Plant Biotechnology 25, 69–75 (2008)

Original Paper

Improved translation efficiency in chrysanthemum and torenia with a translational enhancer derived from the tobacco *alcohol dehydrogenase* gene

Ryutaro Aida[1,*], Takako Narumi[1], Norihiro Ohtsubo[1], Hiroyasu Yamaguchi[1], Ko Kato[2], Atsuhiko Shinmyo[2], Michio Shibata[1]

[1] National Institute of Floricultural Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305–8519, Japan; [2] Nara Institute of Science and Technology, Ikoma, Nara 630–0101, Japan
* E-mail: ryu@affrc.go.jp Tel & Fax: +81-29-838-6822

Received August 16, 2007; accepted November 30, 2007 (Edited by K. Yoshida)

Abstract We investigated the efficiency of the 5′-untranslated region (UTR) of the tobacco *alcohol dehydrogenase* gene (*NtADH*-5′UTR) as a translational enhancer in chrysanthemum (*Chrysanthemum morifolium*) and torenia (*Torenia fournieri*). Three constructs were introduced: control, "ADH−," cauliflower mosaic virus 35S RNA (CaMV 35S) promoter::*β-glucuronidase* (*GUS*); *NtADH*-5′UTR with a spacer, "ADH+S," CaMV 35S promoter::*NtADH*-5′UTR::25-bp spacer::*GUS*; and *NtADH*-5′UTR with no spacer, "ADH+," CaMV 35S promoter::*NtADH*-5′UTR::*GUS*. The highest GUS activity in ADH+S and ADH+ for chrysanthemum was about 45 and 190 times, respectively, and for torenia was 12 and 22 times, respectively, than that for the ADH− plants. *NtADH*-5′UTR enhanced translational efficiency in both species. With the lowest translational efficiency set to 1, the relative translational efficiencies were 1 to 15 (ADH−), 47 to 568 (ADH+S), and 267 to 1360 (ADH+) for chrysanthemum, and 1 to 3 (ADH−), 47 to 114 (ADH+S), and 85 to 226 (ADH+) for torenia. *NtADH*-5′UTR would facilitate practical breeding and fundamental genetic research in chrysanthemum and torenia.

Key words: *Chrysanthemum morifolium*, molecular breeding, ornamental plants, *Torenia fournieri*, translational enhancer.

Genetic transformation is a useful method for plant breeding, and analysis using transgenic plants is also useful for studies of plant physiology. For efficient transgene expression, we require sufficient levels of protein production by the transgenes to modify target characteristics. The development of promoters of higher transcriptional activity is a method for enhancing transgene expression; however, the accumulation of mRNA tends to induce post-transcriptional gene silencing (Vaucheret et al. 1998). Moreover, in this strategy, different promoters of higher transcriptional activity are needed for tissue- and stage-specific expression. On the other hand, the use of translational enhancers would increase protein production with proper amounts of mRNA. Thus appropriate promoters can be used with translational enhancers for a wide range of purposes, including the induction of tissue- or stage-specific gene expression. Therefore, the use of translational enhancers seems to be a desirable method for increasing transgene expression in plants.

The 5′-untranslated region (5′UTR) located upstream of the translational initiation site plays an important role in the translation of the transgene. In one study, the Ω sequence, which represents the 5′UTR region from the tobacco mosaic virus, functioned as a translational enhancer in tobacco protoplasts (Gallie et al. 1987). The Ω sequence also significantly enhanced the expression of the *β-glucuronidase* (*GUS*) gene in tobacco and rice plants (Mitsuhara et al. 1996); however, obvious enhancement was not observed in torenia (Aida and Shibata 1995) and chrysanthemum (R. Aida, National Institute of Floricultural Science, Japan; unpublished data). Other translational enhancers have been found in viruses (Nicolaisen et al. 1992; Meulewaeter et al. 1998; Turner et al. 1999; Matsuda and Dreher 2004; Batten et al. 2006), bacteria (Hook-Barnard et al. 2007), and plants (Yamamoto et al. 1995; Dansako et al. 2003). In addition, the 5′UTR region of the tobacco *alcohol dehydrogenase* gene (a 94-bp fragment; *NtADH*-5′UTR) was reported to be an efficient translational enhancer in *Arabidopsis* and tobacco (Satoh et al. 2004).

