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ORIGINAL PAPER

Evaluation of seed storage-protein gene 5′ untranslated regions in enhancing gene expression in transgenic rice seed

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Abstract 5′ untranslated regions (UTRs) are important sequence elements that modulate the expression of genes. Using the β-glucuronidase (*GUS*) reporter gene driven by the *GluC* promoter for the rice-seed storage-protein glutelin, we evaluated the potential of the 5′-UTRs of six seed storage-protein genes in enhancing the expression levels of the foreign gene in stable transgenic rice lines. All of the 5′-UTRs significantly enhanced the expression level of the *GluC* promoter without altering its expression pattern. The 5′-UTRs of *Glb-1* and *GluA-1* increased the expression of GUS by about 3.36- and 3.11-fold, respectively. The two 5′-UTRs downstream of the *Glb-1*, *OsAct2* and *CMV35S* promoters also increased GUS expression level in stable transgenic rice lines or in transient expression protoplasts. Therefore, the enhancements were independent of the promoter sequence. Real-time quantitative RT-PCR analysis showed that the increase in protein production was not accompanied by alteration in mRNA levels, which suggests that the enhancements were due to increasing the translational efficiencies of the mRNA. The 5′-UTRs of *Glb-1* and *GluA-1*, when combined with strong promoters, might be ideal candidates for high production of recombinant proteins in rice seeds.

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Introduction

Because of progress in modern biotechnology, transgenic plants have become attractive bioreactors for the production of recombinant proteins, including pharmaceuticals and industrial enzymes (Hood and Woodard 2002). Several recombinant industrial enzymes and pharmaceuticals have been commercialized, and the number is expected to increase in the near future (Streatfield 2007). Due to its lower production costs, easy control of production scale, large storage ability, freedom from human- and animal-derived pathogens and lack of ethical issues, the seed system is an ideal plant production platform for the production of recombinant proteins (Takaiwa et al. 2007). However, the low expression and accumulation of recombinant protein is still one of the major limiting factors for commercialization of transgenic plants as reliable protein production systems.

The accumulation of recombinant proteins in plant cells is determined at both transcription and post-transcription levels and concerns many factors, such as strength of the promoter, mRNA stability, translation efficiency, subcellular targeting and deposition of the protein (Streatfield 2007). The primary strategy to increase the production of foreign protein in plant cells is to use a strong promoter because the promoter plays a critical role in transcriptional regulation of gene expression. To date, many researchers have concentrated on developing such promoters, and various strong promoters, both constitutive and tissue-specific expression, have been isolated and characterized (Christensen et al. 1992; Guilley et al. 1982; Lamacchia et al. 2001; Qu and Takaiwa 2004; Qu et al. 2008). Some strong endosperm-specific promoters have been used for production of recombinant proteins in rice seeds (Qu et al. 2005; Yang et al. 2006). However, how to further increase

the expression levels of foreign genes in seeds with the use of strong promoters combined with other factors is still a project for biotechnological researchers.

The 5′ untranslated region (UTR) located upstream of the translational initiation site plays an important role in regulating gene expression at transcriptional and post-transcriptional levels (Bate et al. 1996; Hua et al. 2001). The 5′-UTR of the tomato pollen-specific *LAT59* gene was shown to act as a transcriptional inhibitor to greatly reduce mRNA accumulation without affecting mRNA stability (Curie and McCormick 1997). Elfakess and Dikstein (2008) demonstrated a translational initiation element in the short 5′-UTR that regulated the transcription of mRNAs. The 5′-UTRs regulate gene expression at the post-transcriptional level by modulating mRNA stability and translation efficiency, although most of the factors affecting mRNA stability were reported in 3′-UTRs (Hua et al. 2001). The 5′-UTR of *ntp303* greatly enhances gene translation during pollen tube growth (Hulzink et al. 2002). Alteration of the 5′-UTR increased the expression of GUS in *Aspergillus oryzae* without affecting mRNA levels (Koda et al. 2004). The 5′-UTR of *OsADH* acted as a translational enhancer for *GUS* reporter gene expression in plant cells (Sugio et al. 2008). The expression of *At-P5R* is regulated by its 5′-UTR at both transcriptional and post-transcriptional levels (Hua et al. 2001). Recently, the 5′-UTR of ribosomal protein mRNAs was found to be a binding site for micro-RNA regulating the translation of the genes (Ørom et al. 2008).

