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Short Communication

Effect of the 5′-untranslated region on the expression of transgene in liverwort, *Marchantia polymorpha*

Miho Takemura*, Shingo Nagaya, Haruka Kida

Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan
*E-mail: mtake@ishikawa-pu.ac.jp Tel: +81-76-227-7520 Fax: +81-76-227-7557

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Abstract Transgenic plants have the potential to provide substantial benefits in the production of pharmaceutical compounds, valuable nutrition, and vaccines. Cauliflower mosaic virus 35S promoter is the most general promoter in higher plants, and also used to express the transgene in liverwort. However, some genes, such as those found in other species, are difficult to express in liverwort. We reported previously that the 5′-untranslated region (UTR) of liverwort ADH-like glucose dehydrogenase gene enhanced transient gene expression in liverwort. In this study, we tested the effects of 5′-UTR on the expression of *Gracilaria vermiculophylla* cyclooxygenase (*GvCOX*) gene in stably transformed liverwort. The *GvCOX* gene encodes the enzyme to produce prostaglandins used as pharmaceutical compounds. Cyclooxygenase activity in transgenic liverwort without 5′-UTR was low, although this mRNA was accumulated. In contrast, addition of the 5′-UTR to the *GvCOX* gene significantly increased cyclooxygenase activity in transgenic liverwort. We also found that the boundary sequences between the 5′-UTR and *GvCOX* gene affected mRNA accumulation level. Our system with the 5′-UTR provides a powerful tool for enhancing transgene expression in liverwort.

Key words: cyclooxygenase, transgene expression, transgenic liverwort, translation, 5′-UTR.

Effective expression of the transgene in plants is very important for the analysis of gene function and the production of useful compounds. In higher plants, cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase gene (*NOS*) terminator are widely used for the overexpression of transgene. However, efficiency varies according to the plant species used. It has been reported that several DNA elements function as a translational enhancer to increase gene expression at the translational level. For example, it was shown that GUS activity in tobacco increased 16- to 18-fold by the insertion of the Ω sequence of *tobacco mosaic virus* (Gallie and Kado 1989). Similarly, the 5′-UTR of tobacco *ADH-like UDP glucose dehydrogenase* gene (*NtADH*) increased gene expression by several tenfold in tobacco (Nagaya et al. 2000) and *Arabidopsis* (Nagaya et al. 2010). The 5′-UTR of *Vigna radiata aminocyclopropane-1-carboxylate synthase* (*VR-ACS1*) gene also acts as a translational enhancer (Wever et al. 2010).

The 35S promoter was used to express the transgene in liverwort (Takemura et al. 2013). However, some genes, such as those found in other species, are difficult to express in liverwort (Nagaya et al. 2011). Previously, we isolated and applied the 5′-UTR of the liverwort homolog of *NtADH*-like gene, *MpUDP* (331 bp; Accession number, AB630338) to the liverwort transient gene expression system (Nagaya et al. 2011). We found that the endogenous 5′-UTR and terminator were effective in increasing the transgene expression transiently in liverwort cultured cells. In this study, we created several constructs to investigate the effect of this *MpUDP* 5′-UTR in stably transformed liverwort. We used the *GvCOX* gene as a reporter gene to investigate the efficiency of elements in the production of useful compounds. *GvCOX* gene was isolated from *Gracilaria vermiculophylla* and codes for the cyclooxygenase (COX) to produce prostaglandin (PG) from arachidonic acid (AA) (Kanamoto et al. 2011). In order to express *GvCOX* gene in liverwort, a vector based on the 35S promoter was constructed. Initially, the *p35S-GUS* fragment from pBI221 vector (Clontech, CA, U.S.A.) was inserted into the *Hin*dIII/*Sac*I site of the p35SSRDXG (Oshima et al. 2011) to generate "CaMV35S-GUS-tNOSG". The *GvCOX* coding region amplified by PCR using *Bam*HI and *Sac*I primers (GvCOX F: ggatccATG GTG TTC AAC AAC TTC C and GvCOX R: gagctcTAC ACA GGG TTA TTC TTC, respectively) was inserted into the *Bam*HI/*Sac*I site of "CaMV35S-GUS-

Abbreviations: AA, arachidonic acid; CaMV, cauliflower mosaic virus; COX, cyclooxygenase; *GvCOX*, *Gracilaria vermiculophylla cyclooxygenase*; *MpACT*, *Marchantia polymoprha ACTIN*; *MpUDP*, *Marchantia polymoprha ADH-like UDP glucose dehydrogenase*; *NOS*, nopaline synthase gene; PG, prostaglandin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; UTR, untranslated region.