Chrysanthemum is one of the most popular and important ornamental plants in the world. Genetic transformation would be a powerful tool for breeding of chrysanthemum for characteristics such as new colors. However, transgene expression in chrysanthemum seems

to be insufficient, for example, it has been shown that transgene expression by the CaMV 35S promoter was relatively less in chrysanthemum (Urban et al. 1994; Sherman et al. 1998; Takatsu et al. 2000). The development of a translational enhancer would thus be an important solution to the difficulty of stimulating transgene expression in chrysanthemum. Similarly, torenia is a popular summertime bedding- or ornamental pot-plant. Besides its ornamental use, torenia is a model plant for transformation studies on ornamental characters because it is easy to transform and can flower *in vitro* (Aida and Shibata 2001). In this paper, we studied the use of *NtADH*-5′UTR as a translational enhancer in chrysanthemum and torenia.

Materials and methods

Plant materials and transformation

We used the chrysanthemum (*Chrysanthemum morifolium*) cultivar 'Sei-Marine' and the torenia (*Torenia fournieri*) cultivar 'Crown Violet' in our experiment. Transgenic plants were obtained using an *Agrobacterium*-mediated transformation system described previously (chrysanthemum, Aida et al. 2004; torenia, Aida and Shibata 2001).

Vector plasmids

The vector plasmids used in this study were previously constructed by Satoh et al. (2004). Derivatives of the binary vector pBI121 (Clontech, Mountain View, CA, USA) were used. To simplify the vector construction, the third codon of *GUS* was changed to AGG instead of CGT to generate a *Stu*I site; this substitution has been reported to have less effect on GUS activity (Satoh et al. 2004). As a control plasmid, we used the cauliflower mosaic virus 35S RNA (CaMV 35S) promoter to produce CaMV 35 promoter::*GUS*::nopaline synthase (nos) terminator, described as "ADH−" henceforth. We used a plasmid containing *NtADH*-5′UTR and a 25-bp spacer derived from the pBI121 cloning site to produce CaMV 35S promoter::*NtADH*-5′UTR::25-bp spacer::*GUS*::nos terminator, (described as "ADH+S" henceforth) and a plasmid containing the *NtADH*-5′UTR directly fused with the first codon of *GUS*, to produce CaMV 35S promoter::*NtADH*-5′UTR::*GUS*::nos terminator (described as "ADH+" henceforth).

GUS assay, Southern blot analysis, and real-time PCR

A quantitative and histochemical GUS assay was performed following the procedure reported by Jefferson et al. (1987). The GUS assay buffer contained 20% methyl alcohol to eliminate endogenous GUS activity, as reported by Kosugi et al. (1990). For our quantitative assay, the protein concentration of the plant extracts was determined by means of the dye-binding method of Bradford (1976), using a kit supplied by Bio-Rad Laboratories (Hercules, CA, USA). GUS activity was expressed as picomoles of 4-methylumbelliferone (4-MU) produced at 37 °C per milligram of protein per minute (pmol 4-MU mg^{-1} protein min^{-1}). Plants with GUS activities of more than 100 pmol 4MU mg^{-1} protein min^{-1} were regarded as GUS-positive, because untransformed wild-type plants sometimes showed background levels of 20 to 30 pmol mg^{-1} protein min^{-1} of GUS. For histochemical assay, several slits were cut in each leaf before soaking in the buffer.

Total DNA was extracted from leaf tissue following the method of Hasebe and Iwatsuki (1990). About 20 μg (chrysanthemum) or 5 μg (torenia) of DNA digested with *Hin*dIII was electrophoresed in 0.8% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). *Hin*dIII was used to cut the plasmid at a single site outside the coding region of the *GUS* gene. The coding region of the *GUS* gene was then used as a probe. Southern hybridization was done with a DIG-High Prime and DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics). Blots were finally washed with 0.2× SSC and 0.1% SDS at 68°C.

Total RNA was extracted from leaf tissue with an SV Total RNA Isolation System (Promega, Madison, WI, USA) and cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Levels of *GUS* transcripts were then analyzed using real-time PCR with a SYBR Premix Ex Taq system (Takara Bio, Otsu, Japan). Reactions were carried out in a LightCycler system (Roche Diagnostics). Chrysanthemum *actin* (AB205087) and torenia *actin* (AB330989) were used as the respective constitutive controls. Primer sequences used for the real-time PCR were as follows: forward 5′-AGGGAGGCAAACAATGAATC-3′ and reverse 5′-TTGAACGATCGGGGAAATTC-3′ for *GUS*, forward 5′-ACATGCTATCTTGCGTTTGG-3′ and reverse 5′-CTCTCACAATTTCCCGTTCA-3′ for chrysanthemum *actin*, and forward 5′-TGCAGTAAAGTGTATTGTGGAAG-3′ and reverse 5′-GGAACTATCTGGGTAGGATC-3′ for torenia *actin*.

