Functional expression of the mutants of the chloroplast $tRNA^{Lys}$ gene from the liverwort, *Marchantia polymorpha*, in *Escherichia coli*

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The anticodon of the $tRNA^{Lys}$ gene (trnK) in the liverwort, *Marchantia polymorpha*, was artificially converted to an amber anticodon. This mutant $tRNA^{Lys}$(CTA) gene carrying either the intron or the C_{27}–C_{43} mismatch at the anticodon-stem is not functional in *Escherichia coli*, but without both of them, it does work as a $tRNA^{Lys}$ amber suppressor.

Chloroplast tRNA gene; *Marchantia polymorpha*; Amber suppressor; C_{27}–C_{43} mismatch

1. INTRODUCTION

Expressional machinery of the chloroplast in plants is basically prokaryotic. RNA polymerase, ribosomal proteins, rRNAs and tRNA show a strong homology to those of *E. coli* at the DNA sequence level [1,2], and display the same sensitivity for antibiotics [3]. With respect to the tRNA genes, however, they have 3 major differences when compared with the tRNA genes of *E. coli*. First, all chloroplast tRNA genes have no CCA tail. Second, the stem of some tRNA genes from chloroplast contains one or more mismatches for base-pairing. Finally, like other genes in chloroplast, some tRNA genes are split by the group I or II introns [4].

Interestingly, the trnK (a structural gene for $tRNA^{Lys}$(UUU)) in the liverwort has all these features: it has no CCA tail, a mismatch (C_{27}–C_{43}) at the first pair of the anticodon-stem, and a group II intron composed of 2111 base pairs at between the anticodon-roop and the anticodon-stem [5].

In this paper, it is shown how we converted the anticodon (TTT) of the $tRNA^{Lys}$ gene from the chloroplast to an amber anticodon (CTA) by in vitro mutagenesis. The expression of a set of derivatives originated from this mutant was tested by suppression of the amber mutations in *E. coli*.

2. MATERIALS AND METHODS

E. coli K12 strain, BT3 ($lacZ_{am1000}$ trp_{am} met_{am3} str^r $T6^r_{am}$ $Bf23^r_{am}$) and BT235 ($lacZ_{am1000}$ trp_{am} cys_{am235} str^r) [6], and CA274 ($lacZ_{am125}$ trp_{am}) [7] were used as a host for detection of the amber suppression. JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-pro)/F′ proAB $lacI^q$ lacZΔM15 traD36) and CJ236 (dut ung thi relA; pCJ105(cm^r)) [9] were used for plasmid manipulations. Amber mutants of phage λ, Pam3, Sam7, Pam80, Uam413, and Ram216, used for testing the suppression pattern, were from our laboratory stock.

M9 minimal medium and LB broth [10] were used for cultivation of bacteria.

In order to construct the mutant tRNA gene, a 2307 bp *Sau*3AI-*Hin*cII fragment from liverwort chloroplast DNA, the position 26010–28316 [11], was cloned into pBS^+ plasmid, which was purchased from Stratagene. Primer 1, GGTAGAGTACTACTCGGCTCTAAAGTGCGAGTTGG, for converting the anticodon; primer 2, GAGTACTCGGCTCTAAACCGACGAGTTCCGGGA, for deleting the intron; primer 3, CTTCATCCGAGGAGTTCCGGG, and primer 4, GATTTATTTAAAAAATTGTTTAGAGTTTTTAAACAGTC, for converting the anticodon-stem and the exon binding sequence (EBS), respectively, were synthesized by using ABI 380B DNA synthesizer. In vitro mutagenesis was performed as described in [9]. For a deletion of the intron region, the primer 4 was hybridized to ssDNA, and then the complementary strand was synthesized with Klenow-enzyme. Looped out intron and unhybridized ssDNA were digested with mung-bean nuclease and the products were transformed to JM109 competent cells. All the mutant tRNA genes, which were ligated downstream of the lac promoter on pUC18, were confirmed by DNA sequencing.