The 5′-UTR regulates mRNA stability and translation efficiency by its secondary structure, the sequence context surrounding the AUG codon, and upstream AUG or open-reading frames (Koda et al. 2004; Zou et al. 2003). In tobacco, the primary sequence and secondary stem-loop structure of the *psbA* 5′-UTR are important for determining mRNA stability and translation efficiency; alteration of the structure significantly reduced mRNA level and translation efficiency (Zou et al. 2003). The insertion of secondary structures into the 5′-UTR of yeast *PGK1* mRNA inhibited translation and stimulated decay of PGK1 (Muhlrad et al. 1995).

The 5′-UTR plays an important role in determining transcription level and translation efficiency; therefore, it is frequently added to mRNA templates as a translation enhancer to increase protein production (Hulzink et al. 2002; Sugio et al. 2008). To optimize translation, any sequences located immediately around the translation start site should be modified to fit the consensus initiation sequence, which varies between plant species (Joshi et al. 1997). The 5′ leader sequence (omega) of tobacco mosaic virus and Kozak consensus sequence (ACCAAUGG) are often used to boost recombinant protein levels and can increase protein expression by 10–300-fold, as high as that with other endogenous UTR sequences (Falcone and Andrew 1991; Gallie et al. 1987; Kozak 1991). However, little attention has been paid to the role of rice-seed storage-protein 5′-UTRs in regulating recombinant protein production in rice endosperm. To enhance recombinant protein yield in transgenic crops, 5′-UTRs that promote high translation rates in seed endosperm must be found to improve the production of foreign proteins.

We aimed to investigate whether the 5′-UTRs of rice-seed storage-protein genes affect gene expression and to evaluate their potential in enhancing foreign gene expression in seeds of stable transgenic rice lines. These genes included three glutelin genes, *GluA-1*, *GluA-2* and *GluC*; two prolamine genes, 10 kDa, 16 kDa; and a 26-kDa globulin gene, *Glb-1* (Qu and Takaiwa 2004; Qu et al. 2008). The 5′-UTRs we tested could enhance the expression level of the reporter gene by increasing translation efficiency. The 5′-UTRs might be ideal candidates for high production of valuable recombinant proteins in transgenic rice.

Materials and methods

Plasmid construction

The constructs used in the experiment are summarized in Fig. 1. The endosperm-specific *GluC* promoter without 5′-UTR (p*GluC*) was amplified from the *GluC* promoter with its own 5′-UTR reported previously (Qu et al. 2008) with the primers *GluC*F and *GluC*R. The 5′-UTRs of *GluA-1*, *GluA-2*, 10 kDa prolamine, 16 kDa prolamine and *Glb-1* were linked downstream of p*GluC* by PCR. The p*GluC* and p*GluC*-5′-UTRs were introduced upstream of *GUS* in the binary vector pGPTV-35S-HPT (Qu and Takaiwa 2004). p*Glb-1* and p*Glb-1-GluA-1*-5′-UTR were constructed by the same strategy amplified from the *Glb-1* promoter with its own 5′-UTR reported previously (Qu and Takaiwa 2004). The sequences of 5′-UTRs and primers used for construction are listed in Table 1 and Table S1, respectively.

Production of transgenic plants

Transgenic rice plants (*Oryza sativa* cv. Kitaake) were produced by *Agrobacterium*-mediated transformation as described previously (Qu and Takaiwa 2004). After confirmation by PCR, the transformants were transplanted in a greenhouse.

Analysis of GUS expression

For histochemical analysis, roots, leaves and stalks cut into 5-mm sections and maturing seeds at 17 days after

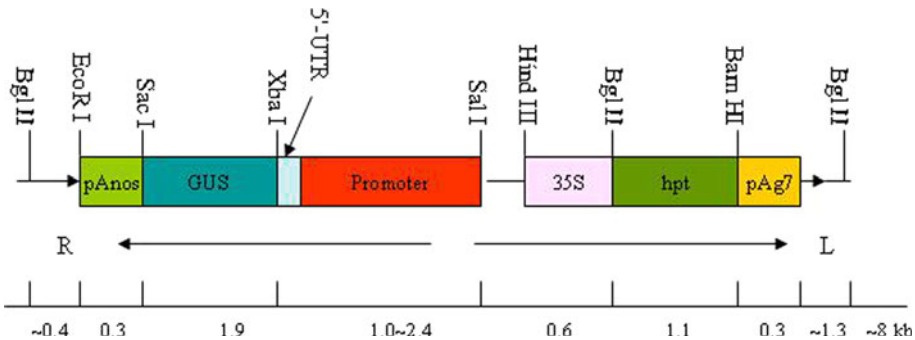

Fig. 1 Schematic diagram of the chimeric gene construction for rice transformation. The promoter and 5′ untranslated regions (UTRs) of various rice-seed storage-protein genes were fused between two of the three restriction sites, *Hin*dIII, *Sal*I and *Xba*I, followed by the *GUS* reporter gene and the Nos terminator. Promoter refers to the *GluC* or *Glb-1* promoters. 5′-UTR refers to the 5′-UTRs of *Glb-1*, *GluA-1*, *GluA-2*, *GluC*, 16 kDa prolamine and 10 kDa prolamine genes