Results and discussion

Enhanced GUS activity with NtADH-5'UTR

In chrysanthemum, we obtained 14 (ADH−), 4 (ADH+S), and 16 (ADH+) transformants. Figure 1A shows GUS activity in the leaves of chrysanthemum transformants about 2 months after regeneration. The number of GUS-positive plants were 4 (ADH−), 4 (ADH+S), and 9 (ADH+). The activity of the GUS-positive plants ranged from 231 to 492, 1090 to 22 000, and 1330 to 93 400 for ADH−, ADH+S, and ADH+, respectively. Figure 2 shows typical results from the histochemical GUS assay of the chrysanthemum transformants. Transformants containing the *NtADH*-5′UTR showed dark precipitates, representing higher levels of GUS activity. On the other hand, plants containing ADH+ exhibited no visible precipitate.

Addition of *NtADH*-5′UTR before the start codon of the *GUS* gene obviously increased GUS activity in the chrysanthemum transformants, with the highest activity observed among the ADH+S and the ADH+ plants (at levels about 45 and 190 times the value in the ADH−

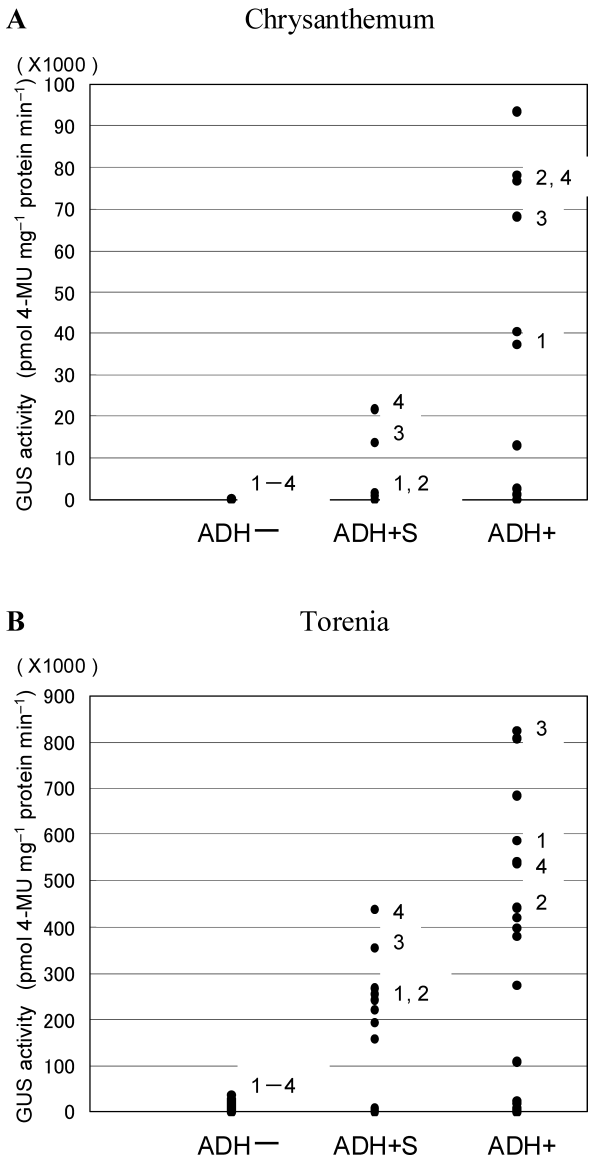

Figure 1. GUS activity in the leaves of (A) chrysanthemum and (B) torenia transformants. ADH−, control plasmid (CaMV 35S promoter::*GUS*::nos terminator); ADH+S, *NtADH*-5′UTR with a 25-bp spacer (CaMV 35S promoter::*NtADH*-5′UTR::25-bp spacer::*GUS*::nos terminator); ADH+, *NtADH*-5′UTR (CaMV 35S promoter::*NtADH*-5′UTR::*GUS*::nos terminator). Each dot represents the GUS activity of a single transformant. Plants selected for further analysis are indicated by numbers beside the dots, and these numbers correspond to the plants shown in Figures 2 and 3 and in Table 1. Addition of *NtADH*-5′UTR obviously increased the GUS activity in the transformants.

plants, respectively). We confirmed the existence of the *GUS* gene by PCR even in the GUS-negative plants (data not shown). In a previous study, chrysanthemum transformants tended to silence the integrated *GUS* gene, with only 9.6% (11 of 115) of the putative transgenic plants containing 35S/*GUS* exhibiting GUS activity (Aida et al. 2004). In the present report, the proportions of GUS-positive transformants were 29, 100, and 56% for ADH−, ADH+S, and ADH+, respectively. Addition of *NtADH*-5′UTR enhanced the level of GUS activity and this seems to have increased the proportion of GUS-positive plants.