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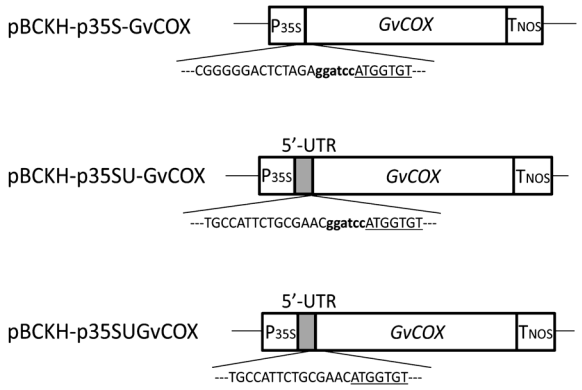

Figure 1. Schematic representation of plasmids containing the *GvCOX* gene. P_{35S}, CaMV 35S promoter fragment; 5′-UTR, 5′-UTR of *MpUDP*; *GvCOX*, *GvCOX* coding region; T_{NOS}, *NOS* terminator fragment. The sequences below show those of the junction between CaMV35S promoter (pBCKH-p35S-GvCOX)/5′-UTR of *MpUDP* (pBCKH-p35SU-GvCOX and pBCKH-p35SUGvCOX) and *GvCOX* gene. The 5′ end sequences of the *GvCOX* gene are underlined. The *Bam*HI site is indicated by lower case.

tNOSG" to generate "CaMV35S-GvCOX-tNOSG". We also constructed "CaMV35S-MpUDP 5′-UTR-GvCOX-tNOSG" as described previously (Takemura et al. 2013). Then we constructed the plasmid without the *Bam*HI site between 5′-UTR and the *GvCOX* and designated "CaMV35S-MpUDP 5′-UTR (no space)-GvCOX-tNOSG". The plasmids "CaMV35S-GvCOX-tNOSG", "CaMV35S-MpUDP 5′-UTR-GvCOX-tNOSG" and "CaMV35S-MpUDP 5′-UTR (no space)-GvCOX-tNOSG" were cloned into the binary vector pBCKH (Oshima et al. 2011) by using the Gateway LR reaction (Life technologies) and designated pBCKH-p35S-GvCOX, pBCKH-p35SU-GvCOX and pBCKH-p35SUGvCOX, respectively (Figure 1). These vectors were transformed into *Agrobacterium tumefaciens* ($C58rif^r$) by electroporation. Transgenic liverworts were generated as previously described (Ishizaki et al. 2008).

The insertions of the transgene in the obtained Hyg^r plants were confirmed by the genomic PCR with the primers for amplification of *GvCOX* fragment. As a result, we obtained approximately thirty transgenic liverworts with each construct. The levels of *GvCOX* mRNA were variable, transgene expressions could not be detected in approximately half of the transgenic liverworts. A similar phenomenon, called gene silencing, was reported in plants (Matzke and Matzke 1998; Nagaya et al. 2005). Since we focused on the effect of 5′-UTR at the translational level, transgenic liverworts expressing *GvCOX* mRNA were selected and used in the following analysis.

We first confirmed transgene insertion into liverwort chromosome by Southern blot analysis. Total DNA was isolated from liverwort thalli using the cetylmethylammonium bromide method (Kim et al. 2002). Five micrograms of genomic DNA were digested with *Eco*RI or *Hin*dIII. The DNA was fractionated by electrophoresis in a 0.8% agarose gel and blotted onto Hybond-N nylon membrane (GE Healthcare). The 400 or 610 bp fragments of *GvCOX* gene were amplified and DIG-labeled with PCR DIG probe synthesis kit (Roche, Basel). This fragment was used as a probe to detect the *GvCOX* gene. The blotted membranes were hybridized in DIG Easy Hyb Granules buffer (Roche) at 42°C with a probe and washed with SSC and SDS buffer. Then hybridized probes were immunodetected with anti-DIG-AP Fab fragments and visualized with a chemiluminescent substrate (CDP-Star, Roche). Analysis of blots was performed by Imageanalyzer (LAS-3000, FUJIFILM, Tokyo). One to five transgene insertions were detected (Table 1), and transgene copy numbers varied among independent transgenic lines.

To investigate the expression levels of transgene in

Table 1. Copy numbers of the transformants.

