Mitochondrial Tyrosyl Transfer Ribonucleic Acid in Soybean Seedlings

RON L. MENG AND LARRY N. VANDERHOF
Department of Botany, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Soybean seedlings were examined for the presence of mitochondrial tRNA. Tyrosyl transfer tRNAs from whole cells, from a well-characterized mitochondrial preparation, and from a snake venom phosphodiesterase-treated mitochondrial preparation, were compared by reverse phase chromatography. It was concluded that none of the three previously reported tRNA Tyr species were mitochondrial. Rather, a fourth tRNA Tyr species, eluting somewhat later, was of mitochondrial origin. Mitochondrial tRNA Tyr was chromato graphically similar to Escherichia coli tRNA Tyr.

Transfer RNA and tRNA synthetases have previously been purified from mitochondria. Barnett and his colleagues (2, 3) pioneered in this area when they studied species specificity in the aminoacyl transfer reaction led to the discovery of the localization of certain tRNAs and their synthetases in Neurospora crassa mitochondria (4–8, 12–14). Mitochondrial tRNA and/or synthetases have also been discovered in yeast (1, 10, 11, 18, 21, 29), Tetrahymena (22, 23), and rat liver (9, 21). Suyama reported that “a large portion of mitochondrial tRNA is not transcribed from mitochondria DNA” in Tetrahymena; it is clear, however, that mitochondrial DNA does transcribe tRNA Leu and tRNA Phe in yeast (11, 18). Gross et al. (17) have reported the involvement of a nuclear gene in the production of the mitochondrial leucyl-tRNA synthetase of Neurospora.

Three distinct tRNA Tyr species have been detected in higher plant tissue (26; also, see “Discussion”). When cells in the pea seedling root went from the dividing to the nondividing stage the amount of tRNA Tyr III decreased noticeably with respect to tRNA Tyr I and II. Even though care was taken in the homogenization to avoid breakage of mitochondria, it was considered possible that one of the three species might have mitochondrial origin, and, hence, be involved in this reported tRNA population change. The following experiments with soybean seedlings show that this is not the case; there is a single mitochondrial tRNA Tyr, similar, but not identical, to E. coli tRNA Tyr, and distinct from the three cytoplasmic species, as determined by reverse phase chromatography.

MATERIALS AND METHODS

Plant Tissue. Soybean seeds (Glycine max, var. Hawkeye 63) were germinated in moist vermiculite in the dark at 30°C and 80% humidity. After 72 hr the cotyledons and plumule were discarded, and the remainder of the seedling was stored in ice-cold 1% sucrose for not longer than 2 hr before use for extraction of tRNA, enzymes, or mitochondria.

Extraction of Mitochondria. A modified method of Flowers and Hanson (16) was used. Tissue was diced with razor blades in 1.5 vol homogenization medium (0.4 M sucrose, 50 mM KH2PO4, 5 mM Na2EDTA, adjusted to pH 7.6 with crystalline tris). A PT10-35 Polytron homogenizer (generator PT20/ST, setting 3) was used to further disrupt the cells. The homogenate was filtered through four layers of cheesecloth, then centrifuged 10 min at 1,000g. The supernatant fluid was recenterfuged 7 min at 31,000g. The resulting pellet was suspended in 0.4 M sucrose and centrifuged 10 min at 1,000g. The supernatant fluid was saved, the pellet resuspended in 0.4 M sucrose, centrifuged 10 min at 1,000g, and that supernatant fluid combined with the first. The combined supernatant fluids were underlaid with 0.6 M sucrose in a centrifuge tube and centrifuged 25 min at 10,000g. The supernatant fluid was discarded and the mitochondria-rich pellet was immediately quick-frozen at −80°C and stored at −20°C for future tRNA extraction. When mitochondria from 1.5 kg tissue were accumulated, a tRNA extraction was performed. All procedures were done at 0–4°C.

Treatment of Mitochondria with Venom Phosphodiesterase. Prior to quick freezing, the final mitochondrial pellet (see above) was suspended in 10 mm tris-HCl, pH 7.5, containing 0.3 mg/ml snake venom phosphodiesterase (Worthington Biochemical Corporation) and 0.2 M sucrose. The suspension was incubated at 37°C for 6 min, heated to 80°C for 3 min to inactivate the enzyme, and immediately mixed with phenol for tRNA extraction. This treatment was modified from Barnett and Brown (7).

Transfer RNA Extraction. Transfer RNA was extracted from whole tissue as previously described (25). For extraction of RNA from mitochondria, the method was identical except that it was possible to omit the final diethylaminoethyl cellulose chromatography step. Mitochondria from 1.5 kg tissue were initially suspended in 10 ml of 10 mm tris-HCl, pH 7.5, and 20 ml of prepared phenol prior to homogenization. Commercial E. coli tRNA (Schwarz Bio Research, Inc.) was used.

