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A cell-free yellow lupin extract containing activities of pseudouridine 35 and 55 synthases*

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Plant cytoplasmic tyrosine tRNA was pseudouridylated at three different positions: 35, 39 and 55. These pseudouridines were introduced by three different enzymes – pseudouridine synthases. Variants of the *Arabidopsis thaliana* pre-$tRNA^{Tyr}$ were constructed that allow to monitor specifically pseudouridylation at different nucleotide positions. Using such RNAs to assay pseudouridine synthesis we have prepared an extract from *Lupinus luteus* cv. *Ventus* seeds containing activities of at least Ψ35 and Ψ55 synthases. This is the first report describing the preparation of the lupin seed extract that specifically modifies plant pre-$tRNA^{Tyr}$ transcribed by T7 RNA polymerase. U35 is converted to Ψ35 only in an intron-dependent manner, while pseudouridylation of U55 is insensitive to the presence or absence of an intron.

RNA maturation is a complex multistep process in which the primary transcript of a gene serves as a substrate for a large number of different enzymes. Cytoplasmic mature transfer RNA appears as a product of different enzyme activities that are involved in the maturation of pre-tRNA 5′- and 3′-ends, splicing and nucleoside modifications. While maturation of the 5′- and 3′-ends takes place in the nucleus [1] and the splicing occurs at the nuclear envelope [2], nucleoside modifications are introduced in different compartments of the cell: in the nucleus, in the cytoplasm and in organelles [3]. Several modified nucleosides in tRNA molecules are introduced only at defined steps of the tRNA maturation pathway. Some of them appear only before intron excision (in the nucleus) [4, 5], other only after intron removal (in the cytoplasm or in the organelles) [6]. There are, however, also some

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Abbreviations: bp, base pair(s); DTT, dithiothreitol; nt, nucleotide(s).

modified nucleosides the appearance of which is insensitive to the presence or absence of an intron [7]. Their introduction can occur in the nucleus (e.g. yeast N^2,N^2-dimethylguanosine [3]) or in the cytoplasm (queuosine [3]). An example of a modified nucleoside that is introduced at the stage of intron-containing tRNA precursor is pseudouridine, found in the middle of the GΨA (or QΨA) anticodon at position 35 in all eukaryotic cytoplasmic tyrosine tRNAs studied so far [4]. An example of a modified nucleoside that is introduced only after intron excision is Ψ32 in yeast cytoplasmic $tRNA^{Leu}$ (CAA) [7]. Pseudouridine 55 in the TΨC arm is an example of a modified nucleoside introduced into different pre-tRNAs in yeast and vertebrates in a manner insensitive to the presence of an intron [8]. While data that describe the tRNA maturation process involving nucleoside modification in yeast and vertebrates accumulate, the analogous processes that take place in plants remain almost unknown. A major handicap for studying plant tRNA maturation are the difficulties with the preparation of homologous *in vitro* transcription system. Last year the group of Sugiura reported the preparation of the first *in vitro* polymerase III plant-specific nuclear extract from tobacco cultured cells [9]. However, T7 RNA polymerase *in vitro* transcription assay could serve as a powerful tool since it can produce large amounts of a plant tRNA precursor that can be used in further observations on the maturation process [10]. A cell-free pre-tRNA processing and splicing system from wheat germ has been established [11].

Here we describe the preparation of a lupin seed extract that specifically modifies plant pre-$tRNA^{Tyr}$ transcribed by T7 RNA polymerase, giving pre-$tRNA^{Tyr}$ molecules almost fully pseudouridylated at the 35th and the 55th position. To our knowledge, this is the first report describing the preparation of such extract from lupin seeds. Furthermore, pseudouridylation that takes place in the middle position of the plant pre-$tRNA^{Tyr}$ anticodon is introduced only at the stage of an intron-containing precursor while the conversion of U55 to Ψ55 in the TΨC arm is insensitive to the presence or absence of an intron, as described in the case of yeast and vertebrates.

MATERIALS AND METHODS

Enzymes and reagents

RNase T2 was obtained from Calbiochem or Sigma, [α-^{32}P]NTPs (spec. act. 29.6 TBq/mmol), [α-^{35}S]dATP (spec. act. > 37 TBq/mmol) and [5-^{3}H]UTP (spec. act. 492 Gbq/mmol) were from Amersham (U.K.), T7 DNA polymerase sequencing kit from Pharmacia, and Muta-gene site-directed mutagenesis kit from Bio-Rad. T7 RNA transcription was carried out using a T7 RNA transcription kit from Promega. Restriction enzymes, Taq DNA polymerase and all other enzymes were from Boehringer Mannheim. Other reagents were from USB. Yellow lupin seeds cv.*Ventus* were obtained from the Plant Breeding Station in Wierzonka (Poland).