In torenia, we obtained 24 (ADH−), 19 (ADH+S), and 24 (ADH+) transformants. Figure 1B shows GUS activity in the leaves of the torenia transformants 1 to 2 months after regeneration. All of the transformants showed GUS activity, and the activities ranged from 275 to 37 200, 275 to 440 000, and 1520 to 825 000 for ADH−, ADH+S, and ADH+, respectively. The GUS activities of the torenia transformants were thus generally higher than those of the chrysanthemum transformants. In the torenia transformants, the presence of *NtADH*-5′UTR also increased GUS activity, as was the case with chrysanthemum. The highest GUS activity in torenia transformants containing ADH+S and ADH+ was 12 and 22 times that in the ADH− plants, respectively.

Satoh et al. (2004) showed that transiently transformed tobacco BY2 (or *Arabidopsis* T87) protoplasts containing ADH+S and ADH+ constructs showed 29 (or 31) and 96 (or 61) times the GUS activity of the corresponding ADH− transformants. In this paper, we showed that *NtADH*-5′UTR also enhanced transgene expression in chrysanthemum and torenia.

GUS activities varied among the transformants of both chrysanthemum and torenia; however, the chrysanthemum transformants showed relatively lower GUS activity compared with the torenia transformants. Even the chrysanthemum transformants containing ADH+ showed GUS activity similar to or slightly higher than torenia transformants containing ADH−. In chrysanthemum, previous reports indicated that the 35S promoter had a low ability to stimulate transgene expression (Urban et al. 1994; Sherman et al. 1998; Takatsu et al. 2000). Several promoters for transgene expression in the leaf tissues of chrysanthemum have been proposed, such as potato *Lhca3.St.1* (Annadana et al. 2001), chrysanthemum *UEP1* (Annadana et al. 2002), chrysanthemum *rbcS1* (Outchkourov et al. 2003), chrysanthemum *cab* (Aida et al. 2004), and tobacco *EF1α* (Aida et al. 2005). All these studies reported a higher expression than the 35S promoter. The reported average and highest GUS activities in the leaf tissues of chrysanthemum transformants were about 25 000 and 140 000 pmol mg^{-1} protein min^{-1} for potato *Lhca3.St.1* (the average was calculated using only GUS-positive plants) (Annadana et al. 2001), 900 and 1400 for chrysanthemum *UEP1* (Annadana et al. 2002), 17 000 and 70 000 for chrysanthemum *rbcS1* (Outchkourov et al. 2003), 1100 and 27 000 for chrysanthemum *cab* (Aida et al. 2004), and 570 and 14 000 for tobacco EF1α (Aida et al. 2005). In the present study, the level of GUS activity in the chrysanthemum transformants containing ADH+ (18 700 and 93 400 for the average and highest activity, respectively) was comparable to that in plants

