Supplementary Results

In vitro import of the TYMV tRNA-like structure into isolated mitochondria

Mitochondrial import of the TYMV tRNA-like structure (TLS) was tested in vitro in controlled conditions. The Arabidopsis thaliana tRNA\(^{\text{Ala}}\)\(^{\text{(UGC)}}\) was used as a positive control for uptake, as alanine tRNAs are part of the cytosolic tRNAs naturally imported into mitochondria in plants (1). The A. thaliana tRNA\(^{\text{Ala}}\) and the TYMV TLS RNA were synthesized in vitro with T7 RNA polymerase in the presence of [\(\alpha\)-\(\text{32P}\)]UTP. The transcripts radiolabeled to a similar specific activity (Figure S1, lanes 1 and 4) were used as substrates for import into isolated Solanum tuberosum organelles. Previous experiments (2) showed that in vitro tRNA uptake into mitochondria is ATP-dependent, with an optimum around 5 mM ATP. Indeed, in the presence of 1 mM ATP, only a small amount of tRNA\(^{\text{Ala}}\) or TYMV TLS was incorporated into the organelles (Figure S1, lanes 2 and 5). Conversely, a strong increase in the mitochondrial uptake of both transcripts was observed when the ATP concentration in the import medium was raised to 5 mM (Figure S1, lanes 3 and 6). Altogether, these assays established that the TYMV TLS can be imported into isolated plant mitochondria with the same ATP-dependence and the same efficiency as a regular tRNA.

![Figure S1](image.png)

**Figure S1.** In vitro RNA import assays. In vitro-synthesized radiolabeled tRNA\(^{\text{Ala}}\) transcript and TYMV TLS transcript were incubated with isolated potato mitochondria in the presence of 1 mM ATP (lanes 2 and 5) or 5 mM ATP (lanes 3 and 6). The organelles were subsequently treated with RNase and washed. The mitochondrial nucleic acids were extracted, fractionated by polyacrylamide gel electrophoresis and analyzed by autoradiography. An aliquote of tRNA\(^{\text{Ala}}\) transcript (lane 1) and an aliquote of TYMV TLS transcript (lane 4) were run on the gel in parallel with the import samples. Marker sizes are indicated in nucleotide numbers (nt).

Aminoacylation properties of the passenger RNA-PKTLS RNAs

The PSTY-PKTLS, PSTY-PKTLS\(_{\text{met}}\) and Rzatp9-L-PKTLS RNAs were synthesized in vitro and used as substrates for aminoacylation assays in the presence of enriched total and mitochondrial enzymatic extracts from Nicotiana tabacum BY-2 cell suspensions. PSTY-PKTLS and Rzatp9-L-PKTLS showed high \([\text{\(^{\text{3}}\)H}]\)valine accepting activity with both types of extract (Figure S2, a and b) but were not aminoacylated in the presence of labeled methionine (not shown). Conversely, as expected from earlier results with the corresponding TYMV TLS variant (3), PSTY-PKTLS\(_{\text{met}}\) could be charged with methionine (Figure S2c) but not at all with valine.
Proteomic analyses of *N. tabacum* cells expressing the Rzatp9-L-PKTLS chimeric ribozyme

Proteomic analyses with transformed *N. tabacum* cells harvested at day 4 after onset of the estradiol treatment detected no significant early changes in the mitochondrial protein patterns versus untreated cells (Figure S3), indicating, together with the *in organello* protein synthesis pattern (Figure 6c in the main paper), that the expression of the ribozyme directed against atp9 did not lead to pleiotropic non-specific effects. Electrophoresis of membrane fractions on Blue-Native polyacrylamide gels (BN-PAGE) showed that the profile of the mitochondrial oxidative phosphorylation complexes was unchanged (Figure S3a). Western blot analyses with antibodies against NAD9 (Complex I subunit, mitochondrially-encoded), ATP1 (ATP synthase subunit, mitochondrially-encoded), ATP2 (ATP synthase subunit, nuclear-encoded) and the voltage-dependent anion channel (VDAC-1, outer membrane marker) revealed similar levels of these polypeptides in estradiol-treated and untreated transformed cells (Figure S3b).
Supplementary Methods