Aminoacyl-tRNA Synthetase Extraction. Aminoacyl-tRNA synthetases were extracted from plant tissue as previously described (25) except that 0.4 M sucrose was omitted from the homogenization medium to enhance the release of mitochondrial synthetases. Synthetases were extracted from freshly grown E. coli MRE 600 RNAse− (Brenner's phosphate buffer with M9 salts, 37°C with aeration, harvested at A0.5 = 0.7, mid- to late-log phase) by a modification of the methods of Kelmers et al. (19) and Waters et al. (28). Twelve gram of cells (wet weight) were dispersed in 24 ml of 10 mm tris-HCl, pH 7.5, containing 10 mm MgCl2 and 1 mm diithiothreitol, and ho-
mogenized with a Polytron at full speed for 1 min. Two volumes of the homogenization buffer were mixed with the homogenate, followed by centrifugation at 30,000g for 40 min. The supernatant fluid from this high speed centrifugation was dialyzed overnight against three changes of the homogenization buffer. The dialysate was mixed with streptomycin sulfate (0.1 g per ml) for 180 min to precipitate nucleic acids, then centrifuged 10 min at 30,000g. The supernatant fluid was adjusted to pH 7.5 with 0.1 N KOH. Ammonium sulfate was added to 65% saturation, and the resultant precipitate was collected by centrifugation (10 min at 30,000g) and dissolved in 20% glycerol containing 10 mM HEPES, pH 7.5, and 0.5 mM dithiothreitol for storage at -20 C.

**Aminoacylation and Reverse Phase Chromatography of tRNA.** These procedures have been previously described (26). One minor change was the size of the reverse phase chromatography II column: it was 0.8 × 244 cm. Both tRNA from total tissue and tRNA from mitochondria were aminoacylated with radioactive tyrosine with an enzyme preparation from total tissue. *E. coli* tRNA was charged with *E. coli* synthetase. In all cases where [3H] tyrosyl-tRNA was co-chromatographed with [14C] tyrosyl-tRNA, comparative experiments were repeated with a reversal of [14C]- and [3H]-tyrosine to avoid artifacts due to contaminated radioactive amino acid preparations.

**Electron Microscopy.** The final mitochondrial pellet was suspended in 0.4 M sucrose containing 50 mM KH2PO4, pH 7.0, and 2.5% glutaraldehyde. The suspension was centrifuged at 30,000g for 10 min. The resultant pellet was washed four times by suspending in 0.4 M sucrose containing 50 mM KH2PO4, pH 7.0. The pellet was treated 4 hr with 2% osmium tetroxide, dehydrated, imbedded in Epon, sectioned on a diamond knife, and poststained with uranyl acetate and lead citrate. Photographs were taken on an RCA EMU-4 electron microscope.

**Fig. 1.** Typical section of mitochondria preparation from soybean seedlings. × 40,000.
The tRNA present in the final mitochondrial pellet from 200 g of tissue. It was determined that the pellet contained 1.7 × 10^5 bacteria and 1.3 × 10^6 mitochondria, or 1 bacterium per 7.7 × 10^6 mitochondria. The amounts of total tRNA and tRNA^* prepared from 100 g of tissue were determined. Seventy-eight A_m units 4S tRNA were obtained. Two per cent, or 1.56 A_m units, of this amount accepted tyrosine. Mitochondria from 100 g of tissue, after RNAse treatment, yielded 1.8 A_m units tRNA, or 2.3% of the total tRNA. Nine per cent, or 0.16 A_m units, of the mitochondrial tRNA accepted tyrosine. Mitochondrial tRNA^* represents 0.21% of the total tRNA and 10% of the total tRNA^*.

When tRNA from total tissue was charged with ^14C-tyrosine and co-chromatographed with ^3H-tyr-tRNA from a mitochondrion-rich preparation, none of the three previously reported tRNA^* species (26) were found to be more highly concentrated in the mitochondrial preparation; however, a new species was detected in the mitochondrial preparation, eluting somewhat later than the three major species of the total tissue preparation (Fig. 2A). This new species was detected only if the synthetase enzymes were extracted from the total tissue in the absence of 0.4 M sucrose.

