ravel *et al*., 1969). Upon the binding of aa-tRNA to the A site of the ribosome, EF-Tu catalyses the hydrolysis of GTP, and EF-Tu:GDP is released from the ribosome. EF-Ts catalyses the nucleotide exchange reaction promoting the formation of EF-Tu:GTP from EF-Tu:GDP (Miller and Weissbach, 1970). The guanine nucleotide exchange reaction occurs through the formation of an intermediate EF-Tu:Ts complex. GTP then replaces EF-Ts in the complex and the EF-Tu:GTP complex binds aa-tRNA permitting another round of ribosome binding.

The sequences of EF-Tu and EF-Ts from numerous prokaryotes are now known (Sprinzl, 1994). Eukaryotes have translational systems in the mitochondria as well as in the cell cytoplasm, and plants have a third protein synthesizing system in chloroplasts. The GTP-dependant binding of aa-tRNA to the ribosome and the subsequent release of the elongation factor as a GDP complex is conserved among prokaryotes, eukaryotes and organelles. In general, the mitochondrial and chloroplast translational factors are more closely related to their bacterial counterparts than to the corresponding cytoplasmic factors. Mammalian mitochondrial EF-Tu (EF-Tu_{mt}) has significant sequence identity (ca. 60%) to the corresponding prokaryotic factors. By contrast, the sequence of EF-Ts is less conserved than that of EF-Tu, and distinct schemes are observed for the interactions of EF-Tu and EF-Ts in different systems (Blank *et al*., 1995). Bovine EF-Tu_{mt} binds guanine nucleotides significantly less tightly than do the bacterial factors (Cai *et al*., 2000a). The activity of *E. coli* EF-Tu can be stimulated by both *E. coli* EF-Ts and EF-Ts_{mt}. In contrast, the activity of EF-Tu_{mt} is not stimulated by *E. coli* EF-Ts (Bullard *et al*., 1999). EF-Tu_{mt} readily forms ternary complexes with mitochondrial or bacterial aa-tRNA and delivers them to the A site of the ribosome. However, although *E. coli* EF-Tu can form ternary complexes with mitochondrial aa-tRNAs, it has little activity in promoting the binding of these complexes to the A-site of either *E. coli* or mammalian mitochondrial ribosomes (Kumazawa *et al*., 1991).

In higher plants, mitochondrial elongation factors have not been characterized biochemically and only partial sequences arising from several plant genomes including *Arabidopsis* and barley are available. In general, the plant sequences have not been clearly identified as EF-Ts and have not been clearly assigned to a chloroplast or mitochondrial localization. This paper describes the identification of EF-Ts_{mt} from *L. esculentum* (LeEF-Ts_{mt}) based on its homology to the previously characterized EF-Ts from bacterial and animal mitochondrial systems. The abilities of LeEF-Ts_{mt} to stimulate polypeptide synthesis with *E. coli* EF-Tu and to promote guanine nucleotide exchange with the bacterial factor are shown. The localization of LeEF-Ts_{mt} to mitochondria rather than chloroplasts is clearly demonstrated. This report represents the first identification of a mitochondrial elongation factor from higher plants.

Materials and methods

Materials

GDP was obtained from Pharmacia-LKB Biochemicals. Poly(U) and *E. coli* tRNA were from Boehringer Mannheim. Arabinose, glycerol, phenylmethylsulfonyl fluoride, phospho(enol)pyruvate and pyruvate kinase were from Sigma. The Ni-NTA resin was purchased from Qiagen. Nitrocellulose membrane filter paper type HA (0.45 μm pore size) was from Millipore. [^{3}H]GDP was obtained from Amersham Bioscience. [^{14}C]Phenylalanine was obtained from DuPont-New England Nuclear and [^{14}C]Phe-tRNA was prepared as described by Ravel and Shorey (1971) with aminoacyl-tRNA synthetases prepared by the method of Muench and Berg (1966). The *E. coli* ribosomes and elongation factors EF-Tu, EF-Ts and EF-G were purified with methods described previously (Zhang *et al*., 1996, 1998).

Expression and purification of LeEF-Ts_{mt}

The coding sequence for tomato EF-Ts_{mt} from clone ER49 (Zegzouti *et al*., 1999) lacking the signal peptide (amino acid residues 63–391) was fused to the sequence encoding a His-tag in vector pBAD-TOPO (Invitrogen). The recombinant protein was expressed in *E. coli* after induction by 0.02% L(+) arabinose for 4 h at 37 °C. Cells (5 g) were collected by low-speed centrifugation and extracted by grinding with twice the cell weight of alumina in an ice-cold mortar. The extract was resuspended in 50 ml of buffer A (50 mM Tris-HCl pH 7.6, 40 mM KCl, 7 mM $MgCl_2$, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride and 7 mM 2-mercaptoethanol) containing 5 μg/ml DNase I and 10 μM GDP. After centrifugation at 28 000 $\times$ g for 15 min, 600 μl of a 50% suspension of Ni-NTA resin was added and the sample was mixed by inversion at 4 °C for 60 min. The mixture was poured into a 5 ml Qiagen fritted column and washed 4 times with 10 ml of buffer B (50 mM Tris-HCl pH 7.6, 1 M KCl, 7 mM $MgCl_2$, 10% glycerol, 10 mM imidazole and 7 mM 2-mercaptoethanol) containing 10 μM GDP. The protein retained by the resin was eluted using three washes of 1 ml each of buffer C (buffer B containing 150 mM imidazole and 10 μM GDP). An aliquot was removed for analysis by SDS-PAGE. The remainder of the sample was dialysed for 2 h at 4 °C twice against 450 ml of buffer D (20 mM Hepes-KOH pH 7.6, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and 10 μM GDP). The

sample was fast frozen in a dry ice-isopropanol bath and stored at −70 °C until use. Protein concentrations were determined by the Bradford method (Bradford, 1976).