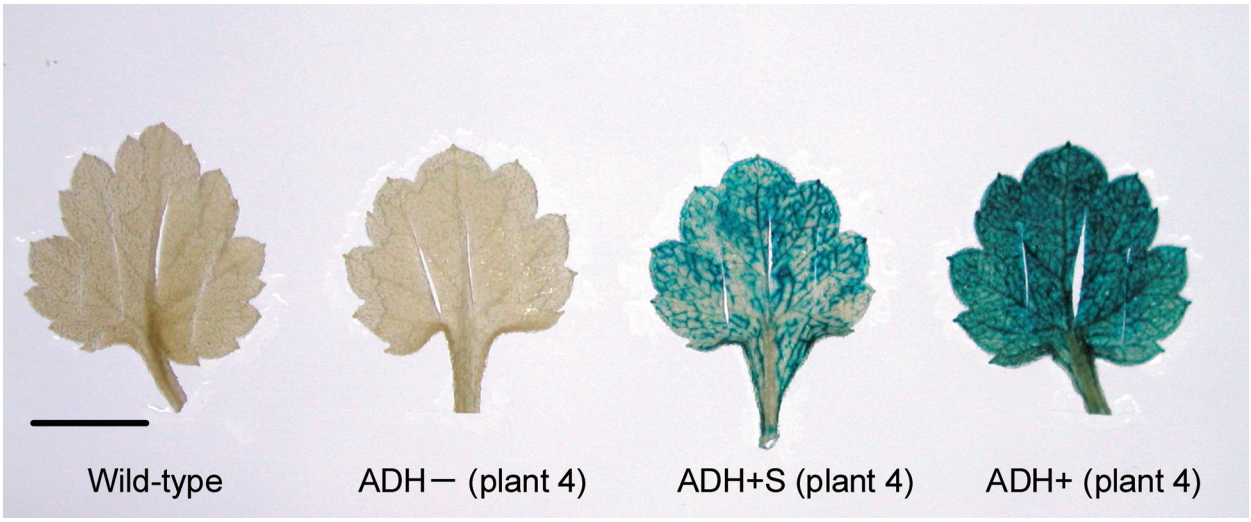

Figure 2. Histochemical GUS assay of the chrysanthemum transformants. Leaves about 10 mm wide were used for the assay. Blue precipitate represents GUS activity. Transformants containing the *NtADH*-5′UTR clearly showed darker precipitates than the plants containing ADH−. Scale bar stands for 5 mm.

containing potato *Lhca3.St.1* promoter (Annadana et al. 2001), which was the most efficient promoter for transgene expression in the leaf tissues of chrysanthemum that had previously been reported. Thus, the CaMV 35S promoter containing *NtADH*-5′UTR will be a useful tool for stimulating constitutive transgene expression in chrysanthemum. The *NtADH*-5′UTR can be used with many kinds of promoters, such as inducible and tissue- or developmental-stage-specific ones. For example, when modifying flower color, the expression of pigment-related genes could be controlled using a petal-specific promoter containing *NtADH*-5′UTR.

We previously reported that the highest level of GUS activity (15 000 to 17 000 pmol 4-MU mg^{-1} protein min^{-1}) was similar in torenia transformants with or without the Ω sequence (Aida and Shibata 1995). Similarly, the Ω sequence barely enhanced the expression of GUS in chrysanthemum (R. Aida, National Institute of Floricultural Science, Japan; unpublished data). *NtADH*-5′UTR showed higher levels of transgene enhancement than the Ω sequence in both chrysanthemum and torenia. *NtADH*-5′UTR would thus be useful for stimulating transgene expression in chrysanthemum and torenia.

For both chrysanthemum and torenia, we selected four lines from each group of transformants (i.e., ADH−, ADH+S, and ADH+), and used these lines for additional measurements of quantitative GUS activity, Southern blot analysis, and real-time PCR. The GUS-positive chrysanthemum in the ADH− and ADH+S groups were used because we obtained only four transformants in both groups. In the other groups, the transformants with relatively higher GUS activity (Figure 1) were selected.

Copy number of the GUS gene in transgenic plants

All the plants that we examined contained the *GUS* gene (Figure 3). Digestion of each vector with *Hin*dIII cuts the plasmid at a single site outside the coding region of the *GUS* gene (before the 35S promoter). The bands detected might have been cut by *Hin*dIII at sites in both the vector and the genome. All the examined plants showed one or more bands, indicating single- or multiple-copy integration of the *GUS* gene into their genome.

In chrysanthemums containing ADH− or ADH+S, the plants with higher GUS activity tended to show more bands. In contrast, all four transformants containing ADH+, selected based on their higher GUS activity, showed a single band. In chrysanthemum, it seems that the ADH+ transgene was able to express higher GUS activity even with a single copy, whereas overproduction of GUS protein from multiple copies of the ADH+ gene might result in a negative effect on the growth of transgenic plants.

The torenia transformants containing ADH− showed several bands; however, the plants containing ADH+S or ADH+ showed only a single band. In torenia, we also selected four transformants with higher GUS activity from 24 (ADH−), 19 (ADH+S), and 24 (ADH+) GUS-positive plants. Transformants containing ADH− should have multiple copies of the transgene for higher GUS activity; however, those containing ADH+S and ADH+ exhibit higher GUS activity in spite of containing only a single copy of the transgene. It is possible that the integration of multiple copies of ADH+S or ADH+ might lead to excessively high expression of GUS and subsequent mortality of the transformants.

