Plant Biotechnology 32, 309–316 (2015)
DOI: 10.5511/plantbiotechnology.15.1103a

Original Paper

Compatibility of translational enhancers with various plant species

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Received August 3, 2015; accepted November 3, 2015 (Edited by K. Hiratsuka)

Abstract Translational enhancers are effective tools to increase the expression level of transgenes in plant cells, and some candidate elements are in use today. However, knowledge about suitable elements for a given plant species are limited. We aim here to make a catalogue of translational enhancers, and evaluated the effectiveness of 5′-UTRs of *Nicotiana tabacum alcohol dehysrogenase* gene (*NtADH* 5′-UTR), *Arabidopsis thaliana ADH* gene (*AtADH* 5′-UTR), *Oryza sativa ADH* gene (*OsADH* 5′-UTR), Ω derived from tobacco mosaic virus, and tobacco etch virus leader (TEVL) in various plant species. We found that the *OsADH* 5′-UTR is functional in all plant species tested, and that Ω and TEVL are effective in eudicots but not in monocots. From these results, we speculate that the degree of translational enhancement of any element in a given plant species is closely correlated with the phylogenic position of the species.

Key words: *Alcohol dehydrogenase* gene 5′-untranslated region, omega, phylogeny, tobacco etch virus leader, translational enhancer.

Currently, rapid progress is being made in the field of plant biotechnology. Transgenic crops including soy, maize, and cotton with tolerance to herbicides, and those with increased resistance to insects, viruses, and abiotic stresses, have been developed, some of which are in commercial use. Genetically modified flower color-altered varieties of flower crops such as carnation and rose are also available for consumers (Tanaka et al. 2009). In addition, plant-made vaccine antigens and biopharmaceuticals are under development (Paul and Ma 2011). In May 2012, United States Food and Drug Administration (FDA) approved a carrot cell culture–based recombinant taliglucerase alfa for treatment of type 1 Gaucher's disease, which is the first example of practical application of plant-made pharmaceuticals (PMPs) for human use (Maxmen 2012).

To obtain a large amount of recombinant proteins in transgenic plants, it is necessary to optimize the expression cassette of the desired transgene. For example, in order to achieve high accumulation of the mRNA of a foreign gene which is stably integrated it into nuclear genomes, the use of transcriptional terminator derived from *Arabidopsis thaliana heat shock protein 18.2* gene has been effectively used in *Arabidopsis thaliana*, rice, lettuce, and tomato (Hirai et al. 2011; Matsui et al. 2011; Nagaya et al. 2010). Furthermore, an improved version of the terminator has been developed (Matsui et al. 2014). At the post-translational level, optimization of subcellular localization of a recombinant protein is important, and sending a recombinant protein into the vesicular transport pathway often leads to successful accumulation of the protein (Yoshida et al. 2004). At translational levels, optimization of the 5′-untranslated region (5′-UTR) is important, because nucleotides just upstream of the initiating AUG have an impact on translational initiation (Sugio et al. 2010), and some 5′-UTRs are reportedly function as translational enhancers (Carrington and Freed 1990; Gallie et al. 1987; Matsui et al. 2012; Satoh et al. 2004; Sugio et al. 2008).

Translational enhancers in plant cells are often found in UTRs of plant viral RNAs. Examples are the 5′-UTR of tobacco mosaic virus RNA, called omega (Ω) (Gallie et al. 1987), that of tobacco etch potyvirus RNA (TEVL, Carrington and Freed 1990), and 3′-UTR of carnation Italian ringspot virus (Nicholson et al. 2010). These three elements have been found to fulfill their functions through a cap-independent translation initiation mechanism (Carrington and Freed 1990; Gallie et al. 1987; Nicholson et al. 2010). Translational enhancers are also found in 5′-UTRs of plant cellular mRNAs such as *Vigna radiata aminocyclopropane-1-carboxylate synthase* gene (Wever et al. 2010), *Nicotiana sylvestris psaDb* gene (Yamamoto et al. 1995), and *alcohol dehydrogenase* genes (Satoh et al. 2004; Sugio et al. 2008), *A. thaliana AGP21*

Abbreviations: LUC, luciferase; UTR, untranslated region.
This article can be found at http://www.jspcmb.jp/
Published online December 5, 2015

gene (Matsui et al. 2012). Translational enhancers have been studied mainly using experimental model plants such as tobacco (*Nicotiana tabacum*), *A. thaliana*, and rice (*Oryza sativa*). We have previously reported that 5′-UTR of *N. tabacum alcohol dehydrogenase* gene (*NtADH* 5′-UTR) is functional in some eudicots including tobacco (*N. tabacum*), *A. thaliana*, chrysanthemum (*Chrysanthemum morifolium*), torenia (*Torenia fournieri*), and lettuce (*Lactuca sativa*) (Aida et al. 2008; Matsui et al. 2006, 2009; Nagaya et al. 2010; Satoh et al. 2004; Sugio et al. 2008) and it is not functional in a monocot rice (*O. sativa*) (Sugio et al., 2008); however, more detailed knowledge about translational enhancers in crop plants is limited.

In this study, we evaluated five known translational enhancers, *NtADH* 5′-UTR (Satoh et al. 2004), *O. sativa ADH* 5′-UTR (Sugio et al. 2008), *A. thaliana ADH* 5′-UTR (Sugio et al. 2008), Ω (Gallie et al. 1987), and TEVL (Carrington and Freed 1990), in various plant species in order to make catalog of translational enhancers, or in other words, to obtain deeper knowledge about the compatibility of a given translational enhancer with a given plant species. *O. sativa* (*Os*) *ADH* 5′-UTR reportedly enhances the expression of *β*-glucuronidase (GUS) by 9 times in *O. sativa* and by about 50 times in *A. thaliana* and tobacco (Sugio et al. 2008). *AtADH* 5′-UTR enhances the expression of GUS by about 100 times in *A. thaliana* and tobacco, but is not functional in rice cells (Sugio et al. 2008). We employed polyethyleneglycol (PEG)-mediated transient assay system using protoplasts derived from plant materials, because this method is generally applicable to any plant species. Among the translational enhancers tested, *OsADH* 5′-UTR was functional in all plant species tested. In our experimental system, Ω and TEVL were effective in eudicots, but in monocots, even acting as suppressors. We also found that in a fern, *Equisetum arvense*, all five translational enhancers exerted moderate effect, intermediate between monocots and eudicots. Interestingly, Korean houttuynia (*Houttuynia cordata*), belonging to magnoliid, has a preference for translational enhancers similar to monocots.

