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CHANGES IN LEUCYL-*t*RNA SPECIES DURING AGEING OF DETACHED SOYBEAN COTYLEDONS*

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Abstract—During ageing of detached soybean cotyledons quantitative changes in certain species of leucyl-*t*RNA occur. Of the six leucyl-*t*RNAs obtained by Freon column fractionation there is an increase in the relative amount of leucyl-$tRNA_{5,6}$ and a decrease in the amount of leucyl-$tRNA_1$ with age. The amount of leucyl-$tRNA_{5,6}$ increases from 6% of the total in dry seeds to 20% in 8-day-old cotyledons. Our results suggest that the relative amounts of leucyl-$tRNA_{5,6}$ probably arise either by, (a) modification of some pre-existing *t*RNA, a process unaffected by partial inhibition of RNA synthesis, protein synthesis, or methylation of RNA, or (b) a preferential destruction of leucyl-$tRNA_{1-4}$.

INTRODUCTION

AGEING, a form of cellular differentiation, might be regulated at the level of specific *t*RNAs and synthetases.[1] The process of ageing and eventually death might result from a loss of certain translational capacities. Inactivation or lack of synthesis of specific *t*RNAs or aminoacyl synthetases corresponding to a restricted group of code words could result in cell deterioration. Specific inhibition of *t*RNA aminoacylation or binding to ribosomes, could also impair protein synthesis and result in ageing.

Modification of some *t*RNA species greatly affects its capacity to function in protein synthesis.[2] Thus modification of existing *t*RNA species may regulate protein synthesis even in the absence of RNA synthesis. Therefore, we initiated a study to determine whether changes in *t*RNA species occur by modification of the nascent RNA. This preliminary report indicates an apparent lack of a requirement for RNA synthesis, protein synthesis and RNA methylation for the age-related changes in leucyl-*t*RNAs.

RESULTS

Detached soybean cotyledons were chosen for this study to determine age-related changes in leucyl-*t*RNAs without influences from other parts of the plant.

Soluble RNA extracted from soybean cotyledons at various stages of ageing were charged with ^{3}H-leucine to determine possible differences in amino acid acceptor activity. The results showed little or no difference in the amount of leucine (cpm) charged per 100 μg *s*RNA from the various preparations (data not included). Since soybean cotyledon *s*RNA can be separated into 6 distinct fractions on a Freon column (Fig. 1; see also ref. 7), it seemed desirable to determine whether changes in individual leucyl-*t*RNAs could be

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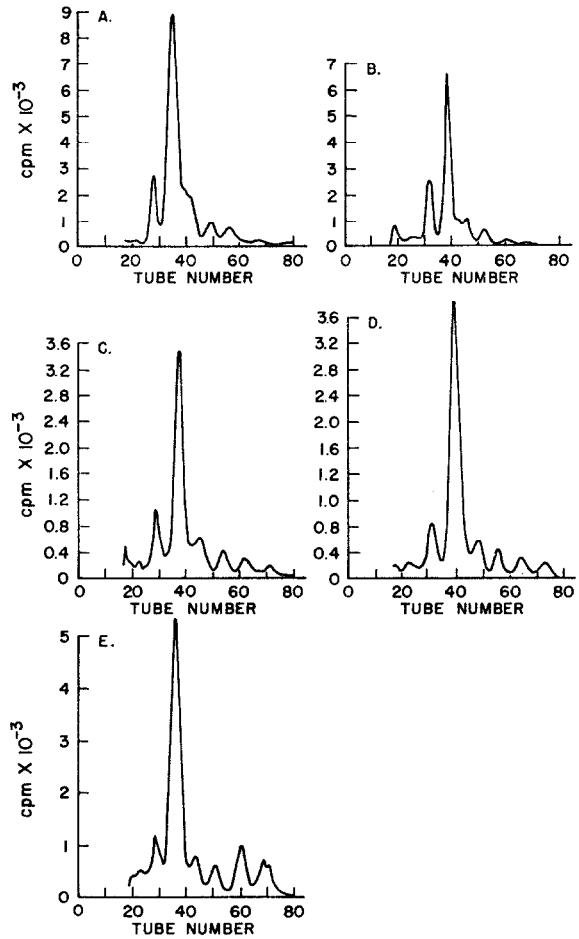

FIG. 1. FREON COLUMN ELUTION PROFILES OF LEUCYL-*t*RNA FROM SOYBEAN COTYLEDONS AT DIFFERENT STAGES OF CULTURE (AGEING).
A—Dry seed; B—20-hr soaked seeds; C—2-day-old cotyledon; D—4-day-old cotyledon; E—8-day-old cotyledon. ^{14}C-L-leucine was used for charging *s*RNA.