Preparation of yellow lupin S-23 extracts

Extract I. Extract I was prepared according to Stange & Beier [11] with some modifications: 10 g of yellow lupin seed meal was ground with 10 g of sea sand in a pre-cooled mortar until a fine powder was obtained. Then 30 ml of extraction buffer (10 mM Tris/acetate, pH 7.6, 3 mM $Mg(OAc)_2$, 50 mM KOAc, 1 mM dithiothreitol (DTT)) was added stepwise and grinding was continued at 4°C until a smooth paste was obtained. The extract was centrifuged twice for 10 min at 23000 ×***g*** and the precipitate discarded. The supernatant (crude extract) was fractionated with streptomycin sulfate. The supernatant was brought to 3% saturation by adding 20% stock of streptomycin sulfate and mixed for 30 min. The mixture was centrifuged at 13000 × ***g*** for 15 min. The supernatant was collected and the

precipitate discarded. Glycerol was added to the collected supernatant to a concentration of 10% (v/v). Then the supernatant fraction was dialysed for 2 h against 100 volumes of a buffer containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol. All the operations were carried out at 4°C.

Extract II. Extract II was prepared according to Guranowski & Pawełkiewicz [12] with some modifications: 10 g of yellow lupin seed meal was ground with 10 g of sea sand in a pre-cooled mortar until a fine powder was obtained. Then 30 ml of extraction buffer (10 mM potassium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol, 0.1 mM EDTA and 10% (v/v) glycerol) was added stepwise and grinding was continued at 4°C until a smooth paste was obtained. The extract was centrifuged twice for 10 min at 23000 × *g* and the precipitate discarded. Then the supernatant fraction was dialysed for 20 h against 100 volumes of extraction buffer. The dialysate was clarified by low speed centrifugation and the supernatant, designated the S-23 extract, was stored at 4°C. All the operations were carried out at 4°C.

Construction of *Arabidopsis thaliana* $tRNA^{Tyr}$ gene derivatives

The *A. thaliana* $tRNA^{Tyr}$ gene pATY2T7 containing a 12 bp intron was mutagenized using the method of Kunkel *et al.* [13]. The mutations were always confirmed by DNA sequencing according to Hattori & Sakaki [14]. Two