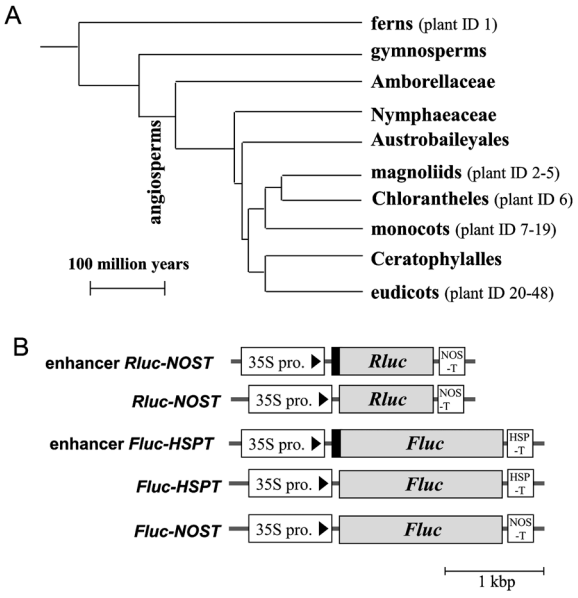

Figure 1. (A) A simplified phylogenic tree of plant species used in this study. This tree was generated following APGII (The Angiosperm Phylogeny Group, 2003; http://www.mobot.org/MOBOT/research/APWeb/welcome.html). Names of taxa are followed by brevity codes used in this study. (B) Schematic representation of plasmids used in this study. 35S, Cauliflower mosaic virus 35S RNA promoter; NOS-T, transcription terminator from *Agrobacterium tumefaciens nopaline synthase* gene; HSP-T, transcription terminator from *A. thaliana heat shock protein 18.2* gene; *Rluc*, *luciferase* gene derived from *Renilla reniformis*; *Fluc*, *luciferase* gene derived from firefly (*Photinus pyralis*). *Fluc-NOST* was constructed using pBI221, while all other plasmids were constructed using pRI909. Open reading frames and translational enhancers are indicated with gray and black, respectively. Predicted nucleotide sequences of the 5′-end of mRNA are as follows: *Rluc-NOST*, *Rluc-HSPT*, and *Fluc-HSPT*, cgcccggguaccaug (initiating aug underlined); enhancer *Rluc-NOST*, and enhancer *Fluc-HSPT*, cgcccggguaccxx..xxaug (x corresponds to nucleotides for translational enhancers, initiating aug underlined); *Fluc-NOST*, cgggggacucuagaggaucuccuaggaagcuuuccaug (initiating aug underlined).

Materials and methods

Construction of plasmids for DNA transient expression

Sequences of all oligo nucleotides used in this study are listed in Supplemental Table S1. DNA fragments for cauliflower mosaic virus 35S promoter (35S) was PCR amplified using primers 35S XbaI-F and 35S KpnI-R and *CaMV35S-Rluc-HSP* (Nagaya et al. 2010) as a template. Resulting fragments were digested with *Xba*I and *Kpn*I, and inserted into *Xba*I–*Kpn*I gaps of pRI909 (Takara Bio, Otsu, Japan). A transcriptional terminator derived from *Agrobacterium tumefaciens nopaline synthase* gene (NOS-T) was digested from pBI121 (Clontech, Palo Alto, CA) with *Sac*I and *Eco*RI, and inserted into *Sac*I–*Eco*RI gaps of 35S-containing pRI909. DNA fragments for *luciferase* gene derived from *Renilla reniformis* (*Rluc*) were PCR amplified using primers Rluc KpnI-F and Rluc SacI-R and *CaMV35S-Rluc-HSP* (Nagaya et al. 2010) as template. Resulting fragments were digested with *Kpn*I and *Sac*I, and inserted into *Kpn*I–*Sac*I gaps of pRI909 containing 35S and NOS-T (*Rluc-NOST* in Figure 1B). DNA fragments for enhancer-fused *Rluc* were generated by an overlap extension PCR method. First, DNA fragments for translational enhancers plus 5′-terminal part of *Rluc* gene in their 3′-terminal part were generated by PCR. Oligo nucleotides used in these steps were NtADH KpnI-F, NtADH Rluc-R, AtADH KpnI-F, AtADHmod Rluc-R, OsADH KpnI-F, OsADH Rluc-R, Ω KpnI-F, Ω Rluc-R, TEVL KpnI-F, and TEVL Rluc-R. Second, PCR was performed using reaction mixtures containing following oligo nucleotides: forward primers (NtADH KpnI-F, AtADH KpnI-F, OsADH KpnI-F, Ω KpnI-F, or TEVL KpnI-F), Rluc SacI-R primer, PCR-amplified

Rluc fragments, and aforementioned enhancer-*Rluc* fragments. Resulting DNA fragments were inserted into *Kpn*I–*Sac*I gaps of *Rluc-NOST* to generate enhancer *Rluc-NOST* (*NtADH* 5′-UTR *Rluc-NOST*, *AtADHmod* 5′-UTR *Rluc-NOST*, *OsADH* 5′-UTR *Rluc-NOST*, Ω *Rluc-NOST*, TEVL *Rluc-NOST*) (Figure 1B). DNA fragment for transcriptional terminator derived from *Arabidopsis thaliana heat shock protein 18.2* gene (Nagaya et al. 2010) was PCR amplified using HSPT SacI-F and HSPT EcoRI-R, digested with *Sac*I and *Eco*RI, and inserted into *Sac*I-*Eco*RI sites of *Rluc-NOST* to generate *Rluc-HSPT*.

To generate *Fluc-NOST*, DNA fragments for the *Fluc* gene, amplified using primers Fluc BglII-F and Fluc-R, were digested with *Bgl*II and *Sac*I, and inserted into *Bam*HI–*Sac*I gaps of pBI221 (Clontech). To generate *Fluc-HSPT*, DNA fragments for the *Fluc* gene, amplified using primers Fluc KpnI-F and Fluc SacI-R, were digested with *Kpn*I and *Sac*I, and inserted into *Kpn*I–*Sac*I gaps of *Rluc-HSPT*. In *Fluc-HSPT*, fourth Ala (gcc) of Fluc protein was changed to Val (gtc) in order to introduce *Aat*II site (gacgtc, underlined "t" is a base that was substituted) into coding region of *Fluc*. To generate enhancer *Fluc-HSPT* construct, DNA fragment for enhancer plus 5′ part of *Fluc* containing *Aat*II site was PCR amplified using a forward primer (OsADH KpnI-F, Ω KpnI-F, or TEVL KpnI-F) and a reverse primer (OsADH AatII-R, Ω AatII-R, or TEVL AatII-R). The resulting DNA fragment was digested with *Kpn*I and *Aat*II, ligated with *Aat*II–*Sac*I fragment of *Fluc*, and inserted into *Kpn*I–*Sac*I gaps of *Rluc-HSPT* to generate enhancer *Fluc-HSPT* (*OsADH* 5′-UTR *Fluc-HSPT*, Ω *Fluc-HSPT*, TEVL *Fluc-HSPT*) (Figure 1B).

Plant materials

Plant materials used in this study are listed in Table 1. Plants were grown either in the field, in the greenhouse under natural sunlight at room temperature (15–20°C), or in sterile conditions in an incubator. For sterile culture, surface-sterilized seeds were germinated on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar, and plants were grown under a 16-h light/8-h dark photoperiod at 25°C. Suspension cells of rice (*Oryza sativa* cv. Nipponbare, plant ID 10), *Arabidopsis thaliana* (ecotype Columbia T87, plant ID 30), and tobacco (*Nicotiana tabacum* L. cv. BY2, plant ID 43) were cultured in liquid modified MS medium (MS medium supplemented with 200 mg l^{-1} KH_2PO_4, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine-HCl, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, and 3% sucrose, pH 5.7) with shaking, under continuous light at 22°C for rice and T87, under continuous dark at 27°C for BY2.

