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Viral and chloroplastic signals essential for initiation and efficiency of translation in *Agrobacterium tumefaciens*

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ABSTRACT

The construction of high-level protein expression vectors using the CaMV 35S promoter in concert with highly efficient translation initiation signals for *Agrobacterium tumefaciens* is a relatively less explored field compared to that of *Escherichia coli*. In the current study, we experimentally investigated the capacity of the CaMV 35S promoter to direct GFP gene expression in *A. tumefaciens* in the context of different viral and chloroplastic translation initiation signals. GFP expression and concomitant translational efficiency was monitored by confocal microscopy and Western blot analysis. Among all of the constructs, the highest level of translation was observed for the construct containing the phage T7 translation initiation region followed by the chloroplastic Rubisco Large Subunit (rbcL) 58-nucleotide 5′ leader region including its SD-like sequence (GGGAGGG). Replacing the SD-like (GGGAGGG) with non SD-like (TTTATTT) or replacing the remaining 52 nucleotides of rbcL with nonspecific sequence completely abolished translation. In addition, this 58 nucleotide region of rbcL serves as a translational enhancer in plants when located within the 5′ UTR of mRNA corresponding to GFP. Other constructs, including those containing sequences upstream of the coat proteins of *Alfalfa Mosaic Virus*, or the GAGG sequence of T4 phage or the chloroplastic atpI and/or PsbA 5′ UTR sequence, supported low levels of GFP expression or none at all. From these studies, we propose that we have created high expression vectors in *A. tumefaciens* and/or plants which contain the CaMV 35S promoter, followed by the translationally strong T7 SD plus RBS translation initiation region or the rbcL 58-nucleotide 5′ leader region upstream of the gene of interest.

1. Introduction

Initiation of translation in *Escherichia coli* involves base pairing between a purine-rich Shine–Dalgarno (SD) domain at the 5′ untranslated region (5′ UTR) of mRNA, and the complementary anti-SD sequence at the 3′ end of 16S rRNA [1]. There are distinct sequence elements of the translation initiation region known to contribute to its efficiency [2]: the initiation codon, the Shine–Dalgarno (SD) sequence [3,4] as well as regions upstream of the SD sequence and downstream of the initiation codon, described as enhancers of translation [5]. The distance between the SD sequence and the initiation triplet has a marked effect on the efficiency of translation [6]. The 6-nucleotide consensus SD AGGAGG core sequence causes the highest level of protein synthesis.

Chloroplasts have their own translation system, which exhibits strong homologies to that of prokaryotes. This is consistent with the presence of a Shine–Dalgarno (SD) sequence (GGAGG) located within 12 nucleotides of the AUG initiation codon of many plastid genes [7]. Moreover, the sequence near the 3′ end of the plastid 16S rRNA contains a highly conserved polypyrimidine-rich region (CCUCC) complementary to the SD sequence as in bacteria. Over 90% of higher plant chloroplast genes encoding polypeptides possess an upstream sequence similar to the bacterial SD sequence. Spacing of these chloroplast SD-like sequences is less conserved, ranging from −2 to −29 nucleotides [8]. Translation of several chloroplast mRNAs is also regulated in response to light as well as to some nuclear-encoded factors. In this regard, it is interesting to study how well chloroplastic translational machinery function in Eubacteria such as *E. coli* and *Agrobacterium tumefaciens*.

The transfer of T-DNA from *Agrobacterium* into the plant genome represents a natural horizontal gene transfer across kingdom barriers and implicates a closer evolutionary relationship between *Agrobacterium* and plants than between any other Eubacterial organism (such as *E. coli*) and plants. The aim of the present study

Abbreviations: CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; NOS, nopaline synthase; SD, Shine–Dalgarno sequence; RBS, ribosome binding site; rbcL, Rubisco Large Subunit.

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is to investigate the sequence determinants responsible for efficient translation in *A. tumefaciens*, which on the one hand is highly similar to *E. coli* in terms of its dependency on the SD sequence for the translation, while on the other hand is also mechanistically similar to chloroplast genes such as the large subunit of the Rubisco in its dependence on the 5′ upstream control region. Also, the essential molecular determinants for the design of an ideal Agrobacterial expression vector are considered.