3. RESULTS

3.1. *Expression of the chloroplast tRNA mutants constructed in vitro in* E. coli

Plasmids used in this study are shown in Fig. 1. pLY113 contains a wild-type trnK gene. pLY123 contains a mutant tRNA gene, in which the anticodon was changed to an amber anticodon. pLY223 contains a mutant with the amber anticodon but without an entire intron region. Those genes have been inserted under the lac promoter on each plasmid. *E. coli* strain CA274 was transformed with those plasmids. If the mutant tRNA genes are functionally expressed, Amp^r transformants

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Abbreviations: ssDNA, single-stranded DNA; IBS, intron binding sequence; EBS, exon binding sequence

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(a)

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LY113     GGGTTGCTAACTCAATGGTAGAGTACTCGGCTTTTAA---2111bpIVS---CCGACGAGTTCCGGGTTCGAGCCCCGGGCAACCCA
LY123     ::::::::::::::::::::::::::::::::CTA::---2111bpIVS---::::::::::::::::::::::::::::::::::
LY223     ::::::::::::::::::::::::::::::::CTA::    no IVS    ::::::::::::::::::::::::::::::::::
LY223-2   ::::::::::::::::::::::::::::::::CTA::    no IVS    ::::G:::::::::::::::::::::::::::::
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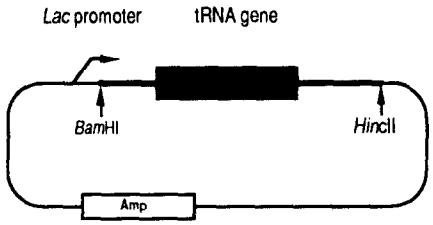
(b)



(c)

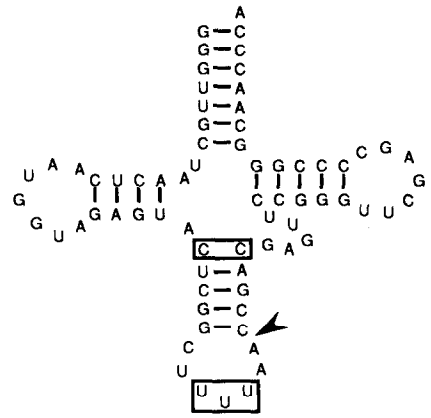


Fig. 1. (a) Sequence of the tRNA gene region. LY113, a wild-type trnK gene with 30 bp at the 5′- and 57 bp at the 3′-flanking region; LY123, the anticodon was removed; LY223, the intron was removed; LY223-2, C_{43} at the anticodon-stem was changed to G_{43}. (b) Structure of plasmids. Each tRNA gene described in (a), was inserted into the *Bam*HI-*Hin*cII sites of pUC18. They are transcribed by a lac promoter in pUC18. (c) A clover-leaf structure model of a wild-type trnK gene of the chloroplast. The anticodon and the C_{27}–G_{43} mismatch in the anticodon-stem is enclosed in squares. An arrow indicates the position of the intron.

should grow on the minimal lactose plates, showing the suppression of two amber markers of CA274. In experiments, no growth was observed in all 3 cases.

3.2. *Isolation of the Sup$^+$ mutants from pLY223*

CA274 carrying pLY223 were plated on the minimal lactose plates containing ampicillin, and spontaneous Lac^+Trp^+ colonies were isolated. To eliminate the mutants on the host chromosome, plasmid DNAs were extracted and re-transformed to the original CA274 bacteria. 8 out of 16 clones examined exhibited amber suppressor activity. To demonstrate whether or not the mutations in those plasmids were dropped in the tRNA gene, their DNA sequences were determined. In all mutant clones, C residue which corresponds to the position 43 of the tRNA molecule was changed to G, restoring a normal C–G pair at the anticodon-stem. We designated those plasmids and the suppressors as pLY223-2 and supMp, respectively.

3.3. *Are the chloroplast tRNA^{Lys}(CUA) molecules charged with lysines in* E. coli*?*

To test this, *E. coli* strains carrying the well-known amber mutations were transformed with pLY223-2 and the suppression spectrum of supMp was compared with the authentic suppressors, as shown in Table I. SupMp shows the same suppression pattern as supD and supG. On the other hand, bacteria carrying pLY223-2 were infected with a set of amber mutants of phage λ, and their growth was examined. Table II represents the results, showing that the suppression pattern by supMp is the same as that by supG, which is derived from a $tRNA^{Lys}$ gene of *E. coli*. From these experiments, it was supposed that the mutant $tRNA^{Lys}$(CUA) encoded by the supMp on pLY223-2 could be charged with a lysine in *E. coli*.