PEG-mediated transient expression

PEG-mediated transient expression analysis was performed according to our previous report (Matsui et al. 2009), with modifications as follows: As starting material for protoplast preparation, either shoots, leaves, leaf buds, petals, flower buds, whole plant body, or cultured cells were used (Table 1). For *E. arvense*, magnoliids, and monocots, protoplastization steps were slightly modified, that is, plant materials were pretreated with 600 mM mannitol instead of 500 mM mannitol followed by incubation in protoplastization enzyme solution containing 600 mM mannitol instead of 400 mM. Amount of *Rluc* plasmids used for one tube is indicated in Table 1. Amount of *Fluc* plasmid was 1.5-fold of *Rluc* plasmid. After transfection, culture was performed in the dark at 25°C for 5 or 6 h in plasmid transfection assays, and for 1 h in mRNA transfection assays. For measurement of luciferase activities, all protoplasts were precipitated and added with passive lysis buffer (Promega, Madison, WI) followed by gentle shaking. Luminescence using Dual-Luciferase Reporter Assay System (Promega) was monitored by a luminometer (Lumat LB9501, Berthold, Germany).

Results

Evaluation of NtADH 5′-UTR in various plant species

In order to determine which translational enhancer is effective in which plant species, we first evaluated the translational enhancer 5′-UTR of *N. tabacum alcohol dehydrogenase* gene (*NtADH* 5′-UTR) in various plant species. In order to systematically select plant materials, a phylogenic tree generated by Angiosperm Phylogeny Group (APG) was referred to. The phylogenic tree was generated based not on morphology but on nucleotide sequence information of genes including plastid genes *rbcL* and *atpB*, and nuclear-coded 18S rDNA (The Angiosperm Phylogeny Group 2003, http://www.mobot.org/MOBOT/research/APWeb/welcome.html). Four magnoliids (plant ID 2 to 5), thirteen monocots (plant ID 7 to 19), and twenty-nine eudicots (plant ID 20 to 48) from angiosperms, and one fern (*Equisetum arvense*) (plant ID 1) were used as materials (Figure 1A and Table 1).

We employed a polyethyleneglycol (PEG)-mediated transient assay system, because this method is generally applicable to any plant species and any plant organ that protoplasts can be prepared. *Luciferase* derived from *Renilla reniformis* (*Rluc*) was used as a reporter gene, and expression plasmids for *Rluc* with or without *NtADH* 5′-UTR were constructed (Figure 1B). To normalize the transfection efficiencies between different transfection events, expression plasmid for firefly (*Photinus pyralis*) *luciferase* was also constructed (*Fluc-NOST* in Figure 1B). Luc activity was quantified 5 or 6 h post-transfection, because activities of both Rluc and Fluc were in linear rise in this time point (Supplemental Figure S1). An enhancement degree of Rluc expression by *NtADH* 5′-UTR was calculated for each plant species (Figure 2). In all eudicots analyzed, Rluc activity obtained with *NtADH* 5′-UTR *Rluc-NOST* (enhancer *Rluc-NOST*) was higher than *Rluc-NOST*. In monocots, the degree of enhancement was relatively lower than in eudicots, and even abatement rather than enhancement was

Table 1. Plant materials used in this study.

Biological classification								Experimental conditions		
Supraordinal clasification			Order	Family	Plant ID	Scientific names	Common names	Starting materials	*Growth condition	*Rluc* plasmids transfected (μg)
Fern			Equisetales	Equisetaceae	1	*Equisetum arvense*	field horsetail	**shoots	G	2
Angiosperms	Magnoliids		Piperales	Pipearceae	2	*Peperomia ferreyrae*	peperomia	leaves	G	2
			Piperales	Saururaceae	3	*Houttuynia cordata*	Korean houttuynia	leaves	S	3
			Magnoliales	Magnoliaceae	4	*Magnolia kobus*	northern Japanese magnolia	flower buds	F	3
			Laurales	Calycanthaceae	5	*Chimonanthus praecox*	wintersweet	flower buds	F	3
			Chloranthales	Chloranthaceae	6	*Chloranthus japonicus*	chloranthus	leaves	G	1.5
	Monocots	Commelinids	Commelinales	Commelinaceae	7	*Tradescantia ohiensis*	common spidewort	petals	F	3
			Commelinales	Commelinaceae	8	*Murdannia keisak*	wart-removing herb	leaves	G	1.5
			Poales	Poaceae	9	*Oryza sativa* cv. Nipponbare	rice	shoots	S	1
			Poales	Poaceae	10	*Oryza sativa* cv. Nipponbare	rice	cultured cells	S	1
			Poales	Poaceae	11	*Secale cereale*	rye	shoots	S	1
			Poales	Poaceae	12	*Zea mays*	maize	shoots	S	1
			Asparagales	Orchidaceae	13	*Vanilla planifolia*	vanilla	leaves	G	1
			Asparagales	Alliaceae	14	*Allium tuberosum*	chinese chive	shoots	S	2
			Asparagales	Alliaceae	15	*Allium cea*	onion	shoots	S	1.5
			Asparagales	Amaryllidaceae	16	*Lycoris radiata*	red spider lily	leaves	G	2
			Liliales	Liliaceae	17	*Lilium* sp.	liliy	leaves	F	1.5
			Alismatales	Araceae	18	*Monstera adansonii*	monstera	leaves	G	1.5
			Alismatales	Araceae	19	*Wolffia globosa*	rootless duckweed	whole organs	G	1.5
	Eudicots		Ranunculales	Papaveraceae	20	*Papaver nudicaule*	poppy	leaves	S	1
		Core eudicots Rosids Fabids	Cucurbitales	Cucurbitaceae	21	*Cucumis sativus*	cucumber	leaves	S	1.5
			Rosales	Rosaceae	22	*Rosa multiflora*	Japanese rose	leaf buds	F	2
			Rosales	Rosaceae	23	*Kerria japonica*	Japanese kerria	petals	F	1.5
			Fabales	Fabaceae	24	*Pisum sativum*	garden pea	leaves	S	1.5
			Fabales	Fabaceae	25	*Vicia angustifolia*	common vetch	leaves	F	1
			Oxalidales	Oxalidaceae	26	*Oxalis corniculata*	yellow wood sorrel	leaves	S	2
			Malpighiales	Violaceae	27	*Viola×wittrockiana*	garden pansy	leaves	S	1
		Malvids	Malvales	Malvaceae	28	*Hibiscus cannabinus*	kenaf	leaves	S	1
			Brassicales	Brassicaceae	29	*Arabidopsis thaliana* ecotype Columbia	thale cress	leaves	S	1
			Brassicales	Brassicaceae	30	*Arabidopsis thaliana* ecotype Columbia T87	thale cress	cultured cells	S	0.3
			Brassicales	Brassicaceae	31	*Cardamine hirsuta*	hairy bittercress	leaves	S	1.5
			Sapindales	Sapindaceae	32	*Cardiospermum halicacabum*	balloon vine	leaves	S	2
			Myrtales	Onagraceae	33	*Oenothera laciniata*	cutleaf evening-primrose	leaves	S	1
			Saxifragales	Crassulaceae	34	*Sedum rubrotinctum*	sedum	leaves	S	2
			Caryophyllales	Caryophyllaceae	35	*Dianthus barbatus*	pink	leaves	S	1.5
			Caryophyllales	Portulacaceae	36	*Talinum crassifolium*	coral flower	leaves	S	1.5
			Caryophyllales	Amaranthaceae	37	*Celosia argentea*	plumed cockscomb	leaves	S	1.5
		Asterids Lamids	Ericales	Primulaceae	38	*Primula malacoides*	primula	leaves	S	1
			Gentianales	Rubiaceae	39	*Galium spurium* var. *echinospermon*	false cleavers	leaves	S	1
			Lamiales	Lamiaceae	40	*Plectranthus scutellarioides*	coleus	leaves	S	1
			Lamiales	Scrophulariaceae	41	*Torenia fournieri*	torenia	leaves	S	1
			Solanales	Solanaceae	42	*Nicotiana tabacum* L. cv. SR1	tobacco	leaves	S	1
			Solanales	Solanaceae	43	*Nicotiana tabacum* L. cv. BY2	tobacco	cultured cells	S	0.3
		Campanulids	Asterales	Campanulaceae	44	*Platycodon grandiflorus*	Chinese bellflower	leaves	S	1
			Asterales	Asteraceae	45	*Chrysanthemum carinatum*	tricolor-chrysanthemum	leaves	G	1
			Asterales	Asteraceae	46	*Lactuca sativa* cv. green wave	lettuce	leaves	S	0.5
			Apiales	Apiaceae	47	*Daucus carota*	carrot	leaves	S	1
			Apiales	Apiaceae	48	*Anethum graveolens*	dill	leaves	S	1

