Leucyl Transfer RNA Synthetase Changes during Soybean Cotyledon Senescence

MICHAEL D. BICK* AND BERNARD L. STREHLER

Department of Biological Sciences, University of Southern California, Los Angeles, Calif. 90007

Communicated by Paul C. Zamecnik, October 29, 1970

ABSTRACT Previous work has shown the complement of leucyl transfer RNA (tRNA^{Leu}) isoaccepting species present in soybean cotyledons to change during cotyledon senescence. Of the six tRNA^{Leu} species resolved by Freon-column chromatography, the capacity of species 1-4 to accept leucine is decreased between 5 and 21 days of age. Parallel changes in tRNA^{Leu} synthetase activity are reported here during the same time period. There is a reduction in the capacity of the synthetase from 21-day-old cotyledons to aminoacylate tRNA^{Leu} 1-4, while the capacity to charge tRNA^{Leu} 5-6 remains high. When tRNA^{Leu} derived from 5-day-old cotyledons is maximally charged with the 21-day synthetase, the subsequent addition of synthetase from 5-day-old cotyledons promotes additional charging, primarily of species 1-4. These results suggest the presence of tRNA^{Leu} "subspecies" within the individual peaks resolved on a Freon column, differing in synthetase specificity.

There is now considerable evidence that cell-specific protein synthesis is regulated, at least in part, at the level of mRNA translation (1-4). Both the concentration of specific tRNA molecules and of the corresponding aminoacyl-tRNA synthetases may play critical roles in limiting translational capacities. Specifically, tRNA changes have been demonstrated during differentiation of animal cells (5-8), in higher plants (9-11), in neoplastic cells (12, 13), and in the same cell type while it produces different proteins (14); and, differences have been demonstrated for amino acid-specific tRNA synthetases of different tissues (10, 15). Reduced and/or altered synthetic capacities of cells may have direct implications in biological aging (15, 16). Limiting quantities of specific tRNAs and/or synthetases may restrict a cell's capacity to read certain genetic code words, and the loss in specific code reading abilities concerned with the synthesis of essential cellular components would lead to deleterious effects on long-term cell function.

In the present studies, the senescing soybean cotyledon was used as a model system to determine changes in the translational machinery during aging. This organ is generally considered to senesce during the early stages of plant development while providing the growing embryonic axis with the hydrolytic products of its own constituents. We have previously shown that the complement of tRNA^{Leu} isoaccepting species changes during cotyledon senescence. In this paper we report a reduction in the capacity of tRNA^{Leu} synthetase to acylate certain of these isoaccepting species during a similar time period. Results are presented that are most readily in-

terpreted as evidence for coeluting subspecies of tRNA^{Leu} which possess different charging specificities.

MATERIALS AND METHODS

Transfer RNA and synthetase preparation

Soybean seeds (*Glycine max* L. var Hawkeye) were allowed to imbibe water overnight, sown in moist vermiculite, and placed in a dark, humid growth chamber maintained at 29-30°C. After appropriate time intervals, cotyledons were excised, placed on ice, washed briefly with ice-cold deionized water, and were then used for either tRNA or synthetase extraction.

Transfer RNA was isolated by the method of Anderson and Cherry (10) except that prior to the final dialysis the tRNA was deacylated by incubation in a small amount of 1 M Tris buffer (pH 9.1) for 30 min at 37°C. The tRNA was then precipitated by the addition of 2 volumes of 95% ethanol and 0.1 volume of 2M potassium acetate (pH 5.5), collected by centrifugation, dissolved in a small amount of deionized water, and was dialyzed overnight against water containing 5 mM 2-mercaptoethanol.

The method of Anderson and Cherry (10) was also used for the synthetase preparation, except that homogenization was performed in a Sorvall Omni-Mixer at high speed. The final preparation was stored at -20°C in 40% glycerol without loss of activity for up to 4 months.

The aminoacylation assay was performed as described (11), except that the leucine concentration was adjusted to 0.01 mM.