Table 1 Sequence and structural features of 5′-UTRs

5′-UTR	Sequence	Length (nt)	ΔG (kcal mol^{-1})[a]
Glb-1	TTGTCTGATTGATCATCA	18	0.3
GluA-1	CCAAGTTCATTAGTACTACAACAAC	25	0.8
GluA-2	AAAAGCATTCAGTTCATTAGTCCTACAACAAC	32	1.1
GluC	AAACTAAGTGAATAACT	17	0.1
16 kDa	ACCCTTACTGAAAAATCACAAACATCAAAACG TTATAAGAGTTCTCTAGCATCCATCACATAGCC	65	−1.2
10 kDa	ATCATCCTCAACAATATTGTCTACACCATCTGG AATCTTGTTTAACACTAGTATTGTAGAATCAGCA	67	−6.5

[a] ΔG is the minimum free energy of the mRNA secondary structure

flowering (DAF) sectioned longitudinally with a razor blade were stained with X-Gluc (5-brom-4-chloro-3-indolyl glucuronide) as described previously (Qu and Takaiwa 2004). Fluorometric assays of GUS activities were conducted as described previously (Qu and Takaiwa 2004), with maturing seeds at 17 DAF used as samples. Three seeds from each independent transgenic plant were assayed.

Real-time quantitative RT-PCR

Total RNA was extracted from rice developing seeds as described previously (Qu et al. 2005). Before cDNA synthesis, RNA was treated with DNase (Takara) to remove possible DNA contamination. Reverse transcription was performed in 20 μl reactions containing 1 μg total RNA according to the manufacturer's instructions (RT-PCR kit, Promega, Madison, WI, USA). Real-time quantitative RT-PCR was performed in a 20 μl total volume containing 5 μl of 50× diluted reverse transcripts, 0.4 μM each primer and 10 μl SYBR® GreenMaster mix (Toyobo, Osaka, Japan) on an Mx3000p™ QPCR System. The reaction conditions were 95°C for 1 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. The generated melting curve was employed as a significant parameter to check the specificity of the amplified fragment. To quantify expression level of target gene (*GUS*), the rice *Act-1* gene (GeneBank accession number: X16280) was selected as endogenous control gene. Relative GUS mRNA level was calculated as the ratio of the PCR product quantity of *GUS* to that of rice *Act-1*. Primers used for this experiment were as follows: q*GUS*F, 5′-CACAGCCAAAAGCCAGACAG-3′; q*GUS*R, 5′-ATGACGACCAAAGCCAGTAAAGTAG-3′; q*Act*F, 5′-TCAACCCCAAGGCCAATC-3′; q*Act*R, 5′-CACCATC ACCAGAGTCCAACA-3′.

Each real-time quantitative RT-PCR assay for the same group was carried out for three biological replicates and each for three technological repeats in separate experiments. The relative quantification method (Delta–Delta CT) was used to evaluate quantitative variation between the replicates examined.

Transient expression assay

To create p35S-*Glb-1*- and p35S-*GluA-1*-5′-UTR constructs, 5′-UTR was linked to 35S promoter by PCR with *35S*F and *35S-Glb-1*R or *35S-GluA-1*R as primers (Table S1) containing *Hin*dIII and *Xba*I sites, respectively. The amplified DNA fragments were inserted into pBI221 to obtain expression vector of p35S-*Glb-1*- and p35S-*GluA-1*-

5′-UTR. A 1.4-kb rice *OsAct2* promoter (GeneBank accession number: EU155408.1) was isolated by PCR from rice genomic DNA with *Act*F and *Act*R as primers (Table S1) containing *Hin*dIII and *Xba*I sites, respectively. The amplified DNA fragment was digested with *Hin*dIII and *Xba*I and cloned into pBI221 to obtain p*Act*. p*Act-Glb-1*- and p*Act-GluA-1*-5′-UTR constructs were created similarly, with the primers *Act*F and *Act-Glb-1*R or *Act-GluA-1*R, respectively.