Plant species are listed in an order that reflects a phylogenic tree of APG. * Plants were grown in the field (F), in the greenhouse (G), or in sterile condition in incubators (S). ** Vegetative organs, but not porophytes, were used.

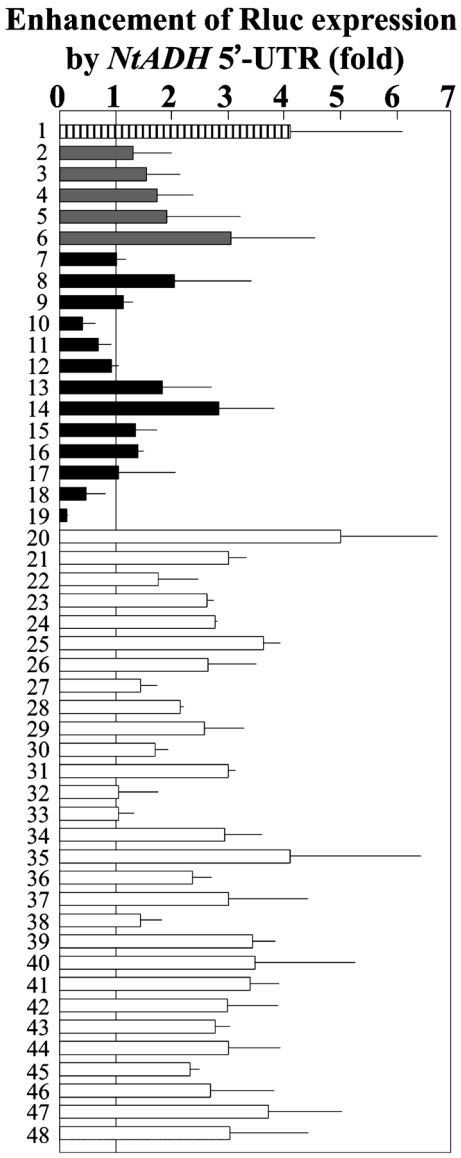

Figure 2. Evaluation of *NtADH* 5′-UTR in various plant species. Protoplasts prepared from each plant species were transfected with *Rluc-NOST* or *NtADH-Rluc-NOST* plasmid together with *Fluc-NOST* plasmid for normalization of transfection efficiency. Means and SDs obtained from at least three independent preparations of protoplasts are shown for each plant species. *E. arvense* is indicated with vertical lines, and those from magnoliids, monocots, and eudicots are indicated by gray, black, and white bars, respectively.

observed in some monocot species (plant ID 10, 11, 12, 18, and 19). Average enhancement by *NtADH* 5′-UTR for eudicots (2.72±0.89) was significantly higher than for monocots (1.17±0.74) ($p<0.01$) or magnoliids (1.91±0.68) ($p<0.05$) (Supplemental Figure S2). In order to evaluate this expression assay system, we also performed a transient expression assay using in vitro synthesized *Rluc* mRNA in *N. tabacum* (plant ID 43), *A. thaliana* (plant ID 30), and *O. sativa* (plant ID 10), according to the procedure of a previous report (Matsuura et al. 2013). The *Rluc* mRNA contained 5′-cap and 3′-poly A (49 adenines). There was a positive correlation between degree of enhancement obtained in the DNA transient assay and that obtained in the RNA transient assay (Supplemental Figure S3). Overall, *NtADH* 5′-UTR exerted a moderate effect in some monocot species, such as *Allium tuberosum* (plant ID 14 in Figure 2), and initial materials (organs) for protoplast preparation of closely related plant species affected the enhancement degrees by *NtADH* 5′-UTR (compare 9: leaf and 10: suspension culture, and 29: leaf and 30: suspension culture).

Evaluation of OsADH 5′-UTR, AtADH 5′-UTR, Ω, and tobacco etch virus leader sequence

Four other known translational enhancers were also evaluated in order to make a catalogue of translational enhancers. In this study, modified *AtADH* 5′-UTR (*AtADH*mod 5′-UTR), in which nucleotides immediately upstream AUG (−3 to −1) was changed to AAG for efficient translational initiation (Sugio et al. 2010), was used. We also evaluated translational enhancers found in virus mRNAs, Ω derived from tobacco mosaic virus (Gallie et al. 1987) and tobacco etch virus leader sequence (TEVL; Carrington and Freed 1990). We constructed expression plasmids for enhancer-fused *Rluc-NOST* (Figure 1B). Plant species were selected for analyses taking into account phylogenic positions and degrees of enhancement by *NtADH* 5′-UTR. The effect of each 5′-UTR in each plant species was calculated (Figure 3A and Supplemental Figure S4). In eudicots and a magloniid *Magnolia kobus* (plant ID 4), all five translational enhancers were effective, while in monocots and magnoliid *Houttuynia cordata* (plant ID 3), Ω and TEVL were less effective than other *ADH* 5′-UTRs and even worse than a control 5′-UTR. In fern *E. arvense* (plant ID 1), Ω and TEVL were more effective than control UTR, and less effective than *NtADH* 5′-UTR and *OsADH* 5′-UTR.

Effects of three representative translational enhancers, *OsADH* 5′-UTR, which seemed to be a universal enhancer, and Ω and TEVL, which were not effective in monocots, were also re-evaluated using *Fluc* as a reporter gene. This time, Rluc without the special 5′-UTR was co-transfected for normalization of transfection efficiencies (*Rluc-NOST*, Figure 1B). In *A. thaliana* (plant ID 30) and tobacco (plant ID 43), all three enhancers functioned well as was the case for the *Rluc* reporter (Figure 3B). In *H. cordata* (plant ID 3) and rice (plant ID 10), Ω and TEVL were less effective than *OsADH* 5′-UTR or control 5′-UTR as was the case for the *Rluc* reporter (Figure 3B). In *E. arvense* (plant ID 1), TEVL was as functional as *OsADH* 5′-UTR, and Ω was less effective than control 5′-UTR. We also evaluated *OsADH* 5′-UTR in stable transformants of tobacco culture cells (plant ID 43) according to the procedure of a previous report (Matsui