Polymerization assay

The function of LeEF-Ts_{mt} in protein synthesis was assayed by its ability to replace *E. coli* EF-Ts in stimulating the activity of *E. coli* EF-Tu in the poly(U)-directed polymerization of phenylalanine with *E. coli* ribosomes and EF-G. Assay mixtures (0.1 ml) contained 50 mM Tris-HCl pH 7.8, 70 mM KCl, 6.2 mM $MgCl_2$, 0.1 mM spermine, 1 mM dithiothreitol, 2.5 mM phospho(*enol*)pyruvate, 0.17 units of pyruvate kinase, 0.5 mM GTP, 12.5 μg poly(U), 35 pmol of [^{14}C]Phe-tRNA, 35 μg of *E. coli* ribosomes, saturating amounts of *E. coli* EF-G and the indicated amounts of *E. coli* EF-Tu and LeEF-Ts_{mt}. All dilutions were done with buffer D. Incubation was carried out for 30 min at 37 °C and the amount of label incorporated into polypeptide was determined by hot trichloroacetic acid precipitation (Graves *et al*., 1980). Blanks representing the amount of label retained on the filter in the absence of EF-Tu or LeEF-Ts_{mt} have been subtracted from each value.

Nucleotide exchange assay

The ability of LeEF-Ts_{mt} to promote guanine nucleotide exchange with *E. coli* EF-Tu was monitored basically as described previously (Schwartzbach and Spremulli, 1989). Increasing concentrations of the purified recombinant LeEF-Ts_{mt} protein were incubated in the presence of 60 units of *E. coli* EF-Tu in 120 μl of 50 mM Tris-HCl pH 7.5, 80 mM NH_4Cl, 80 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol and 2 μM [^{3}H]-GDP (150 cpm/pmol). Incubation was performed at 0 °C for 10 min and the amount of nucleotide exchange was determined by a nitrocellulose filter binding assay (Miller and Weissbach, 1970).

Protoplast isolation and transient expression of a LeEF-Ts_{mt}-GFP fusion protein

A fusion construct LeEF-Ts_{mt} and GFP was constructed as follows. The sequence corresponding to full length of LeEF-Ts_{mt} protein (391 amino acids) was amplified by PCR with Vent polymerase (Clontech) with primers LeEF-Ts_{mt}-5′ (5′-TGGAAGATGGTGTTTTATCGTGGTG-3′) and LeEF-Ts_{mt} -3′ (5′-AGCAGCATTAGCCAAAGGTTCAC-3′) designed from the ER49 cDNA. The N-terminal portion of LeEF-Ts_{mt} (101 amino acids) was amplified with the primers LeEF-Ts_{mt}-5′ and LeEF-Ts_{mt} -N3′ (5′-CTCGATATCCCAATTGCTAGTGAC-3′). The C-terminal portion of LeEF-Ts_{mt} (290 amino acids) was amplified with primers LeEF-Ts_{mt}-C5′ (5′-GCTGCTCAGAAAGACCTAAGA-3′) and LeEF-Ts_{mt}-3′. The amplified fragments were cloned into the pGreen vector (John Innes Centre, Norwich, UK) in frame with the GFP coding sequence fused at the C-terminus. This construct was expressed under the control of a dual cauliflower mosaic virus (CaMV) 35S promoter (Figure 4A). Protoplasts used for transfection were obtained from suspension cultures of tobacco BY-2 cells protoplasts according to the method described previously (Bouzayen *et al*., 1988) or were isolated from sunflower hypocotyls according to the protocol of Chanabé *et al*. (1989). Protoplasts were transfected by a modified polyethylene glycol method as described (Abel and Theologis, 1994). Typically, 0.2 ml of protoplast suspension (0.5×10^6) were transfected with 50 μg of sheared salmon sperm carrier DNA and 30 μg of the appropriate plasmid DNA (35S::GFP, 35S::LeEF-Ts_{mt}-GFP, 35S::LeEF-Ts_{mt}-N′-GFP or 35S::LeEF-Ts_{mt}-C′-GFP). Transfected protoplasts were incubated at 25 °C for 16 h. All transient expression assays were repeated at least three times. In order to stain mitochondria, the mitochondria-specific dye MitoTracker Orange CMH_2TMRos (Molecular Probes, Eugene, OR) was used. Transformed BY-2 protoplasts were treated by 500 nM MitoTracker Orange CMH_2TMRos for 30 min at 37 °C. Confocal fluorescent images were obtained on a confocal laser scanning microscope (Leica TCS SP2, Leica DM IRBE; Leica Microsystems, Wetzlar, Germany). The samples were illuminated with an argon ion laser (488 nm wavelength) for GFP, a green HeNe laser (543 nm) for MitoTracker fluorescence and a red HeNe laser (633) for chloroplast visualisation. The emitted light was collected at 500–525 nm, 550–610 nm and 640–670 nm for GFP, MitoTracker and chlorophyll respectively.

Results and discussion

Sequence analysis of the LeEF-Ts_{mt} gene

The partial cDNA encoding EF-Ts_{mt} (ER49) was initially isolated from tomato fruit among other ethylene-regulated clones (Zegzouti *et al*., 1999). The full-

