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RESEARCH ARTICLE

Cloning and characterisation of an endosperm specific *glutelin B1* promoter from Sri Lankan rice (*Oryza sativa* L. ssp. *indica*) variety Bg 250

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Abstract: Glutelins are the primary source of energy storage in the endosperm of rice grains. Among the glutelin promoters, *Glutelin B-1* (*GluB-1*) is widely studied and used in transgenic rice plants to express recombinant proteins in the endosperm. In this study, three regions: 350 bp, 1308 bp and 2300 bp of the *GluB-1* promoter were PCR amplified from the genomic DNA of Bg 250 rice variety. The amplified fragments were cloned into pGEM®-T Easy vector for characterisation of *GluB-1* promoter. Each region of *GluB-1* promoter was separately cloned into the promoterless binary vector pCAMBIA1391Z harbouring the *β-glucuronidase* (GUS) reporter gene. Putative transgenic plants were generated by *Agrobacterium*-mediated gene transformation and confirmed by PCR using nopaline synthase terminator primers. All *GluB-1* promoter constructs showed expression of the GUS gene in the endosperm of T_0 transgenic plant seeds. The 1308 bp *GluB-1* promoter revealed the highest expression as determined by the GUS assay. This indicates the potential of this promoter for expression of recombinant proteins in rice endosperm.

Keywords: *GluB-1* promoter, transgenic rice.

INTRODUCTION

The total protein content of rice varies from 8 % - 18 % among different varieties (Shewry, 2007). The rice endosperm is the major site for protein storage. Therefore, the endosperm is a unique platform for the expression of foreign recombinant proteins. Moreover, the rice endosperm tissue occupies more than 90 % of the total seed weight, and represents a stable storage organ that enables the plant to accumulate high amounts of recombinant products (Takaiwa *et al*., 2007). The use of the endosperm for protein expression has many advantages compared to other expression systems in terms of the cost, large storage ability, ease of controlling the scale of production and the high level of safety in terms of storage of recombinant proteins (Horvath *et al*., 2000). The rice seed system, as a bioreactor to produce recombinant proteins, has been proven to be a prominent success (Katsube *et al*., 1999).

The promoter plays a major role in determining the temporal and spatial expression pattern and the transcript level of a gene, which finally governs the amount of expressed recombinant protein (Qu *et al*., 2008). Some strong constitutive promoters, such as the cauliflower mosaic virus 35S promoter (CaMV35S), nopaline synthase (NOS) promoter and maize ubiquitin promoter (P*ZmUbi*) are widely used to express protein in transgenic rice seeds (Battraw & Hall, 1990; Cornejo *et al*., 1993). Although these promoters have strong activity, they might lack the peak in late seed development that is characteristic of seed-storage protein promoters, which may be required for high-level recombinant

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expression (Drakakaki *et al*., 2000). In addition, continuous expression of a foreign gene at a high level in all tissues may cause detrimental effects to the host plant. Therefore, a strong endosperm specific promoter could be used to get stable expression of foreign genes than constitutive promoters (Choi *et al*., 2003). The use of seed-specific promoters has resulted in a high level of protein expression leading to recombinant protein accumulation in seeds (Takaiwa, 2013). In early 2000, the major limitation of using the endosperm to obtain the required level and pattern of expression of recombinant proteins was the lack of strong endosperm specific expression promoters (Qu *et al*., 2008). Recently, Jeong and Jung (2015) reported the presence of 14 endosperm-specific promoters in rice. Among them the glutelin gene promoters of rice are ideal to express foreign genes in rice endosperm because rice glutelin accounts for ~80 % of the total rice seed storage protein.

Genome-wide studies have revealed that there are at least 13 glutelin genes (Qu *et al*., 2008); hence the high percentage of glutelin expression. The majority of studies on rice glutelin promoters have been focused on identifying *cis* regulatory elements involved in endosperm specificity and expression patterns (Zheng *et al*., 1993; Croissant-Sych & Okita, 1996; Takaiwa *et al*., 1996; Yoshihara & Takaiwa, 1996; Wu *et al*., 1998; Washida *et al*., 1999).

