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LUPIN CHLOROPLAST AND MITOCHONDRIAL tRNA POPULATIONS AND THEIR GENES*

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Transfer RNAs isolated from lupin chloroplasts and mitochondria were compared by two-dimensional gel electrophoresis. Twenty chloroplast and 24 mitochondrial tRNA species were identified. The saturation hybridization between lupin chloroplast DNA and ^{125}I -labelled lupin chloroplast tRNAs pointed to the presence of about 34 tRNA genes in lupin chloroplast DNA. The number of mitochondrial tRNA genes estimated by the same method was about 30 genes. *EcoRI* restriction digest of lupin mitochondrial DNA probed with ^{32}P -labelled lupin mitochondrial tRNAs revealed only a small number of positive restriction fragments. Some of these mitochondrial restriction fragments hybridized with ^{32}P -labelled chloroplast tRNA.

Chloroplasts and mitochondria contain a complement of transfer RNAs (tRNAs) that function specifically in organellar protein synthesis and are encoded by organellar DNAs. Studies based on two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and hybridization techniques led to characterization of chloroplast tRNA (ct-tRNA) population from various plants and to the estimation of the number of tRNA genes [1 - 8]. The determination of complete sequence of chloroplast DNA (ct-DNA) from liverwort and tobacco has widened our knowledge about tRNA gene organization and confirmed the previous results on the number of tRNA genes encoded by the chloroplast genome [9, 10].

So far, the information available on plant mitochondrial tRNAs (mt-tRNAs) and their genes is rather scarce. The limited data exist on the overall genomic organization of plant mt-tRNA genes and the sequence of

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these genes ([11 - 14] and references therein). The plant mt-tRNAs sequenced up till now namely tRNA^{Phe}, tRNA^{Trp}, tRNA^{Pro}, two tRNAs^{Tyr}, tRNA^{Met}, tRNA^{Met}_m, come only from *Phaseolus vulgaris* [13].

There is evidence that the two tRNA^{Leu} from bean mitochondria and their cytoplasmic counterparts are identical except for one post-transcriptional modification, and that at least eight cytoplasmic tRNAs are imported from cytoplasm into bean mitochondria [15, 16].

The aim of our work is the comparison of the population of lupin organellar tRNAs by two-dimensional polyacrylamide gel electrophoresis, the evaluation of the number of organellar tRNA genes as well as the comparison of hybridization of mt-tRNAs and ct-tRNAs with the *EcoRI* digested mt-DNA.

MATERIALS AND METHODS

Plant material. Lupin seeds (*Lupinus luteus* cv Topaz) were from Experimental Station Wiatrowo (Poland).

Enzymes. T₄ polynucleotide kinase was from Boehringer. *E. coli* tRNA-nucleotidyltransferase was a gift from Dr. Sprinzl. Snake venom phosphodiesterase was from PL-Biochemicals. Chloroplast and mitochondrial aminoacyl-tRNA synthetases were obtained as previously described [17]. *E. coli* strain MRE 600 was used for preparation of aminoacyl-tRNA synthetase [17].

Isolation of chloroplast DNA. Chloroplasts were isolated by differential centrifugation from 15 - 18-day-old leaves and green cotyledons kept for two days in the dark [18, 19]. DNase treatment was omitted. The chloroplasts were purified further by the use of 30, 45, 60% sucrose gradient during 35 min of centrifugation at 100 000 × *g*. The layer between 30 - 45% sucrose concentration was collected. The final purification was done by Sephacryl S-1000 column chromatography [18].

Isolation of chloroplast tRNAs. Chloroplast tRNAs were prepared by the phenol method as previously described [17] except that phosphate buffer was replaced with 10 mM Tris/HCl buffer, pH 7.5, containing 1% SDS.

Isolation of mitochondrial DNA. Mitochondria were isolated from 5 - 7-day-old etiolated hypocotyls essentially according to Synenki *et al.* [20] and Brennicke [21], respectively, in a medium containing 0.45 M sucrose. After disrupting the tissue, the homogenate was filtered through four layers of gauze, then one layer of 200 μm nylon netting. The mitochondrial pellet was resuspended in the initial medium, centrifugated at 2000 × *g* and subsequently submitted to a gradient of 0.6, 1.2, 1.45 and 1.8 M sucrose and centrifuged for 35 min at 100 000 × *g*. Two layers of mitochondria formed between 1.8 and 1.45 M sucrose were collected. Mt-DNA was finally purified by Sephacryl S-1000 column chromatography [18].