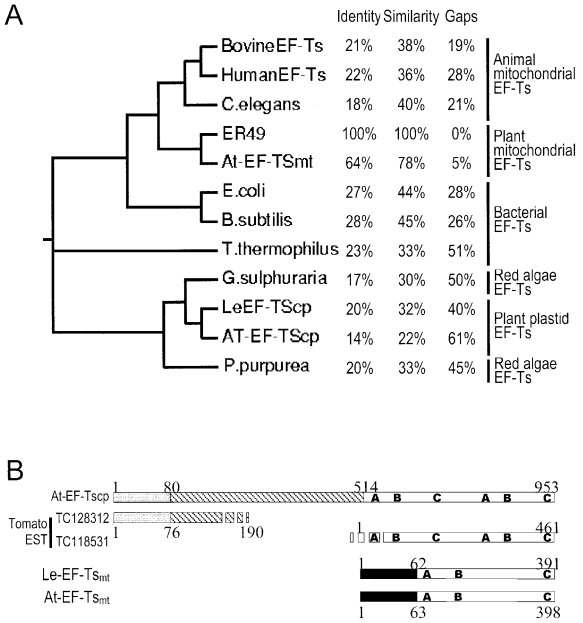

Figure 1. Phylogenetic tree and structural organisation of mitochondrial and chloroplastic plant EF-Ts. A. Phylogenetic tree of LeEF-Ts_{mt} and homologous proteins. The web interface providing the ClustalW program (Thompson *et al.*, 1994) at http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html, was used to construct the phylogenetic tree with the neighbor-joining method. The branches were not drawn proportional to genetic distances. The percentage of identical or similar residues and of gap insertions between LeEF-Ts_{mt} and each sequence was calculated with protein sequences including the targeting signal with the default options of the software package STRETCHER ((Myers and Miller, 1988). Accession numbers in GenBank are: *A. thaliana* At-EF-Ts_{mt}, At4g11120.1; *A. thaliana* EF-Ts_{cp}, At4g29060; *L. esculetum* LeEF-Ts_{mt}, F096247; *B. taurus* EF-Ts_{mt}, L37935; *H. sapiens* EF-Ts_{mt}, L37936; *C. elegans* EF-Ts_{mt}, BAA13470; *E. coli EF-Ts*, P02997; *B. subtilis* EF-Ts, B69727; *T. thermophilus* EF-Ts, X83598; *G. sulphuraria* chloroplast EF-Ts, P35019; *P. purpurea* chloroplast EF-Ts, P51248. The *L. esculentum* LeEF-Ts_{cp} corresponds to a chimaeric sequence made from the two partial EST that can be accessed in TIGR ESTs database under the accession numbers TC128312 and TC118531. B. Schematic representation of mitochondrial and chloroplastic EF-Ts of tomato and *Arabidopsis*. Black boxes represent the mitochondrial targeting signal peptide, grey boxes the chloroplastic targeting signal peptide, hatched boxes a domain with homology to ribosomal protein S1. The numbers indicate the amino acid position. A, B and C represent the EF-Ts1 and 2 signatures and the conserved signature within the dimerization domain respectively, as indicated in Figure 2.

length cDNA was thereafter isolated and named LeEF-Ts_{mt} (accession number AF096247). The full-length clone has a total length of 1.7 kb with an open reading frame of 1.2 kb encoding a protein of 42.8 kDa (391 amino acids). Homology searches using the protein databases revealed significant homologies with conserved domains of numerous EF-Ts from prokaryotes and eukaryotic organelles (Figure 1). Sequence homology calculations taking into account the signal peptide indicated that LeEF-Ts_{mt} had strongest homology with the putative EF-Ts_{mt} from *Arabidopsis thaliana* (64% identical and 78% similar residues in pair-wise alignment). Homology with EF-Ts_{mt} from mammalian organisms was weak (21% identity with *Bos taurus*, 22% with *Homo sapiens*). The percentage identity to red algae EF-Ts and putative plant plastid EF-Ts was also low (20% identity, for example, with tomato LeEF-Ts_{cp} and *Porphyra purpurea*). The identities with bacterial EF-Ts of *E. coli* and *Thermus thermophilus* were 27% and 23%, respectively. Clearly, EF-Ts sequences, while recognizable throughout various species, have not been highly conserved.

A phylogenetic tree of the EF-Ts family (Figure 1A) based on the sequence alignments shows that this factor falls into two main and highly divergent groups of proteins. Plant mitochondrial and chloroplastic EF-Ts belong to two highly distant clusters that also include bacteria and algae, which is consistent with their endosymbiotic origin. Within the mitochondrial group, animal (*B. taurus*, *H. sapiens* and *Caenorhabditis elegans*) and plants (tomato EF-Ts_{mt} and an *Arabidopsis* EST sequence) constitute two closely related sub-groups.

The bacterial sub-group represented by *E. coli* and *Bacillus subtilis* exhibits significant similarity with the animal and plant mitochondrial EF-Ts. Chloroplastic EF-Ts constitute a second and distinct group that includes the closely related tomato and *Arabidopsis* putative EF-Ts as well as red algae (*P. purpurea* and *G. sulphuraria)*. The *T. thermophilus* EF-Ts is phylogenetically located between chloroplastic and mitochondrial EF-Ts.

EF-Ts_{mt} and EF-Ts_{cp} have been identified in *Arabidopsis*, rice, *Medicago* and wheat. Mitochondrial EF-Ts proteins are always smaller than chloroplastic EF-Ts with a mitochondrial targeting signal peptide directly fused to the EF-Ts domains. Chloroplastic EF-Ts are longer proteins, with a chloroplast targeting peptide fused to the EF-Ts domains via a protein domain and which shares homologies with the ribosomal protein S1 (Figure 1B). The typical 'EF-Ts component' of the chloroplastic proteins contains twice the EF-Ts signatures and is organized similarly to red algae EF-Ts. A long sequence within the sub-domains-C-a and -N (see Figure 2) is lacking in both red algae and chloroplastic EF-Ts as compared to mitochondrial EF-Ts from animals and plants. The tomato EST database contains two ESTs with strong homologies

to the N-terminal and C-terminal part of *Arabidopsis* EF-Ts_{cp}. These two ESTs may result from partial sequencing of the same gene. These data indicate that at least some plants, including tomato, have used separate EF-Ts for chloroplasts and mitochondria.

