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Short Communication

Running title:

Translation of *psbC* mRNAs starts from GUG

Corresponding author:

Masahiro Sugiura

Graduate School of Natural Sciences, Nagoya City University, Yamanohata, Mizuho,

Nagoya 467-8501, Japan

Phone: +81-52-781-7137

Fax: +81-52-781-7196

E-mail: sugiura@nsc.nagoya-cu.ac.jp

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Translation of *psbC* mRNAs starts from the downstream GUG, not the upstream AUG, and requires the extended Shine-Dalgarno sequence in tobacco chloroplasts

Hiroshi Kuroda[1], Haruka Suzuki[1], Takahiro Kusumegi[2, 4], Tetsuro Hirose[2, 5], Yasushi Yukawa[1], Masahiro Sugiura[1,2,3]*

[1]*Graduate School of Natural Sciences, Nagoya City University, Yamanohata, Mizuho, Nagoya 467-8501, Japan*

[2]*Center for Gene Research, Nagoya University, Nagoya 464-8602, Japan*

[3]*Sugiyama Human Research Center, Sugiyama Jogakuen University, Nagoya 464-8662, Japan*

Abbreviations:

bp, base pairs; mGFP, modified green fluorescent protein; nt, nucleotides; SD, Shine-Dalgarno; UTR, untranslated region.

Footnotes:

[4]*Present address: Toyota Motor Corporation, Toyota, Toyota 471-8572, Japan*

[5]*Present address: National Institute of Advanced Industrial Science and Technology, Tokyo 135-0064, Japan*

Plastid *psbC* gene encodes a CP43 subunit of Photosystem II. Most *psbC* mRNAs of many organisms possess two possible initiation codons, AUG and GUG, and their coding regions were generally annotated from the upstream AUG. Using an chloroplast *in vitro* translation system, we show here that translation of the tobacco plastid *psbC* mRNA initiates from the GUG. This mRNA possesses a long Shine-Dalgarno(SD)-like sequence, GAGGAGGU, 9-nucleotides upstream of the GUG. Point mutations in this sequence abolished translation, suggesting that a strong interaction between this extended SD-like sequence and the 3'-end of 16S rRNA facilitates translation initiation from the GUG.

Keywords:

GUG codon - *In vitro* - *psbC* - Shine-Dalgarno sequence - Tobacco - Translation.

Plastids carry their own genome and gene expression system. The plastid genome of higher plants contains about 120 genes in which about 80 code for polypeptides (Sugiura 1992). Several genes contain multiple possible initiation codons and generally the longest open reading frames were assigned as protein-coding regions. The plastid translation apparatus is similar to the eubacterial system, whereas about two-thirds of the plastid mRNAs lack the Shine-Dalgarno (SD)-like sequence at the proper position (Bonham-Smith and Bourque 1989, Sugiura et al. 1998). This situation makes difficult to assign initiation codons of plastid mRNAs. One of the methods to define initiation codons is N-terminal sequencing of corresponding proteins. The N-terminus of a number of chloroplast proteins has thus far been determined to be either a methionine residue or a non-methionine residue because of the N-terminal excision (Giglione and Meinnel 2001, Giglione et al. 2004). Translation initiation sites are simply defined to be corresponding AUGs if N-terminal amino acids are methionine residues. Otherwise, conclusive definition is difficult when two or more possible initiation codons are located close to each other.

The *psbC* gene encodes the CP43 subunit of photosystem II and partially overlaps with the upstream *psbD* gene in land plants (Shinozaki et al. 1986, Yukawa et al. 2005). These two genes are cotranscribed with the downstream *psbZ* (ORF62) to produce long transcripts, which are then processed into multiple overlapping mRNAs (Yao et al. 1989, Sexton et al. 1990, Kawaguchi et al. 1992, Chen et al. 1995). In addition, *psbC* alone is transcribed from its own promoter within the *psbD* coding region (Yao et al. 1989). The *psbC* genes of many organisms generally include two possible initiation codons, ATG and GTG, within the overlapping region. A mutagenesis study of *psbC* translation in the cyanobacterium *Synechocystis* sp. PCC 6803 showed that the GTG codon is the sole initiation codon (Carpenter et al. 1990). In *Chlamydomonas reinhardtii* and *Synechococcus* sp. PCC 7942, a GTG is the initiation codon since no upstream ATG exists (Rochaix et al. 1989, Carpenter et al. 1990). However, many *psbC* gene annotations in databases still assign ATG as the initiation codon and no direct evidence for the initiation codon of *psbC* mRNAs has been reported in higher plant chloroplasts.

