Identity elements and aminoacylation of plant tRNATrp

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ABSTRACT
Mutation of the Arabidopsis thaliana tRNATrp anticodon or of the A73 discriminator base greatly diminishes in vitro aminoacylation with tryptophan, indicating the importance of these nucleotides for recognition by the plant tryptophanyl-tRNA synthetase. Mutation of the tRNATrp anticodon to CUA so as to translate amber nonsense codons permits tRNATrp to be aminoacylated by A. thaliana lysyl-tRNA synthetase. Thus, translational suppression by tRNATrp observed in plant cells includes significant incorporation of lysine into protein.

INTRODUCTION
Highly specific interactions between tRNAs and their cognate aminocyl-tRNA synthetases help ensure the fidelity of translation. tRNAs contain discrete sets of nucleotides (identity elements) required for tRNA recognition by cognate and to prevent recognition by non-cognate aminocyl-tRNA synthetases (reviewed in 1–3). Considerable progress has been made in determining these identity elements for different tRNAs from Escherichia coli and yeast. Most frequently, they are concentrated within the anticodon loop and/or the acceptor stem, rendering tRNAs into palimpsests of the earliest genetic code (4). However, there are differences in tRNA recognition between species. For example, nonsense amber suppressors derived from tRNATyr are charged with tyrosine in E. coli but with leucine in yeast (5) and suppressors derived from tRNATrp are charged with glutamine in E. coli, but in Saccharomyces cerevisiae there is no change in aminoacylation specificity (6–8). Only sparse evidence is available about identity elements in plant tRNAs (9). Here we document several identity elements of plant tRNATrp and determine that mutation of C35 in the tRNATrp anticodon to form an amber suppressor tRNA promotes misacylation by plant lysyl-tRNA synthetase. Translational suppression by this tRNATrp in plant cells causes the introduction of lysine at amber nonsense codons.

MATERIALS AND METHODS
Preparation of template DNAs and in vitro transcripts
The anticodon of tRNATrp (10) was changed to CUA (amber), UCA (opal), CCC (Gly) and CCG (Arg) and the discriminator base A73 was changed to G73 by oligonucleotide-directed mutagenesis (Chameleon Mutagenesis Kit, Stratagene). The DNAs were amplified by PCR with a 5’ primer including the T7 RNA polymerase promoter sequence d(CAGTAATACGACT- ACTATAGGATTCGCGCA) and 3’ primer including a BstNI restriction site d(CCCTGGTGAACCGACGTAATCG). In vitro transcription of these DNAs after digestion with BstNI yields unmodified tRNA transcripts with a 3’-CCA end (11). The T7 transcript of tRNATrp (12) was produced using the same procedure.

Aminoacylation of tRNA transcripts
In vitro transcripts of tRNAs were prepared using the Ribomax ™ System (Promega). Aminoacylation of tRNA transcripts was performed at 37°C in an aminoacylation mixture containing 25 mM Tris–HCl, pH 8.0, 1 mM ATP, 2 mM MgCl2, 1 mM spermine, 0.1 mM DTT, amino acids (as specified) and tRNA transcript (as specified).

Translational suppression of firefly luciferase
The preparation and transfection of carrot (Daucus carota) protoplasts were performed as described previously (10). A β-glucuronidase reporter construct was included as an internal standard to normalize transfection efficiency (10). Transfected protoplasts were incubated at room temperature for 24 h, pelleted at 200 g for 15 min, the supernatant was removed and protoplasts were resuspended in 100 μl of cell culture lysis reagent (Promega). An aliquot of 20 μl of cell extract was mixed with 100 μl of luciferase assay reagent (Promega). The reaction mix was placed in a luminometer (model 3010; Analytical Scientific Instruments, Alabama, CA) and counted for 10 s. β-Glucuronidase assays were performed as described by Jefferson (13).

RESULTS AND DISCUSSION
Identity elements of plant tRNATrp
Previously we described the isolation of seven tRNATrp genes from the nuclear genome of Arabidopsis (10,14). All but one of these genes have identical coding regions, with the tRNATrp2 gene having a single C→T substitution in position +5 (where +1 is the position of the first nucleotide of the mature tRNA) (14). The anticodon of tRNATrp2 was changed by oligonucleotide-directed mutagenesis to CUA (amber), UCA (opal), CCC (Gly) and CCG (Arg) codons and the discriminator base A73 was changed to G73 (Fig. 1).
Figure 1. Predicted transcripts of tRNA^{Trp1} and tRNA^{Trp2} genes from A.thaliana. The anticodon mutations and the acceptor stem differences are indicated.

Using conditions where a tRNA^{Trp2} transcript was efficiently aminoacylated with tryptophan, transcripts with single nucleotide substitutions in the anticodon were not detectably aminoacylated (Fig. 2). Thus, as with prokaryotic and yeast tRNA^{Trp}, the nucleotides of the plant tRNA^{Trp} anticodon are important identity elements (15–19). The tRNA^{Trp1} transcript was aminoacylated at a rate only slightly above that observed with the tRNA^{Trp2} transcript (data not shown) indicating that the fifth nucleotide of the acceptor stem, which differs between tRNA^{Trp1} and tRNA^{Trp2}, is only a minor identity element. This is also the case for Bacillus subtilis tRNA^{Trp} (18). The plant tRNA^{Trp} discriminator base is also extremely important for aminoacylation, for mutation of A73 to G73 greatly reduces aminoacylation of the tRNA^{Trp2} transcript (Fig. 2). In contrast, G73 is required for aminoacylation by the prokaryotic tryptophanyl-tRNA synthetase (15–17).