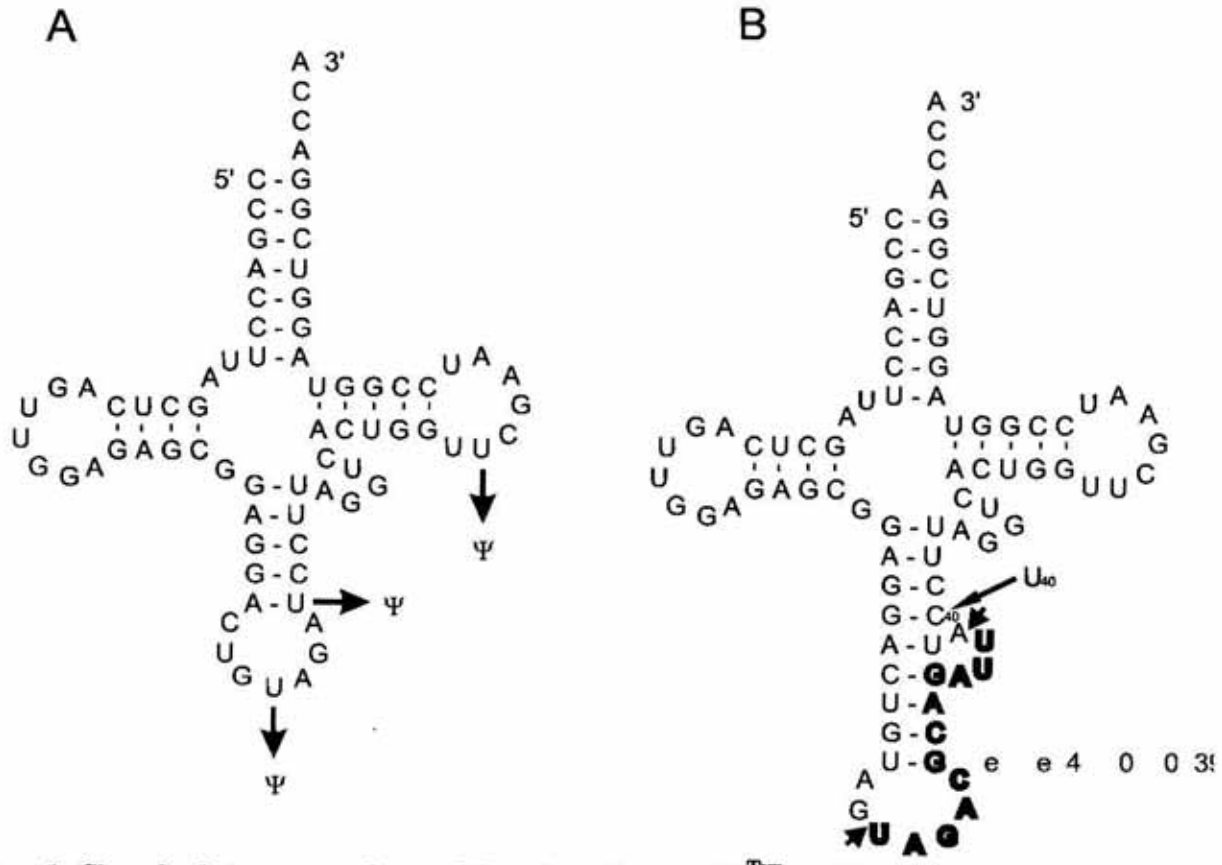

Figure 1. Cloverleaf structures of unmodified *A. thaliana* $tRNA^{Tyr}$ and its primary, intron-containing transcript.

(A) *A. thaliana* unmodified $tRNA^{Tyr}$. Pseudouridylated positions in the mature tRNA are marked. (B) pre-$tRNA^{Tyr}$ containing an intron. Long arrow shows the variant of pre-$tRNA^{Tyr}$ in which C40 has been substituted by U40. Short arrows point to the splicing-sites and the intron is shown in bold letters. The pre-$tRNA^{Tyr}$ variant deprived of the intron represents the cloverleaf structure identical with the structure shown in (A).

mutants of $tRNA^{Tyr}$ gene were constructed: pATY2T7U40 – in which C40 was replaced by U40, and pATY2T7ΔI – in which a 12 nt long intron was deleted (Fig. 1). Both mutants and the wild type *A. thaliana* $tRNA^{Tyr}$ gene were inserted under T7 RNA polymerase promoter and terminated by *Bst*NI restriction site using the PCR technique as described in [10].

T7 RNA transcription of pre-$tRNA^{Tyr}$ substrates using [5-^{3}H]UTP, [α-^{32}P]ATP, [α-^{32}P]UTP or [α-^{32}P]CTP

Plasmids carrying the $tRNA^{Tyr}$ gene or its variants were digested with *Mva*I (an isoschizomer of *Bst*NI) before transcription.

[^{3}H]RNA transcription

Transcription was performed in 20 mM Tris/HCl, pH 7.8, 20 mM $MgCl_2$, 40 mM NaCl, 4 mM spermidine, 10 mM DTT, 2 mM each ATP, CTP, GTP, 1.5 mM UTP, 40 nM linearized plasmid, 500 units/ml T7 RNA polymerase, and 200 μCi/ml of [5-^{3}H]UTP at 37°C for 8–10 h.

[^{32}P]RNA transcription

$tRNA^{Tyr}$ labelled with [α-^{32}P]ATP: transcription was performed in the T7 RNA polymerase buffer, 10 mM DTT, 1 unit/μl Rnasin, 660 μM each CTP, GTP, UTP, 44.4 μM ATP, 0.1 μg/μl linearized plasmid, 2.5 μCi/ml of [α-^{32}P]ATP and 1 unit/μl T7 RNA polymerase at 37°C for 1.5 h.

$tRNA^{Tyr}$ labelled with [α-^{32}P]UTP: transcription was performed in the T7 RNA polymerase buffer, 10 mM DTT, 1 unit/μl Rnasin, 0.5 mM each ATP, CTP, GTP, 120 μM UTP, 0.1 μg/μl linearized plasmid, 5 μCi/ml of [α-^{32}P]UTP and 1 unit/μl T7 RNA polymerase at 37°C for 1.5 h.

$tRNA^{Tyr}$ labelled with [α-^{32}P]CTP: transcription was performed in the T7 RNA polymerase buffer, 10 mM DTT, 1 unit/μl Rnasin, 0.5 mM each ATP, GTP, UTP, 12 μM CTP, 0.1 μg/μl linearized plasmid, 2.5 μCi/ml of [α-^{32}P]CTP and 1 unit/μl T7 RNA polymerase at 37°C for 1.5 h.

All RNA samples were purified by phenol/chloroform extraction, ethanol precipitation and were further purified on 10% PAGE/8 M urea.

In vitro pseudouridine formation assay

Reactions with [5-^{3}H]U $tRNA^{Tyr}$ were carried out in 80 μl reaction mixture containing 50 mM Tris/HCl, pH 7.8, 10 mM NH_4Cl, 5 mM DTT, 10 mM $MgCl_2$, 10–15 pmol of [5-^{3}H]U $tRNA^{Tyr}$ transcripts and 20 μl of S-23 extract. Incubation was at 37°C for 1.5 h. Reactions were stopped by addition of 95 μl of reaction mixtures to 1.0 ml of 12% active charcoal suspended in 0.1 M HCl. Samples were stirred and allowed to stand at room temperature for 5 min, then centrifuged to remove the charcoal; the supernatant was passed through a Millex-Gp filter assembly (0.22 μm). Usually a 0.5 ml sample was counted [15]. One unit of activity is the amount of enzyme catalysing the release of 1 pmol of ^{3}H to the supernatant in 30 min at 37°C.