Among all glutelin promoters of rice, glutelin B-1 (*GluB-1*) promoter is the most widely studied (Sarker *et al*., 2015). It is used as an endosperm specific promoter for expressing foreign genes in rice seeds. The full promoter region of *GluB-1* is around 2335 bp (Le & Takaiwa, 2004). However, a 1.3 kb region of this promoter has been widely used for the expression of transgenes in rice seeds with satisfactory results (Goto *et al*., 1999). The 197 bp minimal promoter, upstream of the start site confers endosperm specific expression. This minimal promoter contains GCN4, prolamin box (PROL), AACA and ACGT core motifs. The GCN4 and PROL motifs are conserved in many seed storage protein genes including wheat, barley, rice and maize (Müller & Knudsen, 1993; Vicente-Carbajosa *et al*., 1997). The GCN4 motif, prolamin box and AACA motifs are recognised by the bZIP Opaque-2(O2) (Wu *et al*., 1998), DNA-binding one zinc finger (DOF) class of zinc finger proteins (Mena *et al*., 1998) and MYB transcription factors, respectively (Suzuki *et al*., 1998) for seed specific expression. To date, identifying putative *cis* regulatory elements in promoters has been the main focus in endosperm-specific promoter studies. Nucleotide substitution mutations of the *cis* regulatory elements in the 197 bp promoter significantly reduces the promoter activity and alters the expression pattern of endosperm. Therefore, it was confirmed that a combination of the *cis* regulatory elements is a minimal requirement for endosperm specific expression (Wu *et al.,* 1998).

Different sizes (1.3 kb, 2.3 kb and 2.4 kb) of the *GluB-1* promoter have been used in various transgenic approaches to produce higher levels of ectopic gene expressions. These include: β-carotene in golden rice, *human lactoferrin (hLF)* gene in Bulgarian barley, soybean *ferritin* gene in rice, over expression of *GABA* in transgenic rice cell lines, artificial avidin in rice and over expressed TPC7 antigen in rice (Goto *et al*., 1999; Katsube *et al*., 1999; Yoza *et al*., 2005; Kamenarova *et al*., 2007; Akama *et al*., 2009; Wang *et al*., 2013). All *GluB-1* promoters showed seed specific expression in the aleurone and sub aleurone layers (Wu *et al*., 1998).

Cloned promoter regions become dysfunctional due to reasons such as promoter homology and mutations in *cis*-acting elements. The functionality of a promoter can be analysed by cloning with a reporter gene such as GUS and transferring the construct into the plant. The expression of the reporter gene gives an indication of the activity of the promoter, both quantitatively and qualitatively.

The present study focused on characterisation of the *GluB-1* promoter isolated from Bg 250. The 350 bp, 1308 bp and 2300 bp lengths of the *GluB-1* promoter were isolated, cloned and characterised to determine the GUS expression in transgenic rice seeds with a view to identifying the most suitable promoter length for transgenic expression in endosperm.

METHODOLOGY

Cloning of different lengths of *GluB-1* promoter

Genomic DNA was extracted from Bg 250 rice variety using the CTAB method (Sun *et al.,* 2010). Primers were designed to PCR amplify different lengths of the *GluB-1* promoter (Table 1) based on the *Oryza sativa japonica* promoter sequence (Accession number AY427569). Each forward primer (GluB-1F350, GluB-1F1308 and GluB-1F 2300) and reverse primer (GluB-1CR) contained *Bam*HI and *Eco*RI restriction sites respectively at the 5' end. The final concentrations of the PCR components were; 50 ng of template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer and 1 U of GoTaq® DNA polymerase (Promega, USA). The PCR conditions were as follows: initial denaturation at 95 ºC for 5 min and 35 cycles of denaturation at 95 ºC for 1 min, annealing for 30 s and an extension at 72 ºC.

Table 1: List of primers for the amplification of different lengths of the *GluB-1* promoter

Primer name	Primer sequence 5' to 3'
GluB-1F$_{350}$	*GGATCC*CTCAAGCATAAGAGCTTTATGGTGC
GluB-1F$_{1308}$	*GGATCC*ACTGAATAGATCTCGATTTTTGAGGAATTT
GluB-1F$_{2300}$	*GGATCC*ACAGATTCTTGCTACCAA
GluB-1CR	*GAATTC*AGCTATTTGTACTTG CTTATGGAAACTTAACCT

The annealing temperature and the time of extension are given in Table 2. The final extension was carried out at 72 ºC for 10 min.