Multiple alignments performed with EF-Ts from species representative of the plant, microbial and animal kingdoms (Figure 2) indicates that LeEF-Ts_{mt} contains the two consensus motifs (EF-Ts 1 and 2 PROSITE accession numbers PS01126 and PS01127) that are characteristic of EF-Ts from a variety of bacteria and subcellular organelles. Further, LeEF-Ts_{mt} is organized into domains that are similar to those observed in *E. coli* EF-Ts (Figure 2). These include the N-terminal domain, the subdomain N and subdomain C of the core of the protein, the dimerization domain and the C-terminal module. A 3-dimensional model of LeEF-Ts_{mt} (Figure 3) was created with Swiss Model (Peitsch *et al*., 1995; Guex and Peitsch, 1997). This model indicates that the overall shape of LeEF-Ts_{mt} is very similar to that of the *E. coli* factor. The N-terminal domain contains important residues of EF-Ts that are in contact with EF-Tu in the nucleotide exchange reaction (starred residues in Figure 2) (Kawashima *et al*., 1996; Zhang *et al*., 1998). LeEF-Ts_{mt} possesses some, but not all, of these critical amino acids (Figure 2). LeEF-Ts_{mt} has a small insertion of 8 amino acids in the N-terminal domain. This insertion is located on the back of this domain away from the region of EF-Ts that interacts with EF-Tu (arrow in Figure 3, left panel).

LeEF-Ts_{mt}, like bovine EF-Ts_{mt}, has an insertion of 21 or 22 amino acids just past the second EF-Ts conserved signature sequence within subdomain N of the core of *E. coli* EF-Ts. This insertion is again modelled to be present on the back of EF-Ts away from the side interacting with EF-Tu (Figure 3). It has been shown that subdomain N of the core is largely responsible for the higher strength of the interaction between bovine EF-Ts_{mt} and *E. coli* EF-Tu (Zhang and Spremulli, 1998). LeEF-Ts_{mt} has an additional insertion of 14 amino acids in subdomain C of the core which is absent in the bovine homolog, but partially present in *A. thaliana* EF-Ts_{mt}. Secondary structure predictions place this insertion in the middle of the β-sheet in subdomain C of the core (Figure 2). This region was difficult for Swiss-Model to build but is reflected in larger loops in this region of LeEF-Ts_{mt} (Figure 3). LeEF-Ts_{mt} is predicted to contain the domain referred to as the dimerization domain. This region is involved in protein-protein contacts in the crystals of the *E. coli* EF-Tu:Ts complex. However, in solution, EF-Ts functions as a monomer. The region referred to as the dimerization domain is fairly well conserved in all organisms (Figure 2) except in *B. taurus* EF-Ts_{mt}. It should be noted that in some alignments bovine EF-Ts_{mt} contains the insertion in subdomain C of the core of EF-Ts seen in LeEF-Ts_{mt} but lacks the dimerization domain observed in most species.

As indicated in Figure 2, LeEF-Ts_{mt} has a signal sequence at the N-terminus that is predicted to direct this protein into mitochondria. The signal sequence is predicted by MitoProt II and TargetP to be 62 amino acids long with a molecular mass of 6.9 kDa (Claros and Vincens, 1996). This sequence has a number of characteristics (arginine at -2 and -3 and serine at $+1$) that are present in a high percentage of mitochondrial signal peptides (Zhang *et al*., 1998). In addition, it is rich in hydroxylated amino acids and has many more basic residues (8 Lys and Arg) compared to a single acidic residue, a general characteristic of mitochondrial import signal peptides (Claros and Vincens, 1996). The signal peptide also contains at its C-terminal end a well conserved cleavage site RRY$*$SAE (Sjöling and Glaser, 1998). Predictions for the targeting of two sequences of an EF-Ts from tomato and *Arabidopsis* have been made using two different prediction programs, TargetP and Predotar. The data presented in Table 1 indicate a high probability for mitochondrial targeting of the tomato LeEF-Tsmt and the *Arabidopsis* At4g11120.1 sequence and a high probability for chloroplastic targeting of partial sequences of tomato (LeTC128312) and *Arabidopsis* (At4g29060). The only chloroplast EF-Ts that has ever been biochemically characterized is from *Euglena gracilis* (Spremulli and Spremulli, 1987). This protein is twice the size of the bacterial and mitochondrial factors (Fox *et al*., 1981). Algal forms that are plastid-encoded have been sequenced but these represent a different class from those in which the gene has been transferred to the nucleus. The *Arabidopsis* chloroplast EF-Ts is 953 amino acids long before cleavage of the signal sequence. This observation is in keeping with the biochemical data showing that the mature *Euglena* protein is quite large (about 70 kDa) (Fox *et al*., 1981). The bacterial and mature mitochondrial EF-Ts are generally 200–300 amino acids long. This is in agreement with the idea that the tomato EF-Ts isolated in this study is mitochondrial and not chloroplastic. It is 391 amino acid as a precursor with a predicted 62 residue import signal. Although predictions suggest that LeEF-Ts_{mt} is local-