Phenoxyacetylsuccinimide derivatization procedure

Transfer RNA was acylated in a 2-ml reaction mixture containing 45 A_{260} units of tRNA, 4 mg of 21-day synthetase protein, and [^3H]leucine. The reaction was allowed to proceed for 35 min; tRNA was then precipitated by the addition of 2 volumes of 95% ethanol and 0.1 volume of 2 M KAc, and collected by centrifugation. Aminoacyl-tRNA was derivatized by Peterkofsky and Jesensky's (17) modification of the method of Gillam, *et al.* (18). Aminoacyl-tRNA was dissolved in 4 ml of 0.1 M triethanolamine buffer (pH 4.0) containing 0.01 M MgSO_4 at 4°C, and 0.4 ml of tetrahydrofuran containing 10 mg of phenoxyacetic acid *N*-hydroxysuccinimide ester. The pH was immediately raised to 8.0 by the addition of 1 N NaOH, the reaction was stirred for 10 min, and the pH was lowered to 4.5 by the addition of 1 N acetic acid. Transfer RNA was subsequently precipitated with ethanol and KAc, collected by centrifugation, and dissolved in a small amount of deionized water. Aliquots were subsequently

* NICHD Predoctoral Trainee, The Gerontology Center, USC (Training grant 52-2403-5945).

TABLE 1. Comparison of the relative amounts of tRNA^{Leu} isoaccepting species present in cotyledons of different ages*

Age of tRNA (days)	Peak					
	1	2	3	4	5	6
2	10.3	66.0	8.3	6.8	3.9	4.5
5	13.2	57.6	9.2	7.3	5.2	6.7
15	7.2	53.6	6.6	5.8	12.4	14.4
21	11.3	37.5	10.2	7.9	14.6	18.5

* tRNA (11 A₂₆₀) was acylated in a 1-ml reaction and fractionated on a Freon column. A synthetase preparation from 5-day cotyledons was used in every case. The amount of radioactivity in each peak was summed and expressed as per cent of the total radioactivity in the six peaks. The total radioactivity eluted from the column was in excess of 5×10^4 cpm (initial specific activity of [¹⁴C]leucine was 311, of [³H]leucine 58 Ci/mmol).

used for aminoacylation with 5-day-old synthetase and [¹⁴C]leucine.

Chromatography of tRNA

Differences in tRNA^{Leu} and synthetase charging were detected by Freon-column chromatography (19), as previously described (10, 11). The column (90 × 2.5 cm) was developed with a 2-liter linear salt gradient from 0.38 to 0.75 M NaCl. Fractions of 10 ml were collected at a flow rate of 1.3 ml/min at room temperature.

RESULTS

Complementarity of changes in tRNA^{Leu} and synthetase

Previous studies from this laboratory (11) have shown that the relative amounts of the six tRNA^{Leu} isoaccepting species of cotyledons change between 2–15 days of plant growth. These studies have now been extended to plants 21-days old, about the maximum lifespan of the cotyledon under the growth conditions employed. These data are summarized in Table 1. A synthetase preparation derived from 5-day-old cotyledons was used routinely. The key changes observed are a decrease in the relative amount of peak 2, with sharp increases in the relative amounts of peaks 5 and 6. The total leucine-acceptor activity in the 21-day-old cotyledons was reduced to about 50% of that characteristic of 5-day-old cotyledons (Table 2).

Fig. 1 shows the capacity of synthetase preparations from 5- and 21-day-old cotyledons to aminoacylate 5- and 21-day

FIG. 1. Charging kinetics of tRNA^{Leu} and synthetase from 5- and 21-day-old cotyledons. 11 A₂₆₀ units of tRNA was used in each reaction, with about 1 mg of synthetase protein. The prefix numbers refer to the age (days) of the cotyledons from which enzyme or tRNA were prepared.