Table 2: Annealing temperatures and extension times of primers for the amplification of different lengths of the *GluB-1* promoter

Combination of primers	Annealing temperature (ºC)	Extension time
GluB-1F$_{2300}$ and GluB-1CR	54	2 min and 30 s
GluB-1F$_{1308}$ and GluB-1CR	58	1 min and 30 s
GluB-1F $_{350}$ and GluB-1CR	58	30 s

The amplified *GluB-1* promoter fragments (350 bp, 1308 bp and 2300 bp) were eluted and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The eluted DNA was then separately cloned into pGEM®-T Easy vector (Promega, USA) according to the manufacturer's instructions. Randomly selected clones were used to extract recombinant plasmids. They were then digested by *Eco*RI (Promega, USA) to confirm the presence of the insert. Subsequently, the recombinant clones were custom sequenced using vector specific primers, SP6 and T7 (Macrogen, Korea) to confirm the promoter sequences.

Promoter sequence analysis

The sequence dataset was analysed using BioEdit software (version 7.0) while the homology searches were performed using BLAST at the NCBI website (*http://blast.ncbi.nlm.nih.gov*). PLACE software (*http://www.dna.affrc.go.jp/PLACE/*) was used to detect *cis* regulatory elements in the *GluB-1* promoter sequence (Higo *et al*., 1999).

Construction of plant transformation vectors

The binary vector pCAMBIA1391Z was used in this study (Cambia, Australia). Sequence confirmed different lengths of the *GluB-1* promoter containing recombinant pGEM®-T Easy vector and pCAMBIA1391Z vector were double digested with *Eco*RI and *Bam*HI. Double digested promoter inserts and the pCAMBIA1391Z vector were ligated using T4 DNA ligase (Promega, USA) and separately transformed into *E. coli* (JM109) competent cells according to standard protocols (Sambrook & Russel, 2000). The recombinant clones were confirmed by colony PCR using specific primers (Table 1). All promoter constructs (Figure 1) were then transformed into *Agrobacterium* GV3101 by the freeze thaw method (Holsters *et al*., 1978). The transformants were selected by colony PCR using specific primers (Table 1).

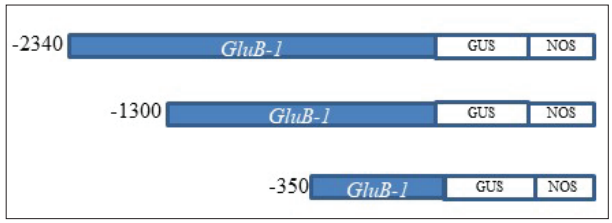

Figure 1: Binary vector pCAMBIA1391Z constructs of the *GluB-1* promoter of different lengths

Regeneration of transgenic rice

The different media used for the regeneration of transgenic rice plants were prepared as reported by Kajendran *et al*., in 2019. Rice variety Bg 250 was used to transform the constructs carrying different lengths of *GluB-1* promoter. De-husked mature seeds were surface sterilised and plated directly on callus induction medium. The plates were then incubated at 28 ºC in

the dark for 21 days for the callus to grow. Meanwhile, *Agrobacterium tumefaciens* carrying vector constructs of different *GluB-1* promoter lengths were separately cultured in LB broth medium containing kanamycin (50 mg/L) and rifampicin (50 mg/L) at 28 ºC until OD_{600} reached 1.0. The bacteria were then harvested by centrifugation (3250 g) and the pellet was re-suspended in *Agrobacterium* re-suspension medium. The fully grown calli were immersed in the above re-suspended *Agrobacterium* for 10 min. The excess *Agrobacterium* suspension was removed by filtration and the calli were blotted dry prior to transferring them into the co-cultivation medium. After 3 days, the calli were washed with sterilised distilled water followed by repeated washing with aqueous solutions of cefotaxime (containing 1 g/L, 750 mg/L and 500 mg/L cefotaxime, respectively) to remove bacteria (*Agrobacterium*). The calli were then allowed to proliferate in callus induction medium. After 3 – 4 weeks, proliferated calli were transferred into hygromycin (Sigma, USA) selection medium. Healthy calli were sub cultured on shoot generation medium for 2 – 3 weeks in the dark. The calli were then incubated under light to generate shoots. Fully-grown shoots were sub cultured on root generation medium to induce root growth. The one-month-old plants were then transferred to sterile soil. Acclimatised plants continued to grow in the green house. The transformed plants were confirmed by PCR analysis using *Nos* terminator primers under the following PCR conditions: initial denaturation at 95 ºC for 5 min and 35 cycles of denaturation at 95 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 30 s followed by a final extension at 72 ºC for 5 min.