Transformants		Copy number
pBCKH-p35SGvCOX	a	1
	b	2
	c	1
	d	1
	e	3
	f	2
	g	3
	h	4
	i	2
	j	2
	k	2
	l	2
pBCKH-p35SU-GvCOX	a	1
	b	1
	c	3
	d	2
	e	4
	f	3
	g	4
	h	3
	i	1
	j	4
	k	1
	l	3
	m	2
	n	1
	o	4
	p	4
pBCKH-p35SUGvCOX	a	5
	b	1
	c	1
	d	1
	e	2
	f	1
	g	2
	h	1
	i	4
	j	3
	k	3
	l	1

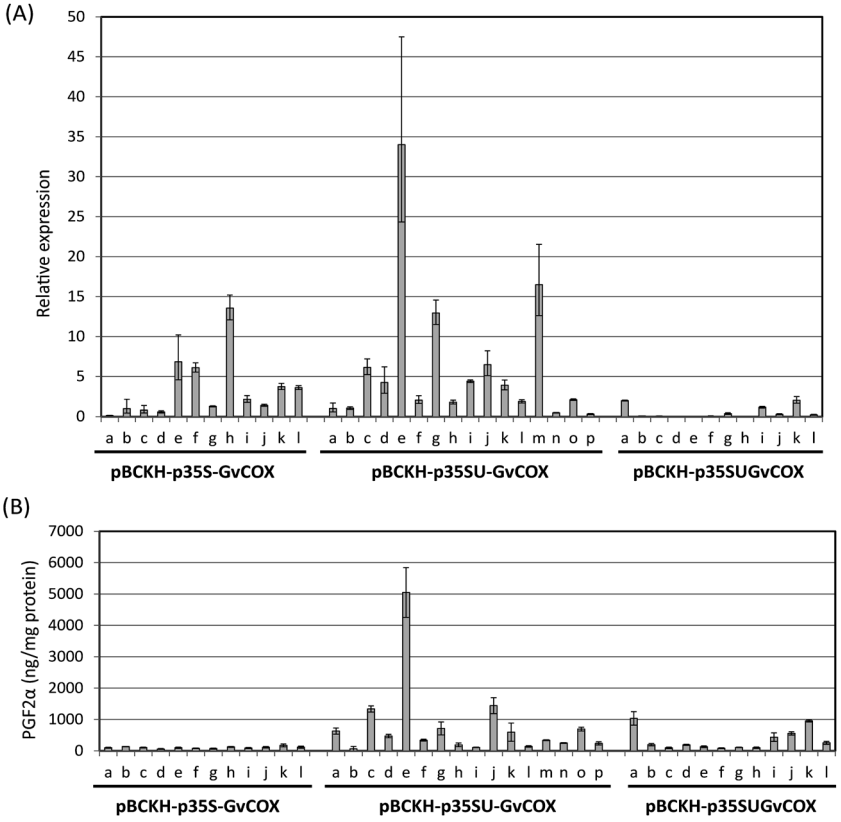

Figure 2. COX activity and mRNA accumulation in transgenic plants. (A) Real-Time PCR analysis. The value relative to the expression level of the transformant pBCKH-p35SU-GvCOX-a is shown. Results are the mean±SD of the three experiments. (B) COX activity. Results are the mean±SD of the three experiments.

the transgenic liverworts, we performed Real-Time PCR analysis as described previously (Takemura et al. 2013). The value relative to the expression level of the transformant pBCKH-p35SU-GvCOX-a was calculated. The expression levels of pBCKH-p35SU-GvCOX transformants ranged from 0.31 (pBCKH-p35SU-GvCOX-p) to 34.00 (pBCKH-p35SU-GvCOX-e) and were slightly higher than those of pBCKH-p35S-GvCOX which varied between 0.13 (pBCKH-p35S-GvCOX-a) and 13.55 (pBCKH-p35S-GvCOX-h) (Figure 2A). In contrast, the expression levels of pBCKH-p35SUGvCOX transformants were significantly lower than pBCKH-p35S-GvCOX, contrary to expectations (Figure 2A). For example, relative expressions of pBCKH-p35SUGvCOX-b, c, d, e, f, h were lower than 0.1, and 2.03 (pBCKH-p35SUGvCOX-k) was the highest value (Figure 2A).