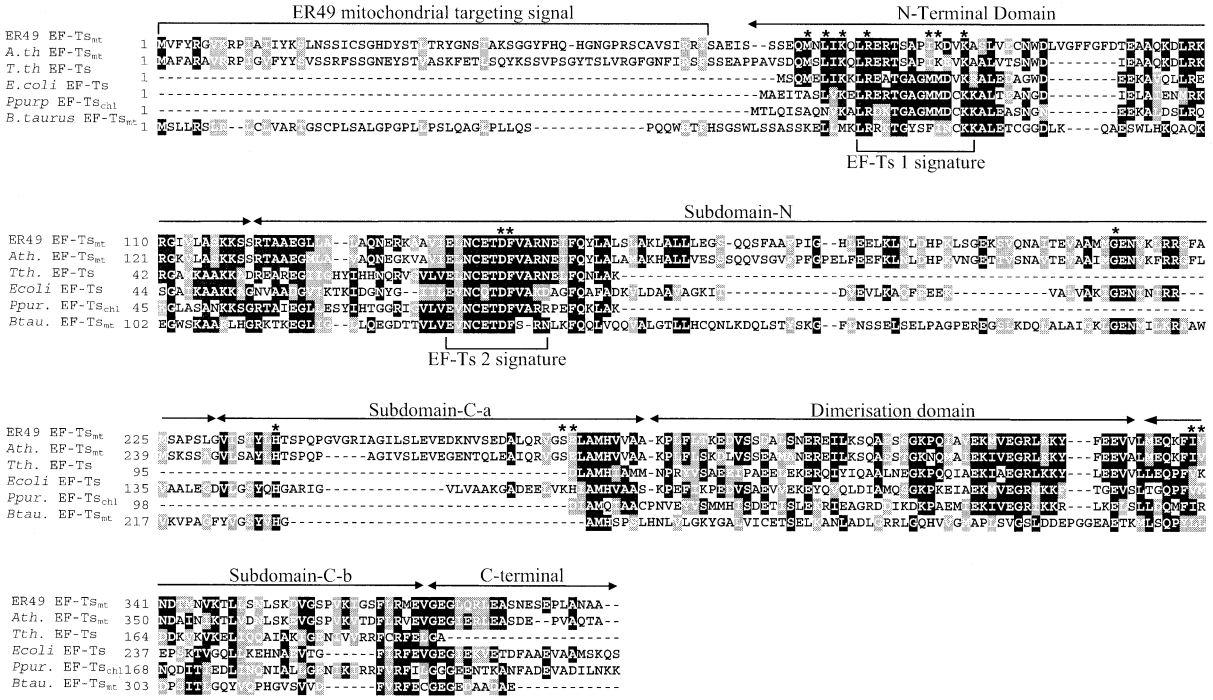

Figure 2. Multiple alignment of LeEF-Ts$_{mt}$ and homologous proteins from *A. thaliana* (GenBank accession number TO1921), *B. taurus* (L37935), *T. thermophilus* (X83598), *E. coli* (P02997) and *P. purpurea* (P51248). Arrows represent *E. coli* EF-Ts domains as defined by Kawashima *et al.* (1996), EF-Ts 1 and 2 correspond to protein signatures defined in the Prosite database (accession numbers PS01126, PS01127; Prosite documentation PDOC00867). Asterisks indicate critical amino acids involved in the interaction of EF-Ts with EF-Tu (Kawashima *et al.*, 1996; Zhang *et al.*, 1996, 1998; Zhang and Spremulli, 1998). Multiple alignment was created with ClustalW (Thompson *et al.*, 1994) at the web interface at http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html.

Table 1. Evaluation of the probability for tomato and *Arabidopsis* EF-Ts to be targeted at the mitochondria and chloroplast using the TargetP and Predotar predicting programs. LeEF-Ts$_{mt}$ and At4g29060 are full-length proteins of 391 and 953 amino acids respectively. LeTC128312 and At4g11120.1 are partial sequences clones corresponding to 190 and 398 amino acids respectively. The accession numbers of the sequences are as described in the legend to Figure 1.

Genes	Target P		Predotar	
	mitochondrial score	plastidial score	mitochondrial score	plastidial score
L. esculentum LeEF-Ts$_{mt}$	0.492	0.037	0.997	0.005
Arabidopsis At4g11120.1	0.503	0.141	0.983	0.001
L. esculentum LeTC128312	0.045	0.692	0.221	0.935
Arabidopsis At4g29060	0.008	0.978	0.031	0.794

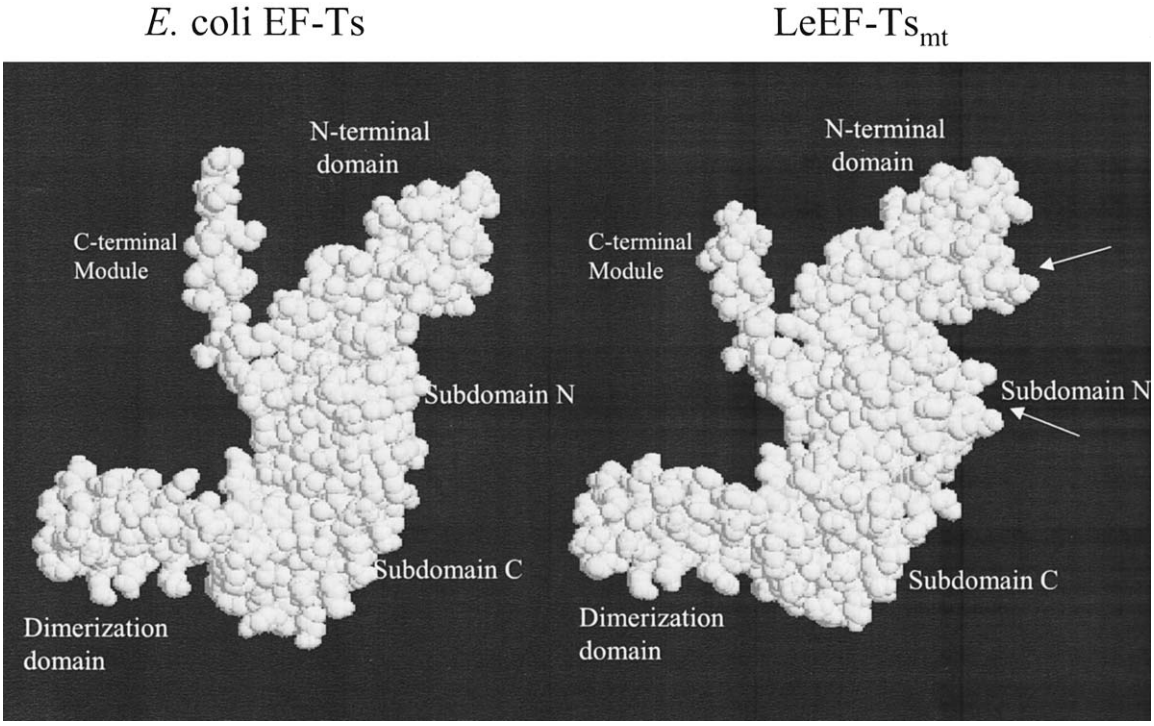

Figure 3. Three-dimensional model of LeEF-Ts$_{mt}$: a 3-dimensional model of LeEF-Ts$_{mt}$ was constructed with Swiss-Model (Peitsch *et al.*, 1995; Guex and Peitsch, 1997) and the coordinates for *E. coli* EF-Ts present in the EF-Tu:Ts complex (Kawashima *et al.*, 1996), coordinate file 1EFUB.pdb in the protein structure database. A space filling model of *E. coli* EF-Ts is shown on the left with the major domains indicated. The predicted structure of LeEF-Ts$_{mt}$ is shown on the right. Arrows indicate two of the regions where insertions occur in LeEF-Ts$_{mt}$ based on the alignments (Figure 2).

ized to mitochondria, the direct demonstration of its localization within this organelle remained necessary (Sjöling and Glaser, 1998).

