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LYSYL *t*RNAs OF *LUPINUS LUTEUS* SEEDS

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Key Word Index—*Lupinus luteus*; Leguminosae; lysyl-transfer RNA isoacceptors.

Abstract—Lysine accepting transfer RNA of lupin seeds and lupin embryo axes can be fractionated into at least 5 species by reversed-phase chromatography (RPC-5). One main and two minor isoacceptors were observed in wheat and barley embryos. Changes in isoaccepting species of tRNA^{lys} were followed in cotyledons of germinating lupin seedlings. Ribosome binding studies revealed that one of the main lupin tRNA^{lys} species recognizes the AAG codon, the second AAA and the third one AAA and AAG.

INTRODUCTION

The multiplicity of isoaccepting *t*RNAs, which is greater than predicted by degeneracy of the genetic code, is still an object of investigation. There are numerous examples for the possible involvement of *t*RNA in various regulatory processes [1] that could account for the observed isoaccepting multiplicity, although firm evidence to that effect is not available. The lysine specific *t*RNAs respond to two codons (AAA and AAG) but in the literature more than two isoaccepting lysine *t*RNAs have been described in eukaryotic systems. In mammalian tissues up to 5 lysyl-*t*RNA were found. However, the pattern of lysyl-*t*RNA depends on the tissue and the rate of growth [2–6]. An extra *t*RNA^{lys} species were observed also in virus transformed cells [7]. Because of the chloroplast and mitochondria-specific *t*RNAs, a still more complicated pattern of lysyl-*t*RNAs might be expected for plant tissues [8]. However, Lea and Norris observed only 3–4 *t*RNAs^{lys} species in maturing wheat grain [9, 10]. Cornelis and Claessens [11] also found only 3 in crown gall tissues from *Nicotiana tabacum*, while in apple and pear [12] or ethylene ripened tomato fruits [13] 4 lysine *t*RNAs were detected.

In lupin seeds one [14] or possibly two [15] *t*RNAs^{lys} were previously observed by methods less efficient than RPC-5. The high resolving power of RPC-5 enabled us now to demonstrate at least 5 lysine specific *t*RNAs in lupin seeds and in cotyledons of young lupin seedlings.

RESULTS AND DISCUSSION

The procedure used for the preparation of crude *t*RNA from lupin seeds, lupin cotyledons, embryo axes of lupin, wheat and barley germ *t*RNA involved successively phenol extraction, deproteinisation with the chloroform–isoamyl alcohol mixture and finally DEAE-cellulose chromatography. The obtained *t*RNA preparations were contaminated with only a small amount of 5S RNA as checked by polyacrylamide electrophoresis according to Loening [16]. The lysine acceptor activity of the *t*RNA was at least 40 pmol/A_{260} unit.

Experiments on aminoacylation kinetics of unfractionated lupin *t*RNA with lysine performed with crude lupin synthetase, gave the same level of *t*RNA esterification as when partially purified enzyme was used. Also, the elution patterns of lys-*t*RNA^{lys} from RPC-5 columns were always the same, independent of the enzyme preparation (crude or purified) used for aminoacylation. The sp. act. of the crude enzyme preparation from 6-day-old cotyledons towards seed and cotyledon *t*RNAs was lower than that prepared from seeds; nevertheless the pattern of lys-*t*RNA^{lys} obtained after aminoacylation with cotyledon enzyme was the same as with the enzyme from seeds. Hague and Kofoid [17] made similar observations for black-eyed pea synthetases. The optimal condition of aminoacylation of *t*RNA with lysine were at pH 8.2 (Tris–HCl buffer). ATP/Mg ratio = 1:2.5 at 37°. The plateau of aminoacylation was obtained after 10 min of incubation.

