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Electroporation of Small RNAs into Plant Protoplasts: Mitochondrial Uptake of Transfer RNAs

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To study tRNA import into plant mitochondria, we have set up a system to follow the fate *in vivo* of tRNA transcripts introduced into plant protoplasts by electroporation. Conditions were optimized for maximum tRNA uptake into potato protoplasts. We have shown that *in vitro* synthesized tRNA transcripts are poor substrates due to rapid degradation leading to low efficiencies of transfer and short life in protoplasts. Labelled natural tRNAs were more efficiently electroporated into protoplasts and they remained stable during protoplast culture. We have observed import into mitochondria of total and purified cytosolic tRNAs in protoplasts but the process was not specific for the tRNA species which are normally imported.

In plant mitochondria three kinds of tRNAs, with different genetic origins, participate in the translation machinery. These tRNAs are coded for by so-called "native" mitochondrial tRNA genes, by chloroplast tRNA genes inserted in the mitochondrial genome and by nuclear tRNA genes (1,2). The import into mitochondria of tRNAs coded for by nuclear genes was demonstrated by expressing heterologous and mutated tRNA genes in transgenic plants (3). The tRNAs expressed from the nuclear transgenes were shown to be present in mitochondria. While valuable information concerning the mechanism of tRNA import into mitochondria can be gained by using transgenic plants, the production of these plants is a time consuming process. Moreover, there is a limit to the extent of modifications that can be tolerated by a tRNA gene without affecting its expression. On the other hand, *in vitro* import of tRNAs into purified plant organelles has not yet yielded conclusive results. As an alternative, we are developing an in-vivo system to analyze import into mitochondria of labelled tRNAs introduced into plant protoplasts by electroporation. This approach would allow us to identify the elements in the tRNA sequence which are determinant in its ability to be imported into mitochondria *in vivo.* We present here results indicating that tRNAs could be introduced into plant protoplasts by electroporation. Labelled tRNAs were subsequently found in mitochondria but their transport was apparently non-specific. The potential use of this system is discussed.

MATERIAL AND METHODS

Protoplast preparation and electroporation. Protoplasts were prepared from potato (Solanum tuberosum L.) cell suspension cultures in MS medium containing vitamins and 0.5 $mg.1^{-1}$ 2-4D. Cells in rapid growth phase (three days post-transfer) were digested overnight at 25°C with cellulase (0.2% w/v) and macerozyme (0.2% w/v) in the culture medium supplemented with 0.4 M mannitol. Protoplasts were sieved through a 100 μm mesh and washed twice in electroporation buffer (10 mM HEPES (Hydroxyethyl piperazine ethanesulfonic), pH 7.2; 150 mM NaCl; 4 mM $CaCl_2$; 0.2 M mannitol), counted and suspended in electroporation solution at a concentration of $2–3 \times 10^6$ $protoplasts.ml^{-1}$. Electroporation was performed using a BioRad Gene Pulser on 800 μl of suspension at a concentration of 2×10^6 to 3.5×10^6 protoplasts/ml in cuvettes with a 4 mm electrode gap. Labelled RNAs (1 to 5×10^6 cpm (counts per minute)) were added to the protoplast mixture a few seconds prior to applying the electric pulse to prevent degradation. Protoplasts were left on ice for 10 min following the electric pulse. To test for viability protoplasts were observed by epifluorescence microscopy after addition of fluorescine diacetate (60 nM final).

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Preparation of RNA. [^{32}P] UTP labelled tRNA transcripts were obtained by runoff transcription of a cloned tRNA(Phe) gene (a gift from Dr. I. Small, (4)). Transcripts were prepared as described (5). Unincorporated nucleotides were separated from labelled transcripts by centrifugation through a 1 ml Sephadex G-50 column. Specific activity of labelled RNAs was measured by Cerenkov counting using a scintillation counter. Cytosolic tRNAs were extracted from potato suspension cells and labelled at their 3′ end using yeast tRNA nucleotidyl-transferase (2). Sequencing of tRNAs was done as previously described (6).