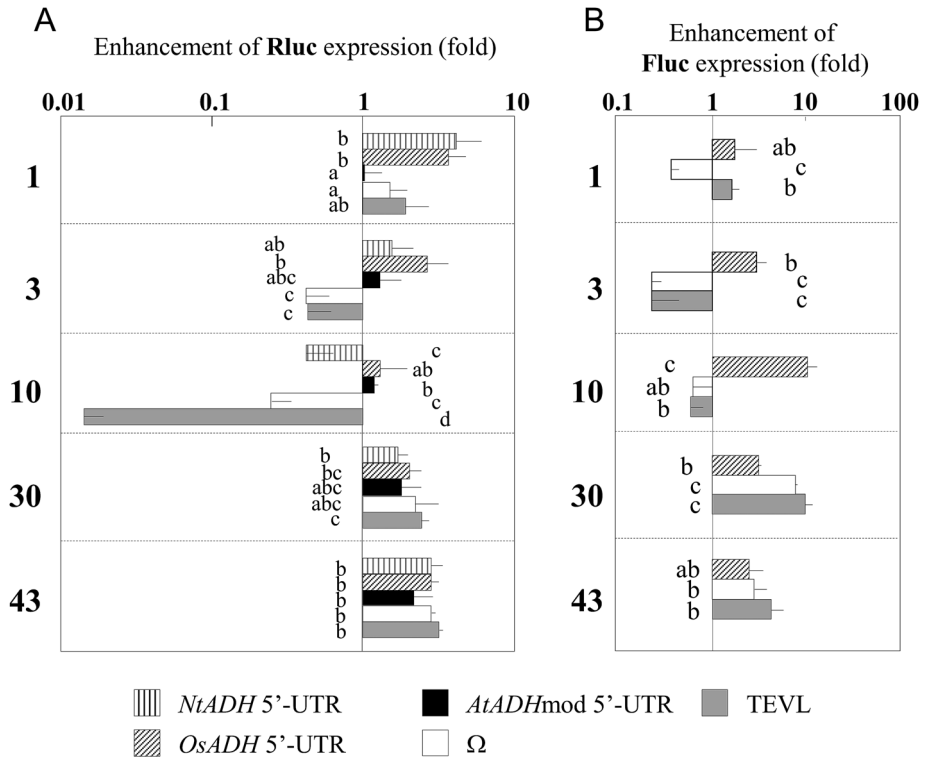

Figure 3. Evaluation of five translational enhancers in plant species. (A) Enhancement of Rluc expression by translational enhancers. Protoplasts prepared from each plant species were transfected with *Rluc-NOST* or enhancer (*NtADH* 5′-UTR, *OsADH* 5′-UTR, *AtADH*mod 5′-UTR, Ω, TEVL) *Rluc-NOST* plasmid together with *Fluc-NOST* plasmid for normalization of transfection efficiency. (B) Enhancement of Fluc expression by translational enhancers. Protoplasts prepared from each plant species were transfected with *Fluc-HSPT* or enhancer (*OsADH* 5′-UTR, Ω, TEVL) *Fluc-HSPT* plasmid together with *Rluc-HSPT* plasmid for normalization of transfection efficiency. Means and SDs are shown for each plant species. Data of *NtADH* 5′-UTR, *OsADH* 5′-UTR, *AtADH*mod 5′-UTR, Ω, and TEVL are indicated, with vertical lines, with diagonal lines, by black, by white, and by gray, respectively. Values are presented on logarithmic scales. Significance tests were performed for each plant species; values not significantly different at a probability of 0.05 are highlighted with the same letter, a, b, or c. Data for *Rluc-NOST* (value is 1) are not shown as bars in these figures, but are not significantly different from bars indicated by "a". Numbers on the left of graphs represent plant IDs.

et al. 2014), and found that enhancement degree (3-fold) is almost the same as that in transient expression system (Supplemental Figure S5).

Discussion

Previous analysis of Ω has revealed that the poly (CAA) motif plays an important role in translational enhancement (Gallie and Walbot 1992), and a 102 kDa protein binding to the poly (CAA) was purified from wheat germ extract (Tanguay and Gallie 1996). The protein is encoded by *heat shock protein 101* (*HSP101*) gene (Wells et al. 1998). Using transgenic yeast, tobacco, and even monocot wheat, HSP101 was proven to have the ability to mediate Ω-dependent translational enhancement (Wells et al. 1998). Meanwhile, mechanism analysis of TEVL revealed that the pseudoknot PK1 region in TEVL plays an important role in translational enhancement (Zeenko and Gallie 2005), and the wheat eIF4G binds to PK1 (Ray et al. 2006). At normal cap-dependent translation initiation, eIF4G binds to polyA-bound PABP and eIF4E, and mediates recruitment of 40S ribosome subunits to mRNA (reviewed by Groppo and Richer 2009). At cap-independent translation initiation mediated by TEVL, it is supposed that eIF4G directly binds to PK1 and recruit ribosome subunits. Considering that transacting factors HSP101, for Ω, and eIF4G, for TEVL, are both well conserved in the plant kingdom, and that monocot-derived proteins can mediate enhancement in certain experimental conditions (Ray et al. 2006; Tanguay and Gallie 1996; Wells et al. 1998), and that compatibility to a given translational enhancer closely correlates with a phylogenic position of the plant species, the reason for the ineffectiveness of Ω and TEVL in monocots might not be that trans-activating factors themselves are not functional, but that basal translation systems in monocots are somewhat different from those of eudicots. The variation in the effectiveness of *NtADH* 5′-UTR indicates that factors other than relatedness of the plant also affects it; however, it is still considered that there is some degree of correlation between the effect of *NtADH* 5′-UTR (enhancement degrees) and the phylogenic position of a plant species.

In this report, we also found that a magnoliid, *H. cordata*, but not *M. kobus* has a preference for translational enhancers similar to monocots. We have

reported that the 5′-UTR of *A. thaliana AGP21* gene is useful for raising expression levels of transgenes in eudicots, but not in monocots (Matsui et al. 2012). We also found that *AtAGP21* 5′-UTR does not enhance the expression of Fluc in *H. cordata* (data not shown). Our data may give some insight into phylogenetic relationships between magnoliids, monocots, and eudicots. In APGII, a clade including magnoliids and monocots is sister to a clade including eudicots and ceratophylalles (The Angiosperm Phylogeny Group, 2003). On the other hand, in APGIII, magnoliids are considered to be a sister to a clade including monocots and eudicots (The Angiosperm Phylogeny Group, 2009). Our results seem to be consistent with APGII classification, and the incompatibility with Ω and TEVL might have been acquired after split of eudicots-(monocots/magnoliids).

The results of this study will be helpful in choosing appropriate translational enhancers, depending on the host plant species. We have many choices of translational enhancers for eudicots, such as TEVL, Ω, *AtADH* 5′-UTR, *NtADH* 5′-UTR, and *AtAGP21* 5′-UTR, but monocot-compatible translational enhancers are to date limited to *OsADH* 5′-UTR. Monocots are important as grain crops, and it is important to find more efficient monocot-compatible translational enhancers for future work.

Acknowledgements

This work was supported by a Grant-in-Aid from the Development of Fundamental Technologies for the Production of High-Value Materials Using Transgenic Plants project managed by the Ministry of Economy, Trade and Industry of Japan. The Arabidopsis cell line T87 was obtained from the RIKEN Plant Cell Bank (Tsukuba, Japan).