2. Materials and methods

2.1. Construction of GFP expression plasmids

The binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia) was used in this study. A 35S: sGFP:NOS expression cassette (GenBank EF546437) of size 1.9-kbp was subcloned into this vector through *Hind*III and *Eco*RI sites and designated pC-GFP (Fig. 1A). To create the pCTCR-GFP construct, the translation control region (TCR) [9], comprised of 58 nucleotides of 5′ UTR and 45 nucleotides from the N-terminal coding region of the rbcL gene were synthesized and cloned into pUC57 plasmid (Bio Basic Inc.) Following digestion of pUC57 by *Kpn*I/*Bam*HI and *Xba*I/*Bgl*II, respectively, and gel purification (QIAquick Gel Extraction Kit, QIAgen), rbcL TCR DNA fragments were subcloned into the pC-GFP binary plasmid using the respective restriction sites. All other vectors of the pC-GFP series were produced by ligating double-stranded oligonucleotides into restriction-enzyme digested plasmid DNA with compatible ends (Table 1). Briefly, complementary oligonucleotides synthesized by Eurofins MWG Operon (Huntsville, AL) were mixed in equimolar amounts (50 μM each), boiled and annealed by cooling to room temperature and ligated into a previously restriction enzyme digested pC-GFP vector using T4 DNA ligase (New England Biolabs) according to the manufacturer's protocol. The product of each ligation reaction was used to transform *E. coli* DH5-alpha competent cells and kanamycin (50 μg/ml) resistant bacterial colonies were screened for the presence of the proper recombinant constructs. The presence and accuracy of the inserted gene within the expression cassette in the final recombinant constructs was confirmed by DNA sequencing (The Centre for Applied Genomics, Toronto, Canada) using the GFP-R reverse primer:5′-AAGTCGTGCTGCTTCATGTG-3′.

2.2. Agrobacterium transformation

A modified freeze–thaw method for transformation of *A. tumefaciens* was used as reported previously [10]. After transformation, the cells were resuspended in LB such that all the samples contained a uniform OD_{595} of 1.0. From this, equal culture amounts were in turn taken to perform the downstream RNA, confocal microscopy and Western analyses.

2.3. RNA isolation, reverse transcription and PCR

Total RNA was isolated according to modified method of Abou-Haidar et al. [11] and subjected twice to DNase I treatments (New England Biolabs, NEB). A reverse transcription reaction of each sample was performed on 1 μg of total RNA with 200 units of M-MLV reverse transcriptase (Promega), 200 ng of GFP/16sRNA

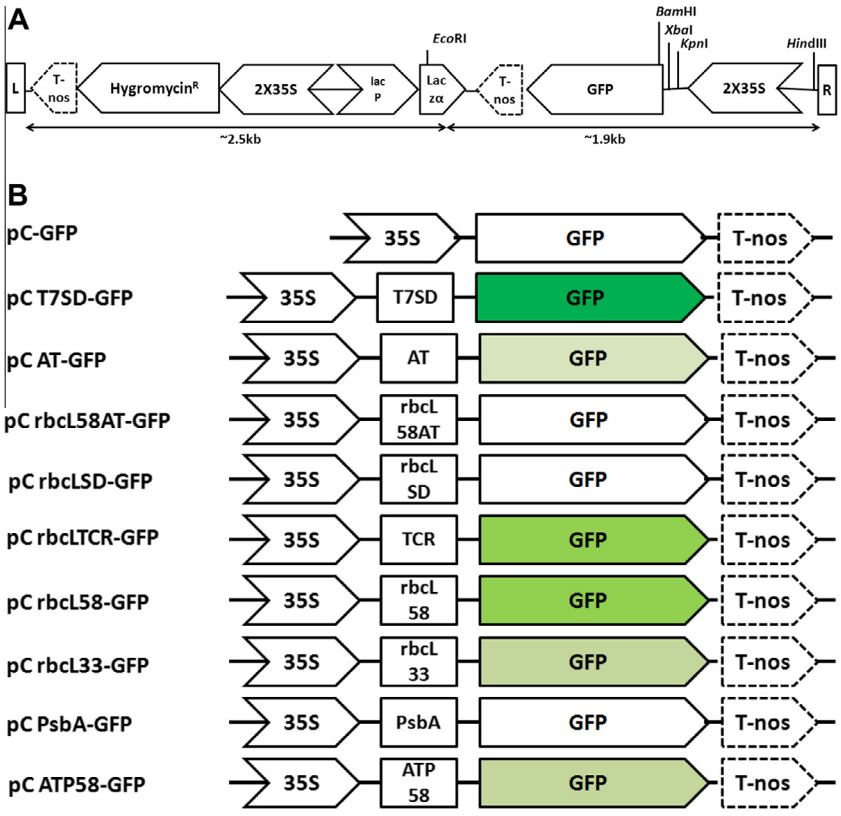

Fig. 1. Physical maps of constructs. (A) Modified pCAMBIA1300 with 35S Promoter, sGFP gene and Tnos terminator cassette. (B) Schematic representation of constructs used in this study. Arrows indicate the direction of transcription and translation. 35S is the CaMV 35S promoter. T-nos: represents the transcription terminator; the box between 35S and GFP contains the different translation initiation contexts. The GFP box is differently colored to reflect the efficiency of its expression. The dark green box representing T7SD shows the highest expression, followed by the rbcL TCR and the rbcL 58 nucleotide 5′ UTR region (light green). Boxes in light green represent marginal GFP expression, while unfilled boxes illustrate no GFP expression. *Note:* Figures not drawn to scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)