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Molecular Cloning and Bioinformatic Analysis of Endosperm Specific Promoter, α-Globulin (AsGlo1)

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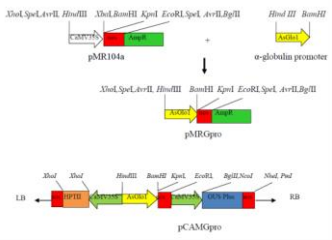
Graphical abstract



Abstract

The α-globulin promoter (AsGlo1) have been successfully amplified from pmCACA:GFP using PCR. The construction of the recombinant plasmid, pMRGpro containing α-globulin promoter was achieved by ligation at *Hind*III and *BamH*I of pMR104a. Cassette containing α-globulin promoter and nos terminator was successfully cloned in pCAMBIA1305.2 and designated as pCAMGpro. Digestion methods were used to confirm the recombinant plasmid. The resulting plasmid was then sequenced and analyzes using bioinformatics tools. The analysis showed a 99% homology with *A. sativa* α-globulin gene promoter (Gene Bank: AY795082.1) and further confirmed using phylogenetic analysis. The result showed a successful cloning of the correct fragment *Avena sativa* α-globulin promoter into an expression vector.

Keywords: Rice endosperm; recombinant protein; endosperm specific promoter; α-globulin promoter

Abstrak

Dalam kajian ini, fragmen promoter α-globulin (AsGlo1) telah berjaya diamplifikasi daripada plasmid, pmCACA:GFP menggunakan kaedah amplifikasi tindak balas berantai polimeras. Pengklonan fragmen promoter α-globulin (AsGlo1) pada tapak enzim penyekatan *Hind*III dan *BamH*I pada plasmid pMR104a, menghasilkan plasmid rekombinan baru dipanggil pMRGpro. Seterusnya, plasmid rekombinan akhir, pCAMGpro yang mengandungi kaset promoter α-globulin dan penamat nos berjaya diklonkan ke dalam pCAMBIA1305.2. Pengesahan plasmid rekombinan ini dilakukan melalui kaedah pencernaan enzim penyekat. Plasmid rekombinan yang diperolehi kemudiannya dijujukan dan dianalisa menggunakan analisa bioinformatik. Keputusan analisa menunjukkan fragmen promoter yang dipencilkan adalah 99% sama dengan *A. sativa* promoter α-globulin (Nombor aksesi Bank Gen: AY795082.1) dan seterusnya disahkan dalam analisa filogenetik. Keputusan ini menunjukkan proses pengklonan fragmen promoter α-globulin ke dalam vektor pengekspresan yang dikehendaki adalah tepat.

Kata kunci: Endosperma padi; protein rekombinan; promoter spesifik endosperma; promoter α-globulin

1.0 INTRODUCTION

Endosperm specific promoters play an important role in the genetic modification of endosperm composition and in using endosperm as bioreactors. Although several endosperm specific promoters have been cloned and used in expression studies, their origin of gene is similar with the transformed plant species. Since α-globulin promoter (AsGlo1) from oat has been shown to drive high expression in barley, it would be interesting to know its effect in rice.

In rice, globulin promoter has been shown to increase targeted protein in endosperm (Hwang *et al*., 2002). The use of tissue-specific promoters have several advantages such as potential to increase recombinant protein stability and deliver targeted gene expression to only specific parts of the plant (Facy, 2009), cost effective and easy agricultural scale-up, high rate of protein synthesis and easy long term storage of recombinant protein (Takaiwa *et al*., 2007). Different research outcomes have illuminated the importance and role of several endosperm-specific promoter to engineer transgenic crops with improved endosperm-specific biosynthesis (Furtado *et al*., 2009, Kawakatsu and Takaiwa, 2010, Vickers *et al.*, 2006). The use of endosperm-specific promoters presents an approach that could solve the potential detrimental effect of non-specific constitutive promoters to the host plant since the foreign gene is continuously being expressed in the entire plant tissues (Qu *et al.*, 2008). The

endosperm-specific expression promoters of other cereals have also received attention from various researchers such as from maize (*Zea mays L.*) (Hu *et al.*, 2011), barley (*Hordeum vulgare L.*) (Choi *et al.*, 2003), rice (Qu and Takaiwa, 2004, Rasmussen and Donaldson, 2006) and wheat (*Triticum aestivum L.*) (Lamacchia *et al.*, 2001,). Therefore, the present study focused on the development of an expression cassette containing endosperm specific promoter (α-globulin) from *Avena sativa* and analyzing the sequences through bioinformatics analysis and the construction of phylogenetic tree.