Protoplast RNA extractions and analysis. Electroporated protoplasts were transferred into an eppendorf tube, treated with RNAse A (1 μg.ml^{-1}) at room temperature for 3 minutes, washed twice in culture medium and lysed in RNA extraction solution (3 M urea, 0.5% SDS (sodium dodecyl sulfate), 12 mM aurintricarboxylic acid). The lysate was extracted with an equal volume of phenol/chloroform (v/v). Nucleic acids were ethanol precipitated at −20°C. Radioactivity present in the dried nucleic acid pellet was measured by Cerenkov counting.

For mitochondrial tRNA extraction, electroporated protoplasts (2.× 107) were pooled, diluted five times in culture medium containing 0.4 M mannitol and incubated 45 min at 26°C. Protoplasts were sedimented at 100 g and resuspended in mitochondria extraction buffer (50 mM Tris-HCl, pH 8; 0.3 M mannitol, 3 mM EDTA; 1 mM β-mercaptoethanol, 0.1% bovine serum albumin). Protoplasts were lysed by five strokes in a Dounce homogenizer and mitochondria were purified essentially as described (7) except that the three-step sucrose gradient was replaced by a two-step gradient (1.6 M and 1 M sucrose) in a 5 ml polypropylene ultracentrifugation tube. The final pellet of mitochondria was resuspended in 50 μl of 0.3 M sucrose, 50 mM Tris-HCl (pH 8), 2à mM EDTA. The outer membrane was disrupted by osmotic shock. Resulting mitoplasts were treated with a mix of three RNAses (20 μg.ml^{-1} RNAseA, 200 U.ml^{-1} RNAse T1 and 0.4 U.ml^{-1}.phosphodiesterase) and washed twice in the same buffer. Mitochondrial tRNAs were extracted as described (3).

Analysis of tRNAs. Nucleic acids extracted from protoplasts and mitochondria were analyzed on a 15% denaturing polyacrylamide gel as described (8). Two-dimensional gel electrophoresis was performed as described (9). Gels were fixed in a 10% acetic acid (v/v), 10% ethanol (v/v) bath, dried and exposed to an X-ray film or to an imaging plate (Fuji). Imaging plates were scanned using a Bio-imaging analyzer (Fujix, Bas 1000) with Fuji's MacBAS software. Bean tRNA(Pro) and tRNA(Leu) were previously purified in our laboratory (8).

RESULTS AND DISCUSSION

To optimize conditions for the electroporation of tRNAs into protoplasts we have used *in vitro* synthesized tRNA transcripts. An *Arabidopsis thaliana* tRNA gene, coding for cytosolic tRNA(Phe) (4) was used as a template to obtain [^{32}P]UTP labelled tRNA-sized transcripts. The tRNA(Phe) gene construct was used because of its high yield of transcripts. The fact that two major tRNA-sized transcripts were obtained from the template (Fig. 2, lane 1) was not an obstacle to its use to optimize electroporation conditions.

Electroporation efficiency was determined by measuring the quantity of labelled transcripts in protoplasts after electroporation either in a total nucleic acid extract or in the bands corresponding to the full-sized tRNA transcripts after polyacrylamide gel electrophoresis. Protoplasts were treated with RNAse A to eliminate non-incorporated tRNA transcripts. To avoid degradation prior to electroporation, transcripts were mixed with protoplasts only seconds before the electric pulse was applied.

Various field strengths and pulse durations were tested. Pulse length was controlled by varying capacitance and resistance. First, capacitance was fixed at 500 μF and resistance at 100 W (giving approximately an 18 ms pulse or time constant) and a field strength varying from 100 to 400 V was applied to the protoplast/tRNA mix. An increase in tRNA uptake by protoplasts was observed with decreasing voltage (Fig. 1), with a maximum uptake at 100 V corresponding to a field strength of 250 volt.cm^{-1}. Lower voltage has not been tested.

With a field strength fixed at 100 volts, a pulse of increasing duration was applied to the sample by using capacitance settings of 125, 250, 500 and 960 μF. Longer pulse durations were obtained by applying two consecutive pulses to the same sample. A decrease in RNA uptake into protoplasts was observed with increasing pulse duration up to a maximum of about 38 ms (Fig. 2). The amount of tRNA transcripts introduced into the protoplasts was proportional to the amount of tRNA transcripts used in the experiment. When the quantity of radiolabelled tRNA was doubled from 1.5 × 10^6 cpm to 3 × 10^6 cpm the amount of incorporated counts also doubled (Fig. 2, lane 8).