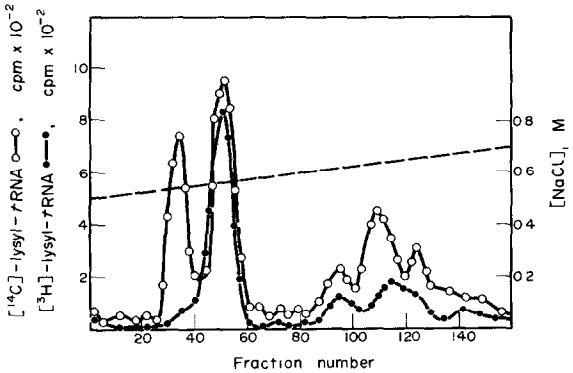

Fig. 1. RPC-5 chromatography of ^{14}C lysyl-*t*RNAs from lupin seed and ^{3}H lysyl *t*RNA from wheat germ. 50 A_{260} units of ^{14}C lysyl-*t*RNA and 30 A_{260} units of ^{3}H lysyl-*t*RNA were applied on a 1 × 50 cm RPC-5 column. The column was eluted with a linear gradient (720 ml total vol.) of 0.5–0.7 M NaCl in the 0.01 NaOAc buffer pH 4.5 containing 0.01 M $MgCl_2$ and 0.001 M EDTA. Fractions of 2.4 ml were collected at a flow rate of 1.2 ml/min

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The amount of charged *t*RNA applied on the column was usually about 50 A_{260} units, carrying 100–150000 cpm of ^{14}C lysine or 300–350000 cpm of ^{3}H lysine. The size of the column, flow rate and volume of gradient solution was always the same. Experimental technique sometimes might fail to distinguish small differences between *t*RNA species, therefore in this study care was taken to obtain maximal reproducibility of the elution profiles from RPC-5 columns. As shown in Fig. 1 3 well-defined and two less well resolved ^{14}C lysyl-*t*RNA peaks eluted at 0.54, 0.56, 0.63, 0.64 and 0.66 M NaCl respectively. Rechromatography of the separated ^{14}C lysyl-*t*RNA peaks showed that they eluted in the same position as originally. To ascertain whether the elution peaks of radioactivity represented intact polynucleotide chains the *t*RNA preparations had been heated to 80° for 5 min and quickly cooled. The elution pattern was the same after this treatment indicating that there were no breaks in the polynucleotide chains. The observed peaks were not artifacts of chromatography nor conformers, since they chromatographed on the same RPC-5 column in the presence of 7 M urea as separate peaks. For better characterisation of lysyl-*t*RNA isoacceptors, ^{14}C lysyl-*t*RNA was digested with RNAse T_1 and the product of digestion was analysed on DEAE cellulose and CM cellulose column as described by Merrick and Dure [18]. Two aminoacyl oligonucleotides produced from a total *t*RNA preparation charged with lysine were observed by chromatography on a CM cellulose column; the aminoacyl-oligonucleotides were not retained on a DEAE cellulose column (Fig. 2).

The major ^{14}C lysyl-oligonucleotide peak was derived from ^{14}C lys-$tRNA_1^{lys}$ and/or ^{14}C lys $tRNA^{lys}$, which clearly indicates that the two isoacceptors have identical 3′ terminal sequences. The minor ^{14}C lysyl-oligonucleotide was derived from one of the minor lysyl-*t*RNAs ($tRNA^{lys}_{3,4\,or\,5}$).

The 3 major lysyl-*t*RNA isoacceptors differ in their coding properties. Individual peaks of lysyl-*t*RNAs collected from RPC-5 column were concentrated by use of DEAE cellulose column then rechromatographed on a small RPC-5 column (0.7 × 19 cm) at pH 4.5. The pooled samples of ^{14}C lysyl-*t*RNAs were precipitated with ethanol and used in a ribosomal binding assay. The ribosomal binding studies were carried out with the