We recently established a highly active *in vitro* translation system from isolated tobacco chloroplasts based on the previous system (Hirose and Sugiura 1996) and analyzed the effect of chloroplast mRNA processing on translation (Yukawa et al. 2007). Fluorescence of a modified green fluorescent protein (mGFP), in place of radioactive amino acids, was used to detect translation products and to measure the efficiency of translation. This method was then applied for measuring the translation rate of several synonimous codons (Nakamura and Sugiura 2007). Here, we show that the translation initiation codon of tobacco *psbC* mRNAs is the downstream GUG, but not the upstream AUG, using the *in vitro* translation system.

Figure 1

Fig. 1 shows sequence alignment of *psbC* portions surrounding the translation initiation sites from several organisms. These sequences are well conserved and include in-frame ATG and GTG, separated by 33 base pairs (bp) except for *Chlamydomonas* and *Synechococcus* 7942. Since the N-terminus of CP43 proteins (*psbC* gene product) was identified as *N*-acetylated, *O*-phosphorylated threonine residue (Ac-T^{P}) in Arabidopsis (Vener et al. 2001), pea (Gómez et al. 2002) and spinach (Michel et al. 1988), N-terminal methionine and glutamic acid residues should be excised from the nascent protein probably by peptide deformylase and other processing activities (Giglione et al. 2004). Therefore, GTG is likely to be the start codon, which is consistent with that of the *Synechocystis* 6803 gene. However, it cannot be ruled out the possibility that both ATG and GTG or ATG alone are initiation codon(s) in higher plant chloroplasts.

To determine the initiation codon of tobacco *psbC* mRNAs, we carried out *in vitro* translation assays using chimeric *psbC* 5'UTR-mGFP mRNAs as described previously (Yukawa et al. 2007). Fig. 2A schematically represents a construct portion consisting of the T7 promoter, a 194- or 230-nucleotide (nt) *psbC* 5' region (from -230 to -1) with its following GTG, the mGFP coding region (no start codon) and the 3'UTR (145 nt) of tobacco *rps16* gene. Template mRNAs were synthesized with T7 RNA polymerase on linearized plasmid DNAs (Fig. 2B). As controls (size markers), we prepared two mRNAs; one allows to translate from the upstream AUG by replacing the *psbC* 5'UTR (upstream 194 nt) with the *psbA* 5'UTR (85 nt) which produces a long mGFP (Fig. 1C, lane 6), and another is translated from the downstream GUG position by deleting the region between AUG and the next to GUG (from -33 to +3) of the above mRNA so that the original mGFP is synthesized (lane 7).

Figure 2

Translation of the mGFP mRNA carrying the 230-nt wild type *psbC* 5' region and the next GUG generated one fluorescent band in native gel (Fig. 2C, lane 1). This mGFP band corresponded with that from the *psbA* 5'UTR-mGFP mRNA as a control (lane 7). If the upstream AUG initiates translation of *psbC* mRNAs, a band with slow migration should be observed at the same position as appeared in lane 6 (the uppermost, dense band). No such band was detected (lane 1). Deletion of a 33 nt part after the AUG and the following GUG also lost the band (lane 2). A point mutation at the AUG (to AaG) did not affect the mGFP synthesis (lane 3) whereas that at the GUG (to GUc) completely abolished translation (lane 4). No translation was observed by the mutation of both AUG and GUG (lane 5). These results demonstrated that the GUG is the sole translation initiation codon of tobacco *psbC* mRNAs as has been reported in *Synechocystis* 6803 (Carpenter et al. 1990). Therefore, the upstream ATG triplet has nothing to do with the translation initiation site, and GTG is conserved as the *psbC* start codon from cyanobacteria to higher plant plastids, supporting the endosymbiotic origin of plastids from an ancestral prokaryote related to cyanobacteria.