The value of total incorporation, estimated by Cerenkov counts measured in the dried nucleic

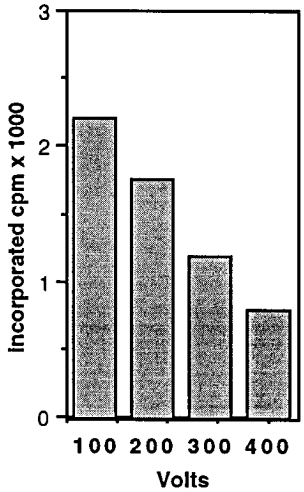

FIG. 1. Voltage dependence of tRNA transcript uptake by protoplasts. Pulses of increasing voltage (100–400 V) and constant length (18 ms) were used to electroporate tRNA transcripts into protoplasts. Transcript uptake into protoplasts was measured by Cerenkov-counting of full-length tRNA transcripts extracted from electroporated protoplasts.

acid pellet, was found to vary from one experiment to the other between 3 and 9% of the total amount of labelled transcripts used.

The highest efficiency of tRNA uptake into protoplasts was obtained with a relatively low field

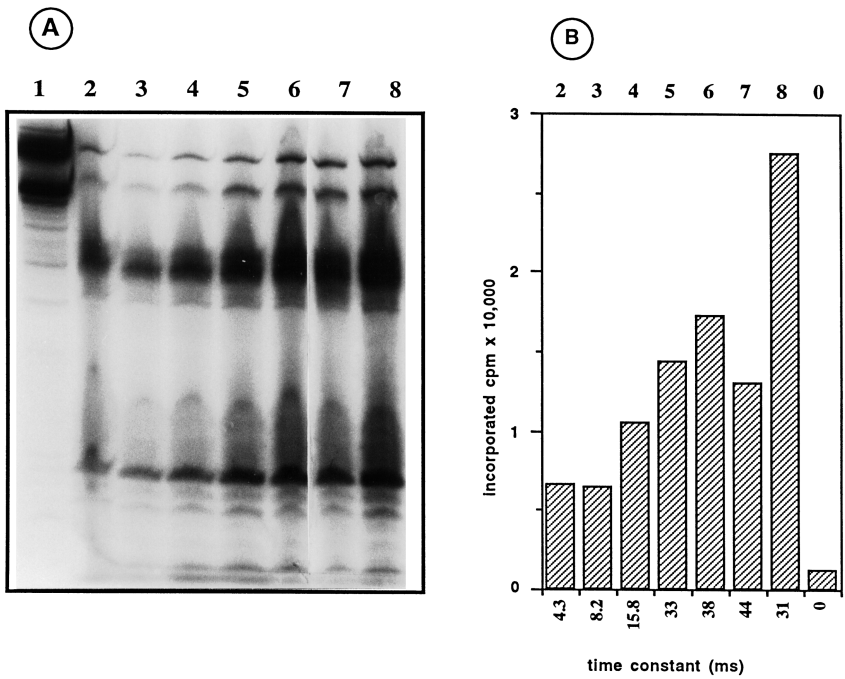

FIG. 2. Pulse length (time constant) dependence of transcript uptake by protoplasts. Pulses of increasing length (4.3 to 44 ms) and constant voltage (100 V) were used to electroporate 1.5×10^6 cpm of transcripts (lane 2–7) or 3×10^6 cpm of transcripts (lane 8) into 2×10^6 protoplasts. Lane 1: transcripts used for electroporation. Lane 0 is the nonelectroporated control. (A) Electrophoresis on a 15% denaturing polyacrylamide gel of nucleic acids extracted from electroporated protoplasts. (B) Transcript uptake into protoplasts was measured by Cerenkov-counting of the gel-bands corresponding to the full-length tRNA transcripts.

strength of 250 V/cm compared to previously described electroporation conditions using DNA and RNA (10,11) but these conditions were very similar to conditions used to electroporate aminoacyl-tRNAs into mammalian cell protoplasts (12).