Transient expression in etiolated rice stem protoplasts and GUS assays were performed as described by Chen et al. (2006). Three replicates were performed in all the GUS assays and the mean and standard deviations were calculated and presented.

Results

Generation of transgenic rice plants containing different 5′-UTRs

To investigate the effects of the 5′-UTR on gene expression in rice seed, the 5′-UTR of six rice-seed storage-protein genes were linked downstream of a *GluC* promoter (Qu et al. 2008) by PCR and then inserted upstream of a *GUS* reporter gene in the binary vector pGPTV-35S-HPT for transcriptional fusion (Fig. 1). The six genes were *GluA-1*, *GluA-2*, *GluC*, *Glb-1*, 10 kDa prolamine and 16 kDa prolamine (Qu and Takaiwa 2004; Qu et al. 2008). All 5′-UTRs differ in length and sequence (Table 1) and were used as the 5′-regulatory element of the *GUS* reporter gene. The different chimeric 5′-UTR-GUS transcription fusions were introduced into the rice cultivar Kitaake via *Agrobacterium*-mediated transformation. The successful insertion of the chimeric gene was confirmed by PCR with genomic DNA isolated from leaves of independent transformants as templates. Six to twenty six positive individuals were obtained from each of the constructions.

5′-UTRs did not affect the endosperm specificity of *GluC* promoter

The expression of *GUS* reporter gene directed by the rice glutelin *GluC* promoter was restricted to the endosperm (Qu et al. 2008). To investigate whether the 5′-UTRs of the rice-seed storage-protein genes affect the expression specificity of the *GluC* promoter, roots, leaves, stalks, and developing seeds from transgenic rice plants harboring various 5′-UTRs were examined by histochemical staining and RT-PCR. The results showed that GUS expression was only detected in the endosperm of all the constructs. Prolonged incubation (overnight) did not result in stained embryos, leaves, sheathes, stalks, or roots of the transgenic rice plants (Fig. 2). Furthermore, the GUS transcripts in these tissues were not detected by RT-PCR (data not shown). Therefore, these 5′-UTRs did not affect the expression specificity of *GluC* promoter.

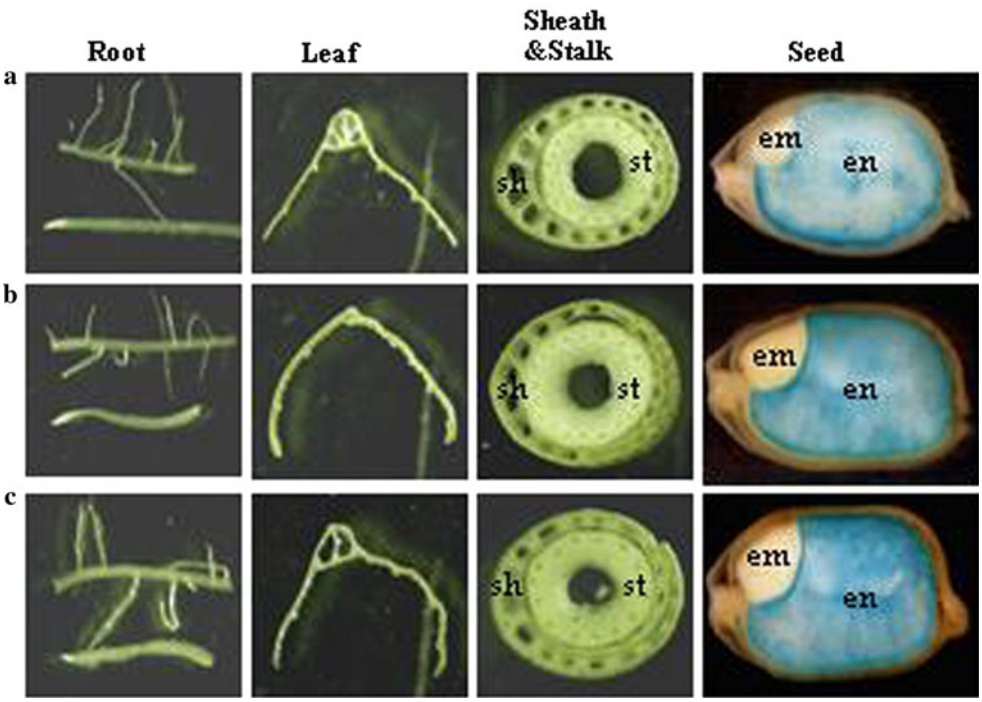

Fig. 2 Histochemical GUS assay in transgenic plant tissues. *em* embryo, *en* endosperm, *sh* leaf sheath, *st* stalk. **a** *GluC* promoter, **b** *GluC* promoter with endogenous 5′-UTR, and **c** *GluC* promoter with *Glb-1* 5′-UTR