Targeting of LeEF-Ts$_{mt}$ to mitochondria

The subcellular localization of LeEF-Ts$_{mt}$ was examined using a protoplast transient expression system and a series of LeEF-Ts$_{mt}$-GFP fusion constructs. Several constructs were prepared including (1) GFP alone, (2) a construct consisting of the full-length LeEF-Ts$_{mt}$ fused to GFP (LeEF-Ts$_{mt}$-GFP), (3) the N-terminal 101 amino acids of LeEF-Ts$_{mt}$ fused to GFP and (4) the C-terminal 290 amino acids of LeEF-Ts$_{mt}$ fused to GFP (Figure 4A). These constructs were all placed under the control of a dual CaMV-35S promoter, transfected into protoplasts and examined for the location of the encoded protein product by confocal microscopy. GFP alone was located all over the cytosolic and nuclear compartments, as expected (Figure 4Ba). However, as indicated in Figure 4Bb, when GFP was fused to full-length LeEF-Ts$_{mt}$, it was targeted to the mitochondria. Fusing GFP to the N-terminal portion of the LeEF-Ts$_{mt}$ including the putative mitochondrial signal peptide was sufficient to restrict the targeting of the GFP recombinant protein to mitochondria (Figure 4Bc). When either LeEF-Ts$_{mt}$-GFP or the N-terminal portion of LeEF-Ts$_{mt}$ was fused to GFP, there was a strict superimposition (merged right image) of the fluorescence induced by GFP (left image) and the staining of the mitochondria with the MitoTracker Orange mitochondria-specific dye (central image). By contrast, the LeEF-Ts$_{mt}$ protein lacking the signal peptide (C-terminal construct, Figure 4Bd) was unable to direct the fusion protein into mitochondria. To exclude the possibility of chloroplast targeting of LeEF-Ts$_{mt}$, the LeEF-Ts$_{mt}$-GFP construct was introduced in sunflower protoplasts that comprised a large number of chloroplasts as compared to BY-2 cells (Figure 4Vb). As expected, the GFP signal did not match with the chorophyll fluorescence, indicating that the LeEF-Ts$_{mt}$ protein could not enter the chloroplasts. Altogether, these data not only show that the LeEF-Ts$_{mt}$ protein is exclusively localized to

the mitochondria but also demonstrate the role of the N-terminal signal peptide in directing the targeting.

Interactions of LeEF-Ts$_{mt}$ with E. coli *EF-Tu and activity in poly(U)-directed polymerization*

The full-length sequence of LeEF-Ts$_{mt}$ minus the signal peptide was expressed in *E. coli* as a His-tagged protein allowing its purification on Ni-NTA resins. This protein is predicted to have a molecular weight of 42 kDa. The initial purification of LeEF-Ts$_{mt}$ from *E. coli* was carried out with the standard protocol for obtaining His-tagged *E. coli* EF-Ts from cells. In this purification scheme, 10 μM GDP is added to all the buffers used. The binding of EF-Ts to EF-Tu is mutually exclusive to the binding of GDP. Thus, buffers containing GDP generally promote the dissociation of the EF-Tu:Ts complex allowing the purification of EF-Ts free of EF-Tu contamination. Analysis of the Ni-NTA-purified LeEF-Ts$_{mt}$ preparation by SDS-PAGE obtained after purification on Ni-NTA agarose indicated the presence of two major bands (Figure 5). The most intense band had a molecular mass of about 41.5 kDa consistent with the amino acid sequence predictions for LeEF-Ts$_{mt}$ (Figure 5, lane 3). The second major band had a molecular mass of about 43 kDa as would be expected for *E. coli* EF-Tu. This observation suggested that a certain fraction of the cellular EF-Tu was retained on the Ni-NTA column by LeEF-Ts$_{mt}$ when preparations were carried out in 10 μM GDP. Similar observations have been made with the mammalian EF-Ts$_{mt}$ (Xin *et al.*, 1997).

To test the possibility that this sample of LeEF-Ts$_{mt}$ was bringing *E. coli* EF-Tu along with it, the sample was tested for the ability to replace *E. coli* EF-Tu in poly(U)-directed polymerization of phenylalanine on *E. coli* ribosomes. This sample was active in the polymerization assay indicating that it contained significant amounts of EF-Tu (data not shown). The presence of *E. coli* EF-Tu in this preparation strongly suggests that LeEF-Ts$_{mt}$ is the tomato mitochondrial EF-Ts and indicates that the plant factor may interact with bacterial EF-Tu more strongly than does *E. coli* EF-Ts. It should be noted that bovine mitochondrial EF-Ts binds *E. coli* EF-Tu about 100-fold more tightly than does *E. coli* EF-Ts (Zhang *et al.*, 1997).

To be able to test the ability of LeEF-Ts$_{mt}$ to stimulate the activity of EF-Tu in protein synthesis more directly, it was essential to remove the contaminating EF-Tu. With the bovine mitochondrial factor, the heterologous *E. coli* EF-Tu:EF-Ts$_{mt}$ complex was dissociated with harsh chemical denaturants. However, it was possible to remove most of the *E. coli* EF-Tu from the LeEF-Ts$_{mt}$ preparation by using very high GDP concentrations during the purification procedure. For these experiments, a preparation of LeEF-Ts$_{mt}$ containing EF-Tu as a contaminant was incubated with 1 mM GDP and then purified on the Ni-NTA resin in the presence of this high concentration of guanine nucleotides. Under these conditions, most of the EF-Tu bound to GDP releasing it from LeEF-Ts$_{mt}$. Analysis of this sample by SDS-PAGE (Figure 5, lane 4) indicated that the majority of the *E. coli* EF-Tu was removed from the LeEF-Ts$_{mt}$ preparation under these conditions. About 50% of the LeEF-Ts$_{mt}$ was recovered in this step.

