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Note

Endogenous promoter, 5′-UTR and transcriptional terminator enhance transient gene expression in a liverwort, *Marchantia polymorpha* L.

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Abstract High expression of transgene is one of the key requirements for the successful establishment of transgenic plants to produce a lot of proteins or metabolites. Although the *cauliflower mosaic virus 35S* (*CaMV35S*) promoter and the *nopaline synthase* (*NOS*) terminator are often used for this purpose, their efficiencies vary to plant species used. In a liverwort, *Marchantia polymorpha* L., the *CaMV35S* promoter and the *NOS* terminator do not show significant enhancing effect of transgene expression. To construct more efficient gene expression system of the liverwort, we employed the transient gene expression assay system. As a result, the endogenous *elongation factor 1α* promoter and *Flowering locus T1* terminator from the liverwort led to enhance transient gene expression, approximately 75 and 3 times, respectively, compared to the *CaMV35S* promoter and the *NOS* terminator. Furthermore, we found that the endogenous 5′-UTR of the liverwort *ADH-like UDP glucose dehydrogenase* (*MpUDP*) yielded an enhancement of 15 times greater than in cases without the *MpUDP* 5′-UTR. These results indicate that DNA elements enhancing gene expression can be obtained efficiently by the transient gene expression assay in a short period of time, promising the application to the transgene expression system for production of proteins and metabolites in the liverwort.

Key words: *ADH-like UDP glucose dehydrogenase*, *elongation factor 1α*, *Flowering locus T1*, liverwort, gene expression.

To produce useful pharmaceutical or industrial compounds with high yield in transgenic plants, the efficient gene expression systems have been developed in higher plants. The liverwort, *M. polymorpha* L., is a unique plant that synthesizes long-chain polyunsaturated fatty acids (LCPUFAs) (Shinmen et al. 1991). Due to the important role of LCPUFAs in human health and nutrition (Demaison et al. 2002; Michaelson et al. 2002), they have been attracting considerable interest. Previous study showed that expression of the liverwort genes encoding the LCPUFA synthesis pathway under control of the *cauliflower mosaic virus 35S* (*CaMV35S*) promoter and the *nopaline synthase* (*NOS*) terminator in transgenic liverwort plants increases only two to 3 fold accumulation of LCPUFAs compared to the wild type liverwort (Kajikawa et al. 2008). This suggests that more efficient expression systems are needed to use transgenic liverworts for production of valuable metabolites such as LCPUFAs. However, it is time and labor consuming to develop efficient transgene expression system generating transgenic plants. In this study, therefore, we employed a rapid transient assay system using the *firefly luciferase* (*Fluc*) gene and the *Renilla luciferase* (*Rluc*) gene as reporter genes. The *Fluc* gene from pGL3 vector (Promega, Madison, WI, USA) was subcloned into the *Nco*I/*Xba*I sites of the "*CaMV35S-Rluc-tNOS*" (Nagaya et al. 2010) to replace the *Rluc* gene and generate "*CaMV35S-Fluc-tNOS*". The sequences of "*CaMV35S-Rluc-tNOS*" and "*CaMV35S-Fluc-tNOS*" are identical except for the coding region of the reporter gene. To evaluate each DNA element on transient gene expression, we carried out the transient expression assay using the liverwort cultured cells. Liverwort suspension cells were grown in 1M51C medium (Ohyama et al. 1988) with constant agitation at 120 rpm and 22°C under a 16 h light/8 h dark photoperiod and transferred into fresh medium every week. The reporter plasmid DNA was introduced into liverwort suspension cells using particle bombardment. Ten ml of 4-day-old liverwort suspension cells was placed on the 1M51C plate (Advantec No. 1, 47 mm diameter, Toyo Roshi Kaisha, Tokyo) by vacuum filtration. The liverwort suspension cells were particle-bombarded with each 500 ng of *Fluc* and *Rluc* plasmid DNA using a BIOLISTIC PDS-