Treatment of the mitochondria with venom phosphodiesterase produced the following results. The total amount of tRNA extractable from the mitochondria decreased rapidly to one-third its initial value after 2 min, then slowly declined from 2 to 10 min (Fig. 3A). If radioactive tRNA, prepared from diced soybean hypocotyls that had been incubated in the presence of ^14C-adenyls, was added to the mitochondrion-phospho-

**RESULTS**

When tRNA is extracted from nongreen plant tissue, it very likely comes from four sources—the cytoplasm, mitochondria, plastids, and contaminating bacteria. It was necessary, therefore, to determine the purity of the mitochondrial preparation. The character of the mitochondrial preparation with respect to plastid contamination is presented in Figure 1. Examination of similar micrographs of sections taken from throughout the final mitochondrial pellet showed no contamination by either plastids or bacteria. Counts of bacteria were also made and compared to the calculated number of mitochondria present in the final mitochondrial pellet from 200 g of tissue.
diesterase mixture, the radioactivity of the subsequent tRNA preparation declined to almost zero after 6 min (Fig. 3B). The decline occurred only in the presence of the esterase.

Co-chromatography of "C-tyrosyl-tRNA\(^{77}\) from mitochondrion-rich preparation with \(^{3}H\)-tyrosyl-tRNA\(^{77}\) from a venom phosphodiesterase-treated mitochondrion-rich preparation gave the profile illustrated in Figure 2B.

When \(E. \ coli\) tRNA was charged with "C-tyrosine and co-chromatographed with \(^{3}H\)-tyrosyl-tRNA\(^{77}\) from venom phosphodiesterase-treated mitochondria, it was apparent that the two species were very similar, but not identical (Fig. 4).

**DISCUSSION**

Three distinct tRNA\(^{77}\) species have been detected in pea seedling roots (26). That none of the three species was artifactual was indicated by several experiments: (a) an aqueous tRNA solution was heated to 85°C, then quick cooled prior to charging to determine the presence of internally damaged (and, hence, chromatographically distinct) molecules (24). In the case of tRNA\(^{77}\) there were no artificial species due to internal damage to the molecules. (b) The enzyme preparation was well characterized with respect to RNase activity (24, 25); no active RNase was present at optimum synthetase conditions. (c) The -CCA termini were determined to be intact in over 90% of the tRNA molecules (25). (d) The "C-tyr-tRNA\(^{77}\) preparation was chromatographed, the three species collected separately, deacylated, recharged, and rechromatographed with no change in their chromatographic properties (J. R. Cowles and J. L. Key, private communication). (e) Finally, and most important, the primary structure of the three tRNA\(^{77}\) species was examined, and it was shown that there were three different 3'-poly nucleotides produced after treatment of tRNA\(^{77}\) with T1 RNase (J. L. Key, personal communication).

Experiments were designed to determine if any of the three tRNA\(^{77}\) species were mitochondrial. Transfer RNA purified from a mitochondrial-rich preparation showed four tRNA\(^{77}\) species—the three species previously reported, plus an additional species which eluted at a somewhat higher concentration (Fig. 2A). This later elution of a mitochondrial species of tRNA\(^{77}\), with respect to cytoplasmic tRNA\(^{77}\), was previously reported in *Neurospora* (14). It appeared that the three original species were cytoplasmic and were present as contaminants in the mitochondrial preparation, and that the fourth species was mitochondrial. These possibilities were tested by treating the mitochondrial preparation with venom phosphodiesterase. Since extractable RNA decreased rapidly to an amount about one-third the original and then leveled off (Fig. 3A), it was concluded that this technique was eliminating the contaminating cytoplasmic tRNA, but not the mitochondrial tRNA. This interpretation was supported by the fact that radioactive tRNA which was added to the mitochondrial-phosphodiesterase mixture was either completely digested after 6 to 10 min, or, if "C-adenine labeling was only of the -CCA terminus, completely stripped of its 3'-terminal adenine.

When tRNA extracted from venom phosphodiesterase-treated mitochondria was co-chromatographed with tRNA from mitochondria which were not treated with ribonuclease, the profile showed (Fig. 2B) that the fourth tRNA\(^{77}\) species remained, while the amounts of tRNA\(^{77}\) I, II, and III were reduced almost to zero. The conclusion was further supported, then, that tRNA\(^{77}\) IV was mitochondrial while tRNA\(^{77}\) I, II, and III were cytoplasmic.

The notion that mitochondria evolved from endosymbiotic procaryotes has received much attention (15, 20, 27). The comparison of *E. coli* tRNA\(^{77}\) and soybean mitochondrial tRNA\(^{77}\) (Fig. 4) showed the two species of tRNA to be chromatographically very similar. This result is in agreement with the vast literature on the similarities between mitochondria and procaryotes of other components of protein synthesis, e.g., ribosome size, sensitivity to drugs, initiation, and is not in disagreement with the theory that mitochondria are, indeed, evolved from endosymbiotic procaryotes.

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**LITERATURE CITED**