The purified LeEF-Ts$_{mt}$ was then tested for contamination by EF-Tu by examining its activity in polymerization in the absence of added EF-Tu. As indicated in Figure 6, polymerization activity of the LeEF-Ts$_{mt}$ sample was low in the absence of added EF-Tu, indicating that this preparation had only trace amounts of EF-Tu contamination. However, when purified *E. coli* EF-Tu was added to the reaction, a strong stimulation of polymerization was observed indicating that LeEF-Ts$_{mt}$ was capable of stimulating EF-Tu in protein synthesis.

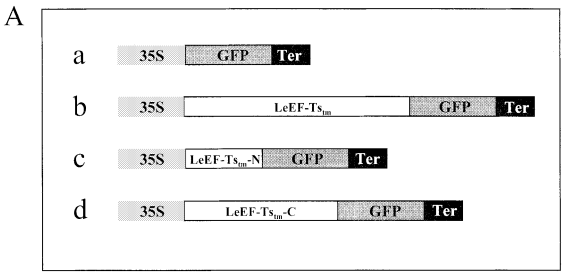

Figure 4. A. Subcellular localization of the LeEF-Ts$_{mt}$-GFP fusion proteins. B, C. Recombinant plasmids were transiently expressed in (B) tobacco BY-2 protoplasts (Bouzayen *et al.*, 1988) or (C) sunflower protoplasts (Chanabé *et al.*, 1989) by a modified polyethylene glycol method. Mitochondria were stained by the mitochondria-specific dye MitoTracker Orange CMH_2TMRos 16 h after transfection. B. Confocal microscopy images in tobacco protoplasts respectively for GFP alone (a), full-length LeEF-Ts$_{mt}$-GFP (b), N-terminal LeEF-Ts$_{mt}$::GFP (c) and C-terminal LeEF-Ts$_{mt}$::GFP (d). The left panel of B indicates GFP fluorescence, the central panel indicates MitoTracker fluorescence, and the right panel merges the two images (GFP fluorescence and MitoTracker fluorescence). C. Confocal images of sunflower protoplast transfected with the full-length LeEF-Ts$_{mt}$-GFP (b). The images correspond, from left to right, to the GFP and chlorophyll fluorescence alone, the two merged colour channels and the DIC image of the same protoplast. Each image represents the projection of 10 to 15 confocal planes. Scale bars indicate 10 μm.

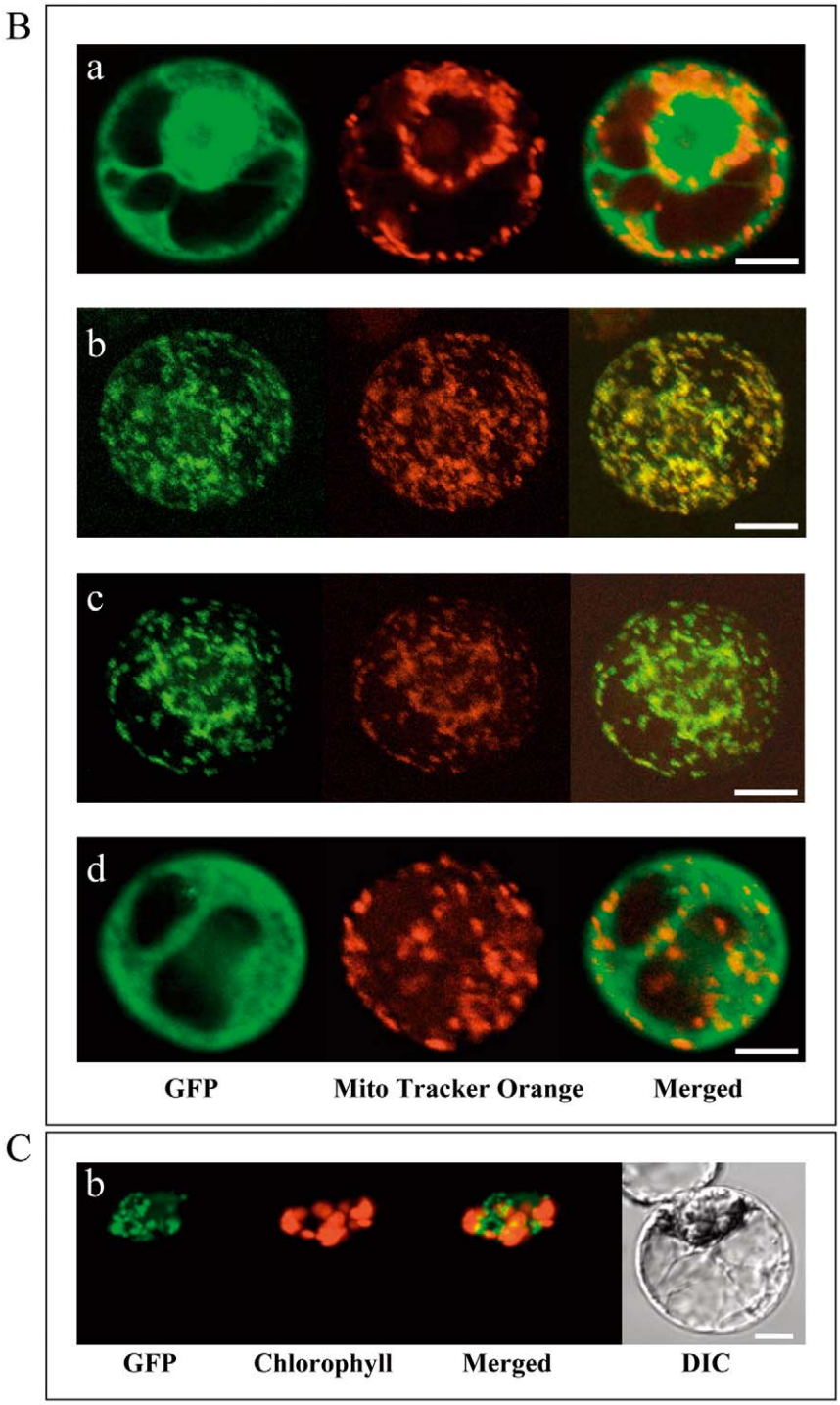

Figure 4. (Continued.)