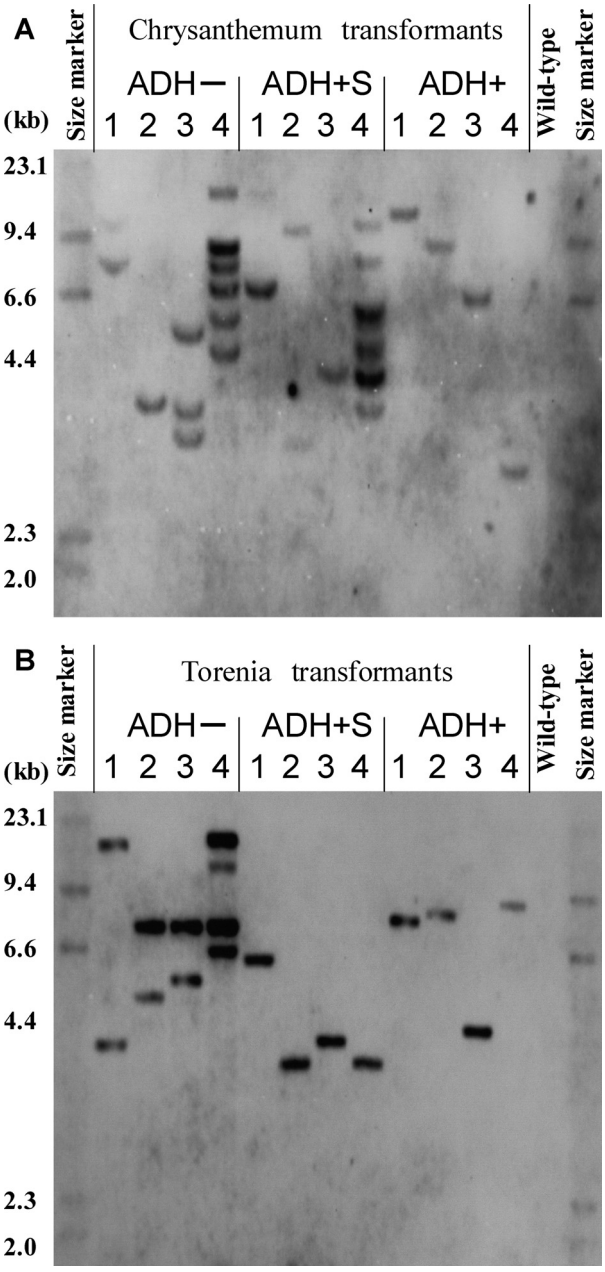

Figure 3. Southern blot analysis of the GUS-positive plants of (A) chrysanthemum and (B) torenia. The coding region of the *GUS* gene was used as a probe. All the plants examined contained the *GUS* gene. The numbers 1 to 4 represent the plants labeled with these numbers in Figure 1.

Enhanced translational efficiency of transgene with NtADH-5′UTR

Table 1 summarizes the GUS activity, relative amount of *GUS* mRNA, and the absolute and relative translational efficiencies of the selected transformants. The ratio of *GUS* mRNA to *actin* mRNA (relative *GUS* mRNA) varied among the transformants, and the ratio was relatively low in the chrysanthemum transformants (0.02 to 1.52) compared with the torenia transformants (0.98 to 11.20). The values of GUS activity divided by the relative amount of *GUS* mRNA was defined as the translational efficiency. When we normalized values by setting the lowest translational efficiency to 1, the resulting relative translational efficiencies were 1 to 15 (ADH−), 47 to 568 (ADH+S), and 267 to 1360 (ADH+) for chrysanthemum, and 1 to 3 (ADH−), 47 to 114 (ADH+S), and 85 to 226 (ADH+) for torenia. The addition of *NtADH*-5′UTR between the promoter and the coding region enhanced the translational efficiency in both chrysanthemum and torenia. Previously, Satoh et al. (2004) reported that the ADH+ construct increased translational efficiency in tobacco transformants (ADH−, 1.0 to 1.4; ADH+, 20 to 44). This suggests that *NtADH*-5′UTR can work as a translational enhancer in a range of plant species, especially dicots.

Based on our observations of the *GUS* constructs, the distance between *NtADH*-5′UTR and the starting codon seemed to be inversely correlated with the translational efficiency. That is, the shorter the distance, the higher the translational efficiency obtained. It is possible that the sequence context around the initiation codon also affects the translational efficiency; however, we did not identify this context in the present study. We hypothesize that *NtADH*-5′UTR should connect directly with the start codon to attain the highest translational efficiency. A site for restriction enzymes is thus needed between *NtADH*-5′UTR and the first codon to facilitate creation of such constructs. In the present study, the distance of 25 bp seemed to work well to enhance translation.