Isolation of mitochondrial tRNAs. The crude mitochondrial pellet obtained from 1 kg of hypocotyls was resuspended in the homogenization medium containing 0.05 M Tris/HCl, pH 8.0, 0.005 M EDTA, 0.005 M mercaptoethanol, 0.2% bovine serum albumin (BSA) and 0.45 M sucrose, centrifuged again at $2000 \times g$ for 10 min to remove remaining chloroplasts and nuclei. Mitochondria were centrifuged through 0.6 M sucrose cushion at $17000 \times g$ for 20 min and resuspended in a medium composed of 0.3 M sucrose, 10 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$ containing 0.3 mg snake venom phosphodiesterase. The suspension was incubated for 8 min at $37^\circ C$, then heated up to $80^\circ C$ for 3 min, and then Triton X-100 0.2% lysed mitochondria were phenol extracted. Mt-tRNAs were purified further by electrophoresis on an 8% polyacrylamide with 4 M urea and a 2.5% stacking gel. The 4S RNA band was located in the u.v. light and recovered [22].

Fractionation and identification of tRNAs. Two-dimensional gel electrophoresis of crude organellar tRNAs was carried out as described by Steinmetz [23]. tRNA [8-9 units as determined at 260 nm) was applied on the gel [24]. The tRNAs were identified by aminoacylation in the presence of either *E. coli*, chloroplast, or mitochondrial aminoacyl-tRNA synthetases and 3H - or ^{14}C -labelled amino acids. Each aminoacylation was carried out in the presence of 19 other cold amino acids and 0.04-0.1 mg of enzymatic protein.

Transfer of DNA fragments to nitrocellulose filters. DNA fragments were transferred from agarose gels to nitrocellulose by the method of Southern [25].

Labelling of tRNAs. The tRNAs were labelled with ^{125}I according to Commerford [26]. Unreacted ^{125}I was removed by Sephadex G-25 filtration. Concentration of the ^{125}I -labelled tRNAs were determined by A_{260} measurements. Radioactivity was measured in a Beckman LS-100 scintillation counter in Biofluor scintillation liquid. Specific radioactivities of the labelled tRNAs were in the range of $2-3 \times 10^6$ c.p.m. μg .

^{32}P -Labelled tRNAs were prepared using T_4 polynucleotide kinase and [γ - ^{32}P]ATP or tRNA nucleotidyl transferase and [α - ^{32}P]ATP [27, 28].

Hybridization experiments. For hybridization of iodinated tRNAs to organellar DNAs, 1 μg samples of ct-DNA and mt-DNA were fixed to 9 mm nitrocellulose filter and incubated in $2 \times SSC$ ($1 \times SSC$ is 0.15 M NaCl and 0.015 M sodium citrate pH 7.0) and 40% formamide, 0.1% SDS at $37^\circ C$ for 32 h with different concentrations of the labelled tRNAs. The filters were washed twice for 10 min each time in $2 \times SSC$ and incubated in $2 \times SSC$ with RNase A (20 $\mu g/ml$) for 30 min at $37^\circ C$, washed again twice with $2 \times SSC$ and counted in Biofluor. The results were corrected for the background counts taken from hybridization of cytoplasmic tRNAs to ct-DNA and mt-DNA. tRNAs isolated from dry lupin seeds were considered cytoplasmic tRNAs.

The ^{32}P -labelled ct-tRNAs and mt-tRNAs were hybridized against restriction fragments of mitochondrial DNA bound to nitrocellulose filter for 16 h at 42°C in 5×SSC, 1×Denhardt sol., 50% formamide, 0.01% SDS. The filter was prehybridized in the same mixture for 2 h. After hybridization, the filter was washed twice for 15 min at the same temperature and with the same solution, then twice for 15 min in 5×SSC with 0.1% SDS at 42°C, and finally once for 60 min in 1×SSC at room temperature. The filter was dried and subjected to autoradiography.