Abbreviations: *CaMV, cauliflower mosaic virus; EF1α, elongation factor 1α; Fluc, firefly luciferase; FT1, Flowering locus T1; NOS, nopaline synthase; Rluc, Renilla luciferase; UDP, ADH-like UDP glucose dehydrogenase;* UTR, untranslated region.
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1000/He delivery System (Bio-Rad, Hercules, CA). The device parameters were as follows: 1.0 μm gold particle; 1,100 p.s.i. rupture disk; 12 cm distance from stopping screen to target tissue; 28 mmHg vacuum pressure. Particle-bombarded cells were collected after growing at 22°C for two days, and ground in liquid nitrogen. Approximately 0.1 g of the resulting powder was suspended in 500 μl of passive lysis buffer (Promega). The mixture was centrifuged, and the supernatant was assayed for renilla and firefly luciferase activity using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions with a luminometer (Lumat LB 9501; Berthold Technologies, Bad Wilbad, Germany) configured to measure light emissions over a 10-s period. In our experiments, FLUC and RLUC activities were over 10^5 and 10^7 relative light units, respectively. These activities were normalized to cobombarded *Rluc* and *Fluc*, respectively, to correct for transfection efficiency and cell viability in each sample. The differences in average LUC activity are significant at $P<0.01$ according to the student's t-test.

Firstly, we focused the promoter. Strong promoters are required for effective transgene expression in plant cells. Although the *CaMV35S* promoter (Odell et al. 1985) is often used, its efficiency depends on plant species. Generally, promoters of highly expressed genes exhibit high transcriptional activity. The eukaryotic translation elongation factor, *EF1α*, plays a central role in protein synthesis by promoting the binding of aminoacyl-tRNA to 80 S ribosomes. Since the *EF1α* genes in plants are highly and constitutively expressed (Curie et al. 1993; Nagaya et al. 2000), there is a possibility that the *EF1α* gene promoter would be more efficient than the *CaMV35S* promoter also in the liverwort. Thus, we compared the liverwort *EF1α* gene promoter to the *CaMV35S* promoter. The analysis of the *Arabidopsis EF1α* promoter has shown that an intron within the 5′-UTR is important for gene expression (Curie et al. 1993). Since the liverwort *EF1α* promoter also has an intron within the 5′-UTR, we employed its promoter region including the entire 5′-UTR. To isolate the *EF1α* promoter from the liverwort, expressed sequence tags (ESTs) homologous to the tobacco *EF1α* coding region were identified by BLAST searches against the EST database of the liverwort (T. Kohchi, personal communication). Up-stream sequences of the liverwort *EF1α* homolog were identified by BLAST searches against the incomplete genome database of the liverwort (T. Kohchi, personal communication). DNA fragment of the liverwort *EF1α* promoter was obtained by PCR from the liverwort genomic DNA. The liverwort *EF1α* promoter (−1281 and +448 relative to the transcription initiation site), designated *MpEF1α* promoter (database accession number, AB630337), was amplified by PCR using primers including *Kpn*I or *Hin*dIII: pEF1f (5′-ggtaccCAAATGAGTCACACACATT-3′) and pEF1r (5′-aagcttCAACCTTTCTGCAGGCACA-3′). The amplified *MpEF1α* promoter was inserted into the *Kpn*I/*Hin*dIII sites of the "*CaMV35S-Fluc-tNOS*" and the "*CaMV35S-Rluc-tNOS*" to replace the *CaMV35S* promoter and to generate "*MpEF1α-Fluc-tNOS*" and the "*MpEF1α-Rluc-tNOS*", respectively.