2.0 MATERIALS AND METHODS

A α-globulin promoter, AsGlo1 coding region from *Avena sativa* was amplified from 4.5 kB plasmid (pmCACA:GFP) was obtained from Dr. Claudia Vickers (Queensland University, Australia) and cloned into an intermediate vector, pMR104a (UKM) containing a NOS terminator. The isolated α-globulin promoter were excised using *Hind*III and *BamH*I by T4 DNA ligase was used to replace the CaMV35S coding region in pMR104a. Amplification of the promoter was achieved by PCR (Promega) using primers F: '5-CACAAACGTGCAAAAGCTTAATTCG-3' and primers R: 5'-GACGGATCCGAGATTGTAGAAGG-3'. The forward primers contained engineered *Hind*III site whereas reverse primers contained *Bam*HI site. PCR was performed under the following conditions; initial denaturation at 94°C at 3 min; (30 cycles), denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min and final extension at 72°C at 10 min. The amplified α-globulin promoter was excised using *Hind*III and *Bam*HI, and ligated by T4 DNA ligase to replace CaMV35S coding region in pMR104a, producing a new recombinant plasmid called pMRGpro (Figure 1). The new recombinant plasmid contained AsGlo1 fragment was then transformed into *E.coli* competent cells and Qiagen Spin Miniprep kit was used to isolate the plasmid. The cassette containing AsGpro:NOS cassette (~1,111 bp) were then digested with the *Hind*III and *Eco*RI and ligated into the expression vector (pCAMBIA1305.2) at the same restriction enzymes. The final expression vector was designated as pCAMGpro. The confirmation of recombinant plasmid was obtained through digestion and PCR. All the PCR mixture was obtained from Promega while restriction enzymes from NEB or otherwise stated. Finally, the AsGlo1 fragment was sequenced and analyzed by bioinformatics. The forward sequence was aligned with the reverse complement of the reverse sequence using the ClustalX alignment mode of Bioedit software. Phylograms of the selected sequences with highest homology to AsGpro was constructed using MEGA5 software (Tamura *et al.*, 2011) for construction of phylogenetic tree.