5′-UTRs enhance GUS expression level in transgenic rice seeds

To investigate the effect of the 5′-UTRs on gene expression, we determined GUS activities in maturing seed (17 DAF) of the transformants containing *GluC* promoter combined with different 5′-UTRs. The GUS activity differed greatly among constructs (Fig. 3). The highest GUS activity was observed from p*GluC-Glb-1*-5′-UTR (26 plants) with 41.19 ± 21.93 pmol 4MU min^{-1} μg^{-1} protein, then p*GluC-GluA-1*-5′-UTR (11 plants), p*GluC*-10 kDa prolamine-5′-UTR (15 plants), p*GluC-GluA-2*-5′-UTR (12 plants), p*GluC*-16 kDa prolamine-5′-UTR (6 plants) and p*GluC-GluC*-5′-UTR (11 plants) with 38.14 ± 22.29, 36.42 ± 20.50, 35.90 ± 22.89, 29.43 ± 6.08 and 26.46 ± 10.50 pmol 4MU min^{-1} μg^{-1} protein, respectively. The GUS activity of p*GluC* was 12.26 ± 10.14 pmol 4MU $min^{-1}\mu g^{-1}$ protein (9 plants) (Fig. 3). GUS activity was increased 2.16-fold (p*GluC-GluC*-5′-UTR) to 3.36-fold (p*GluC-Glb-1*-5′-UTR) comparing to that of p*GluC*. Statistical analysis revealed that the GUS activity was increased significantly ($P < 0.01$) in all constructs bearing various 5′-UTRs. All of the constructions differed only in the 5′-UTRs; therefore, any variation in GUS expression would reflect the effect of the 5′-UTRs alone. All of the 5′-UTRs we tested enhanced the *GluC* promoter activity significantly, suggesting that the enhanced translational accumulation by the 5′-UTR may be a general phenomenon of rice-seed storage-protein genes.

Enhancement of 5′-UTR on GUS expression is independent of promoter

GUS activity of seeds at 17 DAF containing p*Glb-1*, p*Glb-Glb-1*- and p*Glb-1-GluA-1*-5′-UTR was 15.43 ± 5.85 (7 plants), 34.43 ± 11.85 (7 plants) and 27.92 ± 6.37 (6 plants) pmol 4MU min^{-1} μg^{-1} protein, respectively (Fig. 4). Therefore, the 5′-UTRs of *Glb-1* and *GluA-1* could enhance the activity of the *Glb-1* promoter in endosperm. GUS activity of p*Glb-Glb-1*- and p*Glb-1-GluA-1*-5′-UTR was 2.23-fold ($P < 0.01$) and 1.81-fold ($P < 0.01$), respectively, higher than that of *Glb-1* promoter. The enhancement of the two 5′-UTRs on GUS activity of the *Glb-1* promoter was weaker than that of the *GluC* promoter.

The 5′-UTRs of *Glb-1* and *GluA-1* were linked downstream of cauliflower mosaic virus 35S (*CaMV35S*) and rice *OsAct2* promoters, respectively. The transcriptional fusions were made by replacing the *35S* promoter in pBI221 with the chimeric promoters. Five gene fusions of p*35S-Glb-1*-5′-UTR, p*35S-GluA-1*-5′-UTR, p*Act*, p*Act-Glb-1*-5′-UTR and p*Act-GluA-1*-5′-UTR were constructed.