Analysis of GUS gene in transgenic plants

Leaf discs, root and matured seeds from a T_0 transgenic plant (of different *GluB-1* promoters) and non-transgenic Bg 250 rice plants were collected and screened histochemically to analyse the expression of the GUS gene. Transgenic seeds, leaf discs and roots were submerged in fixation buffer (2 % formaldehyde, 0.05 % Triton X-100 and 50 mM sodium phosphate), vacuum infiltrated for 10 min on ice and kept at room temperature (28 ºC) for 10 min. The fixation buffer was removed and washed twice with 50 mM sodium phosphate buffer. Thereafter, the seeds, leaf discs and root samples were stained with 1.5 mM of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM sodium phosphate and 0.1 % Triton X-100 by vacuum infiltrating for 5 – 10 min and kept at 37 ºC overnight in the dark prior to visualisation (Jefferson, 1987; Ratnayake & Hettiarachchi, 2010)

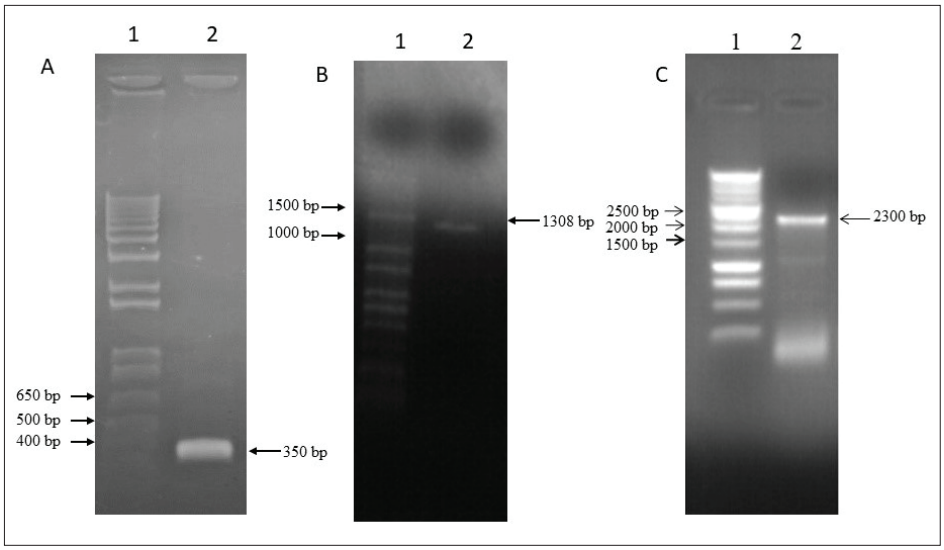

Figure 2: PCR amplification of *GluB-1* promoter. A: lane 1 - 1 kb ladder (Invitrogen, USA); lane 2 - 350 bp *GluB-1* promoter; B: lane 1 - 100 bp ladder (UC Biotech, Sri Lanka); lane 2 - 1308 bp *GluB-1* promoter; C: Lane 1- 1 kb ladder (Promega, USA); Lane 2 - 2300 bp *GluB-1* promoter

RESULTS AND DISCUSSION

Rice is the staple food for millions of people around the world. Hence, genetic engineering has been focused on improving the grain quality of rice (Ye *et al*., 2000). The introduction of additional traits through gene stacking requires the use of promoters with differential seed specificity. However, promoters with diverse sequences need to be used to avoid homology-dependent gene silencing, and hence to ensure stable transgene expression (Stam *et al*., 1997). In addition, patent protection limits the use of non-rice seed promoters in commercial production (Barton & Berger, 2001). Therefore, the aim of the present work was to determine the potential of different lengths of the *GluB-1* promoter (350 bp, 1308 bp and 2300 bp lengths) to drive the expression of a GUS gene. The seeds of a single transgenic plant corresponding to different promoter lengths were selected for the histochemical assay.

Sequence confirmation of different lengths of *GluB-1* promoter

The 350 bp, 1308 bp and 2300 bp length *GluB-1* promoter regions were amplified from genomic DNA of rice variety Bg 250 (Figure 2) and cloned into pGEM®-T Easy Vector.