In vitro pseudouridine formation assay for ^{32}P-labelled pre-tRNAs was carried out as described above for [5-^{3}H]U $tRNA^{Tyr}$. ^{32}P-labelled RNA products were purified by phenol/chloroform extraction, ethanol precipitation and further purified on 10% PAGE/8 M urea.

Quantification of pseudouridine in the ^{32}P-labelled tRNA samples

^{32}P-labelled (100000 c.p.m.) RNAs, obtained as described above, were digested with 0.5 units of RNase T2 for at least 5 h at 37°C in 10 μl of 5 mM ammonium acetate, pH 4.6. The labelled nucleotides were identified by two-dimensional chromatographic analysis on cellulose thin-layer plates as described by Grosjean *et al.* [6]. The efficiency of pseudouridine modification was measured by cutting out the

labelled spots from the TLC plates and counting the radioactivity by the liquid scintillation techniques. Taking into account the amount of label in each of the nucleotide spots (AMP, UMP, GMP, CMP and/or ΨMP) and knowing the relative number of labelled nucleotides per RNA substrate, the number of moles of each or both pseudouridines per mole of pre-tRNA was calculated.

RESULTS

Synthesis of unmodified $tRNA^{Tyr}$ variants

The biosynthesis of Ψ takes place at the polynucleotide level and involves an intramolecular rearrangement of uridine. Examination of the structure of Ψ suggests that it is chemically formed from U by cleavage of the carbon–nitrogen glycosyl bond, followed by rotation of the uracil base and then reattachment at C^5 to yield Ψ. Plant cytoplasmic tyrosine tRNA is pseudouridylated at three different positions: 35, 39 and 55 (Fig. 1). The pseudouridines are introduced most probably by three different enzymes – pseudouridine synthases. Variants of the tRNA substrate were constructed that allowed us to monitor specifically pseudouridylation at different nucleotide positions (Fig. 1). Using such RNAs to assay pseudouridine synthesis, enzymes producing this nucleoside could be identified and purified from *Lupinus luteus* extract. The wild type tyrosine tRNA containing an intron should be pseudouridylated at three positions: in the middle position of the anticodon (35th position) [16], at the 55th position in the TΨC arm and, possibly, at the 39th position [3]. The mutant $tRNA^{Tyr}$ molecule derived from pATY2T7U40 template should contain Ψ35, Ψ55 and possibly Ψ39. The mutant of $tRNA^{Tyr}$ molecule without an intron should be modified at two positions: 39th and 55th. Whether Ψ39 is introduced also at the stage of the intron-containing molecule remains unclear [3]. These calculations were based on the previous structural studies carried out in our laboratory and by others (for review see [3]).

Pseudouridylation of $tRNA^{Tyr}$ variants using lupin S-23 extracts

Yellow lupin seed S-23 extracts were prepared to test the activities of pseudouridine synthases as described in Materials and Methods. We tested two different protocols for plant extract preparation from seeds. Our attempts to prepare extracts containing active pseudouridine synthases from lupin seedlings were unsuccessful. Most probably the level of proteases, despite the large amounts of different protease-inhibitors used, was too high.

Usually extracts from yellow lupin seeds contained 10–15 mg of protein/ml as measured using the Bradford technique. Preliminary tests for Ψ conversion using wild type pre-$tRNA^{Tyr}$ labelled with [5-^{3}H]UTP as a substrate were carried out and the amount of tritium released was calculated. When the different RNA substrates were incubated with a lupin seed extract they all released tritium (Table 1).

Table 1. Plasmids and corresponding RNA transcripts

Plasmid	Nucleosides altered in $tRNA^{Tyr}$ gene	Potential sites of Ψ formation	Release of tritium from RNA incubated with lupin seed extracts
pATY2T7	none, wild type	U35, U39(?)*, U55	+
pATY2T7ΔI	intron deleted	U39, U55	+
pATY2T7U40	C40 → U40	U55 U35, U39(?)*	+

*The asterisk points to the discrepancies that exist for formation of Ψ39 which was found to be negatively intron-dependent for tobacco $tRNA^{Tyr}$ [11] and intron-insensitive in several other cases [3].

The finding that these RNAs release tritium indicates that they may be used to monitor specifically pseudouridylations at particular positions. The specific activity of pseudouridine synthases was measured using [5-^{3}H]U-pre-tRNA^{Tyr} containing an intron giving 0.6 units/μl of extract. Although it was not known at the time whether we monitor Ψ35, Ψ39 or Ψ55 formation, subsequent analysis proved that the isolated extracts contained only Ψ35 and Ψ55 pseudouridine synthase activities. All reactions were carried out using Extract I and Extract II. Both exhibited tritium release activity. For further experiments we decided to use Extract I because of simplicity of its preparation.