We have used run-off transcripts of a tRNA(Phe) gene because it would be possible to modify the primary structure of the tRNA by site directed mutagenesis of the template DNA. This would allow identification of nucleotides which are determinant in the ability of the tRNA to be imported into mitochondria. However, these transcripts turned out to be poor substrates for electroporation because of a high rate of degradation. The migration pattern of radiolabelled tRNA transcripts extracted from the electroporated protoplasts showed extensive degradation after 10 min of incubation on ice (Fig. 2), resulting in a number of bands below the bands corresponding to the tRNA transcripts. The top two bands corresponding to the tRNA gene transcription products were cut out of the gel and counted using a scintillation counter. In the example given in Fig. 2, the amount of full-length transcripts represents approximately 2% of the total amount of labelled transcript extracted from the protoplasts. We have also observed that transcripts were degraded very rapidely prior to electroporation when mixed with the protoplast suspension, probably due to the release of RNAses by lyzed protoplasts. By reducing pre-electroporation time to a few seconds it was possible to avoid massive degradation of the transcripts. High instability of the tRNA transcripts could be due to the absence of modified nucleotides resulting in higher sensitivity to RNAses.

In another set of experiments we have used natural cytosolic tRNAs extracted from potato cells and labelled by incorporation of [^{32}P]ATP at their 3′ end using yeast tRNA nucleotidyl transferase. Uptake of these tRNAs into protoplasts was tested using the conditions of electroporation that were found to be optimal when using *in vitro* tRNA transcripts (100 V, 960 μF, 100 W). The total amount of labelled nucleic acids incorporated into protoplasts was found to be three to ten times higher than when using *in vitro* transcripts. About 20% of these tRNAs remained intact after electroporation (Fig. 3). Decay of tRNAs in protoplasts was monitored over 80 min of culture at 26°C (Fig. 3). The total amount of tRNAs taken up by protoplasts, as well as the proportion of intact tRNAs present at different time points, were measured. A decrease in the total amount of labelled tRNAs was observed over culture time, while the ratio of intact tRNAs vs total labelled RNA did not vary significantly over time. Because of the higher stability of the natural tRNAs it was possible to improve their uptake into protoplasts by delivering two consecutive electric pulses (not shown).

To monitor tRNA uptake into mitochondria a total of 30×10^6 protoplasts were electroporated with 8×10^7 cpm of labelled potato cytosolic tRNAs in the conditions described above and cultured for 45 min at 26°C. Protoplast survival after electroporation and 1-hour culture at 26°C was monitored by counting live cells using fluoresceine diacetate staining. A 40% loss of protoplasts was observed in these conditions. Mitochondria were extracted and purified. Mitoplasts were RNAse treated and nucleic acids were extracted. The nucleic acid pellet extracted from mitochondria contained 6,000 Cerenkov cpm. Labelled tRNAs were separated by two dimensional polyacrylamide gel electrophoresis. A finger print of tRNAs extracted from mitochondria is shown in figure 4 B in parallel with a fingerprint of the same tRNA population before electroporation (Fig. 4 A). It appeared that a majority of the tRNAs that were electroporated into the protoplasts were found in the mitochondrial fraction, with comparable relative intensities on the 2-D gel autoradiograph. At least 16 major spots could be identified in the mitochondrial fraction while only eleven major tRNA species are known to be imported into mitochondria in potato (13). A noticeable exception are tRNA spots located near the migration origin. In this part of the gel there are seven major spots and about 10 minor ones that do not appear in the pattern corresponding to the mitochondrial fraction (Fig. 4 A and B). These tRNAs are in general the larger ones that possess a long extra-loop such as the serine and leucine tRNAs. These two observations suggest that the tRNAs found in the mitochondrial fraction have not been transported into mitochondria via the