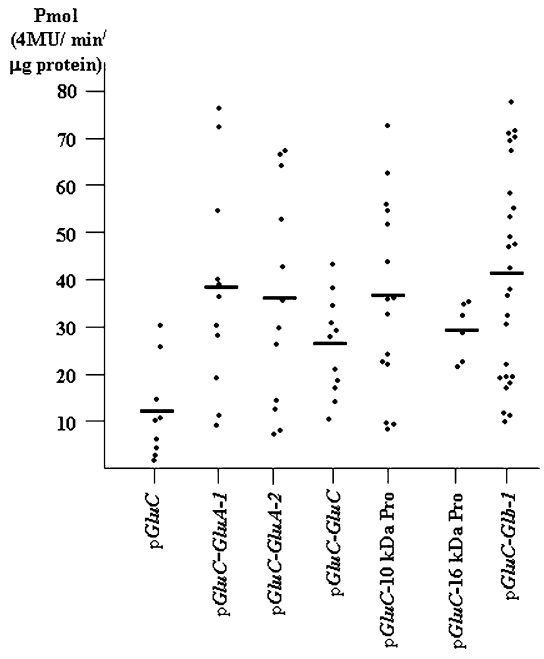

Fig. 3 GUS activity expressed by various 5′-UTRs downstream of the *GluC* promoter in maturing seeds at 17 days after flowering (DAF). GUS activity is expressed as pmol 4MU min^{-1} μg^{-1} protein. *Horizontal bars* represent the average GUS activity. p*GluC*: *GluC* promoter; *GluA-1*, *GluA-2*, *GluC*, *Glb-1*, 10 kDa Pro and 16 kDa Pro represent relative 5′-UTRs downstream of *GluC* promoter

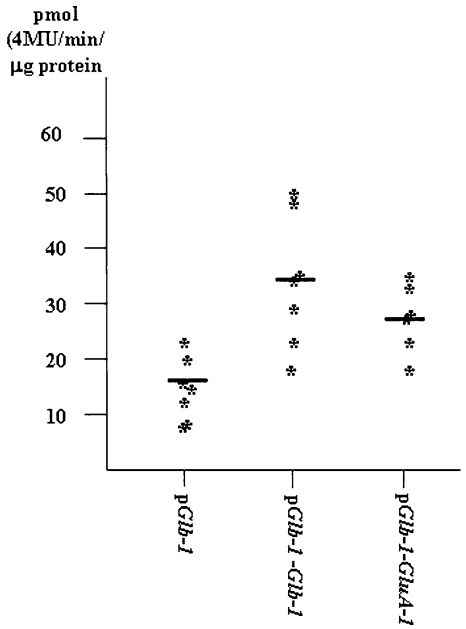

Fig. 4 GUS activity expressed by various 5′-UTRs downstream of *Glb-1* promoter in maturing seeds at 17 DAF. GUS activity is expressed as pmol 4MU min^{-1} μg^{-1} protein. *Horizontal bars* represent the average GUS activity. p*Glb-1*: *Glb-1* promoter; *Glb-1* and *GluA-1* represent relative 5′-UTRs downstream of *Glb-1* promoter

The transcriptional fusions, together with pBI221, were then introduced into etiolated rice stem protoplasts by PEG-mediated transformation, and their GUS activities were measured. As shown in Table 2, the 5′-UTRs of *Glb-1* and *GluA-1* enhanced the activities of the *CaMV35S* and *OsAct2* promoters. GUS activity of p*35S-Glb-1*- and p35S-*GluA-1*-5′-UTR was 6.55-fold ($P < 0.01$) and 3.99-fold ($P < 0.05$), respectively, significantly higher than that of p35S. Similar results were obtained for p*Act-Glb-1*- and p*Act-GluA-1*-5′-UTR, with GUS activity 3.57-fold ($P < 0.01$) and 2.07-fold ($P < 0.05$), respectively, significantly higher than that of p*Act*. Therefore, the enhancement with *Glb-1* and *GluA-1* 5′-UTRs may mainly be independent of the upstream promoter sequences.

The 5′-UTR of *Glb-1* and *GluA-1* affects translational efficiency but not mRNA accumulation

A high level of GUS expression in the presence of the rice-seed storage-protein 5′-UTRs can be explained by a high level of GUS mRNA (because of an increase in transcription or mRNA stability) or by more efficient translation of the GUS mRNA. To distinguish between these two mechanisms, we performed real-time quantitative RT-PCR to quantify the GUS mRNA level at 17 DAF in the transgenic lines transformed with p*GluC-Glb-1*-5′-UTR, p*GluC-GluA-1*-5′-UTR and p*GluC* (5′-UTR-less), with the control of rice *Act-1* mRNA. As shown in Fig. 5, the GUS mRNA level in p*GluC-Glb-1*-5′-UTR transgenic lines was only 16% higher than that of p*GluC*, while almost no difference between p*GluC-GluA-1*-5′-UTR and p*GluC* (only 4% higher). The GUS mRNA level was not altered in the presence of the *Glb-1*- or *GluA-1*-5′-UTR. Therefore, the increased GUS activity with p*GluC-Glb-1*- and p*GluC-GluA-1*-5′-UTR was not caused by increasing the mRNA level but by affecting the translational efficiency of the mRNA.