Identification of pseudouridylated positions in pre-tRNA^{Tyr} transcripts

The formation of Ψ35 can be followed by labelling the synthesised pre-tRNA^{Tyr} containing an intron with [α-^{32}P]ATP and subsequent hydrolysis by RNase T2. The labelled phosphate is transferred from the 5′ positions of the adenosines to the 3′ positions of the nearest neighbour. Under these conditions Ψ35 is labelled, but not the other two pseudouridines present at positions 39 and 55 in plant tRNA^{Tyr} which are followed by a cytidine. The formation of Ψ55 and Ψ39 can be followed by labelling the synthesised pre-tRNA^{Tyr} with [α-^{32}P]CTP. Since both: U55 and U39 will be visible after RNase T2 digestion, a mutant of the intron-containing tRNA^{Tyr} molecule was constructed, in which C40 that immediately follows U39 to be pseudouridylated, was changed to U40. In this case labelling of pre-tRNA^{Tyr} with [α-^{32}P]CTP will visualise Ψ55 while labelling with [α-^{32}P]UTP-Ψ39. The pre-tRNA^{Tyr} without an intron labelled with [α-^{32}P]CTP should show us the presence of Ψ55 and Ψ39.

^{32}P-labelled pre-tRNAs were incubated in lupin seed Extract I under conditions described in Materials and Methods. Table 2 shows the results of identification of pseudouridylated positions in different pre-tRNA^{Tyr} substrates and efficiency of the reactions.

Pseudouridine 35 is introduced efficiently into the wild-type pre-tRNA^{Tyr} containing an intron, while its introduction is inhibited in the case of an intron-less pre-tRNA^{Tyr} substrate (Fig. 2). Although it was long known, that in the case of yeast, vertebrates and insects, the conversion of U35 to Ψ35 absolutely requires the presence of an intron in the pre-tRNA^{Tyr}, here for the first time, it is shown clearly, that this is also true for plant pre-tRNA^{Tyr} and plant pseudouridine 35 synthase.

Analysis of pre-tRNA^{Tyr} mutant C40→U40 labelled with UTP revealed no pseudouridine at the 39th position (not shown). Furthermore, the same molecule when labelled with [α-^{32}P]CTP was fully modified (100% efficiency) at the 55th position (Fig. 3A). In the case of wild type intron-containing pre-tRNA^{Tyr} labelled with [α-^{32}P]CTP we never obtained the efficiency of pseudouridylation higher than 1 mole of Ψ per one mole of pre-tRNA^{Tyr} suggesting that there is no Ψ39 syn-

Table 2. Efficiency of pseudouridine 35 and 55 modification in lupin seed Extract I

Plasmid	Nucleosides altered in tRNA^{Tyr} gene	Labelling	Sites of Ψ formation	Ψ mole/mole pre-tRNA^{Tyr}
pATY2T7	none, wild type	[α-^{32}P]ATP	Ψ35	0.8
pATY2T7	none, wild type	[α-^{32}P]CTP	Ψ55	1.0
pATY2T7ΔI	intron deleted	[α-^{32}P]ATP	Ψ35	0.0
pATY2T7ΔI	intron deleted	[α-^{32}P]CTP	Ψ55	1.0
pATY2T7U40	C40→U40	[α-^{32}P]CTP	Ψ55	1.0
pATY2T7U40	C40→U40	[α-^{32}P]UTP	Ψ39	0.0