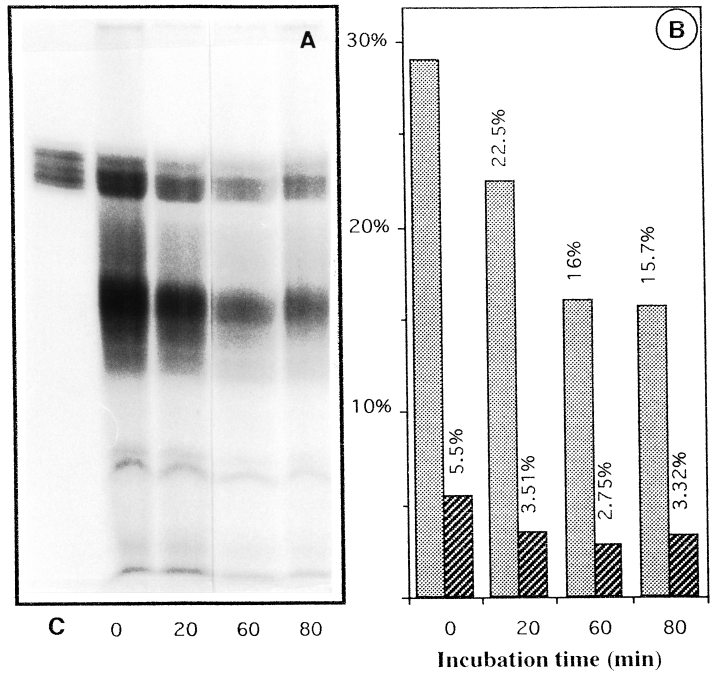

FIG. 3. Decay of electroporated cytosolic tRNAs during protoplast culture. Cytosolic tRNAs labelled at their 3′ end (lane c) were electroporated into protoplasts. (A) Transfer RNAs were extracted after 0, 20, 60 and 80 minutes of protoplast culture and analyzed on a 15% polyacrylamide gel. (B) Graph illustrating percentage of total electroporated tRNAs found in protoplasts (⁙) and the percentage of incorporated tRNAs which are intact at different time points tested (▨).

normal tRNA import apparatus which is apparently highly specific toward the imported tRNA species (1). We could also observe that two tRNA spots have a higher relative intensity in the mitochondrial fraction compared to their relative intensity on the control gel. However these tRNAs were present in insufficient quantities to be identified. The presence of labelled tRNAs in the mitochondrial fraction, resistant to RNAse treatment, could be due to tRNA uptake into mitochondria during the electroporation process. The fact that tRNAs are rapidely degraded outside and inside mitochondria raises the question of whether the labelled tRNAs observed in mitochondria could have been labelled in mitochondria by incorporation of labelled nucleotides released by degradation of electroporated tRNAs. Indeed free nucleotides would easily cross both cytoplasmic and mitochondrial membranes. But released and reincorporated labelled nucleotides should be randomely distributed among endogenous RNAs which is not the case in our experiment as most of the slow migrating tRNA spieces are not labelled.

In order to test our system regarding specificity of tRNA import into mitochondria we have used two purified or partially purified tRNAs. Purified bean cytosolic tRNA(Pro), which is a non imported species in potato, was chosen as a negative control and partially purified bean cytosolic tRNA(Leu), an imported tRNA species in potato was used as a positive control. Figure 5 (lane 2) shows that the fraction containing tRNA(Leu) also contains two other, yet unidentified, tRNA species. The identity of the tRNA in the top band was confirmed by sequencing as being the tRNA(Leu). Purified tRNAs were labelled using tRNA nucleotidyl transferase, mixed together and electroporated into protoplasts. After 45 min at 26°C mitochondrial tRNAs were extracted and analyzed on a 10% polyacrylamide gel (Fig. 5). Figure 5 (lane 1) shows the tRNAs extracted from the mitochondrial fraction of electroporated protoplasts. After extended exposure using an imaging plate, a band corresponding to the lowest band of the tRNA(Leu) fraction was revealed. The band