- A. 5-Enz-5-RNA C. 21-Enz-5-tRNA
B. 5-Enz-21-tRNA D. 21-Enz-21-tRNA

tRNA^{Leu}. 11 A₂₆₀ units of tRNA were used in each reaction; the tRNA concentration was shown to be limiting. The addition of inorganic pyrophosphatase (EC 3.6.1D) to the reaction (8.5 units/ml) did not enhance the maximal extent of charging in any case, indicating a total aminoacylation of tRNA species chargeable by a given synthetase preparation. It can be seen from Fig. 1 that there is not only a reduction in the capacity of tRNA^{Leu} derived from 21-day-old cotyledons to be charged, but there is also a reduction in the capacity of the synthetase from 21-day-old cotyledons to aminoacylate tRNA^{Leu}. Thus, 21-day synthetase is only 33% as effective in charging 5-day-old tRNA as is the 5-day synthetase, and similar results are seen using the 21-day-old tRNA. The total amount of leucine charged in each of these cases is shown in Table 2.

Chromatographic comparison of tRNA^{Leu} synthetase activities

In order to determine the nature of the enzyme changes that occur with advancing cotyledon age, a 5-day-old tRNA preparation was acylated *in vitro* with the 5- and 21-day synthetase

TABLE 2. Leucine acceptor activity of 5- and 21-day-old tRNA and synthetase*

	En- zyme†	RNA†	Total pmol/A ₂₆₀	Picomoles in each peak					
				1	2	3	4	5	6
A	5	5	36.6 ± 3	4.4 ± 0.3	19.2 ± 0.2	3.0 ± 0.2	2.4 ± 0.1	1.9 ± 0.1	2.3 ± 0.6
B	5	21	18.2 ± 2	2.1 ± 0.6	6.8 ± 0.8	1.9 ± 0.4	1.5 ± 0.3	2.6 ± 0.3	3.3 ± 0.4
C	21	5	11.0 ± 1	1.5 ± 0.2	5.8 ± 0.8	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.2
D	21	21	6.0 ± 1	0.6 ± 0.1	1.9 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	1.1 ± 0.2	1.5 ± 0.3
Ratio	A/D		6.1	7.3	10.1	7.5	6.0	1.7	1.5

* Total pmol acceptor activity was determined by maximum charging in an *in vitro* assay. The acceptor activity in each peak was calculated from the total pmol incorporation from the per cent of the total radioactivity in each peak. Values are average of two different column runs.

† Numbers in this column are age (days) of cotyledon from which enzyme or tRNA was prepared.

FIG. 2. Freon-column cochromatography of 5-day-old $\text{Leu-tRNA}^{\text{Leu}}$ acylated with 5-day synthetase (—) and 21-day synthetase (---). $[^3\text{H}]$ leucine was used in the former case, $[^{14}\text{C}]$ leucine in the latter. The inset shows the relative amount of radioactivity in each peak. Radioactivity in each peak was summed and expressed as percent of the total in all six peaks.

preparations, using $[^3\text{H}]$ leucine in one case and $[^{14}\text{C}]$ leucine in the other, as shown in Fig. 2. The relative amount of acceptor activity in each peak is shown in the inset to the figure. The most notable difference is the decreased capacity of the 21-day synthetase to acylate peaks 1-4. The pmol of leucine charged in peaks 5 and 6 is only slightly reduced with the 21-day synthetase (see Table 2).

Similar changes are observed when 21-day-old tRNA is acylated with 5- and 21-day synthetase (Fig. 3). The capacity to charge $\text{tRNA}^{\text{Leu}}_{1-4}$ is even further reduced, and species 5 and 6 become a major portion of the total leucine acceptor activity in the 21-day-old cotyledon. The insets to Figs. 2 and 3 show that the major shift in isoaccepting species is due to both tRNA and synthetase changes in the homologous sys-

FIG. 3. Freon-column cochromatography of 21-day-old $\text{Leu-tRNA}^{\text{Leu}}$ acylated with 5-day synthetase (—) and 21-day synthetase (---), using $[^3\text{H}]$ - and $[^{14}\text{C}]$ leucine, respectively. Inset as described in Fig. 2.