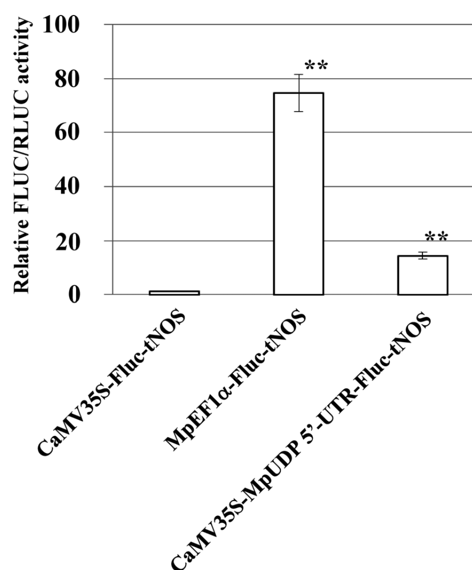

Figure 1. The *MpEF1α* promoter and *MpUDP* 5′-UTR increase FLUC activity. FLUC/RLUC activity of bombarded cells with "*CaMV35S-Fluc-tNOS*", "*MpEF1α-Fluc-tNOS*", or "*CaMV35S-MpUDP 5′-UTR-Fluc-tNOS*" was assayed. "*CaMV35S-Rluc-tNOS*" was used as an internal control in the transient expression assays. FLUC activity was normalized to cobombarded *Rluc* to correct for transfection efficiency and cell viability in each sample. Values relative to FLUC/RLUC with the "*CaMV35S-Fluc-tNOS*" are shown. Mean values with S.D. are shown for three measurements. Significant differences: $^{**}P<0.01$. (student's t-test)

Using *Fluc* gene as a reporter, we compared the transient gene expression efficiency of the *MpEF1α* promoter to that of the *CaMV35S* promoter. As a result, the *MpEF1α* promoter increased FLUC activity approximately 75-fold over the *CaMV35S* promoter (Figure 1).

Secondly, the 5′-UTR is important for the translation of downstream genes, as previously shown by the 16- to 18-fold increase in GUS activity in tobacco by the insertion of the Ω sequence of *tobacco mosaic virus* (Gallie et al. 1989). Similarly, the 5′-UTR of tobacco *ADH-like UDP glucose dehydrogenase* gene (*NtADH* 5′-UTR) increased gene expression in tobacco (Nagaya et al. 2000) and *Arabidopsis* (Nagaya et al. 2010). These elements are known as translational enhancer which enhances gene expression at the translational level. We applied the 5′-UTR of the liverwort homolog of *NtADH* gene to the liverwort transient gene expression system. To isolate 5′-UTR of the liverwort homolog of *NtADH* gene, ESTs homologous to the tobacco *NtADH* coding region were found and upstream sequences of the liverwort *NtADH* homolog were identified as described

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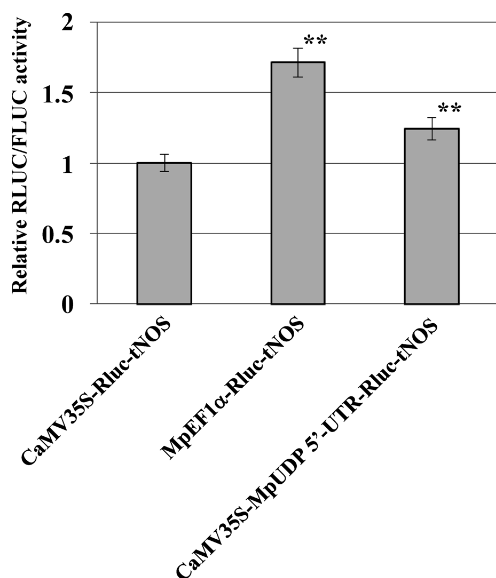