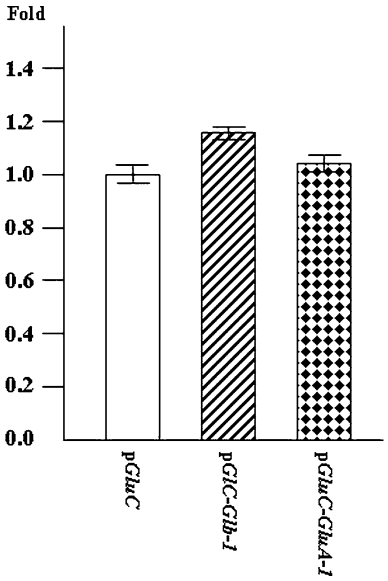

Fig. 5 Relative GUS mRNA levels in maturing seeds at 17 DAF revealed by real-time quantitative RT-PCR. The value of GUS mRNA from the p*GluC* was arbitrarily set to 1 for standard. Results are given as fold ± SD

Discussion

The 5′-UTR is involved in both transcriptional and post-transcriptional regulation of gene expression, having the capacity to enhance the expression level of a downstream reporter gene (Dansako et al. 2003; Gallie et al. 1987). The use of 5′-UTRs to control the expression of foreign gene was suggested to be an effective way to increase the yield of products of interest. To date, several studies have used 5′-UTRs to enhance the expression of a downstream reporter gene (Dansako et al. 2003). However, whether the 5′-UTRs of rice-seed storage-protein genes affect gene expression and whether the effects are constant have not been reported. Using stable transgenic rice lines, we evaluated the potential of six rice-seed storage-protein gene 5′-UTRs on enhancing the GUS expression in rice endosperm. The 5′-UTRs of *GluA-1*, *GluA-2*, *GluC*, *Glb-1*, 10 kDa and 16 kDa prolamine genes significantly enhanced *GluC* promoter activities, with the expression of GUS increased by 2.16–3.36-fold (Fig. 3). The highest GUS activity was obtained with the 5′-UTR of *Glb-1*, then *GluA-1*, the 10 kDa prolamine gene, *GluA-2*, the 16 kDa prolamine gene and *GluC* (Fig. 3). As there is no sequence similarity among them (Table 1), and each fusion was constructed by placing the 5′-UTR downstream of the same promoter, the *GluC* promoter, and the context sequences

Table 2 Relative GUS activity obtained by transient expression analysis in rice protoplasts

Construct	Fold change	Construct	Fold change
p*35S*[a]	1	p*Act*[a]	1
p*35S-Glb-1*-5′-UTR	6.55 ± 0.48**	p*Act-Glb-1*-5′-UTR	3.57 ± 0.35**
p*35S-GluA-1*-5′-UTR	3.99 ± 0.65*	p*Act-GluA-1*-5′-UTR	2.07 ± 0.22*

[a] p*35S*, *CaMV35S* promoter; *pAct*, *OsAct2* promoter

Significance at $*P < 0.05$ and $**P < 0.01$, respectively, in comparisons of p*35S* or p*Act*

around the translation initiation codon of *GUS* reporter gene in the binary vector of pGPTV-35S-HPT were not affected, the differences in GUS expression levels reflected the effect of the 5′-UTRs. Enhancement of translation by the 5′-UTR might be a general phenomenon of rice-seed storage-protein genes. It is noteworthy that the six rice-seed storage-protein gene 5′-UTRs did not alter the spatial specificity of the *GluC* promoter (Fig. 2), since they did not contain the motifs required for endosperm-specific expression, such as the GCN4, AACA and prolamine box (Takaiwa et al. 1996).

Previous reports showed that the promoters of *GluC*, *Glb-1*, 10 kDa prolamine and 16 kDa prolamine genes with endogenous 5′-UTR drove high GUS expression in transgenic rice seeds, and those of *GluA-1* and *GluA-2* were much weaker (Qu and Takaiwa 2004; Qu et al. 2008). We found GUS activity of *GluC* and *Glb-1* promoters without 5′-UTR significantly lower than that with endogenous 5′-UTR (Figs. 3, 4), which indicates that the 5′-UTRs of *GluC* and *Glb-1* played an important role in enhancing the gene expression. It is notable that the GUS activities varied greatly among the individual transformants within each construct (Figs. 3, 4), which might be caused by genomic location of insertion and gene dosage effects (Hobbs et al. 1993).