Conclusion

In this study, we demonstrated that *NtADH*-5′UTR enhances the translational efficiency of transgenes in both chrysanthemum and torenia. Such promoters of high gene expression will be more efficient and will not induce post-transcriptional gene silencing, which is caused by the accumulation of excessive amounts of mRNA (Vaucheret et al. 1998). Even promoters with low efficiency can produce sufficient levels of the target substance if *NtADH*-5′UTR is used. Using *NtADH*-5′UTR would let us enhance the expression of inducible or tissue- and developmental-stage-specific promoters with poor efficiency, thereby facilitating practical breeding and fundamental genetic studies in chrysanthemum and torenia.

Satoh et al. (2004) found that *NtADH*-5′UTR enhanced transgene expression at the translational level in tobacco and *Arabidopsis*; however, it barely worked in rice. In this report, we demonstrated that *NtADH*-5′UTR works as a translational enhancer in two additional species. We thus hypothesize that *NtADH*-5′UTR enhances transgene expression at the translational level in a broad range of plants, especially dicotyledons.

Table 1. GUS activity, amount of *GUS* mRNA, and absolute and relative translational efficiency of the chrysanthemum and torenia transformants.

chrysanthemum					
Construct	Line	GUS activity	Relative *GUS* mRNA	Translational efficiency	Relative efficiency
ADH−	1	328±84	1.35	243	1
ADH−	2	336±21	1.19	282	1
ADH−	3	475±38	0.13	3650	15
ADH−	4	722±201	1.49	485	2
ADH+S	1	569±452	0.05	11400	47
ADH+S	2	2760±829	0.02	138000	568
ADH+S	3	14300±2470	0.31	46100	190
ADH+S	4	21500±354	0.69	31200	128
ADH+	1	23200±12200	0.07	331000	1360
ADH+	2	81900±13600	0.98	83600	344
ADH+	3	82300±22500	0.87	94600	389
ADH+	4	98500±19300	1.52	64800	267
torenia					
Construct	Line	GUS activity	Relative *GUS* mRNA	Translational efficiency	Relative efficiency
ADH−	1	33200±12200	5.20	6380	1
ADH−	2	33300±19500	2.25	14800	3
ADH−	3	44600±13800	7.62	5850	1
ADH−	4	57000±32500	11.20	5090	1
ADH+S	1	297000±48200	1.23	241000	47
ADH+S	2	404000±42100	1.00	404000	79
ADH+S	3	474000±190000	1.08	439000	86
ADH+S	4	596000±144000	1.03	579000	114
ADH+	1	629000±70300	1.46	431000	85
ADH+	2	930000±421000	1.61	578000	114
ADH+	3	1030000±220000	1.02	1010000	198
ADH+	4	1130000±528000	0.98	1150000	226

GUS activity (pmol 4-MU mg^{-1} protein min^{-1}) was measured three times in leaves of the transformants and is presented as an average with standard deviation of these measurements. Relative *GUS* mRNA was calculated as the ratio of *GUS* mRNA to *actin* mRNA. Translational efficiency was calculated as the ratio of GUS activity to the relative amount of *GUS* mRNA. Relative efficiency was calculated based on the value for the transformant with the lowest translational efficiency (chrysanthemum, ADH− plant 1; torenia, ADH− plant 4).

Acknowledgments

We would like to thank Ms. Satoko Ohtawa, Ms. Yumi Ohtsuka, Ms. Kiyomi Shimizu, and Ms. Misako Takahashi (National Institute of Floricultural Science) for producing and handling the transformants.