To elute the hybridized mt-tRNAs, the nitrocellulose filter was placed in double-distilled water twice for 30 s at 95°C. Before the next hybridization step the filter was checked for remaining radioactivity.

Chemicals. Acrylamide, *N,N*-methylene bisacrylamide, Tris base, boric acid, urea were from Serva. Agarose was from Sigma, Sephacryl S-1000 was from Pharmacia. Biofluor was from NEN and [^{32}P]ATP was from Amersham, Na ^{125}I was from the Institute on Nuclear Research (Świerk, Poland).

RESULTS

Total unlabelled chloroplast tRNAs were fractionated by 2-D PAGE into 35 spots. Twenty spots were identified as tRNAs by aminoacylation with either chloroplast or *E. coli* aminoacyl-tRNA synthetases (Fig. 1). These 20 tRNAs are specific for 12 amino acids. A preliminary report of this work was presented at the EMBO-FEBS tRNA Workshop-Strasbourg, July 1980. Meanwhile Mubumbila [29] reported the separation of lupin chloroplast tRNAs by 2-D PAGE. He has localized the position of tRNAs specific for histidine, glycine, alanine and aspartic acid which we have not tested, but we have additionally identified the tRNAs specific for methionine and lysine. Our evidence suggests the presence of two tRNA^{Val} in the lupin chloroplast tRNA population. As it is shown on Fig. 1 a few tRNAs are represented by more than one isoacceptor: tRNA^{Ser}—3, tRNA^{Leu}—3, tRNA^{Ile}—2, tRNA^{Arg}—2, tRNA^{Lys}—2. The amino acids for which tRNAs have not been so far identified are: cysteine, glutamic acid, glutamine and asparagine. Our failure to detect some tRNAs on 2-D PAGE may be due to the inactivation of these tRNAs during electrophoresis, or to the inactivation of the corresponding aminoacyl-tRNA synthetases. Some of the spots on 2-D PAGE are probably not tRNAs since their migration patterns correspond to rather small ribosomal RNAs; other spots might contain degradation products.

It should be pointed out that separation patterns for chloroplast tRNAs were quite reproducible, and the level of amino acids acceptance both by the chloroplast and *E. coli* aminoacyl-tRNA synthetases was similar.

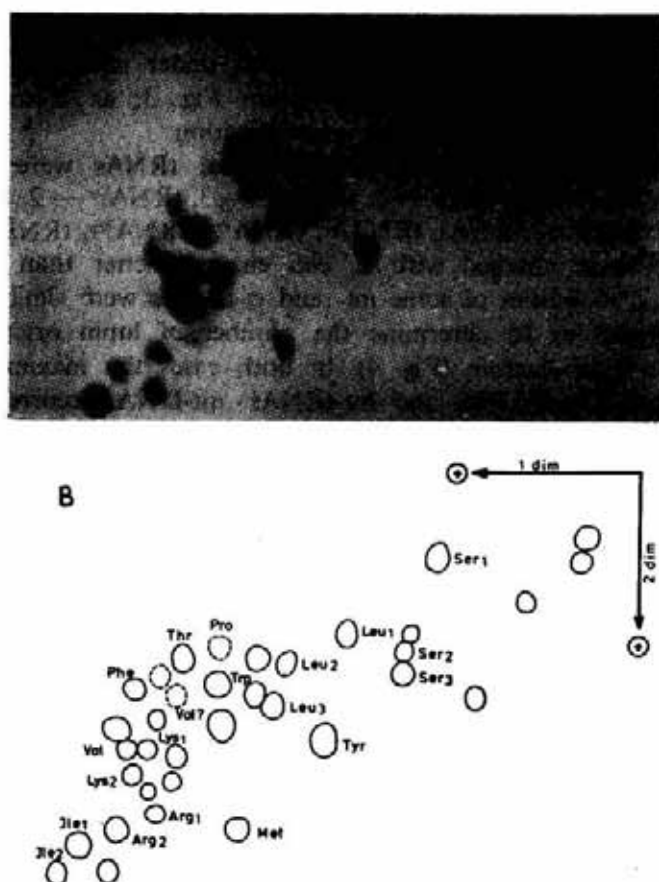


Fig. 1. Fractionation of lupin chloroplast tRNAs by two-dimensional polyacrylamide gel electrophoresis.