detected. All six leucyl-*t*RNA peaks could be detected at different stages of development of the cotyledon ranging from 0 to 8 days (Fig. 1a–e). However, quantitative changes in the amount of certain peaks occurred with age. The greatest change was noted in leucyl-tRNA$_{5,6}$. In dry seeds leucyl-tRNA$_{5,6}$ constitute 6% of the total leucyl-*t*RNA and the proportions were 4, 9, 11 and 20% after 1, 2, 4 and 8 days of culture respectively in the cotyledons. The highest rate of increase in leucyl-tRNA$_{5,6}$ occurred between 20 and 48 hr of culture. During this period there was a doubling in the amount of leucyl-tRNA$_{5,6}$.

Leucyl-tRNA$_1$ also varied with age. This species of *t*RNA decreased as leucyl-tRNA$_{5,6}$ increased, the highest amount of leucyl-tRNA$_1$ being observed in 20-hr cultured cotyledons and the least in those cultured for 8 days. The extent of these variations was smaller than that of species 5 and 6.

There are two possible ways by which leucyl-tRNA$_{5,6}$ could arise, namely by *de novo* synthesis, or by modification of existing leucyl-*t*RNAs. Inhibitors of RNA and protein synthesis were employed to determine whether RNA synthesis and protein synthesis were required for the observed changes in leucyl-*t*RNAs.

Cotyledons (20 g) were soaked in Petri dishes in 20 ml of solution containing 10^{-4} M streptomycin, 10^{-4} M penicillin and 10 μc/ml ^{3}H-uridine with and without 10 μg/ml of 6-methylpurine, an inhibitor of nucleic acid synthesis.[3] After 48 hr, cotyledons were removed, thoroughly washed and total ribonucleic acid was extracted by the phenol-sodium lauryl sulfate method.[4] The amount of nucleic acid extracted from the two treatments was the same but there was a marked difference in the incorporation of ^{3}H-uridine. In control cotyledons the amount of ^{3}H-uridine incorporated was 4350 cpm/mg nucleic acid, but in 6-methylpurine treated cotyledons it was only 2460 cpm/mg nucleic acid. Furthermore, 6-methylpurine treated cotyledons showed a distinct relative increase in leucyl-tRNA$_{5,6}$ (Table 1). This increase in leucyl-tRNA$_{5,6}$ with limited RNA synthesis suggested that either the amount of leucyl-tRNA$_{1-4}$ decreased as a result of 6-methylpurine treatment or that the modification of some RNA to form leucyl-tRNA$_{5,6}$ proceeded unaffected while synthesis of other tRNAs was inhibited.

To determine whether protein synthesis was essential for the change in leucyl-tRNAs, cotyledons were treated with cycloheximide (10 ppm). Incubation of cotyledons in cycloheximide for 48 hr had no effect on the amount of leucyl-tRNA$_{5,6}$ (Table 1).

TABLE 1. EFFECT OF INHIBITORS OF NUCLEIC ACID AND PROTEIN SYNTHESIS ON CHANGES IN LEUCYL-*t*RNAS

Treatment	Relative amount of leucyl-tRNA of each peak (% of total) I	II	III	IV	V	VI
Experiment 1						
Control	17	56	10	7	7	4
6-Methylpurine (10 μg/ml)	13	49	13	9	11	6
Experiment 2						
Control	14	57	10	7	8	3
Cycloheximide (10 ppm)	8	59	12	10	8	3

In each Freon column fractionation 500 μg cotyledon sRNA was charged with ^{3}H-leucine by 1 mg cotyledon enzyme in a total vol. of 1 ml. The results are expressed as the relative amount of leucyl-tRNA in each peak calculated as the percentage of total radioactivity.

Some possible mechanisms for the modification of tRNA are (a) addition of methyl, thiol, or isopentenyl groups to existing tRNA molecules, (b) removal of such groups, or (c) changes in conformation of tRNA structure. Since methylation of tRNA seemed to be a likely modification, and as previous studies[5] had shown that ethionine blocks methylation of tRNA in a plant system, we therefore employed ethionine in this study to determine whether blocking methylation affected the changes in leucyl-tRNAs. However, it was found that ethionine had little effect on leucyl-tRNAs except at concentrations of $2{\cdot}5 \times 10^{-3}$ M and $2{\cdot}5 \times 10^{-2}$ M (Table 2).