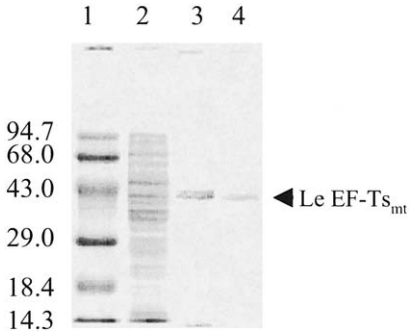

Figure 5. SDS-PAGE analysis of LeEF-Ts$_{mt}$ protein after Ni-NTA affinity purification. Lanes: 1, molecular weight markers; 2, crude extract; 3, protein (500 ng) purified in 10 μM GDP; 4, protein (500 ng) purified in the presence of 1 mm GDP. Proteins were stained with Coomassie Blue.

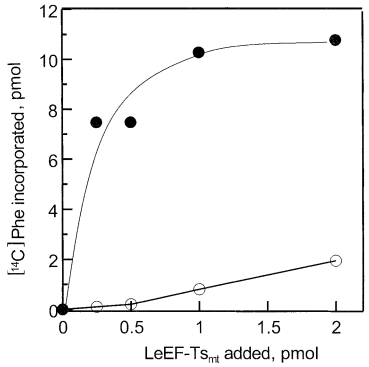

Figure 6. Stimulation of *E. coli* EF-Tu-directed polymerization of phenylalanine by LeEF-Ts$_{mt}$. The incorporation of [^{14}C]Phe into polypeptide was measured as described in Materials and methods. Reaction mixtures contained increasing concentrations of LeEF-Ts$_{mt}$ in the absence of EF-Tu (open symbols). Blanks representing the amount of radioactivity measured in the absence of either EF-Tu or LeEF-Ts$_{mt}$ (less than 0.2 pmol) have been subtracted from each of these values. Alternatively, reaction mixtures were supplemented with a small amount of *E. coli* EF-Tu (closed symbols). The amount of polymerization occurring with EF-Tu alone under the assay conditions used (about 0.5 pmol) has been subtracted from these values.

Nucleotide exchange activity of LeEF-Ts$_{mt}$ *with* E. coli *EF-Tu*

EF-Ts acts as a guanine nucleotide exchange factor promoting the exchange of GDP for GTP on EF-Tu through the formation of an intermediate EF-Tu:Ts complex. The standard way of testing this exchange activity is to monitor the exchange of cold GDP for labelled GDP on EF-Tu. To test the possibility that LeEF-Ts$_{mt}$ was capable of catalysing such a reaction, increasing concentrations of the purified recombinant LeEF-Ts$_{mt}$ were incubated in the presence of *E. coli* EF-Tu:GDP and [^{3}H]GDP. The amount of [^{3}H]GDP bound to EF-Tu was then measured with a nitrocellulose filter binding assay. As indicated in Figure 7, LeEF-Ts$_{mt}$ promoted the nucleotide exchange reaction with *E. coli* EF-Tu. This assay and the polymerization assay described above indicate that LeEF-Ts$_{mt}$ is the mitochondrial factor catalytically equivalent to EF-Ts and that, like bovine EF-Ts$_{mt}$, it is capable of recognizing the prokaryotic EF-Tu.

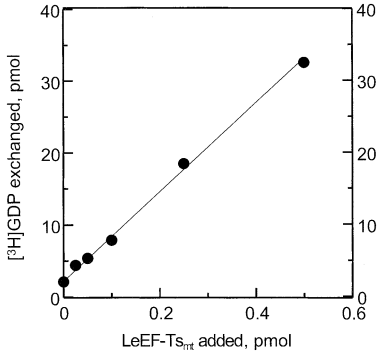

Figure 7. Stimulation of GDP exchange by LeEF-Ts$_{mt}$ using *E. coli* EF-Tu. The stimulation of GDP exchange with EF-Tu was examined as described in Materials and methods. Reaction mixtures contained about 60 units of active EF-Tu and the indicated amounts of LeEF-Ts$_{mt}$. The amount of GDP exchange carried out by EF-Tu alone during the incubation period (about 2.1 pmol) is indicated by the y intercept.

Conclusion

The work presented here describes the isolation and functional characterization of the first mitochondrial elongation factor from higher plants. Previous studies had shown that the LeEF-Ts$_{mt}$ gene is stimulated by ethylene and during fruit ripening (Zegzouti *et al.*, 1999). Tomato fruit, as other climacteric fruit, exhibit a ripening-associated ethylene-dependent climacteric rise in respiration. Despite the increase in respiration, the synthesis of ATP in the mitochondria is reduced due to non-phosphorylating bypasses such as alternate oxidase and uncoupling proteins (Almeida *et al.*, 1999). It is predictable that the reduction in the energetic demand in the mitochondria reduces the need for mitochondrial protein synthesis. Under these conditions, the role of Le-EF-Ts$_{mt}$ in nucleotide exchange for protein synthesis would decrease. However, EF-Ts also plays a role in stabilising the nucleotide free form of EF-Tu in an active status under conditions where nucleotide pools may not be maintained at a sufficient level (Cai *et al.*, 2000b). Hence, we speculate that the stimulation by ethylene of EF-Ts$_{mt}$ during fruit ripen-

ing would occur to stabilize EF-Tu_{mt} for later use or long-term storage in the tissue.

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