FIG. 4. (A) 5-day-old tRNA^{Leu} acylated with 21-day synthetase (1 mg) (O—O); after 32 min 0.4 mg of additional 21-day synthetase was added (●—●). Incubation of 21-day synthetase in a complete reaction minus tRNA (+—+) showed no charging. (B) 5-day-old tRNA^{Leu} acylated with 21-day synthetase (1 mg) (O—O); after 32 min 0.4 mg 5-day synthetase was added (●—●). Incubation of 5-day synthetase in a complete reaction minus tRNA (+—+) showed no charging.

tems (5-tRNA-5-Enz and 21-tRNA-21-Enz), leading to $\text{tRNA}^{\text{Leu}}_{5-6}$ becoming major species in the older cotyledon. The total leucine-acceptor activity in these two species is only slightly decreased, while the acceptance in species 1-4 has decreased to 10-16% of the values in the young cotyledon.

21-day synthetase cannot fully aminoacylate 5-day-old tRNA

The change in the capacity of 21-day synthetase preparations to acylate tRNA^{Leu} was of particular interest since there was no evidence that any tRNA^{Leu} species were lost (as detected by Freon-column chromatography); rather, only the maximal

FIG. 5. Freon-column chromatography of tRNA acylated with both 5- and 21-day synthetase. 5-day-old tRNA was acylated in a 2-ml reaction with 21-day synthetase and $[^3\text{H}]$ -leucine (---) for 35 min. Transfer RNA was then precipitated, derivatized with phenoxyacetic acid *N*-hydroxysuccinimide ester, and acylated with 5-day synthetase and $[^{14}\text{C}]$ leucine (—) for 8 min (maximum amino acid incorporation was achieved in this time period).

TABLE 3. *Effect of tRNA preincubation on charging**

Enzyme†	RNA†	Pre-incubation	Relative amount (%)					
			1	2	3	4	5	6
5	5	None	13.7	59.2	8.7	7.3	5.5	6.1
5	5	21-enz	12.9	58.3	9.1	8.0	5.3	6.3
21	5	None	12.5	48.7	9.6	9.0	9.6	10.1
21	5	5-enz	13.0	49.6	9.4	8.5	9.8	9.4

* 5-day-old tRNA (25 A_{260}) was incubated with 2 mg of synthetase protein (either 5- or 21-day) in 20 μ mol Tris·HCl (pH 7.8), 10 μ mol $MgCl_2$, 0.2% soluble polyvinyl pyrrolidone, 10 μ mol ATP, and 10 μ mol CTP, in a total volume of 2 ml. Incubation was for 30 min at 30°C. Transfer RNA was phenol extracted, precipitated with ethanol, and collected by centrifugation. tRNA^{Leu} was then acylated with the appropriate synthetase preparation and fractionated on a Freon column as described in Table 1.

† Numbers in column refer to age (days) of cotyledons from which enzyme and tRNA were prepared.

extent of charging was affected. To determine the nature of the loss in synthetase activity, 5-day-old tRNA was aminoacylated to a plateau level with 21-day synthetase, then 5-day synthetase was added. Fig. 4 shows that under these conditions the addition of 5-day synthetase promotes further charging, whereas the addition of more of the 21-day synthetase does not produce such an effect. No aminoacylation occurred in reaction mixtures in the absence of added tRNA, ruling out the possibility that significant amounts of tRNA were present in either synthetase preparation. When 5-day-old tRNA was acylated with 5-day synthetase, the subsequent addition of more 5-day synthetase did not promote further charging.

In order to determine which tRNA^{Leu} species were being acylated when 5-day synthetase was added to the completed 5-tRNA-21-enzyme reaction, charging was performed with 21-day synthetase and [³H]leucine for 35 min. The product was precipitated and derivatized with phenoxyacetic acid *N*-hydroxysuccinimide ester to stabilize the aminoacyl bond. After derivatization and isolation, the tRNA was acylated with the 5-day synthetase and [¹⁴C]leucine, and fractionated on the Freon column. The results are presented in Fig. 5, which shows that the ³H label is incorporated into all six species of tRNA^{Leu} with 21-day synthetase, whereas the subsequent acylation with 5-day synthetase results in the charging of only tRNA^{Leu}₁₋₄ (with the ¹⁴C label). Subsequent acylation with 21-day synthetase results in no further charging or incorporation of the ¹⁴C label. Other studies have shown that under the incubation and time conditions employed there is essentially no exchange of labeled leucine. These data would thus suggest that the reduced capacity of the 21-day synthetase to aminoacylate tRNA^{Leu} is due exclusively to a loss in the capacity to fully acylate tRNA^{Leu}₁₋₄.