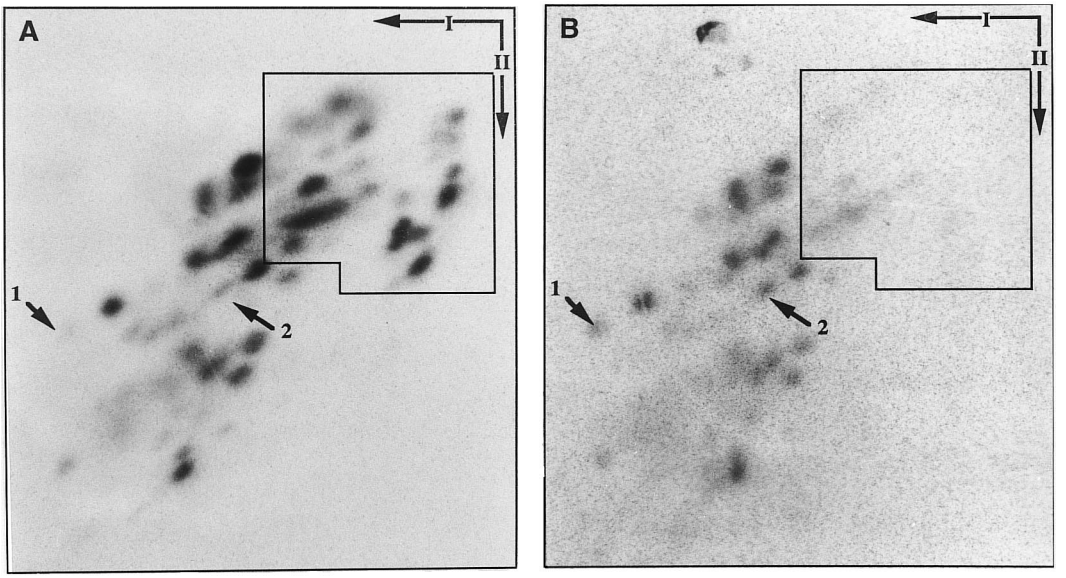

FIG. 4. Analysis of mitochondrial tRNAs of electroporated protoplasts. Two-dimensional gel electrophoresis of control labelled potato cytosolic tRNAs (A) and of the tRNAs extracted from mitochondria of electroporated protoplasts (B). Transfer RNAs absent in the mitochondrial fraction are framed (A). Arrows point to the two tRNA spots that are more intense in the pattern of the the mitochondrial fraction relative to neighbouring spots.

corresponding to the tRNA(Leu), normally imported into mitochondria, was not found in the mitochondrial fraction in our experiments. Partial sequencing of the imported tRNA showed no significant sequence homology with a known tRNA(Leu) but the sequence information obtained was not sufficient to identify this tRNA.

As for the experiment using total cytosolic tRNAs, if in vivo labelling of tRNA had occured, tRNAs would have been labelled randomly. As only one of the tRNA introduced by electroporation can be detected in mitochondria one can perclude this hypothesis.

In these two experiments mitochondrial uptake of tRNAs seems to be related to the gel mobility

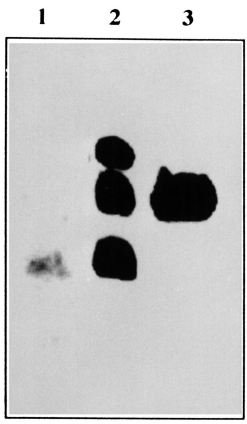

FIG. 5. Electroporation of purified tRNA species into protoplasts. Polyacrylamide gel electrophoresis of purified tRNA proline (lane 3) and partially purified tRNA leucine (lane 2) used for electroporation. Lane 1 corresponds to the tRNAs extracted from mitochondria of electroporated protoplasts. Top band of lane 2 is tRNA(Leu) (CAA). (picture was obtained using a Bio-Imager).

of the tRNAs. When total cytosolic tRNAs were used the slow migrating species near the origin of the gel were not found in mitochondria. With purified tRNA species, only the fastest migrating tRNA was found in mitochondria.

We have set up conditions to electroporate tRNAs into plant protoplasts in order to study the import of tRNAs into plant mitochondria. Such a system was used previously by Tarassov et al. 1992 (14) to study mitochondrial import of tRNA(Lys) in yeast yet it does not seem to be applicable to plant cells because of apparent non specific uptake of tRNAs into mitochondria during the electroporation process. Other attempts to introduce tRNA into mammalian cells by electroporation (12) or by passive diffusion through a perforated membrane (15) have shown that the tRNAs do not necessarily become available to the cell processes (such as translation) because of structural organization of the cell leading to channeling of the substrates. In view of these fact crossing the protoplast membrane may only be the first of a series of obstacles to be overcome by the exogenous tRNA to be taken up by the tRNA import apparatus.

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