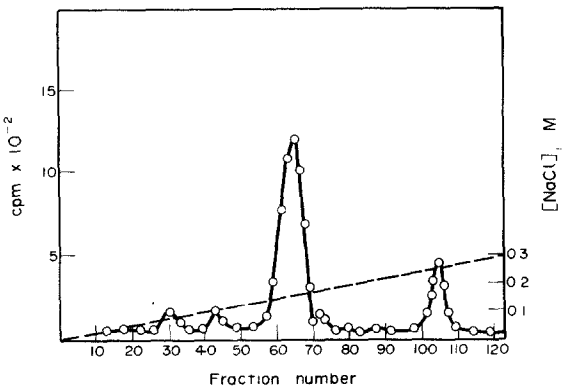

Fig. 2. Elution profiles of ^{14}C lysyl-oligonucleotides from CM cellulose column. 30 A_{260} units of T_1 RNAse digested ^{14}C lysyl-*t*RNA (120000 cpm) were applied on a 1 × 21 cm CM-cellulose column and eluted with linear gradient (200 ml total vol.) 0.0–0.3 M NaCl in 0.01 M NaOAc pH 4.5. Fraction of 2 ml were collected. 0.5 ml of each fraction was mixed with 10 ml Brays mixture [32] and its radioactivity was determined in Beckman LS100 scintillation counter.

triplets ApApA and ApApG or in the presence of poly (A). The results are shown in Table 1. These data show that $tRNA_1^{lys}$ is specific for the codon AAG and $tRNA_2^{lys}$ is specific for the codon AAA. Actually, the coding response of $tRNA_4^{lys}$ is not completely clear. This recognised both the codons AAA and AAG but was bound with half the efficiency of $tRNA_1^{lys}$ or $tRNA_2^{lys}$. It might be due to the incomplete separation of $tRNA_4^{lys}$ from minor lysyl-*t*RNA species. Similarly equal recognition of the two codons was observed by Ortwerth *et al.* [4] for $tRNA_5^{lys}$ from rat liver and mouse leukemic cell, which was also contaminated with some minor components. On the other hand, species having the anticodon UUU can recognize both AAA and AAG as was observed with $tRNA_1^{lys}$ from bakers' yeast [19] and black eyed pea [17].

It should be noted that generally low binding of the 3 lysine *t*RNA species might be caused by a non-optimal concentration of Mg^{2+}. Chiu and Suyama [20] found a very high Mg^{2+} optimum for *Tetrahymena* mitochondrial $tRNA^{lys}$ binding assays which can not be regarded as a unique property of *Tetrahymena* $tRNA^{lys}$.

Table 1. Binding of ^{14}C lysyl-*t*RNA from lupin seeds to wheat germ ribosomes

Aminoacyl-*t*RNA	Template	Amount applied (pmol)	Binding without template (pmol)	Specific binding with template (Δ pmol)
^{14}C lysyl-$tRNA_1^{lys}$	poly(A)	7.8	1.1	0.22
	AAA	7.8	0.62	0.21
	AAG	7.8	0.45	0.98
^{14}C lysyl-$tRNA_2^{lys}$	poly(A)	12.6	1.1	1.5
	AAA	12.6	1.0	0.98
	AAG	12.6	1.0	0.27
^{14}C lysyl-$tRNA_4^{lys}$	poly(A)	8.5	0.51	0.52
	AAA	8.5	0.49	0.51
	AAG	8.5	0 45	0.47

The procedure of ref. [34] was used for ribosomal-bindings assays. The reaction mixture (0.1 ml) contained 0.1 M Tris–acetate pH 7.2 0.02 M MgOAc, 0 05 M KCl, 2.5 A_{260} unit of wheat germ ribosomes, and 0 2 A_{260} unit of triplet of 20 μg of poly(A) Incubations were carried out at 25° for 20 min.