The nucleotide sequences of the amplified promoter (2300 bp) showed 99 % identity to *Oryza sativa indica* group cultivar Shuhui 498 and 98 % identity to *Oryza sativa japonica* group cultivar Nipponbare. Nucleotide sequence of the 2300 bp length of *GluB-1* promoter was deposited in GenBank (accession number MH748577). The endosperm specific *cis* regulatory elements of *GluB-1* were identified by PLACE promoter analysis as shown in Figure 3.

Generation of transgenic plants and expression of GUS

A histochemical GUS assay was performed on the seeds, leaf discs and root from a T_0 transgenic rice plant transformed with each construct to determine the expression of the GUS reporter gene. The presence of blue colour in the embryo indicated the successful insertion of different lengths of the promoter and its ability to express the GUS reporter gene (Figure 4). The intensity of the blue colour was used as a scale for the level of expression of GUS controlled by the promoter. According to the colour intensity, the promoter efficiency was as follows: 1308 bp > 350 bp > 2300 bp length of *GluB-1* promoter. No expression of GUS gene was observed in leaf discs and roots of transgenic plants and the negative control (leaf discs, roots and seeds of non-transgenic rice).

In silico analysis of *GluB-1* promoter

All transgenic rice seeds carrying the promoter/GUS fusions showed expression of the GUS gene. The highest intensity of the colour was observed in transgenic rice seeds containing the 1308 bp promoter/reporter construct indicating higher expression of the GUS gene compared to the other two constructs (Figure 4B).

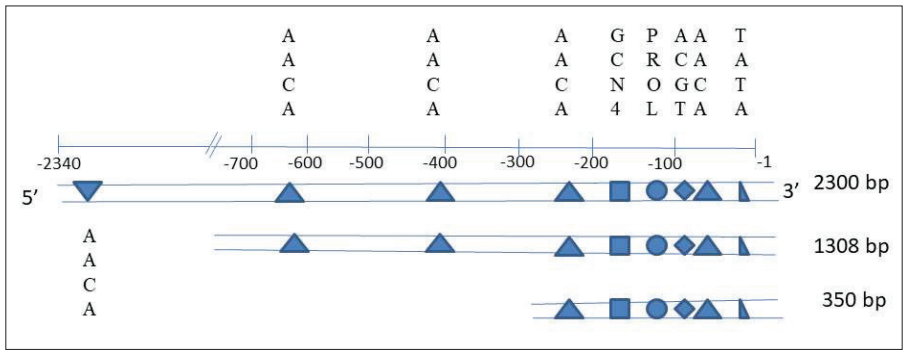

Figure 3: Schematic diagram showing the location of *cis* elements in different lengths of the *GluB-1* promoter. Negative numbers indicate the positions of nucleotides relative to the transcription start site. ▲ - AACA motif; ■ - GCN4 motif; ● - Prolamin box; ◆ - ACGT motif; ▮ - TATA box

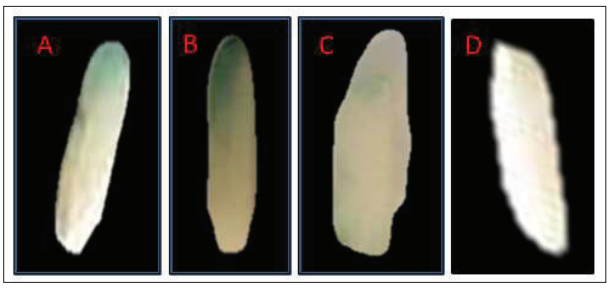

Figure 4: Histochemical analysis of the expression of the GUS gene controlled by different lengths of *GluB-1* promoter in transgenic plant seeds: A - 350 bp *GluB-1* promoter; B - 1308 bp *GluB-1* promoter; C - 2300 bp *GluB-1* promoter; D - negative control (non-transgenic Bg 250 rice variety)