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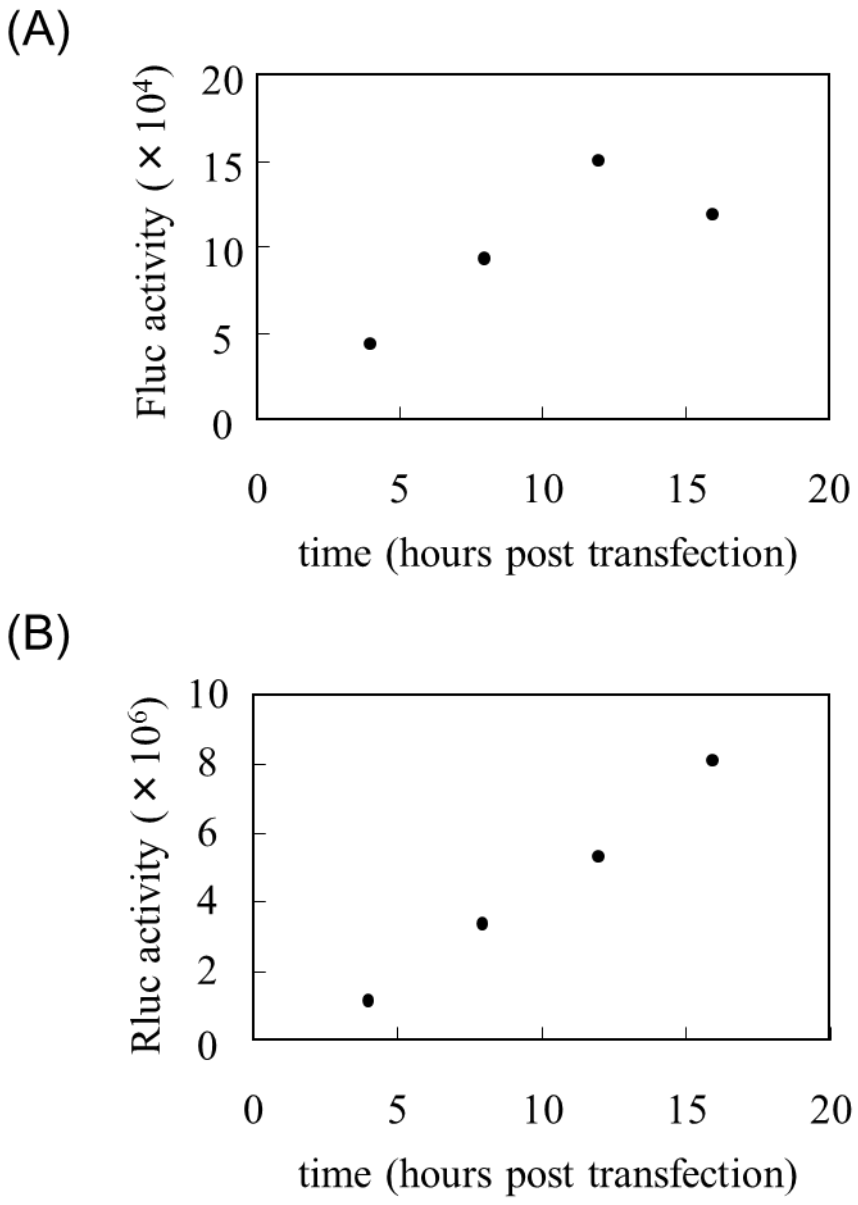
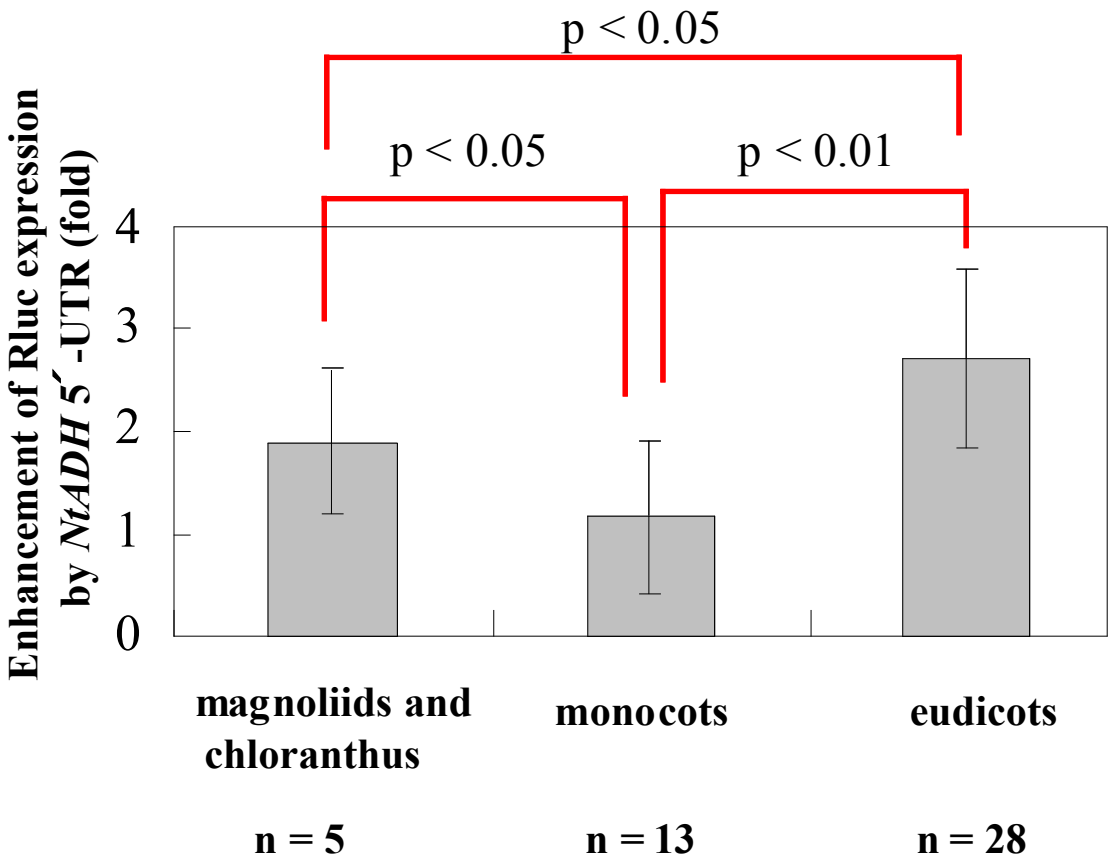
Supplemental figure S1

Time course measurement of luciferase activity. Protoplasts derived from lettuce (*Lactuca sativa* L. cv. green wave; plant ID 46) were co-transfected with *Rluc-NOST* and *Fluc-NOST* plasmids. Protoplasts were incubated for 4, 8, 12, or 16 hours before sampling. Four tubes of protoplast suspension were independently transfected *luc* plasmids, and average values for Fluc activity (A) and those for Rluc activity (B) are indicated in figures below.