GUG and UUG are used as start codons in less than 10% of eubacterial genes and these codons are known to be less efficient in translation initiation than AUG (Kozak 1999). To examine whether the GUG codon can initiate *psbC* translation as efficiently as AUG, we replaced the GUG with AUG and with other codons, and tested translation *in vitro*. As shown in Figure 3, the translation from AUG was ca. 2-fold higher than that from the authentic GUG, indicating that the GUG is not an optimum initiation codon of the *psbC* mRNA (lane 3). UUG could also initiate translation with a low efficiency (lane 4). Translation was hardly detected from CUG (lane 5).

Figure 3

A long SD-like sequence "GAGGAGGU", which is perfectly complementary to the 3'-end of tobacco 16S rRNA (3'-UUUCCUCCACU), is found 9-nt upstream from the GUG initiation codon of *psbC* mRNAs (Figs. 1 and 4). This element is supposed to form a base-paring with 16S rRNA stronger than a typical SD sequence (GGAGG) and hence could facilitate translation initiation from the GUG. To examine this, *in vitro* translation assays with mutated mRNAs were carried out. As shown in Fig. 4, point mutations (mSD1 and mSD2) within the extended SD-like sequence almost completely abolished translation (lanes 3 and 4). This indicates that the extended SD-like sequence is crucial for *psbC* translation. As the *rps19* mRNA possesses an SD-like sequence, GGAG, 8-nt upstream from the GUG and the *ycf15* mRNA, if expressed, has no SD-like sequence in front of the GUG, a long SD-like element seems to be not always essential for translation of GUG-containing mRNAs. The extended SD-like sequence may facilitate coordinated expression of *psbC* and *psbD* whose initiation codon is ATG. In addition, its strong base-pairing with 16S rRNA may prevent translation initiation from the upstream AUG. The fact that *Chlamydomonas* and *Synechococcus* 7942 *psbC* genes possess no upstream ATG and no extended SD-like sequence (see Fig. 1B) support the above hypothesis.

Figure 4

The extended SD-like sequence was also required for translation of the *psbC* mRNA possessing AUG in place of GUG; the mGFP band from aUG was stronger than that from GUG while no or weak band was detected by mutations in the SD-like sequence (lanes 5-7). The requirement of SD-like sequences on translation was reported for three tobacco plastid mRNAs; *rps14* (GGA), *atpE* (GGAG) and *rbcL* (GGAGG) (Hirose et al. 1998, Hirose and Sugiura 2004). All these mRNAs have AUG as the start codon and 3- to 5 bp SD interaction. Since at least a 3-bp SD interaction is generally required in the eubacterial system (Kozak 1999), the necessity of an SD-like element longer than the average for translation of the *psbC* mRNA with AUG is puzzling. Any point mutation within the extended SD-like sequence results in change in predicted secondary structures around the initiation site, suggesting that a certain secondary structure surrounding the start codon is important for translation initiation of *psbC* mRNAs. Translation of the *Chlamydomonas psbC* mRNA requires three nucleus-encoded products which interact with its 5'UTR (Zerges et al. 1997, Zerges et al. 2003). Therefore, it cannot be ruled out the possibility that a protein factor(s) is involved in the recognition of the GUG start codon in tobacco *psbC* mRNAs.

In addition to *psbC*, there are several plastid genes that have multiple possible initiation codons and that have minor initiation codons, such as GUG in tobacco *rps19* and *ycf15* (Yukawa et al. 2005) and UUG in *Chlorella infA* (Wakasugi et al. 1997, Hirose et al. 1999). Therefore, translation initiation sites of these genes should be carefully evaluated. One approach to define an initiation codon is a transplastomic study as reported for *Chlamydomonas atpB* previously (Rimbault et al. 2000). The *in vitro* method shown here is an alternative way to define translation initiation sites and it is faster and less laborious than transplastomic approaches.

Materials and Methods

Nicotiana tabacum var. BY-4 plants were grown on soil in a control growth chamber. Isolation of intact chloroplasts and preparation of chloroplasts extracts were carried out as described previously (Yukawa et al. 2007). The 5' region of tobacco *psbC* gene including 5'UTR and its mutated versions were amplified by PCR using tobacco chloroplast DNA as templates. A hybrid 5'UTR of *psbA* and *psbC* 5' parts was also constructed. These PCR products were digested with *Nhe*I and cloned into the *Stu*I-*Spe*I region of the plasmid pHK309 including the mGFP coding region and the plastid *rps16* 3'UTR (Yukawa et al. 2007). All constructs were confirmed by DNA sequencing. 5' constructs and primers for PCR were listed in Supplementary material. Plasmid DNAs were linearized with *Xho*I (*psbC* constructs) or *Eco*RI (*psbA* constructs) and purified with the Wizard SV Gel and PCR Purification kit (Promega, Madison, WI, USA). RNA synthesis, *in vitro* translation reactions and detection of mGFP fluorescence were performed as described (Yukawa et al. 2007).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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Legends to figures