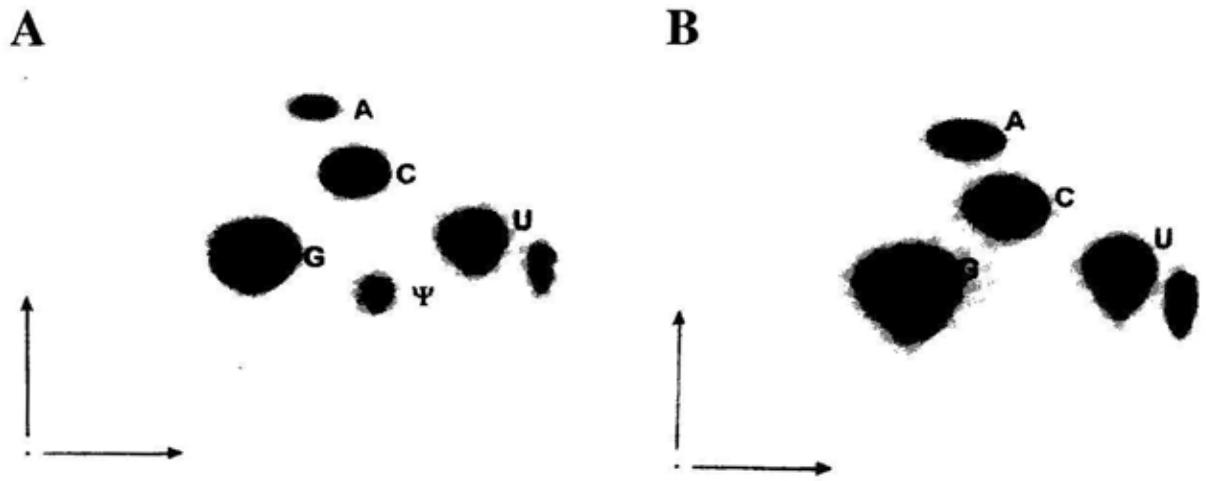

Figure 2. Pseudouridine 35 synthesis in yellow lupin Extract I.

Plasmid DNA of pATY2T7 and pATY2T7ΔI were transcribed using T7 DNA polymerase and [α-^{32}P]ATP. The transcripts were incubated for 1.5 h with yellow lupin Extract I, purified on 8% PAGE/8 M urea and analysed for the presence of Ψ35 after RNase T2 digestion by two-dimensional chromatography on cellulose thin-layer plates as described in Materials and Methods. (A) Autoradiogram of cellulose TLC plate obtained after chromatography of RNase T2 hydrolysate of intron-containing pre-tRNA^{Tyr}. The efficiency of Ψ35 formation was 0.8 mole of pseudouridine per one mole of pre-tRNA^{Tyr}. (B) Autoradiogram of cellulose TLC plate obtained after chromatography of RNase T2 hydrolysate of intron-less pre-tRNA^{Tyr}. The lack of the intron inhibited Ψ35 formation.

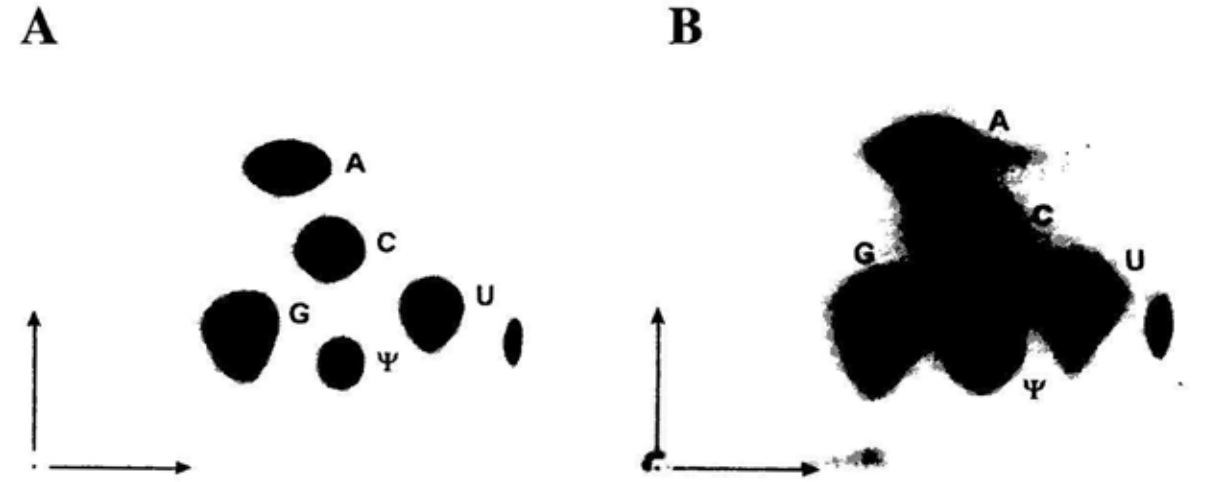

Figure 3. Pseudouridine 55 synthesis in yellow lupin Extract I.

Plasmid DNA of pATY2T7U40 and pATY2T7ΔI were transcribed using T7 DNA polymerase and [α-^{32}P]CTP. The transcripts were incubated for 1.5 h with yellow lupin Extract I, purified on 8% PAGE/8 M urea and analysed for the presence of Ψ55 after RNase T2 digestion by two-dimensional chromatography on cellulose thin-layer plates as described in Materials and Methods. (A) Autoradiogram of cellulose TLC plate obtained after chromatography of RNase T2 hydrolysate of intron-containing pre-tRNA^{Tyr} U40. The efficiency of Ψ55 formation was 1.0 mole of pseudouridine per one mole of pre-tRNA^{Tyr}. (B) Autoradiogram of cellulose TLC plate obtained after chromatography of RNase T2 hydrolysate of intron-less pre-tRNA^{Tyr}. The efficiency of pseudouridine 55 formation was 1.0 mole of Ψ per one mole of pre-tRNA^{Tyr}.