References

Aida R, Shibata M (1995) Comparison of promoter-dependent expression of β-glucuronidase genes in transgenic *Torenia* plants. *Acta Horticulturae* 392: 219–225

Aida R, Shibata M (2001) Transgenic *Torenia fournieri* Lind. (torenia). In: Bajaj YPS (ed) *Biotechnology in Agriculture and Forestry.* Springer Verlag, Berlin, vol 48 Transgenic Crops III pp 294–305

Aida R, Ohira K, Tanaka Y, Yoshida K, Kishimoto S, Shibata M, Ohmiya A (2004) Efficient transgene expression in chrysanthemum, *Dendranthema grandiflorum* (Ramat.) Kitamura, by using the promoter of a gene for chrysanthemum chlorophyll-*a*/*b*-binding protein. *Breed Sci* 54: 51–58

Aida R, Nagaya S, Yoshida K, Kishimoto S, Shibata M, Ohmiya A (2005) Efficient transgene expression in chrysanthemum, *Chrysanthemum morifolium* Ramat., with the promoter of a gene for tobacco elongation factor 1α protein. *JARQ* 39: 269–274

Annadana S, Mlynarova L, Udayakumar M, Jong Jde, Nap JP (2001) The potato *Lhca3.St.1* promoter confers high and stable transgene expression in chrysanthemum, in contrast to CaMV-based promoters. *Mol Breed* 8: 335–344

Annadana S, Beekwilder MJ, Kuipers G, Visser PB, Outchkourov N, Pereira A, Udayakumar M, Jong Jde, Jongsma MA (2002) Cloning of the chrysanthemum *UEP1* promoter and comparative expression in florets and leaves of *Dendranthema grandiflora. Transgenic Res* 11: 437–445

Batten JS, Desvoyes B, Yamamura, Y, Scholthof KBG (2006) A translational enhancer element on the 3′-proximal end of the *Panicum mosaic virus* genome. *FEBS Lett* 580: 2591–2597

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 72: 248–254

Dansako T, Kato K, Satoh J, Sekine M, Yoshida K, Shinmyo A (2003) 5′ untranslated region of the *HSP18.2* gene contributes to efficient translation in plant cells. *J Biosci Bioeng* 95: 52–58

Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TM (1987) A comparison of eukaryotic viral 5′-leader sequences as enhancers of mRNA expression *in vivo*. *Nucleic Acids Res* 15: 8693–8711

Hasebe M, Iwatsuki K (1990) *Adiantum capillus-veneris* chloroplast DNA clone bank: as useful heterologous probes in the systematics of the leptosporangiate ferns. *Am Fern J* 80: 20–25

Hook-Barnard IG, Brickman TJ, McIntosh MA (2007) Identification of an AU-rich translational enhancer within the

Escherichia coli fepB leader RNA. *J Bacteriol* 189: 4028–4037

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. *Plant Sci* 70: 133–140

Matsuda D, Dreher TW (2004) The tRNA-like structure of *Turnip yellow mosaic virus* RNA is a 3′-translational enhancer. *Virology* 321: 36–46

Meulewaeter F, Van Montagu M, Cornelissen M (1998) Features of the autonomous function of the translational enhancer domain of satellite tobacco necrosis virus. *RNA* 4: 1347–1356

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37: 49–59

Nicolaisen M, Johansen E, Poulsen GB, Borkhardt B (1992) The 5′ untranslated region from pea seedborne mosaic potyvirus RNA as a translational enhancer in pea and tobacco protoplasts. *FEBS Lett* 303: 169–172

Outchkourov NS, Peters J, Jong Jde, Rademakers W, Jongsma MA (2003) The promoter-terminator of chrysanthemum *rbcS1* directs very high expression levels in plants. *Planta* 216: 1003–1012

Satoh J, Kato K, Shinmyo A (2004) The 5′-untranslated region of the tobacco *alcohol dehydrogenase* gene functions as an effective translational enhancer in plant. *J Biosci Bioeng* 98: 1–8

Sherman JM, Moyer JW, Daub ME (1998) A regeneration and *Agrobacterium*-mediated transformation system for genetically diverse *Chrysanthemum* cultivars. *J Amer Soc Hort Sci* 123: 189–194

Takatsu Y, Hayashi M, Sakuma F (2000) Transgene inactivation in *Agrobacterium*-mediated chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) transformants. *Plant Biotechnol* 17: 241–245

Turner RL, Glynn M, Taylor SC, Cheung MK, Spurr C, Twell D, Foster GD (1999) Analysis of a translational enhancer present within the 5′-terminal sequence of the genomic RNA of potato virus S. *Arch Virol* 144: 1451–1461

Urban LA, Sherman JM, Moyer JW, Daub ME (1994) High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). *Plant Sci* 98: 69–79

Vaucheret H, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. *Plant J* 16: 651–659

Yamamoto YY, Tsuji H, Obokata J (1995) 5′-Leader of a photosystem I gene in *Nicotiana sylvestris*, *psaDb*, contains a translational enhancer. *J Biol Chem* 270: 12466–124–70