Figure 2. RLUC activity with the *MpEF1α* promoter or the *MpUDP* 5′-UTR. RLUC/FLUC activity of bombarded cells with "*CaMV35S-Rluc-tNOS*", "*MpEF1α-Rluc-tNOS*", or "*CaMV35S-MpUDP 5′-UTR-Rluc-tNOS*" was measured. "*CaMV35S-Fluc-tNOS*" was used as an internal control in the transient expression assays. RLUC activity was normalized to cobombarded *Fluc* to correct for transfection efficiency and cell viability in each sample. Values relative to RLUC/FLUC with the "*CaMV35S-Rluc-tNOS*" are shown. Mean values with S.D. are shown for three measurements. Significant differences: $^{**}P<0.01$. (student's t-test)

above. The liverwort 5′-UTR of *ADH-like UDP glucose dehydrogenase* gene (331 bp), designated *MpUDP* 5′-UTR (database accession number, AB630338), was amplified by PCR using primers including *Hin*dIII or *Nco*I: UTR1f (5′-aagcttGGGCGACCGTCAGGGTTTC-TC-3′) and UTR1r (5′-ccatggTTCGCAGAATGGCAA-CA-3′). The amplified *MpUDP* 5′-UTR was inserted into the *Hin*dIII/*Nco*I sites of the "*CaMV35S-Fluc-tNOS*" and "*CaMV35S-Rluc-tNOS*" to generate "*CaMV35S-MpUDP 5′-UTR-Fluc-tNOS*" and "*CaMV35S-MpUDP 5′-UTR-Rluc-tNOS*", respectively.

As a result of transient assay, the FLUC activity of *CaMV35S-MpUDP 5′-UTR-Fluc-tNOS* gene was approximately 15-fold higher than cases without the *MpUDP* 5′-UTR (Figure 1). These results show that the *MpEF1α* promoter and the *MpUDP* 5′-UTR are effective in contributing to increased expression levels of *Fluc* reporter gene in the liverwort.

To investigate whether the *MpEF1α* promoter and the *MpUDP* 5′-UTR also function in combination with other reporter genes, we constructed plasmids based on the *Rluc* reporter gene. Lower expression of the *Rluc* gene was detected than would be expected based on increase of the *Fluc* gene expression (Figure 2), showing that they were less effective than cases with the *Fluc* reporter gene. This indicates different enhancing effects with different reporter genes. The *MpUDP* 5′-UTR is thought to enhance gene expression at the translational level as same as reported in the Ω sequence and the *NtADH* 5′-UTR (Gallie et al. 1989; Nagaya et al. 2000; Nagaya et al. 2010). However, the *MpUDP* 5′-UTR did not enhance *Rluc* expression (Figure 2), indicating that 5′-UTR might not improve translational efficiency of the *Rluc* gene. The *MpEF1α* promoter including the 5′-UTR also showed no significant effect on *Rluc* expression. Therefore, there is a possibility that the *MpEF1α* promoter contribute to enhance gene expression largely at the translational level but not at the transcriptional level. Further investigation is required to reveal different enhancing effects depending on the reporter gene used.

Finally, another important factor in gene expression is the efficiency of the terminator. The terminator regulates the level of expression by controlling the transcriptional termination and 3′ end processing of mRNA. Different terminators strongly influence the level of gene expression (Ingelbrecht et al. 1989). The *heat shock protein 18.2* (*HSP*) terminator from *Arabidopsis* increases gene expression approximately 2.5-fold higher than with the widely used *NOS* terminator from *Agrobacterium tumefaciens* (Nagaya et al. 2010) and this increase is independent of the specific gene used (Nagaya et al. 2010; Oshima et al. 2011). Moreover, the *NOS* terminator was shown to have low efficiency compared with the endogenous gene terminator in *Arabidopsis* (Nagaya et al. 2010). Therefore, there is a possibility that the liverwort endogenous gene terminators increase gene expression compared to the *NOS* terminator. We investigated the terminator derived from the liverwort endogenous gene and the *NOS* terminator. Since 3′ downstream sequences of the liverwort homolog to the *Arabidopsis Flowering locus T1* (*FT1*) gene were previously obtained (T. Kohchi, personal communication), the terminator of this gene was used. To isolate the liverwort *FT1* terminator, ESTs homologous to the *Arabidopsis FT1* coding region were found and downstream sequences of the liverwort *FT1* homolog were identified as described in *MpEF1α* promoter and *MpUDP* 5′-UTR. The liverwort *FT1* terminator (300 bp downstream of the stop codon), designated *MpFT1* terminator (database accession number, AB630339), was amplified by PCR using primers including *Xba*I or *Bam*HI: tFT1f (5′-tctagaTA-ATACTTCTGACTTCC-3′) and tFT1r (5′-ggatccATG-GTTCATATCATGCCGAT-3′). The amplified *MpFT1* terminator was inserted into the *Xba*I/*Bam*HI sites of the "*CaMV35S-Fluc-tNOS*" and "*CaMV35S-Rluc-tNOS*" to generate "*CaMV35S-Fluc-tMpFT1*" and "*CaMV35S-Rluc-tMpFT1*", respectively.