tRNA-acceptor activity is not being modified by either synthetase

To determine whether the two synthetase preparations differ in RNase activity, or in some other factor which modifies tRNA so as to alter its acceptor activity, aliquots of 5-day-old tRNA were separately incubated with either 5- or 21-day synthetase preparations in the absence of amino acid. After phenol extraction and ethanol precipitation, the tRNAs were acylated with the converse synthetase. The results of these experiments are shown in Table 3. In neither case does incubation of tRNA with one synthetase preparation affect the relative distribution of label among the various peaks when the

tRNA is subsequently acylated with the other synthetase. These experiments rule out differential RNase activities in the two enzyme preparations as a source of the differences observed in peak charging, as well as differences in the removal or addition of the -CpCpA terminus of the tRNA, or other modifications of tRNA acceptor activity.

DISCUSSION

Previous studies in which quantitative and qualitative differences in tRNA charging capacities were demonstrated among different tissues from a given animal or plant source make it evident that cells change in their ability to utilize different tRNAs during development. The occurrence of tRNA and synthetase modulation during ontogeny, and the effect of this modulation on the proteins being produced, was clearly demonstrated by the recent experiments of Ilan (20) with the mealworm *Tenebrio molitor*. The changes in tRNA^{Leu} and the corresponding synthetases reported here are just the converse of Ilan's findings. In both sets of experiments concomitant changes in both tRNA and synthetase occurred. In the case of senescing cotyledons, however, the changes in tRNA^{Leu} acceptor activity and the acylating capacity of the synthetase consists of a loss in certain isoaccepting components present. Although tRNA^{Leu}₁₋₄ are not completely lost, their relative capacity to accept leucine is decreased 6–10-fold. The capacity to control message translation by limiting quantities of tRNA has been demonstrated by Anderson (21). We are presently attempting to determine the relative concentrations *in vivo* of each of the Leu-tRNA^{Leu} species in the young and old cotyledons. The *in vitro* assay measures the maximum acceptor activity for amino acid, but it may be that tRNA is less than 100% aminoacylated *in vivo*, and that the rates for acylating tRNA^{Leu}₁₋₄ *in vivo* are lower than the rates for acylating species 5 and 6. If this were the case, tRNA^{Leu}₅₋₆ species would constitute an even greater portion of the total leucine acceptor activity, particularly in the older cotyledon.

Kanabus and Cherry (22) have reported the fractionation of tRNA^{Leu} synthetase from soybean cotyledons into three peaks of activity on hydroxylapatite. The first peak is specific for acylating tRNA^{Leu}₅₋₆, and the other two charge species 1–4. The data reported here suggest the loss of one of these two synthetase fractions specific for tRNA^{Leu}₁₋₄ in the aging cotyledon. However, this would require some degree of specificity in the capacity of these two synthetases to acylate these tRNA species. That is, each of the first four peaks resolved on

a Freon column might actually consist of tRNA^{Leu} "subspecies," perhaps differing in the degree of methylation or some other modification that affords synthetase specificity. Thus, when 5-day-old tRNA is acylated with 21-day synthetase, the subsequent addition of 5-day synthetase may result in the charging of a group of tRNA^{Leu}₁₋₄ subspecies not previously charged by the 21-day synthetase preparation (which lacks the appropriate synthetase).

Subspecies, if present, may represent organelle (mitochondrial and chloroplastic) tRNAs and their respective synthetases (23). Thus, species 1-4 might be present in both the cytoplasm and in organelles, but the species may differ in subtle modifications depending on their intracellular location, thus giving rise to different synthetase specificities. However, the report of Anderson and Cherry (10) showing tRNA^{Leu}₃₋₄ to be the major mitochondrial species makes it unlikely that the changes reported here are due entirely, if at all, to specific changes of tRNA in organelles.