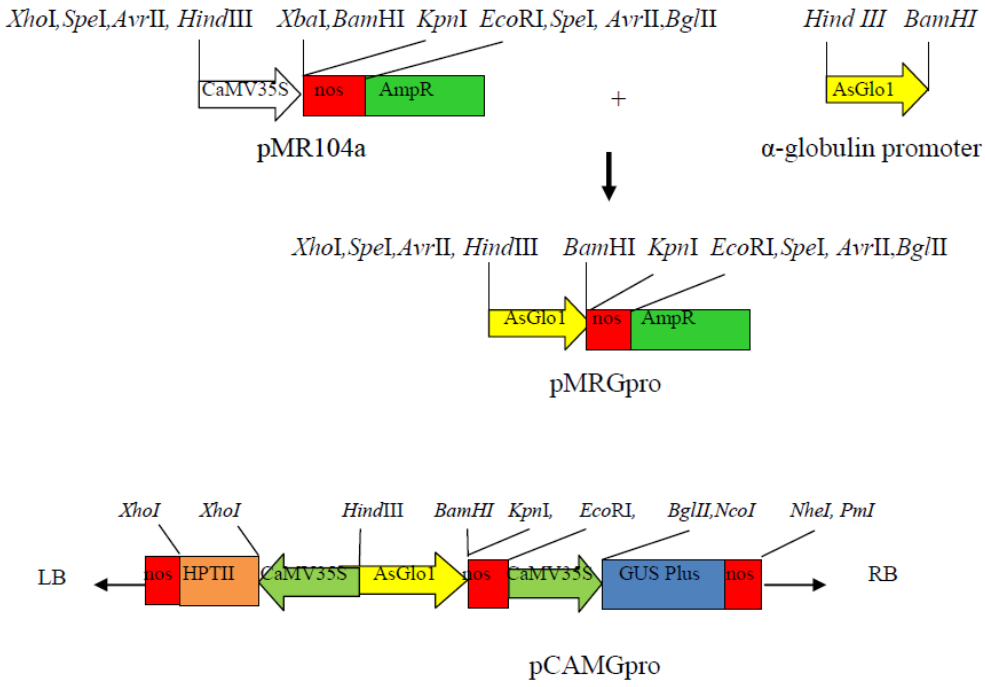


Figure 1 Schematic diagram of pMR104a cloned with AsGlo1 promoter to generate new plasmid, pMRGpro (above). Below show illustrated cassette containing AsGlo1 promoter and NOS terminator inside the pCAMBIA 1305.2 to generate new plasmid, pCAMGpro

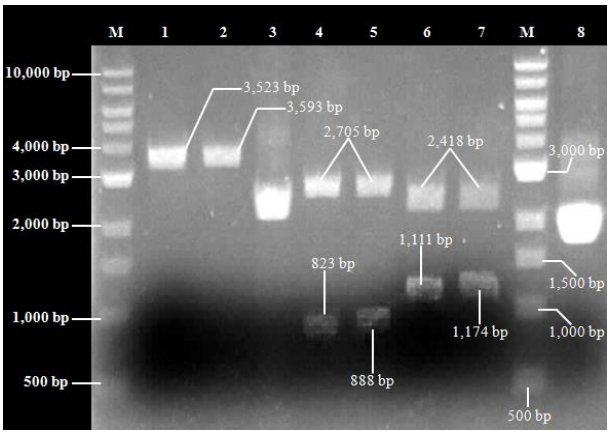
3.0 RESULTS AND DISCUSSION

3.1 Construction of Recombinant Vectors

The potential recombinant colonies of pMRGpro were inoculated in LB media containing 50µg/µl ampicilin and further screened for positive colonies. Single fragment was obtained when pMRGpro (3,523 bp) and pMR104a (3,593 bp) were digested with *Hind*III (lane 1 and 2 of Figure 2A). When pMRGpro was digested with *Hind*III and *BamH*I, two fragments was observed; ~2705 bp band for backbone plasmid and ~823bp band for AsGlo1 fragment (Figure 2A; lane 4). To further confirm that AsGlo1 fragments is successfully inserted into pMR104a replacing the CaMV35S promoter, pMRGpro and pMR104a were

subjected to double digestion with enzyme *Hind*III and *Eco*RI generated 2 fragments; pMRGpro (lane 6) and pMR104a (lane 7). From Figure 2A lane 6 and lane 7 it can be observed that 2 fragments were generated for both pMRGpro and pMR104a. From the double digestion, pMRGpro produced the upper fragment (2,418 bp) representing the pMRGpro backbone without the AsGpro:NOS cassette while ~1111 bp for AsGlo1:NOS cassette (lane 6). While for pMR104a, the upper fragment (2,418 bp) represented the pMR104a backbone while ~1174 bp represented CaMV35S:NOS cassette (lane 7). The cassette difference in AsGlo1:NOS (1,111 bp) and the CaMV35Spro:NOS (1,174 bp), confirms the replacement of the CaMV35Spro with the AsGlo1.

