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Note

High level expression of transgenes by use of 5′-untranslated region of the *Arabidopsis thaliana arabinogalactan-protein 21* gene in dicotyledons

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Abstract For higher expression of a foreign gene in plant cells, it is important to optimize nucleotide sequences corresponding to 5′-untranslated region (5′-UTR), because it usually has great impacts on the expression of the gene mainly at the translational level. In this study, with an aim to find useful 5′-UTRs, thirty nine 5′-UTRs derived from *Arabidopsis thaliana* genes were tested by transient expression of firefly *luciferase* (*Fluc*), and that of *A. thaliana arabinogalactan-protein 21* (*AtAGP21*) gene was selected for further analyses. Its activity was either equaling or surpassing that of known translational enhancer, *A. thaliana alchol dehydrogenase* (*AtADH*) 5′-UTR in dicotyledons, and was further improved by the optimizing sequence context of the initiating codon (−3 to −1 of AUG). Finally, we also found that the modified *AtAGP21* 5′-UTR was useful in recombinant expression of horseradish peroxidase (HRP) in tobacco cultured cells, and the yield was as much as 23 mgl^{-1} culture medium in seven days.

Key words: 5′-UTR, *Arabidopsis thaliana arabinogalactan-protein 21*, horseradish peroxidase.

Recently, there has been rapid progress in the field of plant biotechnology. Transgenic plants with improved agronomic traits are being put into commercial use, and plant-made vaccine antigens and biopharmaceuticals have also been developed. It is now understood that to achieve higher production levels of a target protein, the corresponding transgene cassette should incorporate DNA elements that are expected to beneficially impact the expression of the gene at transcriptional, translational, and post-translational levels. At the translational level, the 5′-untranslated region (5′-UTR) plays an important role. For example, the nucleotides immediately upstream of the initiating AUG (−3 to −1) exert a large influence on the initiation of translation of the reporter protein, *β-glucuronidase* (*GUS*), yielding as much as a 4-fold difference in GUS protein levels between the best and the worst sequences (Sugio et al. 2010). We have also reported that the 5′-UTRs of *alcohol dehydrogenase* (*ADH*) genes function as translational enhancers in plant cells (Satoh et al. 2004; Sugio et al. 2008). For instance, the 5′-UTR of *Arabidopsis thaliana ADH* gene (*AtADH* 5′-UTR) enhanced the expression of GUS by 150- and 87-fold in tobacco (*Nicotiana tabacum*) and *A. thaliana*, respectively (Sugio et al. 2008). In addition to the enhanced expression of a reporter protein, we have demonstrated that the 5′-UTR of the *N. tabacum ADH* gene (*NtADH* 5′-UTR) modulated the expression of the enzyme horseradish (*Armorucia rusticana*) peroxidase in tobacco cultured cells and the expression of a vaccine antigen protein in lettuce (*Lactuca sativa*) (Matsui et al. 2006; Matsui et al. 2009).

Here, we set out to find more effective 5′-UTRs. Accordingly, we first tested thirty nine 5′-UTRs derived from *A. thaliana* genes for their translational activities. Specifically, we used *in vitro* synthesized mRNA encoding firefly (*Photinus pyralis*) *luciferase* (*Fluc*), which contained a candidate 5′-UTR and a 49 base long poly A tail, and *Renilla reniformis luciferase* (*Rluc*) for

Abbreviations: HRP, horseradish peroxidase; HSPT, transcriptional terminator derived from *A. thaliana heast shoch protein 18.2* gene; UTR, Untranslated region.

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normalization. The mRNA was transiently co-transfected into protoplasts of *A. thaliana* cultured cells, then the relative Fluc/Rluc activity was calculated for each 5′-UTR (manuscript in preparation). Among the 5′-UTRs tested, we selected that of *Arabinogalactan-protein 21* (*AtAGP21*; AGI code, At1g55330) for further analyses because the Fluc/Rluc activity was among the highest we observed in these assays, about 2.5-fold higher than that obtained by transfection of *AtADH* 5′-UTR::*Fluc* mRNA. The sequence context of AUG initiation codon (positions −3 to −1) of *AtAGP21* is "uua", which is predicted to be a poor enhancer of translation initiation, and we expected that the translational activity of the *AtAGP21* 5′-UTR would be improved by 2.3-fold by changing "uua" to "aag" (Sugio et al. 2010). Meanwhile, because the sequence context of AUG of *AtADH* 5′-UTR (aua) was originally good, a similar improvement was not expected.