**In vitro RNA import experiments**

The tRNA\textsuperscript{Ala} and TYMV TLS substrates for import assays with isolated mitochondria were synthesized by *in vitro* transcription with T7 RNA polymerase. To generate the templates for transcription, the corresponding DNA sequences were cloned into the pUC18 plasmid with the T7 RNA polymerase promoter at the 5'-end and a BstNI restriction site at the 3'-end. Transcription of the BstNI-digested recombinant plasmids produced the appropriate RNAs with a 3'-CCA end. Transcription was run with a RiboMAX kit (Promega) in the presence of [\textalpha-\textsuperscript{32}P]UTP (800 Ci mmole). Import assays with isolated *S. tuberosum* mitochondria were carried out as described previously (2).

**Aminoacylation reactions**

The PSTY-PKTLS, PSTY-PKTLS\textsuperscript{met} and Rzatp9-L-PKTLS substrates for aminoacylation assays were synthesized as above by *in vitro* transcription with T7 RNA polymerase, using template sequences cloned into the pUC18 plasmid with the T7 RNA polymerase promoter at the 5'-end and a BstNI restriction site at the 3'-end. Enzymatic extracts were derived from *N. tabacum* BY-2 cells harvested 4 days after subculturing. Extracts were prepared from whole cells or from isolated mitochondria. For total extracts, cells were ground in liquid nitrogen and resuspended in 5x enzyme buffer (1x enzyme buffer: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 10% v/v glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with 15 \mu g/ml \textalpha-\textsuperscript{2} macroglobulin, 15 \mu g/ml leupeptin and 40 \mu l/ml of Complete\textsuperscript{TM} (Boehringer-Mannheim) protease inhibitor cocktail. The suspension was centrifuged for 20 min at 50,000 x g. The supernatant was brought to 0.15 M NaCl and loaded onto a DEAE-cellulose column equilibrated with 1x enzyme buffer containing 0.15 M NaCl. The flow-through fraction was submitted to gel-filtration on Sephadex G-50 spin columns equilibrated with 1x enzyme buffer. The excluded fraction was completed with 10 \mu g/ml \textalpha-2-macroglobulin, 10 \mu g/ml leupeptin and 10% v/v propanediol. To prepare organelle extracts, mitochondria were resuspended in 2x enzyme buffer supplemented with \textalpha-2-macroglobulin, leupeptin and protease inhibitor cocktail. Organelles were lysed by 3 freeze/thaw cycles and sonication (3 times 10 sec at 4°C) before proceeding through the same steps as for total extracts. Aminoacylation assays were run as described previously (1) in the presence of 0.1 mM [\textsuperscript{3}H]valine or [\textsuperscript{35}S]methionine and optimal concentrations of enzymatic extracts.

**Protein analyses**

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot experiments were carried out according to standard protocols. Electrophoresis of membrane fractions on Blue-Native polyacrylamide gels (BN-PAGE) was run as described previously (4).
References

1. Maréchal-Drouard, L., Guillemaut, P., Cosset, A., Arbogast, M., Weber, F., Weil, J.H. and Dietrich, A. (1990) Transfer RNAs of potato (*Solanum tuberosum*) mitochondria have different genetic origins. *Nucleic Acids Res.*, **18**, 3689-3696.

2. Delage, L., Dietrich, A., Cosset, A. and Maréchal-Drouard, L. (2003) *In vitro* import of a nuclearly encoded tRNA into mitochondria of *Solanum tuberosum*. *Mol. Cell. Biol.*, **23**, 4000-4012.