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TABLE 2. EFFECT OF ETHIONINE ON LEUCYL-*t*RNAs

Radioactive leucine	Concn of ethionine (M)	Relative amount of leucyl-*t*RNA in each peak (% of total)					
		I	II	III	IV	V	VI
^{14}C	0	13	62	7	8	7	4
^{14}C	$2{\cdot}5 \times 10^{-6}$	12	59	9	7	8	5
^{14}C	$2{\cdot}5 \times 10^{-4}$	14	61	10	7	6	2
^{3}H	$2{\cdot}5 \times 10^{-3}$	12	54	9	8	12	7
^{3}H	$2{\cdot}5 \times 10^{-2}$	13	56	6	8	11	7
^{3}H	0	14	57	10	7	8	3

Soybean cotyledons were cultured in different concentrations of ethionine for 2 days. Freon column fractionations were done with *s*RNA samples from two different treatments, one charged with ^{3}H- and the other with ^{14}C-leucine. Two control *s*RNA preparations (one charged with ^{3}H- and the other with ^{14}C-leucine) were fractionated on the same column. The data are expressed as indicated in Table 1.

DISCUSSION

During ageing, quantitative changes occur in certain leucyl-*t*RNAs. Leucyl-$tRNA_{5,6}$ increase while leucyl-$tRNA_1$ concurrently decreases. However, there may be no direct correlation between these changes. The investigation of the nature of these changes in specific fractions of leucyl-*t*RNA was attempted by the use of various inhibitors. It was learned that 6-methylpurine treatment increased the relative content of leucyl-$tRNA_{5,6}$. Thus, it appeared that RNA synthesis may not be necessary for modification of specific RNA. If leucyl-$tRNA_{5,6}$ are the products of modification of pre-existing *t*RNAs while the formation of leucyl-$tRNA_{1-4}$ is dependent on RNA synthesis, then blocking RNA synthesis would effectively increase the relative population of leucyl-$tRNA_{5,6}$.

Table 1 shows no increase in peaks 5 and 6 with treatment of the tissue with cycloheximide but a large increase was noted with 6-methylpurine treatment. Furthermore, methylation of *s*RNA appears not to be involved in the production of $tRNA_{5,6}$, since high concentrations of ethionine even increased the amount of these *t*RNAs. The fact that these inhibitors (6-methylpurine, cycloheximide and ethionine) do not reduce the relative amount of leucyl-$tRNA_{5,6}$ isolated from the tissue suggests that leucyl-$tRNA_{1-4}$ are preferentially destroyed rather than leucyl-$tRNA_{5,6}$ being synthesized. The results of Bick and Strehler[6] agree with this conclusion.

EXPERIMENTAL

Plant material. Soybean (*Glycine max* L. cv. Hawkeye 63) seeds were soaked overnight in deionized water. The seed coat was then peeled off, embryos removed and the cotyledon halves were incubated in the light (ca. 6000 lx) in trays containing 10^{-4} M streptomycin and 10^{-4} M penicillin G. The cotyledons were soaked for 48 hr in solutions containing the inhibitors.

Extraction of sRNA. Cotyledons (100 g) were homogenized in a Waring Blender with Tris buffer and phenol.[6] 2 N AcOH was used to solubilize *s*RNA.

Aminoacyl-tRNA synthetase preparation. Soybean seeds were germinated and after 5 days synthetase was extracted from the cotyledons by the procedure of Anderson and Cherry.[7]

Freon column fractionation of sRNA. *s*RNA (500 μg) was charged with ^{3}H- or ^{14}C-L-leucine[7] and separated on Freon (RPC-2) columns ($2{\cdot}5 \times 40$ cm) according to the method of Weiss and Kelmers.[8] The elution

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buffer consisted of 0·01 N HOAc adjusted to pH 4·5 with NaOH and 0·01 M $MgCl_2$. Elution of sRNA was accomplished with a one-L-linear gradient of NaCl ranging from 0·4 to 0·8 M. At least 80 fractions of 10 ml each were collected at a flow rate of 2 ml/min. RNA from each fraction was co-precipitated in the cold with 200 μg DNA by the addition of 1 ml of 55% TCA. The samples were collected on glass-fiber filters (Whatman GF/A) and the radioactivity determined in a Packard Scintillation counter. Since preferential self-absorption was observed with ^{3}H-labelled RNA in comparison to ^{14}C-labelled material, all data presented in this paper are from single labelled column fractionations. The calculations presented here are based on the assumption that the *t*RNA charging *in vitro* is complete.

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Key Word Index—Soja max; Leguminoseae; soya bean; leucyl-transfer RNA; ageing.