A. A representative pattern of the methylene stained gels after electrophoretic separation of chloroplast tRNAs using 10% polyacrylamide/4M urea for 38 h at 450 V in the first dimension, and 20% polyacrylamide/4 M urea for 120 h at 350 V in the second dimension.

B. Diagrammatic representation of chloroplast tRNA separation. The amino acids accepted by each spot (as revealed by aminoacylation) are indicated.

Lupin mitochondrial tRNAs separated on 2-D PAGE into approximately 34 spots. Twenty four of them were identified as tRNAs specific for 14 amino acids by aminoacylation with either mitochondrial of *E. coli* aminoacyl-tRNA synthetases (Fig. 2). In some sets of 2-D PAGE differences in the pattern of separated spots were detected. They partly depended on the age of hypocotyls taken for isolation of mitochondria. Despite all the differences recorded, including the presence of spots that were stronger than previously detected, the pattern of mitochondrial tRNA population included always about 20 species (Fig. 2). To ascertain whether any of

the changes in the pattern could result from chloroplast contamination, the mitochondrial preparations were analysed under microscope. A typical section of lupin mitochondria is shown on Fig. 3; as it may be seen, there was no detectable chloroplast contamination.

As in the case of ct-tRNA, mitochondrial tRNAs were represented by a few isoacceptors: tRNA^{Arg} — 3, tRNA^{Leu} — 3, tRNA^{Ser} — 2, tRNA^{Ile} — 2, tRNA^{Tyr} — 2. Some mt-tRNAs, tRNA^{Tyr}, tRNA^{Phe}, tRNA^{Trp}, tRNA^{Lys}, tRNA^{Val} and tRNA^{Met} were charged with *E. coli* enzyme better than others. The electrophoretic mobilities of some mt- and ct-tRNAs were similar. All these results prompted us to determine the number of lupin organellar genes by saturation hybridization (Fig. 4). In both cases the maximum hybridization in ct-tRNAs-ct-DNA and mt-tRNAs-mt-DNA occurred when the tRNA/DNA ratio was about 5.0. In the case of ct-DNA 0.88% of this DNA formed hybrids with ct-tRNA. Assuming that lupin ct-DNA and ct-tRNA are of 97×10^6 Da [30] and 25×10^3 Da, respectively, and knowing the specific radioactivity of tRNA, it is estimated that in lupin chloroplast DNA about 34 genes are present. The number of lupin mitochondrial

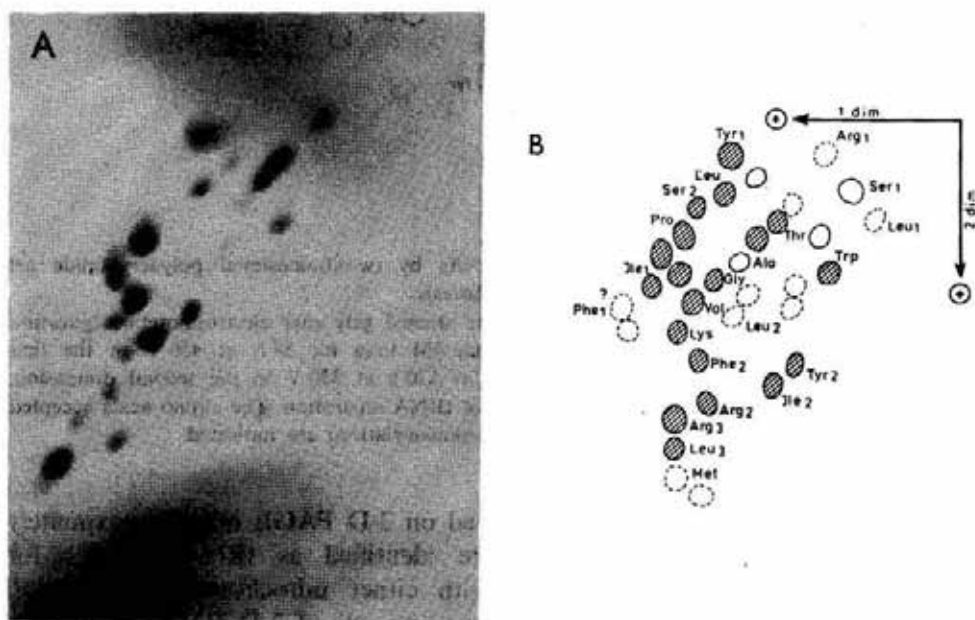


Fig. 2. Fractionation of lupin mitochondrial tRNAs by two-dimensional polyacrylamide gel electrophoresis. The conditions of separation were the same as for ct-tRNAs (cf. Fig. 1).