Next, we analyzed the COX activities of the transformants. Approximately 1.0 g of each liverwort thalli was ground in liquid N_2 and suspended in 2 ml of extraction buffer (100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5 mM PMSF, 1 μM Hematin). The homogenate was applied to COX activity assay as a crude protein extract. The COX activities of the transformants were measured by the method described by Kanamoto et al. (2011). At first, 500 μl of the homogenate was pre-incubated at 20°C for 5 min to make complex COX with hematin. After pre-incubation, it was mixed with 10 μl of 10 mM AA and incubated at 20°C for 10 min. Then, 25 μl of 100 mM $SnCl_2$ was added and incubated at 20°C for 5 min to reduce the initial product, PGH_2, to $PGF_{2\alpha}$. After the addition of 60 ng $PGF_{2\alpha}$-d4 as an internal control to calculate the efficiency of extraction, the reaction buffer was adjusted to pH 3 with 1N HCl. The reaction products were extracted by ethyl acetate, dried by evaporation, and resuspended with ethanol. $PGF_{2\alpha}$ content of this ethanol solution was measured by LC-MS/MS. AA and all PGs were purchased from the Cayman Chemical Co. (Ann Arbor). The COX activities of pBCKH-p35SU-GvCOX transformants ranged from 63 ($PGF_{2\alpha}$ ng/mg protein) (pBCKH-p35SU-GvCOX-b) to 5047 (pBCKH-p35SU-GvCOX-e) (Figure 2B, Figure 3). These values were significantly higher than those of pBCKH-p35S-GvCOX which were between 60 (pBCKH-p35S-GvCOX-d) and 174 (pBCKH-p35S-GvCOX-k) (Figure 2B, Figure 3). On the other hand, the activities of pBCKH-p35SUGvCOX which varied from 83 (pBCKH-p35SUGvCOX-f) to 1032 (pBCKH-p35SUGvCOX-a), were slightly higher than those of pBCKH-p35S-GvCOX (Figure 2B, Figure 3).

Real-Time PCR and Southern blot analysis showed that multiple copies transgenes had positive effects on *GvCOX* expression in transgenic liverwort (Figure 2A,

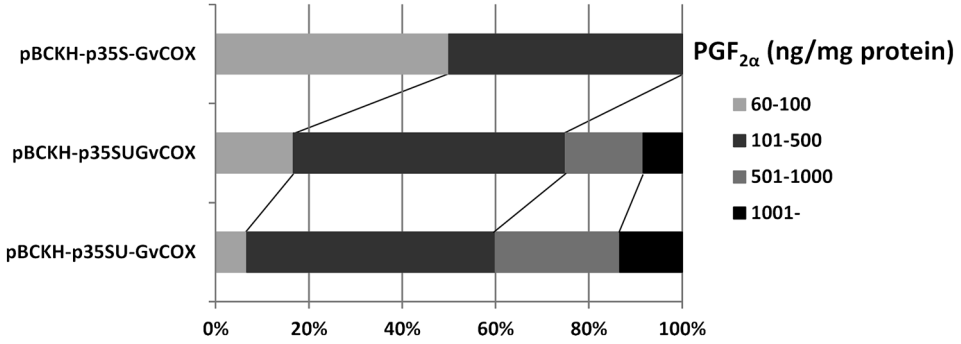

Figure 3. Distribution of COX activities. Transformants were classified into four groups dependent on the COX activity.

Table 1). Since increased transgene copy number has negative effects on transgene expression (Nagaya et al. 2005), gene silencing frequently occurs in plant cells. However, further increase in copy number of transgene may contribute to increase *GvCOX* expression in transgenic liverwort.

Our results shows that addition of 5′-UTR was effective in increasing $PGF_{2\alpha}$ production (Figure 2B, Figure 3). Although mRNA levels in lines pBCKH-p35S-GvCOX-e, f, and h were as same as those in pBCKH-p35SU-GvCOX-c and g, levels of $PGF_{2\alpha}$ in the former were lower than those in the latter (Figure 2). These results suggest that pBCKH-p35S-GvCOX transgene has potential to produce *GvCOX* mRNA; however, inefficient translation of *GvCOX* mRNA leads to the low COX activities. Addition of 5′-UTR is necessary to effectively translate mRNA to protein; however, the effect of 5′-UTR was different between pBCKH-p35SU-GvCOX and pBCKH-p35SUGvCOX. We constructed pBCKH-p35SU-GvCOX first and found that this construct increased COX activity (Takemura et al. 2013). Because Oshima et al. (2011) reported that the Gateway linker sequence in a location similar to the *Bam*HI site negatively affected mRNA accumulation and translational efficiency, we removed the *Bam*HI site immediately before the ATG start codon of *GvCOX* gene in pBCKH-p35SU-GvCOX. However, the resulting construct pBCKH-p35SUGvCOX did not contribute to increased $PGF_{2\alpha}$ production compared with before *Bam*HI site removal (Figure 2). Unexpectedly, *GvCOX* mRNA levels of pBCKH-p35SUGvCOX were significantly lower than those of pBCKH-p35SU-GvCOX (Figure 2A), showing that removal of the *Bam*HI site had a negative effect on *GvCOX* mRNA accumulation. The difference between both constructs is the presence or absence of the *Bam*HI site in front of the ATG start codon of the *GvCOX* gene (Figure 1), suggesting the importance of the boundary sequences or distances between 5′-UTR and ATG codon of *GvCOX* gene. Further experiments will be needed to confirm this.