We constructed expression plasmids for *Fluc* fused with each 5′-UTR (Figure 1), and the *AtAGP21* 5′-UTR was re-evaluated. The *AtAGP21* 5′-UTR, an *AtAGP21*mod 5′-UTR in which the nucleotides −3 to −1 of AUG were changed to "aag" for efficient translation (Sugio et al. 2010), and the *AtADH* 5′-UTR were assayed. In these expression plasmids, the transcription initiation site of the CaMV 35S promoter was designed to correspond to the 5′-end of the 5′-UTR, and no additional nucleotides were inserted between the initiating AUG of *Fluc* and each 5′-UTR. The *Rluc* expression plasmid was used to normalize the transfection efficiency (Figure 1). Protoplasts were co-transfected with *Fluc* and *Rluc* plasmids by the polyethylene glycol (PEG) method (Matsui et al. 2009), then incubated for 6 h. Collected protoplasts were lysed in passive lysis buffer (Promega, Madison, WI), and luminescence was obtained using the dual-luciferase reporter assay system (Promega) monitored for 10 s by a luminometer (Lumat LB9501, Berthold, Germany). In *A. thaliana* cells, the *AtAGP21* 5′-UTR was as effective as the *AtADH* 5′-UTR, and its translational activity was almost doubled by optimization of the sequence context of AUG (Figure 2A). Similarly, in protoplasts prepared from petals of the ornamental flower torenia (*Torenia fournieri*), the *AtAGP21*mod 5′-UTR was about 1.4-fold more effective than the *AtADH* 5′-UTR (Supplemental Figure 1). In cultured tobacco cell protoplasts (*N. tabacum* L. cv. BY2) and in protoplasts derived from leaves of lettuce, the *AtAGP21*mod 5′-UTR was as effective as the *AtADH* 5′-UTR (Supplemental Figure 1). These data suggested that the translational activity of the *AtAGP21*mod 5′-UTR is either equaling or surpassing that of the *AtADH* 5′-UTR in dicotyledons. We also evaluated the *AtAGP21*mod 5′-UTR in the monocotyledon, rice (*Oryza sativa* cv. Nipponbare) (Figure 2B). We previously reported that neither the *NtADH* 5′-UTR nor the *AtADH* 5′-UTR were effective

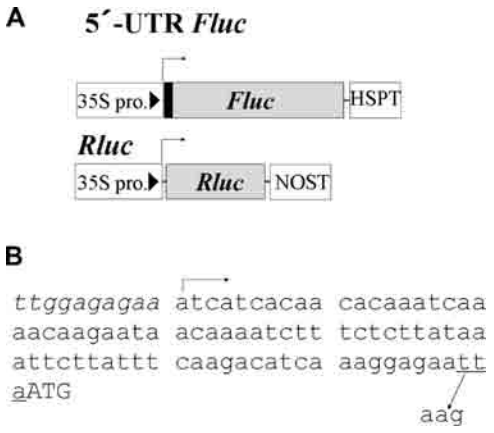

Figure 1. Schematic representation of the examined constructs. *Fluc* was fused with each 5′-UTR (A). The expression plasmid for *Rluc* was co-transfected for normalization efficiency. Nucleotide sequences of DNA fragment corresponding to part of 35S promoter (italics) and 5′-UTR are indicated in (B). In *AtAGP21*mod 5′-UTR, nucleotides −3 to −1 of AUG were changed to "aag". 35S pro., Cauliflower mosaic virus 35S RNA promoter; NOST, transcription terminator from *Agrobacterium tumefaciens nopaline synthase* gene; HSPT, transcription terminator from *A. thaliana heat shock protein 18.2* gene. Transcription start sites are indicated by arrows.