A. A representative pattern of the methylene stained gels.

B. Diagrammatic representation of mitochondrial tRNA separation. Shaded spots represent species of tRNA which were always detectable on the gel.

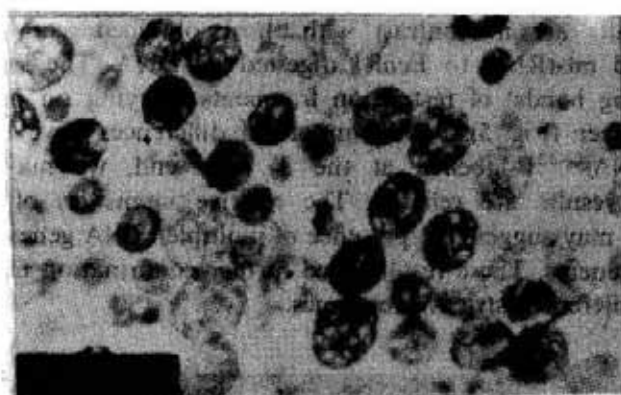


Fig. 3. A thin section of a pellet of the mitochondrial fraction isolated from hypocotyls of dark-grown *Lupinus luteus*. The fraction consists mainly of mitochondria, but remnants of other cell organelles are present: $\times 5000$

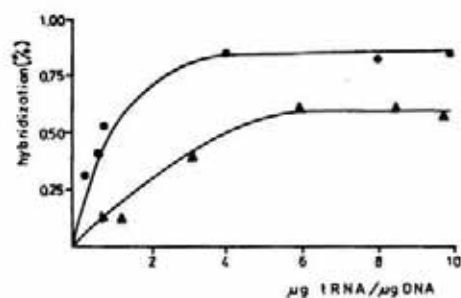


Fig. 4. Saturation hybridization of lupin total ^{125}I -labelled chloroplast tRNA (spec. act. 3.0×10^6 c.p.m./ μg tRNA) with lupin ct-DNA (●) and lupin ^{125}I -labelled mt-tRNA (spec. act. 2.1×10^6 c.p.m./ μg tRNA) with lup n mt-DNA (▲).

tRNA genes calculated from the amounts of hybrids (0.62%) and from molecular weight of mt-DNA [31] approximates 30.

These results are in contrast with those obtained from hybridization of ^{32}P -labelled mt-tRNA to *Eco*RI digested mt-DNA. The limited number (about 6 strong bands) of restriction fragments carrying on putative tRNA genes can be seen (Fig. 5). As no important differences have been observed by using tRNAs ^{32}P -labelled at the 3' or 5'-end, we may expect that the obtained results are reliable. The relative intensities of most of the labelled bands may suggest the presence of multiple tRNA genes on individual restriction fragments. These results need further confirmation in other experiments with different restriction enzymes.