Table 2. Comparison of lysyl-tRNA species present in different aged lupin cotyledons and lupin seeds

Source of tRNA	Relative amount of each $tRNA^{lys}$ species % total				
	$tRNA_1^{lys}$	$tRNA_2^{lys}$	$tRNA_3^{lys}$	$tRNA_4^{lys}$	$tRNA_5^{lys}$
Seeds	17.5	36.2	11.3	25.4	9.6
Cotyledons from 3.5 hr imbibed seeds	26.7	31.1	8.3	20.9	13.0
Cotyledons 3 days	28.5	25.2	11.1	24.2	10.5
Cotyledons 6 days	32.9	59.7	7.4	—	—

The amount of radioactivity in each peak was summed and expressed as % of total counts

We have also examined the number and relative level of isoaccepting lysyl-tRNA at 3 developmental stages of cotyledons by RPC-5 cochromatography with lysyl-tRNA from seeds. Independent of the label (^3H or ^{14}C) of lysyl-tRNAs, the same results were always obtained. The data concerning distribution of radioactivity among the lysyl-tRNA species of developing cotyledons are summarised in Table 2. The proportion between lysyl-tRNA isoacceptors varied to some extent depending on the developing period. Quantitative or qualitative differences in isoaccepting species of different plant cells and tissues have been reported [21–23] but in the case of lysyl-tRNA the results are controversial [18, 21, 22]. The observed increase of $tRNA_1^{lys}$ and $tRNA_2^{lys}$ fractions in cotyledons of germinating lupin seeds is probably caused by the increase in the amount of chloroplast tRNA in these fractions [8, 18] especially as the levels of the chloroplast enzymes increase markedly in germinating cotyledons [24]. The above mentioned findings do not exclude the possibility of the appearance of tRNA species with some alteration in structure induced by special physiological events. This seems to be supported partly by the work of Raba *et al.* [25] on sequencing $tRNA_4^{lys}$ from mammalian cells.

We have noticed also that the amount of lysyl-tRNAs in wheat germ and barley germ is reduced when these tRNAs were aminoacylated with homologous or heterologous synthetases (from lupin seeds) and chromatographed on RPC-5 in the same condition as lupin tRNAs (Fig. 1). In wheat and barley germs one species of lysyl-tRNA is dominant, while in lupin seeds, lupin cotyledons and dry embryo axes 3 main species are observed. It is interesting that the embryo of developing grain of wheat contains at least 3 lysyl-tRNA species, and no significant differences in the relative amounts of lysyl-tRNA were observed at different stages of developing grain [10]. The reduced amount of lysyl-tRNA species in wheat germ and barley germ by comparison with lupin embryo axes, suggests a difference between the species of plants and that appearance or disappearance of some tRNA isoacceptors is stimulated by some metabolic processes.

EXPERIMENTAL

For germinating studies the seeds of *Lupinus luteus* (cv Express) were surface sterilized (with a freshly prepared soln made by filtering a slurry of 6 g CaOCl in 100 ml H_2O) for 10 min, and after thorough washing, left to imbibe in sterile H_2O for 3 hr. Seeds were germinated in daylight at 22° in sterile moist vermiculite. The germinating seedlings were dissected by hand into their component tissues. Harvested cotyledons were immediately frozen in dry ice at −30° and then ground. The meal was stored at 0° for 30 min to remove dry ice and used for extraction of tRNA. Lupin embryo axes were prepared from the dry seeds by hand. Wheat germ and barley germ were obtained from commercial companies.