in rice cells (Satoh et al. 2004; Sugio et al. 2008). The *OsADH* 5′-UTR, which is an effective translational enhancer in rice (Sugio et al. 2008), was used as a control. We found that the *AtAGP21*mod 5′-UTR was far less efficient than the *OsADH* 5′-UTR (Figure 2B) in these experiments. In protoplasts derived from other two monocotyledons, maize (*Zea mays*) and rye (*Secale cereale*), the *OsADH* 5′-UTR functioned as a translational enhancer but the *AtAGP21*mod 5′-UTR was less effective than the *OsADH* 5′-UTR (Supplemental Figure 1). We are currently searching for other translational enhancers that are more effective than the *OsADH* 5′-UTR in monocotyledons.

We applied the *AtAGP21*mod 5′-UTR to production of the useful enzyme, horseradish (*Armorucia rusticana*) peroxidase (HRP), in tobacco cultured cells, BY2. The HRP is highly stable and widely used as a reporter enzyme for a variety of detection procedures, such as enzyme-linked immunosorbent assay and western blotting. We have previously reported that active HRP was secreted into the culture medium from cells expressing the HRP gene without C-terminal vacuolar sorting signal (Matsui et al. 2003), and that the expression level was increased by the use of *NtADH* 5′-UTR (Matsui et al. 2006). This time, transcriptional terminator derived from *A.thaliana heast shoch protein 18.2* gene (HSPT) was used to express HRP, because higher level expression of a transgene is possible using HSPT in various plant species including *A. thaliana*, lettuce, tomato, and rice (Nagaya et al. 2010; Matsui et al. 2011; Hirai et al. 2011). Actually, we found that the HSPT is superior to a transcriptional terminator derived

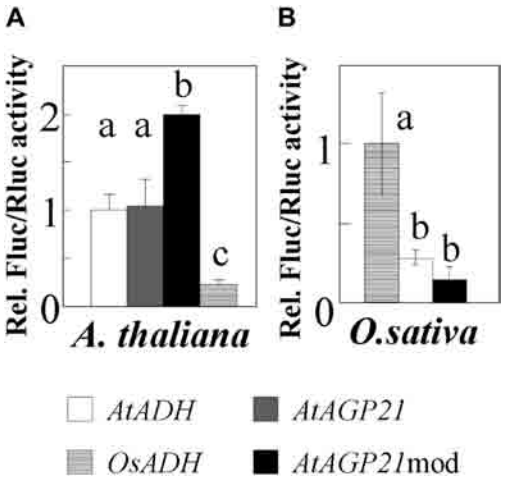

Figure 2. Evaluation of the *AtAGP21*- and *AtAGP21*mod 5′-UTRs. Protoplasts prepared from *A. thaliana* (A) or *O. sativa* (B) were transfected with plasmids using the PEG-mediated transient expression method. 500 ng of the *Fluc* plasmid and 250 ng of the *Rluc* plasmid were used for one transfection of *A. thaliana*. Three μg of the *Fluc* plasmid and 250 ng of the *Rluc* plasmid were used for one transfection of *O. sativa*. The means and SDs of three independent experiments are shown. The Fluc/Rluc activity relative to the *AtADH*- or the *OsADH* 5′-UTR is indicated in the case of *A. thaliana*, and *O. sativa*, respectively. Significance tests were performed, and the values highlighted with the same letter are not significantly different at a probability of 0.05. Bars for the *AtADH*-, the *AtAGP21*-, and the *AtAGP21*mod 5′-UTR are colored with white, gray, and black, respectively. A bar for the *OsADH* 5′-UTR is with horizontal lines.