3.4. *Expression of the mutant tRNA^{Lys}(CTA) gene with the intron*

Because the anticodon-stem of the chloroplast tRNA gene in pLY123 has a C–C mismatch, no suppression may be expected even if the transcripts from pLY123

Table I

Suppression patterns of the supMp and the authentic suppressors on *E. coli* amber mutations

Suppressor gene	Amino acid inserted	*E. coli* amber mutation			
		lac_{am1000}	trp_{am}	cys_{am235}	met_{am3}
supD	Ser	–	+	–	–
supE	Gln	+	+	–	–
supF	Tyr	–	+	+	+
supG	Lys	–	+	–	–
supP	Leu	–	+	+	–
supU	Trp, Gln	+	+	+	+
supMp		–	+	–	–

All suppressors except supG are amber suppressors. supG is an ochre suppressor. Bacteria possessing a specific amber mutation were transformed with pLY223-2, and their growth on minimal plates after 2 days incubation at 32°C were examined. The authentic suppressors were described in [6]. + indicates growth; –, no growth.

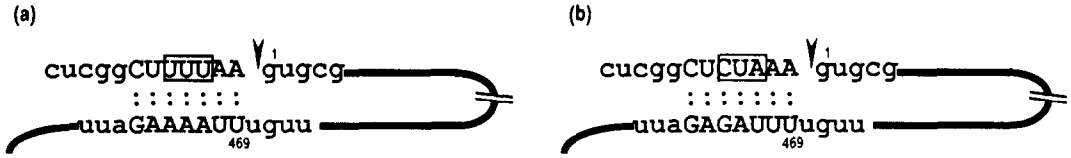


Fig. 2. IBS–EBS pairing model. IBS and EBS are in upper case. The anticodon bases are enclosed in a square. An arrow indicates the exon-intron junction. Numbers are the positions in the intron. (a) LY113 (wild-type); (b) LY123-22.

were spliced in *E. coli*. Moreover, it has been reported that a group II intron contains both an intron binding sequence (IBS) in the 3′ end of the 5′ exon and an exon binding sequence (EBS) within the intron, and that their pairing is necessary for splicing [14]. Fig. 2a shows the IBS–EBS structure model of the trnK intron. Because a putative IBS in most chloroplast tRNA introns overlaps the anticodon region, conversion of the anticodon might inhibit the splicing. From these respects, we introduced another two mutations into the $tRNA^{Lys}$ gene on plasmid pLY123; namely, one mutation restores the mismatch at the anticodon-stem, and the other at the EBS matches with the altered IBS, as shown in Fig. 2b. Regardless of these considerations, this mutant tRNA gene on the plasmid, designated as pLY123-22, could not show the suppressor activity, indicating that no splicing of such an intron occurred in *E. coli*.

4. DISCUSSION

In this paper, we have described how *E. coli* can functionally express an intron-less chloroplast $tRNA^{Lys}$(CTA) gene, where a C_{27}–C_{43} mismatch at the anticodon-stem was corrected to a normal pairing, C_{27}–G_{43}. Considering that many tRNA genes in chloroplast have some mismatch at the same position, we suppose that a translational machinery of the chloroplast could utilize even such tRNAs in the plant cell. In *E. coli*, only one tRNA gene, $tRNA^{Thr}$(CGU), which has a mismatch (G_{27}–A_{43}) at the same position, has been reported [15]. It would be interesting to know whether or not this $tRNA^{Thr}$(CGU) of *E. coli* is functional. Once the mismatch of the chloroplast $tRNA^{Lys}$(CUA) was repaired, *E. coli* can correctly charge a lysine to the mutant tRNA molecule, judging from the suppression pattern.

We analyzed the sequence of 8 Sup^+ mutants isolated independently from pLY223. Interestingly, all of the mutants had the same base change from C_{43} to G_{43}. Nevertheless, an equal frequency of the mutation at the position 27 from C to G could be expected. It is possible that the mutant $tRNA^{Lys}$(CUA), having a pairing G_{27}–C_{43}, is not a good amber suppressor. If this is the case, it could be proposed that only a pairing at the position 27–43 is not sufficient to restore activity in vivo.

Although we have not yet succeeded in detecting the splicing of a group II intron in *E. coli*, the system described in this report may be useful for investigating some *cis*- and/or *trans*-acting requirements for splicing of the group II intron.

Table II

Suppressor patterns of the supMp and the authentic suppressors on λ amber mutations

Suppressor gene	λ amber mutation				
	P_{am3}	S_{am7}	P_{am80}	U_{am413}	P_{am216}
supD	+	–	–	+	–
supG	–	–	–	+	–
supMp	–	–	–	+	–

Suppression was determined by growth of the mutant phages (1×10^6 PFU/spot) spotted onto the lawn of CA274 bacteria carrying pLY223-2. Suppression by the authentic suppressors has been described in: [6] for P_{am3}, P_{am80} and S_{am7}; [11] for U_{am413}; [12] for R_{am216}. The plates were incubated at 37°C overnight. + indicates growth; –, no growth.

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