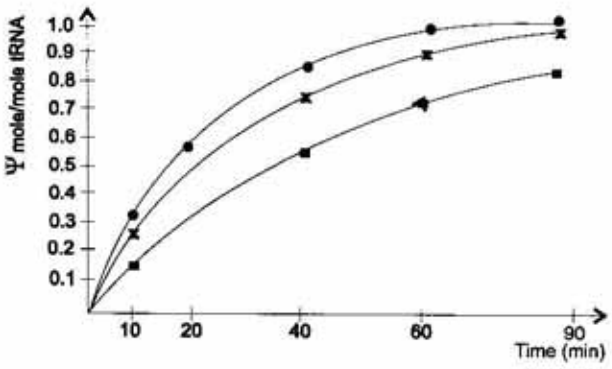

Figure 4. Kinetics of Ψ35 and Ψ55 formation in *A. thaliana* pre-tRNA^{Tyr} and its mutants.

About 2 pmole of appropriate ^{32}P-labelled pre-tRNA^{Tyr} transcripts was incubated with lupin Extract I for the time indicated, and analysed for the relative amount of pseudouridine residues at positions 35 and 55. ■, Wild type pre-tRNA^{Tyr} containing an intron, labelled with [α-^{32}P]ATP – visualisation of Ψ35; ✖, pre-tRNA^{Tyr} containing an intron, U40 labelled with [α-^{32}P]CTP – visualisation of Ψ55; ●, pre-tRNA^{Tyr} without an intron, labelled with [α-^{32}P]CTP – visualisation of Ψ55.

thase activity in the lupin extract. As described above, analysis of the pre-tRNA^{Tyr} U40 containing an intron labelled with [α-^{32}P]CTP revealed full modification of U55 to Ψ55 (Table 2, Fig. 2). The same results were obtained when pre-tRNA^{Tyr} without an intron, labelled with [α-^{32}P]CTP was analysed (Fig. 3B). Our experiments show that the introduction of this modified nucleoside in lupin extract is insensitive to the presence or absence of an intron.

Kinetic data for Ψ35 and Ψ55 formation are shown in Fig. 4.

DISCUSSION

Our results show the presence in lupin extract of at least two enzymatic activities responsible for the pseudouridylation of U35 and U55 in plant pre-tRNA^{Tyr}. We were not able to detect Ψ39 synthase activity in either the lupin extracts (data for lupin Extract II are not shown). There are discrepancies in the literature concerning the substrate requirements for formation of Ψ39, which was found to be negatively intron-dependent in the case of tobacco pre-tRNA^{Tyr} [11] and intron-insensitive in several other cases [3]. Since we did not observe the presence of Ψ39 in pre-tRNA^{Tyr} containing an intron, there are three possibilities to explain this phenomenon: (i) lupin pseudouridine synthase 39 is negatively-dependent on the presence of an intron, as it has been reported in the case of tobacco Ψ39 synthase, (ii) conversion of C40 to U40 inhibited the reaction, (iii) there is no Ψ39 synthase activity in the lupin seeds extract. We did not observe Ψ39 in the intron-containing precursor but it is not present, either, in the intron-less molecule (conclusions are drawn from the fact that both molecules were pseudouridylated only up to one mole of Ψ per mole of pre-tRNA and the mutant molecule of pre-tRNA^{Tyr} U40 was not pseudouridylated at all when labelled with [^{32}P]UT, and exhibited one mole of pseudouridine 55 when labelled with [^{32}P]CTP). It seems to us unlikely that the transition of C40 to U40 would inhibit pseudouridylation at the uridine 39: this position in different tRNAs is frequently pseudouridylated regardless of the nucleotide sequence environment [17]. We think that no Ψ39 synthase is present in the lupin Extract I.

U35 is converted to Ψ35 only in an intron-dependent manner, while pseudouridylation of U55 is insensitive to the presence or absence of an intron. It is unlikely that the two pseudouridines are introduced by the same enzyme. Pseudouridine synthase 35 requires the presence of an intron and a specific nucleotide sequence surrounding U35 to be modified [16]. Pseudouridine synthase 55 is an enzyme that modifies all cytoplasmic tRNAs at the conservative U55 in the TΨC arm, in a structural environment completely different from that for U35 [16,17]. The idea of more than one pseudouridine synthase acting on both sites is supported by the results obtained by Samuelsson & Olsson [18]. Using different mutants of yeast tRNA^{Gly} they have

shown that pseudouridine activities corresponding to positions 13, 32 and 55 in these tRNA substrates could all be separated chromatographically, indicating that there is a separate enzyme for each of these sites. Both Ψ35 and Ψ55 synthases seem to be present in the nucleus. Ψ35 synthase requires the presence of an intron for its activity. Intron removal takes place at the nuclear envelope before mature $tRNA^{Tyr}$ leaves the nucleus. Pseudouridylation has to occur before splicing, also in the nucleus. The activity of Ψ55 synthase is insensitive to the presence of an intron. Since it acts on substrates containing introns, it is possible that it is present also in the nucleus. The last idea is supported by the fact that yeast pre-$tRNA^{Tyr}$, when injected into the *Xenopus laevis* oocyte nucleus, is fully pseudouridylated at the stage of an intron-containing precursor [19].