In this study, we found that the 5′-UTR of the liverwort *MpUDP* gene enhances seaweed *GvCOX* gene expression in stable transgenic liverwort. The 5′-UTR is also effective in expressing mouse *COX* gene in transgenic liverwort (data not shown). Our results indicate the importance of boundary sequences in allowing 5′-UTR to act effectively. The 35S promoter is widely used in plants; however, the 35S promoter itself is not sufficient for effective transgene expression in liverwort due to inefficient gene translation. The highest *GvCOX* expression line obtained, pBCKH-p35SU-GvCOX-e, produced 44 μg/g fresh weight of PGs (Takemura et al. 2013). Since one tablet of PG medicine contains 20 μg of PG, 1 g of the transgenic liverwort is able to produce two tablets of medicine. Our results show that appropriately combining 5′-UTR with the 35S promoter provides a powerful tool for enhancing foreign gene expression in liverwort. In future, 5′-UTR will be coupled with another useful promoter such as a tissue-specific promoter.

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References

Gallie DR, Kado CI (1989) A translational enhancer derived from tobacco mosaic virus is functionally equivalent to a Shine-Dalgarno sequence. *Proc Natl Acad Sci USA* 86: 129–132

Ishizaki K, Chiyoda S, Yamato KT, Kohchi T (2008) *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol* 49: 1084–1091

Kanamoto H, Takemura M, Ohyama K (2011) Identification of a cyclooxygenase gene from the red alga *Gracilaria vermiculophylla* and bioconversion of arachidonic acid to $PGF_{2\alpha}$ in engineered *Escherichia coli*. *Appl Microbiol Biotechnol* 91: 1121–1129

Kim SH, Mizuno K, Fujimura T (2002) Isolation of MADS-box genes from sweet potato (*Ipomea batatas* (L.) Lam.) expressed specifically in vegetative tissues. *Plant Cell Physiol* 43: 314–322

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. *Curr Opin Plant Biol* 1: 142–148

Nagaya S, Kato K, Ninomiya Y, Horie R, Sekine M, Yoshida K, Shinmyo A (2005) Expression of randomly integrated single

complete copy transgenes does not vary in *Arabidopsis thaliana*. *Plant Cell Physiol* 46: 438–444

Nagaya S, Kawamura K, Shinmyo A, Kato K (2010) The *HSP* terminator of *Arabidopsis thaliana* increases gene expression in plant cells. *Plant Cell Physiol* 51: 328–332

Nagaya S, Nakai Y, Kato K, Sekine M, Yoshida K, Shinmyo A (2000) Isolation of growth-phase-specific promoters from cultured tobacco cells. *J Biosci Bioeng* 89: 231–235

Nagaya S, Takemura M, Ohyama K (2011) Endogenous promoter, 5′-UTR and transcriptional terminator enhance transient gene expression in a liverwort *Marchantia polymorpha* L. *Plant Biotechnol* 28: 493–496

Oshima Y, Mitsuda N, Nakata M, Nakagawa T, Nagaya S, Kato K, Ohme-Takagi M (2011) Novel vector systems to accelerate functional analysis of transcription factors using chimeric repressor gene-silencing technology (CRES-T). *Plant Biotechnol* 28: 201–210

Takemura M, Kanamoto H, Nagaya S, Ohyama K (2013) Bioproduction of prostaglandins in a transgenic liverwort, *Marchantia polymorpha*. *Transgenic Res* 22: 905–911

Wever W, McCallum EJ, Chakravorty D, Cazzonelli CI, Botella JR (2010) The 5′ untranslated region of the *VR-ACS1* mRNA acts as a strong translational enhancer in plants. *Transgenic Res* 19: 667–674