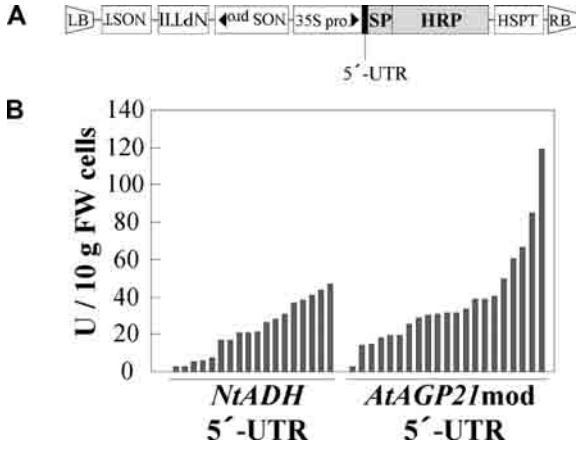

Figure 3. Production of horseradish peroxidase in tobacco cells by use of *AtAGP21*mod 5′-UTR. (A) Schematic representation of the examined constructs. SP, signal peptide for secretion of HRP; NOS pro. *A. tumefaciens nopaline synthase* gene promoter; NPTII, *neomycine phosphotransferase* gene; RB, right border; LB, left border. Either *NtADH* 5′-UTR or *AtAGP21*mod 5′-UTR was fused to HRP. (B) We measured the HRP activity of randomly selected kanamycin-resistant cali.

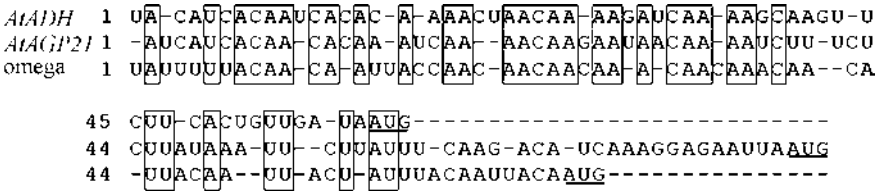

Figure 4. Alignment of the nucleotide sequences of the 5′-UTRs. The nucleotide sequences of the *AtADH*-, *AtAGP21*-, and Ω 5′-UTRs were aligned. The initiating AUG codons are underlined.

from *Agrobacterium nopaline synthase* gene (NOST) for expression of HRP in BY2 cells (Supplemental Figure 2). Stable BY2 transformants were generated using *Agrobacterium tumefaciens* harboring gene for HRP fused with *AtAGP21*mod 5′-UTR or *NtADH* 5′-UTR, and kanamycin resistant cali were randomly picked for quantitative determination of HRP accumulation (Matsui et al. 2006). We found that *AtAGP21*mod 5′-UTR was superior to *NtADH* 5′-UTR. A clone with the highest expression was cultured in 100 ml of liquid LS medium, modified MS medium (Murashige and Skoog 1962) supplemented with 200 mg l^{-1} KH_2PO_4, 100 mg l^{-1} myoinositol, 1 mg l^{-1} thiamine–HCl, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 3% sucrose, pH 5.7, and subcultured every seven days. The HRP was secreted into liquid culture medium, and the yield was 6900 U (corresponding to about 23 mg) l^{-1} in seven days (data not shown). The yield was much higher than that attained by an *E. coli* expression system (~110 μg l^{-1}) or that of a yeast system (5.3 mg l^{-1}) (Lin et al. 1999; Morawski et al. 2000).

In order to get some insights into the *cis*-sequences in the *AtAGP21* 5′-UTR that are important for the enhancement of function, nucleotide sequences of the 5′-UTRs were compared (Figure 4). We found that the *AtAGP21* 5′-UTR and the *AtADH* 5′-UTR contained some CAA repeats. Poly (CAA) is often found in some 5′-UTRs of virus RNA (Gallie and Walbot 1992; Daohong and Said 2004). Such poly (CAA) is reportedly essential for the translational enhancement activity of Ω, a 5′-UTR of tobacco mosaic virus mRNA (Gallie and Walbot 1992), and a *trans*-factor that binds to these sequences was identified (Tanguay and Gallie 1996). The poly (CAA) in the *AtAGP21*- and *AtADH* 5′-UTRs may also govern their activities; this hypothesis should be tested experimentally in the future. We are currently

taking another approach to identify *cis*-sequences that are important for translational enhancement. Using the datasets of translational enhancement activity of 5′-UTRs which were obtained from mRNA transfection assay as mentioned in the opening sentence, we plan to perform *in silico* multivariate analysis to find additional candidate enhancer sequence motifs. The resulting information will help us to synthesize more efficient translational enhancers.

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