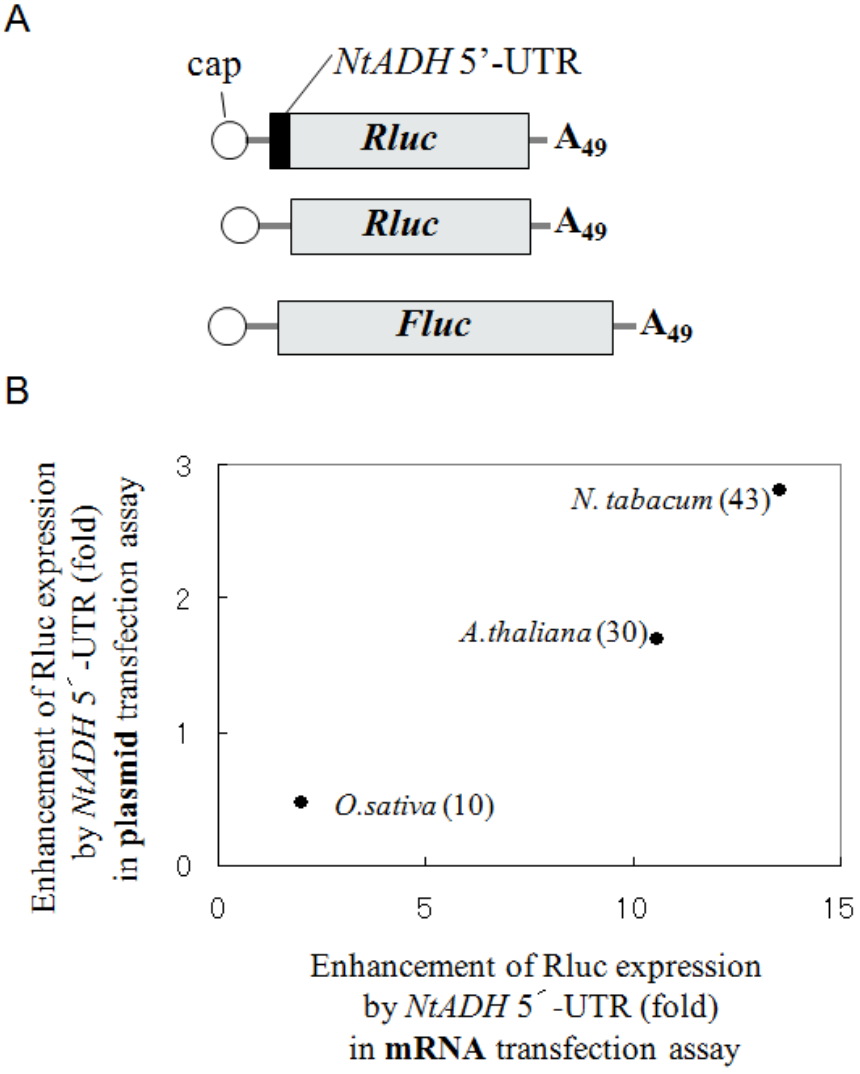
Supplemental figure S2

Degrees of enhancement by *NtADH* 5′-UTR have some correlation with phylogenic positions of plant species. An average value and SD of degrees of enhancement of Rluc expression by *NtADH* 5′-UTR (Figure 2) is indicated for each taxon. Student's *t*-test was performed.



Supplemental figure S3

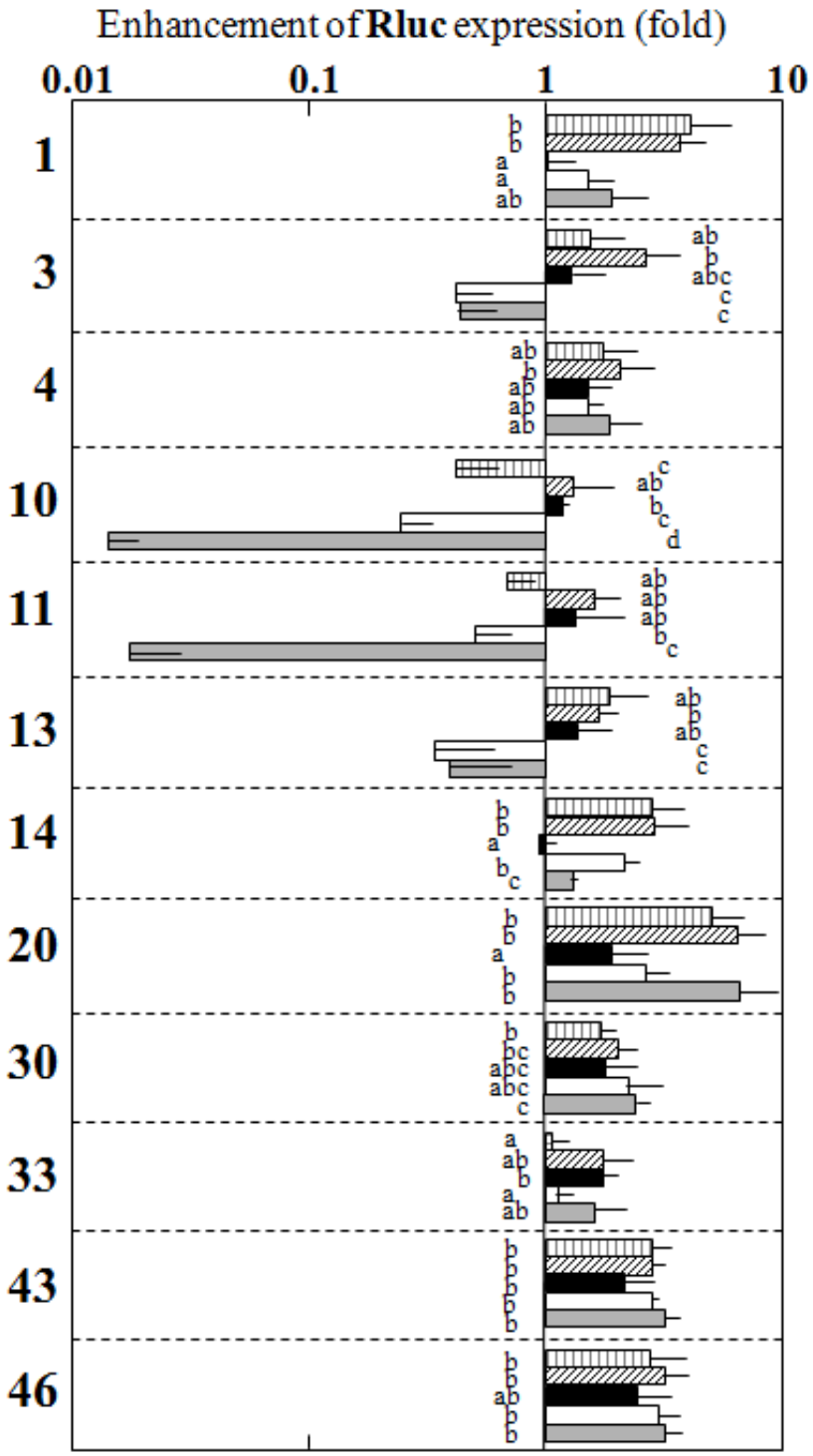
Evaluation of *NtADH* 5′-UTR in RNA transfection assay. (A) Schematic representations of *in vitro* synthesized mRNA. Each mRNA contains 5′ cap and 3′ poly A (49). *NtADH* 5′-UTR-*Rluc* or *Rluc* was co-transfected with *Fluc* for normalization of transfection efficiencies. *O. sativa* (plant ID 10), *A. thaliana* (plant ID 30), and *N. tabacum* (plant ID 43) were used in this assay. After PEG-mediated transfection of mRNAs, protoplasts were incubated for 1 hour at 25°C under dark, and luciferase activities were measured. Predicted 5′ mRNA nucleotide sequences are as follows: ggcccggguaccaug (initiating aug underlined) in cases of *Rluc* and *Fluc*, and ggcccggguacc*tatttaact...aauaa*aug (nucleotides for *NtADH* 5′-UTR are in italics, initiating aug of *Rluc* underlined) in the case of *NtADH* 5′-UTR *Rluc*.