With six peaks of Leu-tRNA^{Leu} resolved on a Freon column and six known codons for leucine, it seems reasonable to suggest that each peak responds to a unique codon or a small group of codons. Should this be the case, the changes reported here in tRNA^{Leu} and synthetase might well limit message translation (to messages rich in the codons corresponding to Leu-tRNA^{Leu}₅₋₆). This would be particularly true if the *in vivo* concentrations of aminoacylated tRNA^{Leu}₁₋₄ are lower than the maximum concentrations achieved *in vitro*. It has been proposed (15, 16) that the initiating events in senescence consist of the loss of certain translational capacities in the mature, post-mitotic phase of the lifespan. The present data are consistent with this hypothesis.

This work was supported by contracts AT (11-1)-113-20 from U.S. Atomic Energy Commission, and GB-7493 from the National Science Foundation.

1. Kano-Sueoka, T., and N. Sueoka, *Proc. Nat. Acad. Sci. USA*, **52**, 1535 (1964).

2. Stent, G. S., *Science*, **144**, 816 (1964).
3. Sueoka, N., T. Kano-Sueoka, and W. J. Gartland, in *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 571 (1966).
4. Kaneko, I., and R. H. Doi, **55**, 564 (1966).
5. Holland, J. J., M. W. Taylor, and C. A. Buck, *Proc. Nat. Acad. Sci. USA*, **58**, 2437 (1967).
6. Lee, J. C., and V. M. Ingram, *Science*, **158**, 1330 (1967).
7. Yang, W. K., and G. D. Novelli, *Proc. Nat. Acad. Sci. USA*, **59**, 208 (1968).
8. Mushinski, J., and M. Potter, *Biochemistry*, **8**, 1684 (1969).
9. Vold, B. S., and P. S. Sypherd, *Proc. Nat. Acad. Sci. USA*, **59**, 453 (1968).
10. Anderson, M. B., and J. H. Cherry, *Proc. Nat. Acad. Sci. USA*, **62**, 202 (1969).
11. Bick, M. D., H. Liebke, J. H. Cherry, and B. L. Strehler, *Biochim. Biophys. Acta*, **204**, 175 (1970).
12. Yang, W. K., A. Hellman, D. H. Martin, K. B. Hellman, and G. D. Novelli, *Proc. Nat. Acad. Sci. USA*, **64**, 1411 (1969).
13. Gallo, R. C., and S. Pestka, *J. Mol. Biol.*, **52**, 195 (1970).
14. Yang, W. K., and G. D. Novelli, *Biochem. Biophys. Res. Commun.*, **31**, 534 (1968).
15. Strehler, B. L., D. D. Hendley, and G. P. Hirsch, *Proc. Nat. Acad. Sci. USA*, **57**, 1751 (1967).
16. Strehler, B. L., *7th International Congress of Gerontology, Vienna* (1966), p. 177.
17. Peterkofsky, A., and C. Jesensky, *Biochemistry*, **8**, 3798 (1969).
18. Gillam, I., D. Blew, R. C. Warrington, M. von Tigerstrom, and G. M. Tener, *Biochemistry*, **7**, 3459 (1968).
19. Weiss, J. F., and A. D. Kelmers, *Biochemistry*, **6**, 2507 (1967).
20. Ilan, J., in *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 787 (1969); Ilan, J., J. Ilan, and N. Patel, *J. Biol. Chem.*, **245**, 1275 (1970).
21. Anderson, W. F., *Proc. Nat. Acad. Sci. USA*, **62**, 566 (1969); Anderson, W. F., and J. M. Gilbert, *Biochem. Biophys. Res. Commun.*, **36**, 456 (1969).
22. Kanabus, J., and J. H. Cherry, *Fed. Proc.*, **29**, 891 (1970); Kanabus, J., and J. H. Cherry, *Plant Physiol.*, **46**, Suppl. 30 (1970).
23. Barnett, W. E., and D. H. Brown, *Proc. Nat. Acad. Sci. USA*, **57**, 452 (1967); Buck, C. A., and M. M. K. Nass, *Proc. Nat. Acad. Sci. USA*, **60**, 1045 (1968); Apler, J. L., *Biochemistry*, **8**, 2285 (1969).