Results of the transient assay indicated that FLUC activity with the *MpFT1* terminator was approximately 3-fold higher than with the widely used *NOS* terminator (Figure 3A). Furthermore, we constructed plasmids with

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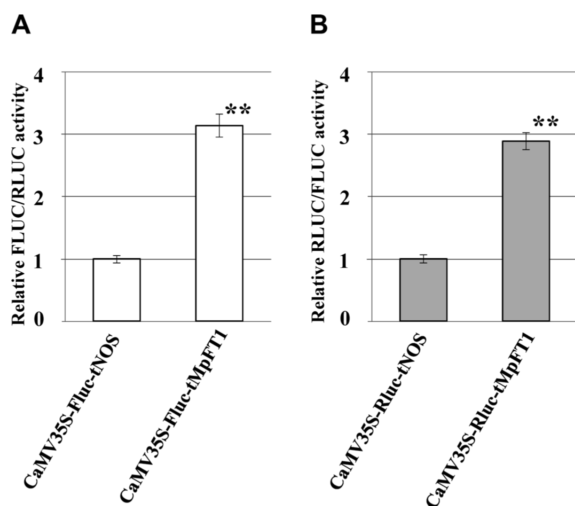

Figure 3. The *MpFT1* terminator increases expression of both *Fluc* and *Rluc* reporter genes. (A) FLUC/RLUC activity of bombarded cells with "*CaMV35S-Fluc-tNOS*" or "*CaMV35S-Fluc-tMpFT1*". "*CaMV35S-Rluc-NOS*" was used as an internal control in the transient expression assays. Values relative to FLUC/RLUC with the "*CaMV35S-Fluc-tNOS*" are shown. (B) RLUC/FLUC activity of bombarded cells with "*CaMV35S-Rluc-tNOS*" or "*CaMV35S-Rluc-tMpFT1*". "*CaMV35S-Fluc-NOS*" was used as an internal control in the transient expression assays. Values relative to RLUC/FLUC with the "*CaMV35S-Rluc-tNOS*" are shown. Significant differences: $^{**}P < 0.01$. (student's t-test)

the *MpFT1* terminator fused to the *Rluc* reporter gene. Results similar to those with the *Fluc* reporter gene were obtained (Figure 3B), showing that the *MpFT1* terminator was able to increase expression of both *Fluc* and *Rluc* reporter genes. Considering the effects of the *MpEF1α* promoter and the *MpUDP* 5′-UTR depending on the reporter gene, the *MpFT1* terminator is expected to be more effective for enhancement of various gene expressions in the liverwort.

In present study, we found no significant enhancing effect of gene expression in the liverwort with the elements reported in higher plants, the *CaMV35S* promoter and the *NOS* terminator. These results suggest that there is species-specificity for the enhancing effect of these elements. On the other hand, endogenous *EF1α* promoter and *FT1* terminator were efficient to express gene highly. This suggests that even when the widely used elements do not have the ability to enhance gene expression, use of the respective DNA elements from endogenous genes in particular plant species is recommended. We show here that the liverwort DNA elements enhance gene expression in transient gene expression system. It is interesting whether the combination of these elements function more effectively and we are currently studying. We are also investigating the effectiveness of these elements in the transgenic liverworts.

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