Preparation of tRNA. tRNA from lupin seeds was prepared as described previously [26]. Crude tRNA was purified further on Sephadex G-100 column (2 × 140 cm) with 0.5 M NaCl, 0.01 M $MgCl_2$ and 2 mM EDTA. The tRNA containing fractions were pptd with EtOH and recovered by centrifugation. tRNA from cotyledons was extracted as follows: 200 g of chilled tissue were mixed with 200 ml of 25 mM Tris–HCl buffer pH 7.5 containing 0.1 M NaCl, 10 mM $MgCl_2$, 0.1% SDS, 1% of bentonite and 5 mM 2-mercaptoethanol and with 200 ml of PhOH containing 2 mM EDTA. The mixture was stirred in the cold for 30 min followed by centrifugation at 10000 *g* for 15 min. The PhOH extraction of the aq. phase was repeated twice more and finally the aq. phase was collected and the nucleic acid pptd by addition of 2.5 vol. of cold 95% EtOH in the presence of 0.1 vol. of M KOAc pH 5. The ppt. was collected by centrifugation and the resulting pellet was extracted with 2.7 M NaCl in a mixture of 10 mM $MgCl_2$, 10 mM EDTA, 7 mM 2-mercaptoethanol and 1% of bentonite for 1 hr. The ppt. was collected by centrifugation and discarded. The aq. phase was extracted twice with an equal vol. of $CHCl_3$–isoamyl alcohol (20:1). tRNA was pptd from the aq. layer by addition of 2.5 vol. of cold EtOH. tRNA was deacylated by incubation of its soln in Tris–HCl buffer (M) pH 8.5 for 35 min at 37° in the presence of 1% of bentonite. This crude tRNA was further purified on DEAE cellulose in 10 mM NaOAc buffer pH 4.5 containing 10 mM $MgCl_2$ and eluted with M NaCl in this buffer. 20 g of dry embryo axes from lupin were used for isolation of tRNA using the above method. tRNA from barley germ was prepared as in ref. [27]. Wheat germ tRNA was isolated by the method in ref. [28], omitting CTA fractionation.

Lysyl-tRNA synthetase. Extraction of crude synthetases from lupin seeds, 6-day-old cotyledons and wheat and barley germs were performed as described in ref. [29] with the exception that 0.1 M Pi buffer was used instead of 10 mM. The fraction precipitating between 35–50% $(NH_4)_2SO_4$ satd was further passed through a Sephadex G-75 column (2 × 50 cm) equilibrated with the same buffer. The first eluted fraction was pooled and used as a source of tRNA synthetases. Partially purified synthetases were obtained after chromatography on the aminohexyl-Sepharose [29].

Transfer RNA aminoacylation assay. The reaction was carried out at 37° in 0.1 M Tris–HCl buffer pH 8.2, 15 mM $MgCl_2$, 6 mM ATP, 2.5 mM 2-mercaptoethanol, 0.25 mg of crude enzyme (or 0.06 mg of purified enzyme), 0.005–0.05 mg tRNA and 2.5 nmol of lysine-[^{14}C] (210 mCi/mmol) or lysine-[^3H] (6 Ci/mmol). Reaction was terminated after 20 min and amino acceptor activity of tRNA was assayed by the filter-paper disc

method [30]. The amount of radioactivity on the filter was determined in a scintillation counter. For RPC-5 chromatography of aminoacyl-*t*RNA, the amount of *t*RNA in the reaction mixture was scaled up to 10 A_{260} units; the amount of other components was also increased by a corresponding amount. 5 reaction mixtures conducted simultaneously were then passed through a small DEAE cellulose column and aminoacyl-*t*RNA was eluted M NaCl in the NaOAc buffer pH 4.5 The sample was then diluted to 0.5 M NaCl and applied directly on RPC-5 column.

RPC-5 chromatography. The packing for RPC-5 chromatography was prepared according to ref. [31]. The column (1 × 50 cm) was operated at 23°. Chromatographic runs were at a flow rate of 1.2 ml/min; 2.4 ml fractions were collected. Aminoacyl-*t*RNA was separated with linear gradient of 0.5–0.7 M NaCl in 0.01 M NaOAc buffer pH 4.5 contained 10 mM $MgCl_2$, 1 mM EDTA. 0.5 ml was taken from every 2nd fraction and counted in Bray's soln [32]. The recovery of the radioactivity from the column was *ca* 90% of that applied. Co-chromatography expts were performed under the same conditions. The input ratio of radioactivity for $^3H/^{14}C$ aminoacyl-*t*RNA was 3:1 Protein content was determined by the method of ref. [31].

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