Although the activity of the *GluA-1* promoter was lower than that of *GluC* and *Glb-1* promoters (Qu and Takaiwa 2004; Qu et al. 2008), its 5′-UTR had higher capacity to enhance the GUS expression than did other 5′-UTRs, except for the 5′-UTR of *Glb-1* (Fig. 3). In contrast, the activity of 10 kDa prolamine gene promoter was higher than that of *Glb-1* and *GluA-1* promoter (Qu and Takaiwa 2004; Qu et al. 2008); however, the activity of the *GluC* promoter with the 10 kDa prolamine 5′-UTR was lower than that with *Glb-1* and *GluA-1* 5′-UTRs. These results suggest that a 5′-UTR derived from a weak promoter does not mean low capacity in enhancing gene expression. These results were consistent with those reported previously (Dansako et al. 2003). The 5′-UTRs of *Glb-1* and *GluA-1* significantly enhanced GUS expression level when fused to either endosperm-specific promoters (*GluC* and *Glb-1*) in stable transgenic rice (Figs. 3, 4) or constitutive promoters (*CaMV35S* and *OsAct2*) in transient expression protoplasts (Table 2). Therefore, the enhancement of the rice-seed storage-protein 5′-UTRs, at least the 5′-UTRs of *Glb-1* and *GluA-1*, is independent of the upstream promoter sequences. Similar results were reported for 5′-UTRs of *lat*52 and *Lox*1 (Bate et al. 1996; Rouster et al. 1998). Thus, higher yield of recombinant proteins in transgenic rice seed might be achieved by combining the 5′-UTRs with strong promoters, although we tested only two of the six 5′-UTRs.

It is reported that the 5′-UTR might enhance gene expression by increasing transcription rate, mRNA stability or translation efficiency (Hua et al. 2001; Koda et al. 2004; Zou et al. 2003). Hulzink et al. (2002) reported that the 5′-UTR of *ntp303* enhanced translation of luciferase (LUC) with the LUC activity about 50-fold as high as that of the control of *syn99* though the relative mRNA level was increased 2.72-fold. Our real-time quantitative RT-PCR results showed no significant difference (only 16 and 4% higher) in mRNA level among the constructs containing 5′-UTRs of *Glb-1* and *GluA-1* and the 5′-UTR-less construct (Fig. 5), which suggests that the enhancement effect might take place mainly at the translational level, although we did not measure the transcription or degradation rate of the mRNA. Similar results were also reported in 5′-UTRs of *Arabidopsis HSP18.2* and human *Hsp70* (Dansako et al. 2003; Vivinus et al. 2001).

The translation efficiency of mRNA could be affected by the stability of the secondary structure within the 5′-UTR. The average length of 5′-UTRs is relatively constant over diverse taxonomic classes and ranges between 100 and 200 nt (Mignone et al. 2002). Translation of GUS mRNAs possessing long 5′-UTRs with high amounts of secondary structure is inefficient but is greatly enhanced by truncation of the long 5′-UTR or by precluding a secondary structure (Kozak 1991), which leads to low stability (ΔG values < -33 kcal mol^{-1}) and would allow for efficient progress of the scanning ribosomes to the initiator AUG (Koromilas et al. 1992). The aim of this study was not to determine the whole sequences of the rice-seed storage-protein gene 5′-UTRs but to evaluate their effects on foreign gene expression. The six 5′-UTRs we used are in general short, ranging from 17 to 67 nt (Table 1), though their whole sequences might be longer. The expression enhancement is not associated with the length of the 5′-UTRs in our study for that the length of the 5′-UTRs of *GluA-1* and *Glb-1* was 25 and 18 nt, respectively, whereas that of the 10 kDa prolamine was 67 nt (Table 1). Calculation of the stability of secondary structure within each of the 5′-UTRs by the MFOLD program (Zuker 2003) showed that the ΔG values of the six 5′-UTRs were lower than -33 kcal mol^{-1}, with almost no ΔG value difference among the 5′-UTRs (Table 1). The length or the intrinsic stability of secondary structure within the 5′-UTRs is not sufficient to explain the translational efficiency we observed.

Plant seed systems have been used as an ideal bioreactor for the production of recombinant proteins (Takaiwa et al. 2007). However, low level of expression and accumulation is considered as one of the most important limiting factors for commercialization of transgenic plants as reliable recombinant protein production systems. The 5′-UTRs reported here, especially those of *Glb-1* and *GluA-1*, may be ideal candidates for optimizing the production of a large amount of foreign protein in plant seed systems if combined with strong promoters and/or *cis*-elements. The

5′-UTRs of *Glb-1* and *GluA-1*, together with endosperm-specific promoters, are being used for production of useful recombinant protein in rice seeds.

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