The expression of genes is regulated by the combinatorial interactions of multiple *cis* elements in the promoter. Transcription factors specifically bind to the *cis* elements that are necessary for transcription initiation of genes. Analysis of the *GluB-1* promoter by the PLACE promoter analysis tool revealed the cloned promoters to contain additional AACA motifs upstream of the -197 bp minimal promoter (Figure 3). Wu *et al*. (1998), demonstrated that the deletion of an extra AACA motif at -212 bp resulted in an eightfold reduction of promoter activity. It is noteworthy that the 350 bp, 1308 bp and 2300 bp promoters used in this study were derived from the same *GluB-1* promoter sequence, which was able to express the GUS gene. However, the promoter activity was not the same according to the observed colour intensity in histochemical analysis. The promoter activity can be affected by the pattern of the *cis* elements in the promoter, insertion position and gene dosage of transgenes integrated into the transgenic plant. It appeared that the 1308 bp *GluB-1* promoter was stronger than 350 bp and 2300 bp *GluB-1* promoters. The only notable difference in the sequence between the 350 bp promoter and the 1308 bp promoter is the presence of two additional AACA motifs in the 1308 bp promoter starting at position -413 bp (Figure 3). These extra AACA motifs are likely to be responsible for the increased level of GUS expression in the 1308 bp *GluB-1* promoter (Figure 4B).

The GUS activity of the 2300 bp promoter construct was much less compared to the other two constructs (Figure 4C). It may be due to the presence of an AACA motif found in reverse orientation (at -2192 bp) in this construct. Reverse oriented motifs can reduce the GUS activity (Yoshihara *et al*., 1996; Wu *et al*., 2000). Yoshihara and co-workers in 1996 reported that the reverse constructs (GCN4 and AACA motifs) decreased the relative GUS activity to about 0.7 in seeds and Wu *et al*. (2000) constructed a vector containing reverse oriented sequence between the -245 and -145 bp region containing GCN4 and AACA motifs. This construct was able to direct GUS gene expression in the transgenic rice seeds, although at a lower level than that obtained by the same fragment in the normal orientation, confirming the above. However, CaMV 35S promoter constructs (-343 to -46, -209 to -46, and -168 to -46) with either the forward or the reverse orientation of *cis* elements had similar GUS expression levels in leaves of *Nicotiana tabacum* (Fang *et al*., 2007).

The GUS expression observed for *Japonica* spp. (Le & Takaiwa, 2004; Sarker *et al*., 2015) under the control of the 1300 bp and 2300 bp length *GluB-1* promoters were contradictory to the results observed in the present study. GUS gene expression was 10 times higher with the 2300 bp *GluB-1* promoter compared to the 1300 bp promoter. It has been attributed to the presence of an extra ACGT motif in the 2.3 kb promoter. However, this additional ACGT (GTACGTG) motif was absent in Nipponbare (*japonica*; Accession number: AY427569), Bg 250 (*indica*; Accession number: MH748577) and Shuhui 498 (*indica*) rice cultivars. Comparative analysis of the *GluB-1* promoter nucleotide sequence of Nipponbare, Bg 250 and Shuhui 498 revealed that one AACA motif (at - 413 bp) was absent in Shuhui 498. Further, other additional motifs such as SORLIP 1(GCCAC) at -357 bp, SORLIP 2 (GGGCC) at -875 bp and MybSt1 (GGATA) at -228 bp were found within the proximal region of the *GluB-1* promoter *indica* sequences (Bg 250 and Shuhui 498), which were absent in the *japonica* sequence. These motifs have been identified in various plant promoters with the potential to increase promoter activity (Baranowskij *et al*., 1994; Hudson and Quail, 2003; Jiao, 2005). An additional GATA box (at -227 bp) and E-box (at -1086 bp) were also located within the *GluB-1* promoter *indica* sequences (Bg 250 & Shuhui 498). Previous studies on seed specific promoters suggest that these motifs enhance the expression and tissue specificity of the genes (Kim *et al*., 2006).

The variation in expression between transgenic rice plants could also be due to 'position effects' that depend on the chromosomal location of transgene insertion, co-suppression, and/or the presence of multiple copies of transgenes (Tang *et al*., 2003; Donnarumma *et al*., 2011). However, Nagaya and co-workers reported that position effects are not a major cause of variability of transgene expression in the random integration of a single copy of the transgene in *A. thaliana* (Nagaya *et al*., 2005).

CONCLUSION

In this study the *GluB-1* promoter of rice variety Bg 250 was fully sequenced and *in-silico* analysed. Combined results of motif analysis and colour intensity of the GUS assay revealed that additional necessary *cis* elements for seed specific promoter were found within the 1308 bp *GluB-1* promoter. Furthermore, a higher expression of GUS in the endosperm was observed with the 1308 bp *GluB-1* promoter compared to the 350 bp and 2300 bp *GluB-1* promoter. Therefore, the 1308 bp *GluB-1* promoter appears to be the most suitable promoter for the production of recombinant proteins in the endosperm of rice seeds.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this study

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