Supplemental figure S4

Evaluation of the level of Rluc expression enhancement by five translational enhancers in different plant species. Protoplasts prepared from each plant species were transfected with *Rluc-NOST* or enhancer (*NtADH* 5′-UTR, *OsADH* 5′-UTR, *AtADH*mod 5′-UTR, Ω, TEVL) *Rluc-NOST* plasmid together with *Fluc-NOST* plasmid for normalization of transfection efficiency. Five or six hours post transfection, protoplasts were collected, luciferase activity analyses performed, and fold enhancement of Luc expression calculated. We performed triplicate transfections for one preparation of protoplasts for a translational enhancer, and this was repeated at least three times. Means and SDs are shown for each plant species. Data of *NtADH* 5′-UTR, *OsADH* 5′-UTR, *AtADH*mod 5′-UTR, Ω, and TEVL are indicated with vertical lines, diagonal lines, black, white, and gray, respectively. Values are presented on logarithmic scales. Significant tests were performed for each plant species; values not significantly different at a probability of 0.05 are highlighted with the same letter, a, b, or c. Data for *Rluc-NOST* (value = 1) are not shown as bars in this figure, but are not significantly different from bars indicated by “a”. Numbers on the left of the graph represent plant IDs.

Supplemental figure S4 continued



Supplementary figure S5

Evaluation of *OsADH* 5´-UTR using stable transformants. Cultured cells from transgenic tobacco (*N. tabacum* cv. BY2; plant ID 43) were generated using *Agrobacterium tumefaciens* expressing *Fluc-HSPT* or *OsADH* 5′-UTR *Fluc-HSPT*. Kanamycin-resistant calluses on solidified LS medium were randomly picked for Fluc activity measurement. An average value and SD from 14 independent clones is indicated for each construct.

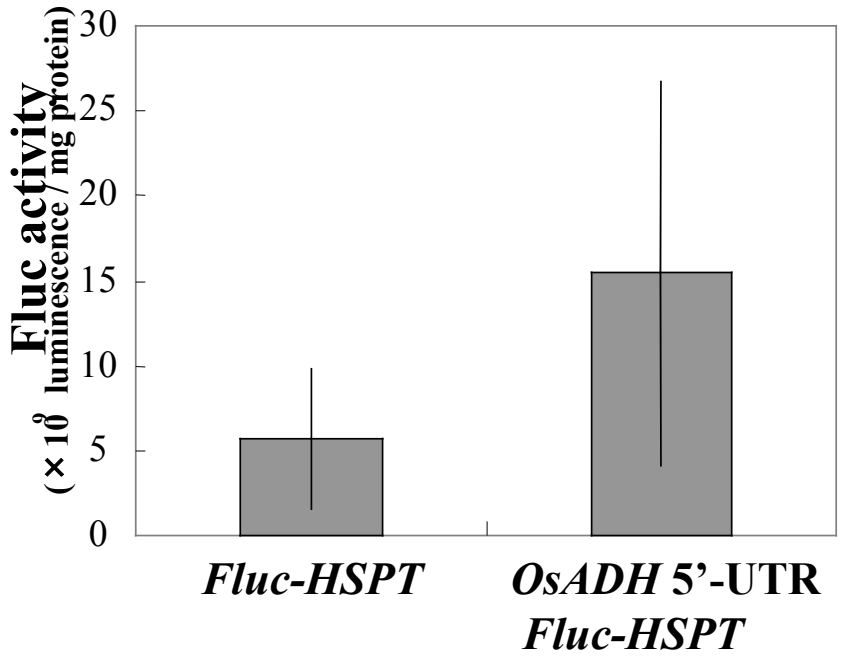


Table S1 Origonucleotides used in this work.		
names of oligonucleotides	**sequences (5′ to 3′)**	**comments**
35S XbaI-F	aaatctagattagccttttcaatttcag	*Xba* I site underlined
35S KpnI-R	aaaggtacccgggcgtgttctctccaaatgaaa	*Kpn* I site underlined
Rluc KpnI-F	aaaggtaccatggcttccaaggtgtacgac	*Kpn* I site underlined
Rluc SacI-R	aaagagctcttactgctcgttcttcagc	*Sac* I site underlined
NtADH KpnI-F	aaaggtacctatttaactcagtattcagaaac	*Kpn* I site underlined
NtADH Rluc-R	*caccttggaagccat* ttatttttcttgatt	nucleotides corresponding to *Rluc* gene are in italics
AtADH KpnI-F	aaggtaccatcacaatcacacaaaactaac	*Kpn* I site underlined
AtADHmod Rluc-R	*caccttggaagccat* cttcaacagtgaagaacttg	nucleotides corresponding to *Rluc* gene are in italics
OsADH KpnI-F	aaggtaccgaattccaagcaacgaactgcg	*Kpn* I site underlined
OsADH Rluc-R	*gtacaccttggaagccat* taatccccctctttttcaaagaac	nucleotides corresponding to *Rluc* gene are in italics
OsADH AatII-R	*ttt gacgtc ttccat* taatccccctctttttcaaag	nucleotides corresponding to *Fluc* gene are in italics, *Aat* II site underlined;
Ω KpnI-F	aaggtacctatttttacaacaattaccaacaac	*Kpn* I site underlined
Ω Rluc-R	*gtacaccttggaagccat* tgtaattgtaaatagtaattgtaat	nucleotides corresponding to *Rluc* gene are in italics
Ω AatII-R	*ttt gacgtc ttccat* tgtaattgtaaatagtaattg	nucleotides corresponding to *Fluc* gene are in italics, *Aat* II site underlined;
TEVL KpnI-F	aaggtacctcgagaattctcaacacaacatat	*Kpn* I site underlined
TEVL Rluc-R	*gtacaccttggaagccat* ggctatcgttcgtaaatggtg	nucleotides corresponding to *Rluc* gene are in italics
TEVL AatII-R	*tt gacgtc ttccat* ggctatcgttcgtaaatggtg	nucleotides corresponding to *Fluc* gene are in italics, *Aat* II site underlined;
HSPT SacI-F	aagagctcatatgaagatgaagatgaa	*Sac* I site underlined
HSPT EcoRI-R	aagaattcactagtcttatctttaatcata	*Eco* RI site underlined, *Spe* I site double underlined
Fluc BglII-F	aagatctcctaggaagctttcc*atggaagacgccaaaaacat*	*Bgl* II site underlined, nucleotides corresponding to *Fluc* gene are in italics
Fluc-R	gagctcaaattcgatcgaattctctagaa*ttacacggcgatctttccgc*	*Sac* I site underlined, nucleotides corresponding to *Fluc* gene are in italics
Fluc KpnI-F	aggtacc*atggaa gacgtc aaaaacataaa*	*Kpn* I site underlined, *Aat* II site double underlined, nucleotides corresponding to *Fluc* gene are in italics
Fluc SacI-R	agagctcactagt*ttacacggcgatctttccgc*	*Sac* I site underlined