The preparation of the lupin extract exhibiting the activity of both Ψ35 and Ψ55 synthase is the first step in the purification and isolation of the two enzymes and their genes.

REFERENCES

1. Hopper, A.K. & Martin, N.C. (1992) Processing of yeast cytoplasmic and mitochondrial tRNAs; in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*. (Jones, E.W., Pringle, J.R. & Broach, J.R., eds.) pp. 99–141, Cold Spring Harbor Laboratory Press, NY.

2. Peebles, C.L., Gegenheimer, P. & Abelson, J. (1983) Precise excision of intervening sequences from precursor tRNAs by a membrane-associated yeast endonuclease. *Cell* **32**, 525–536.

3. Grosjean, H., Szweykowska-Kulinska, Z., Motorin, Y., Fasiolo, F. & Simos, G. (1997) Intron dependent enzymatic formation of modified nucleotides in eukaryotic tRNAs: A review. *Biochimie* **79**, 293–302.

4. Johnson, P.F. & Abelson, J. (1983) The yeast $tRNA^{Tyr}$ gene intron is essential for correct modification of its tRNA product. *Nature* **302**, 681–687.

5. Szweykowska-Kulińska, Z., Senger, B., Keith, G., Fasiolo, F. & Grosjean, H. (1994) Intron-dependent formation of pseudouridines in the anticodon of *Saccharomyces cerevisiae* minor $tRNA^{Ile}$. *EMBO J.* **13**, 4636–4644.

6. Grosjean, H., Droogmans, L., Giege, R. & Uhlenbeck, O.C. (1990) Guanosine modifications in runoff transcripts of synthetic transfer RNA^{Phe} genes microinjected into *Xenopus* oocytes. *Biochim. Biophys. Acta* **1050**, 267–273.

7. Strobel, M.C. & Abelson, J. (1986) Effect of intron mutations on processing and function of *Saccharomyces cerevisiae* SUP53 tRNA *in vitro* and *in vivo*. *Mol. Cell Biol.* **6**, 2662–2673.

8. Melton, D.A., DeRobertis, E.M. & Cortese, R. (1980) Order and intracellular location of the events involved in the maturation of spliced tRNA. *Nature* **284**, 143–148.

9. Yukawa, Y., Sugita, M., Sugiura, M. (1997) Efficient *in vitro* transcription of plant nuclear tRNA(Ser) genes in a nuclear extract from tobacco cultured cells. *Plant J.* **12**, 965–970.

10. Szweykowska-Kulińska, Z., Krajewski, J. & Wypijewski, K. (1995) Mutations of *Arabidopsis thaliana* pre-$tRNA^{Tyr}$ affecting pseudouridylation of U35. *Biochim. Biophys. Acta* **1264**, 87–92.

11. Stange, N. & Beier, H. (1987) A cell-free plant extract for accurate pre-tRNA processing, splicing and modification. *EMBO J.* **6**, 2811–2818.

12. Guranowski, A. & Pawełkiewicz, J. (1977) Adenosylhomocysteinase from yellow lupin seeds. *Eur. J. Biochem.* **80**, 517–523.

13. Kunkel, T.A., Roberts, J.D. & Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.

14. Hattori, M. & Sakaki, Y. (1986) Dideoxy sequencing method using denaturated plasmid templates. *Anal. Biochem.* **152**, 232–238.

15. Nurse, K., Wrzesiński, J., Bakin, A., Lane, B.G. & Ofengand, J. (1995) Purification, cloning and properties of the tRNA Ψ55 synthase from *Escherichia coli*. *RNA* **1**, 102–112.

16. Szweykowska-Kulińska, Z. & Beier, H. (1992) Sequence and structure requirements for the biosynthesis of pseudouridine (Ψ35) in plant pre-tRNA^{Tyr}. *EMBO J.* **11**, 1907–1912.

17. Sprinzl, M., Steegboren, C., Hubel, F. & Steinberg, S. (1996) Compilation of tRNA sequences and of tRNA genes. *Nucleic Acids Res.* **24**, 68–72.

18. Samuelsson, T. & Olsson, M. (1990) Transfer RNA pseudouridine synthases in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 8782–8787.

19. Nishikura, K. & De Robertis, E.M. (1981) RNA processing in microinjected *Xenopus* oocytes. Sequential addition of base modifications in a spliced transfer RNA. *J. Mol. Biol.* **145**, 405–420.