Fig. 1. Alignment of sequences around *psbC* translation initiation sites. **A.** *psbC* genes that contain two potential initiation codons, ATG and GTG. *Synechocystis* sp. PCC 6803 (*S.* 6803). Potential translation initiation codons, ATG and GTG, are marked in bold. The underlined TAA is the stop codon of overlapping *psbD*. Asterisks indicate identical bases. Determined N-terminal amino acid sequences of the *psbC* products (CP43) from Arabidopsis, spinach and pea are shown on the top. The N-terminus threonine (Ac-T^P) is *N*-acetylated and *O*-phosphorylated. The second line shows a simple conversion from tobacco DNA to amino acids (#, stop codon). **B.** *psbC* genes that contain only one potential initiation codon GTG. The GTG is marked in bold. Predicted amino acid sequences are also shown. *Chlamydomonas* (*C. reinhardtii*) and *Synechococcus* sp. PCC 7942 (*S.* 7942). Note that *Chlamydomonas psbC* is not overlapped with its *psbD*.

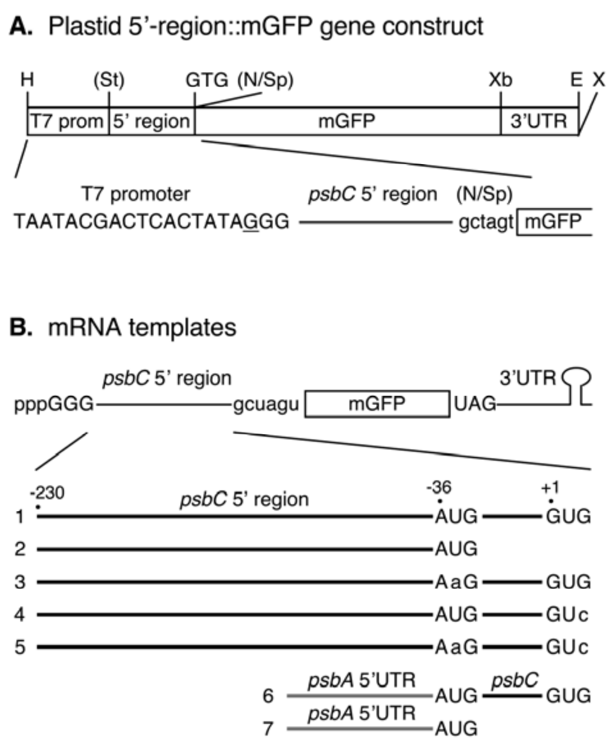
Fig. 2. Determination of the *psbC* translation initiation site. **A.** Schematic representation of the test 5'UTR-mGFP gene construct, which contains the T7 promoter (T7 prom), the 5' region from tobacco *psbC*, GTG, mGFP coding region and the 3'UTR of tobacco *rps16*. The underlined G of the T7 promoter is transcription initiation site. Restriction enzymes: E, *Eco*RI; N, *Nhe*I; Sp, *Spe*I; St, *Stu*I; X, *Xho*I. Restriction enzymes in parentheses were removed during plasmid construction. **B.** mRNA templates. Mutagenized nucleotides are shown in lower case. As control mRNAs, the *psbA* 5'UTR (85 nt) was linked to the *psbC* segment. Nucleotide positions relative to the first G of the GUG as +1 were indicated. Numbers on the left of mRNAs correspond to lanes in Fig. 2C. **C.** Synthesized mGFP. Translation reactions (50 μl) were carried out at 28 ˚C for

2 h with 20-pmole mRNA and 10 A280/ml chloroplast S30 as described previously (Yukawa et al. 2007). In the case of *psbA* constructs, 5-pmole mRNA was added to the reaction mixture. After the reaction, proteins were separated by 12.5% native polyacrylamide gel electrophoresis and mGFP fluorescence was detected by a Molecular Imager Fx (Bio-Rad). Lane C represents a control reaction without addition of test mRNA. A faint band below the major band in lane 6 is probably a truncated mGFP.