Genes encoding tRNA^{Trp}_{CCU} (amber), tRNA^{Trp}_{CCA} (ochre) and tRNA^{Trp}_{CCA} (opal) were introduced into carrot protoplasts together with firefly luciferase reporter genes containing amber, ochre andopal codons, respectively, so as to measure translational suppression. The luciferase amber reporter gene was suppressed by tRNA^{Trp}_{CCU} between 0.5 and 5% in different assays; the level of suppression of the ochre reporter gene by tRNA^{Trp}_{CCA} was always several fold less, and suppression of the opal reporter gene by tRNA^{Trp}_{CCA} was not detected (Fig. 3; 10,20). In light of the undetectable in vitro aminoacylation of these tRNAs by tryptophanyl-tRNA synthetase, the significant capacity of tRNA^{Trp}_{CCA} to suppress amber nonsense mutations could be due to the aminoacylation of these tRNA^{Trp} species in vivo by other aminoacyl-tRNA synthetases.

tRNA^{Trp}_{CCU} is charged with lysine in vitro and encodes lysine in vivo

Concomitant with these studies, we observed that a luciferase reporter gene (am-206), whose activity relies upon incorporation of Lys at a site important for function (12), was suppressed in vivo as efficiently by tRNA^{Trp}_{CCU} as was a luciferase reporter gene (am-4) with an amber mutation at a site which tolerates a variety of amino acids (10; Fig. 4). This suggests that tRNA^{Trp}_{CCU} might be charged with lysine in vivo. Furthermore, co-transfection of the gene for tRNA^{Trp}_{CCU} with the gene for A.thaliana lysyl-tRNA synthetase increased translational suppression of the reporter gene (Fig. 4), also consistent with the notion that tRNA^{Trp}_{CCU} is charged by the lysyl-tRNA synthetase. (Cloning and expression of the gene for A.thaliana lysyl-tRNA synthetase will be described elsewhere.). In E.coli, tRNA^{Trp}_{CCU} suppressors are charged efficiently with Trp or Gln in vitro and insert predominantly Gln during translation in vivo (6, 7), but for S.cerevisiae tRNA^{Trp}_{CCU} there is no change in aminoacylation specificity (8). To confirm that the plant tRNA^{Trp}_{CCU} is indeed aminoacylated by lysyl-tRNA synthetase, we measured its charging in vitro with purified A.thaliana lysyl-tRNA synthetase. Remarkably, the tRNA^{Trp}_{CCU} transcript was aminoacylated to a 3-fold greater extent than a tRNA^{lys}_{CCU} transcript (Fig. 5), indicating that tRNA^{Trp} and tRNA^{lys}_{CCU} share features important for recognition. A likely

Figure 2. Aminoacylation of tRNA^{Trp2} transcripts with tryptophan. Assays were performed with aminoacylation mixtures containing 30 µM L-[3H]Trp (34 Ci/mmol), wheat germ extract (Promega) and 4 µM tRNA transcript.
explanation for the observed preference for tRNA$^{\text{Trp}_{CUA}}$ is that it contains A73, which we believe to be preferred over G73 by *A.thaliana* lysyl-tRNA synthetase (A.Topin and W.Folk, unpublished results).

These data demonstrate that the anticodon and the discriminator base A73 are major identity elements of plant tRNA$^{\text{Trp}}$, consistent with observations of others that the anticodon of tRNA$^{\text{Trp}}$ is phylogenetically conserved as an identity element (15–19) and with the suggestion that the replacement of G73 in prokaryotic tRNA$^{\text{Trp}}$ by A73 in eukaryotic tRNA$^{\text{Trp}}$ mirrors evolutionary changes in the specificity of the respective tryptophanyl-tRNA synthetases (18). As sequences in the anticodon-binding domain of the *E.coli* tryptophanyl-tRNA synthetase that distinguish between C35 and U35 also transmit information to the opposite end of the enzyme so as to modulate amino acid binding (19,21), it would be interesting to determine whether this interaction has been preserved in the plant tryptophanyl-tRNA synthetase, concomitant with the evolutionary change in specificity for the discriminator base. Additionally, we have shown that plant tRNA$^{\text{Trp}_{CUA}}$ is charged with lysine in *vivo* and promotes translation of lysine at amber codons in *vivo*. This contrasts with observations made using analogous tRNA$^{\text{Trp}_{CUA}}$ suppressors in *E.coli* and yeast, which are charged preferentially by glutamine or tryptophan, respectively (8,15), suggesting that the plant lysyl-tRNA synthetase differs in its specificity for the anticodon nucleotides. A consequence of this is that translational suppression by tRNA$^{\text{CUA}}$ observed in plant cells (10,20) includes significant incorporation of lysine into protein.

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