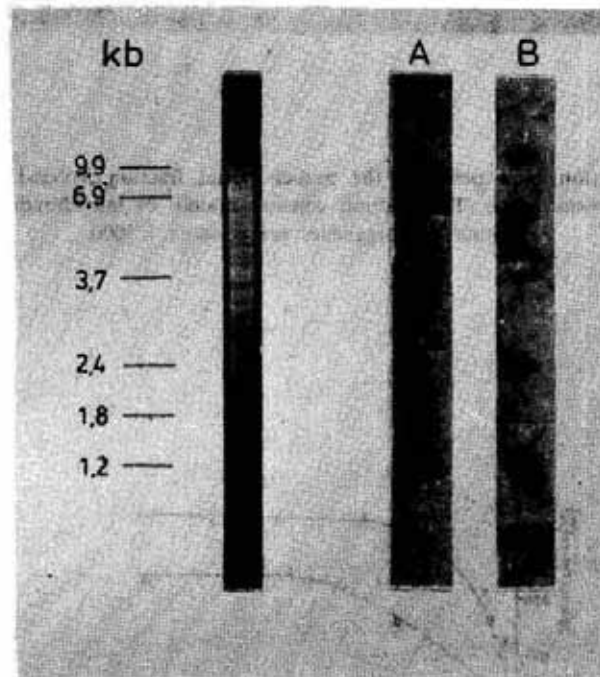


Fig. 5. Patterns of *Eco*RI restriction fragments of mt-DNA on 1% agarose, and autoradiograms showing the hybridization of purified ^{32}P -labelled lupin mt-tRNAs (A) and lupin ct-tRNAs (B) to the restriction digest of mt-DNA. Numbers to the left denote the positions of size marker.

When mitochondrial tRNAs were removed from the hybrids with mt-DNA and the DNA was probed with ^{32}P -labelled ct-tRNAs, some other *Eco*RI fragments gave positive results of hybridization. Only 2-3 fragments show positive hybridization to both ct- and mt-tRNAs. This may reflect either the contamination of mt-DNA with ct-DNA, or the existence of some

homology to the probed ct-tRNAs. The fragments of mt-DNA with the highest homology to ct-tRNAs are probably occupied by chloroplastic tRNAs transferred to mt-DNA. These data agree well with the observed extent of direct cross-hybridization of mt- and ct-DNAs (unpublished)

DISCUSSION

There has been considerable interest in studying plant organellar tRNAs and their genes as one could expect that the population of organellar tRNAs might be more conserved than the population of cytoplasmic tRNAs. Indeed, the number of tRNA genes as well as the comparable number of isoacceptors estimated within some groups of plants, e.g. legumes, despite several differences may indicate some conservation of chloroplast tRNA population. The mitochondrial tRNA population is more heterogeneous. The presence of reiterated tRNA genes, pseudogenes, "native" and "promiscuous" tRNA genes in the mt-DNA of different higher plants [11, 12] may cause their differential expression. The imported tRNAs may also contribute to the heterogeneity of the mt-tRNA population [15].

The data for lupin chloroplast tRNA are quite similar to those published for other plants, but the data for lupin mitochondria present a more complex pattern. So far there was only one indication that the mt-tRNA population may contain numerous species [15]. No large scale 2-D PAGE separation or identification of mt-tRNA species has been presented. On the assumption that lupin mt-tRNA is not contaminated with ct-tRNA we may suppose that a number of species identified in 2-D PAGE may come from an imported tRNA, like in bean mitochondria [15], or from transcribed chloroplast tRNAs inserted in mt-DNA. The presence of different portions of the chloroplast genome including tRNAs in plant mt-DNA is well documented [32], but only two examples of their transcription have been reported so far [14, 33]. Generally it is known that the insert chloroplast genes are not expressed in mitochondria [34]. The number of lupin mt-tRNA genes estimated from saturation hybridization experiments are not quite comparable with the data obtained for the digested lupin mt-tRNA. Studies in wheat mt-DNA [11] gave similar results and suggested the presence of a smaller number of tRNA genes. The obtained number of potential lupin tRNA genes may be overestimated not only because of reiterated tRNA genes, but also of the presence of pseudo-genes and degraded rRNAs. They may significantly affect the quantitation of tRNA genes in mt-DNA. Even though our tRNA preparation did not contain detectable amounts of degraded rRNA this possibility still exists.

It has been established that sequence homologies exist between ct- and mt-tRNAs and their genes. The hybridization studies pointed to the

presence of chloroplast DNA sequences in the lupin mitochondrial genome. The question arises what percentage of lupin mt-DNA has a base sequence complementary to lupin ct-tRNAs. The hybridization results presented in this paper provide support for the existence of high, moderate or low degree of homology between mt- and ct-tRNA genes [11]. The experiments reported were carried out with total chloroplast tRNAs and mt-tRNAs so the sequence mismatching in the hybrids could not be determined. The direct sequence analyses which are carried out at present should help in elucidating these problems.

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