3. Dreher, T.W., Tsai, C.H. and Skuzeski, J.M. (1996) Aminoacylation identity switch of *turnip yellow mosaic virus* RNA from valine to methionine results in an infectious virus. *Proc. Natl. Acad. Sci. USA*, **93**, 12212-12216.

4. Giege, P., Sweetlove, L.J., Cognat, V. and Leaver, C.J. (2005) Coordination of nuclear and mitochondrial genome expression during mitochondrial biogenesis in *Arabidopsis*. *Plant Cell*, **17**, 1497-1512.
Table S1. Primers used for reverse transcription, PCR and real-time PCR reactions in various experiments.

| Experiments | Primer name | Sequence 5' > 3' |
|-------------|-------------|------------------|
| Gene constructs | TLS-HDV5P | CATCGGAACAGGATCGGACTG |
| | HDV3PX | GTCTCTAGACTCCCTTAGCCAT |
| | TLS5PB | GACGGAATCCCCGACACCTG |
| | RsLTLS5PH | GTCAAAGCTTCCAATACCCCTGAGCTTGGGAAATAGCAGCT |
| | HDV3PE | AGCAAGAAATCTCCTCTAGGGCAATGGTTCG |
| RT-PCR | TLS3P | TGGTTCCGATGACCCTCGGA |
| | HDV3P | CTCCCTTAGGATCGGATG |
| | PS5P | CGCGGAGATCCGGGCAATG |
| | PRO5P | ATGAATCTCGAATTCCTAGCAGCTTATAGGCAATGGTTCG |
| | PRO3P | CAGATTCTGAAATTCCTAGCAGCTTATAGGCAATGGTTCG |
| | CYSSP | GAAATCTCAGCTTATAGGCAATGGTTCG |
| | CPS2P | CGCGGAGATCCGGGCAATG |
| | Rzatp95P | CCAATACTCAGGCACTTTGCTG |
| | TLSmP | AGGGGCCCAGATCTGGC |
| Real-time RT-PCR | RzATP9-TLS-FW | CCGAAGTGATGTTGAGGTAATA |
| | RzATP9-TLS-RV | CGTAAACTCGGAGCTTACAGAT |
| | ATP9-1-66NT-FW | GCAACAGTGATCAATTGATCAGC |
| | ATP9-1-66NT-RV | TGGCTACAGATCTGCTCAAGG |
| | ACTIN-1FW | GCAGAAGGCGTAACACAGGT |
| | ACTIN-1RV | CGGAGAATCTGATAGGGACAG |
| | RPL2-1FW | CTGCAAAAGCGTAAAGGCC |
| | RPL2-1RV | CGCGGAGATCGGCAATG |
| | PCR templates for transcription | Atcibatps | AGCGAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | Atcibatpas | GTACAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | Ntcibatps | AGCGAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | Ntcibatpas | CAAAGCCCAAATAGGCAATAC |
| | linatps | ATGAGCTTTTGGCCAATAGCAGCTTATGAAAGTTAGCAATGTCAGGAGG |
| | TLS3P | TGGTTCCGATGACCCTCGGA |
| | rzsats | AGCGAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | ext5P | AGCGAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | FLOE | atp9tot3P-ROX | ROX-CTAACGGACTTTAGAATAGGAGAT |
| 5'-RACE | RNA primer | CGACGCGGAGACACGCCACGAGTGAGGAGGATTTG |
| | atp9tot3P | GTACAGAATTTCAATGACTCTACTAGAGTTGATCCGATAG |
| | Generacer3P | CGCGGAGATCCGGGCAATG |
| | Northern | ext5Pnor | CTCGAGCTATGCGACACTCAGGC |
| | TLS3P | TGAGCTTGTTGAGGACTCTGAG |
| | atp9nor5P | CTCTGAGCCATCAGCTTACTG |
| | atp9nor3P | TCGATGAGGAAATAGGAGAT |
| | 18Snor5P | TGGTGCTATGCCATCAGCTTACTG |
| | 18Snor3P | TCGATGAGGAAATAGGAGAT |