Fig. 3. Effect of mutagenesis of the *psbC* initiation codon on translation. **A.** Synthesized mGFP. Translation reactions and mGFP detection were carried out as described in Fig. 2C. Wild-type (GUG) and altered codons are shown above the gel pattern. **B.** Quantification of translation efficiencies. Translation efficiencies are shown relative to the wild-type value as 100%.

Fig. 4. Effect of point mutations in the extended SD-like sequence of *psbC* mRNAs on translation. **A.** Schematic representation of test 5'UTRs. Nucleotides GAGGAGGU of the mRNA are complementary to 3'-end of 16S rRNA (underlined). Wild type and mutagenized SD-like sequences were shown as wtSD and mSD, respectively. Altered nucleotides are shown in lower case. Nucleotide positions relative to the first G of the GUG as +1 were indicated. **B.** Synthesized mGFP. Translation reactions and mGFP detection were carried out as described in Fig. 2C.

Fig. 1



A. Plastid 5'-region::mGFP gene construct
H
(St)
GTG (N/Sp)
Xb
E X
T7 prom
5' region
mGFP
3'UTR
T7 promoter
psbC 5' region
(N/Sp)
TAATACGACTCACTATAGGG
gctagt
mGFP
B. mRNA templates
pppGGG
psbC 5' region
gcuagu
mGFP
UAG
3'UTR
-230
psbC 5' region
-36
+1
1 AUG GUG
2 AUG
3 AaG GUG
4 AUG GUc
5 AaG GUc
6 psbA 5'UTR AUG psbC GUG
7 psbA 5'UTR AUG

Fig. 2

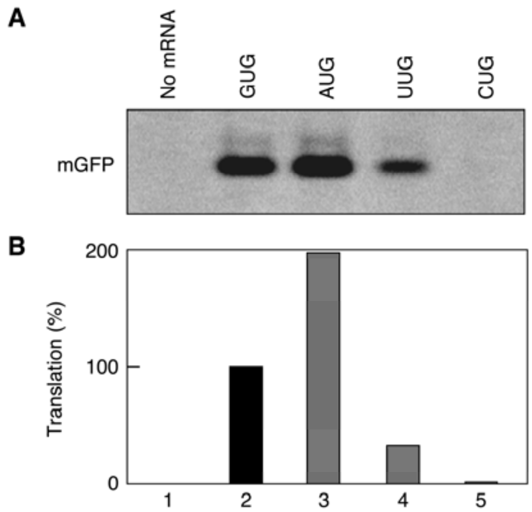

A
No mRNA
GUG
AUG
UUG
CUG
mGFP
B
200
100
0
Translation (%)
1
2
3
4
5

Fig. 3

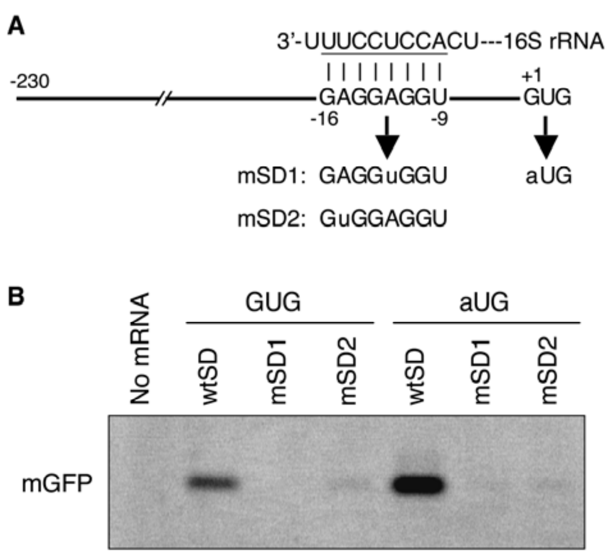

A
3'-UUUCCUCCACU---16S rRNA
-230
GAGGAGGU
-16
-9
+1
GUG
mSD1: GAGGuGGU
aUG
mSD2: GuGGAGGU
B
No mRNA
GUG
aUG
wtSD
mSD1
mSD2
wtSD
mSD1
mSD2
mGFP

Fig. 4