A



B

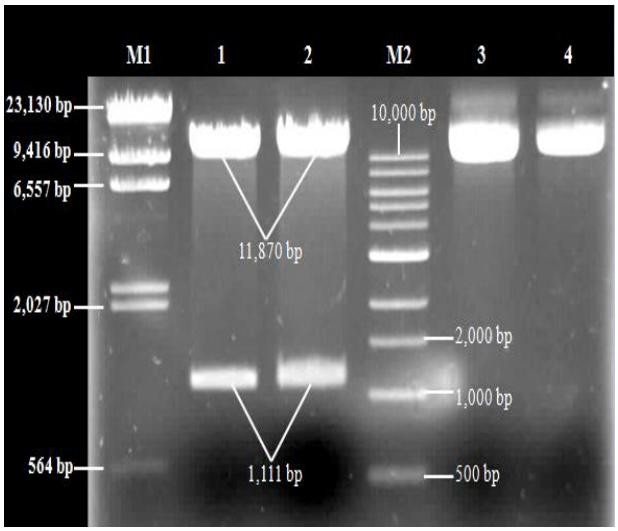


Figure 2 Gel electrophoresis (1% w/v) shows (A) confirmation of recombinant plasmid, pMRGpro. Lane 1 & 2: Single fragment of pMRGpro and pMR104a digested with *Hind*III respectively, Lane 3: Undigested pMR104a, Lane 4: Fragment AsGlo1 promoter (below) from pMRGpro when digested with *Hind*III and *BamH*I, Lane 5: Two fragments show pMR104a without CaMV35S (upper) and fragment CaMV35S (below) when digested with *Hind*III and *BamH*I, Lane 6: pMRGpro digested with *Hind*III and *Eco*RI, Lane 7: pMR104a digested with *Hind*III and *Eco*RI, Lane 8: Undigested pMRGpro and M: 1 Kb ladder. (B) Digestion of 2 colonies pCAMGpro with *Hind*III and *EcoR*I. Lane 1 and 2: pCAMBIA1305.2Gpro digested with *Hind*III and *EcoR*I; Lane 3 and 4: Undigested pCAMBIA1305.2Gpro. M1: Lambda DNA-HindIII digest and M2: 1 Kb DNA marker

The construction of final expression vector was achieved by ligation of AsGlo1:NOS cassette and pCAMBIA1305.2 at site of *Hind*III and *Eco*RI. From Figure 2B, digestion with *Hind*III and *Eco*RI generated two bands (lane 1 and 2) with upper band (~11,870 bp) representing the pCAMGpro backbone without the cassette (pCAMBIA1305.2) while the lower band is the AsGlo1:NOS cassette (~1,111 bp). The amplification of the GUS*Plus* gene was performed using the forward primer, GUS_F (5'CGCCGATGCAGATATTCGTA3') and the reverse primer, GUS_R (5' ATTAATGCGTGGTCGTGCAC 3') at an annealing temperature of 58°C for 1 min and gave the expected band size of approximately 789 bp. This confirms that the pCAMGpro recombinant plasmid was formed from the pCAMBIA1305.2 backbone containing the GUS *Plus* gene (Figure 3).

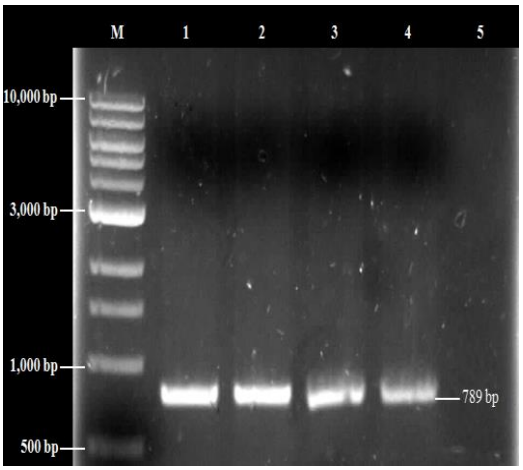


Figure 3 Gel electrophoresis (1% w/v) shows screening of pCAMGpro by amplification of the *β-glucuronidase* gene. Lane 1–4: GUS*Plus* gene fragment (789 bp), Lane 5: Negative control. M; 1Kb ladder

3.2 Bionformatic Analysis and Phylogenetic Tree

The present study shows amplified sequence was at highest query coverage (98%) and sequence homology to *A. sativa* (AsGlo1) gene, promoter region (Acc: AY795082.1) and *A. sativa* 12S α-globulin seed storage protein gene, complete cds (Acc: J05485.1) (Table 1).

Table 1 BLAST results closest to AsGpro obtained from NCBI

Accession Number	Name of sequences	Query Coverage	Maximum Identity
AY795082.1	*A. sativa* (Glo1) gene, promoter region	98%	99%
J05485.1	*A. sativa* 12S α-globulin seed storage protein gene, complete cds	98%	96%
X68648.1	*A. sativa* pseudogene for 12S seed α-globulin	61%	86%

The optimal tree with the sum of branch length = 0.53005192 is shown in Figure 4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsentein, 1985).

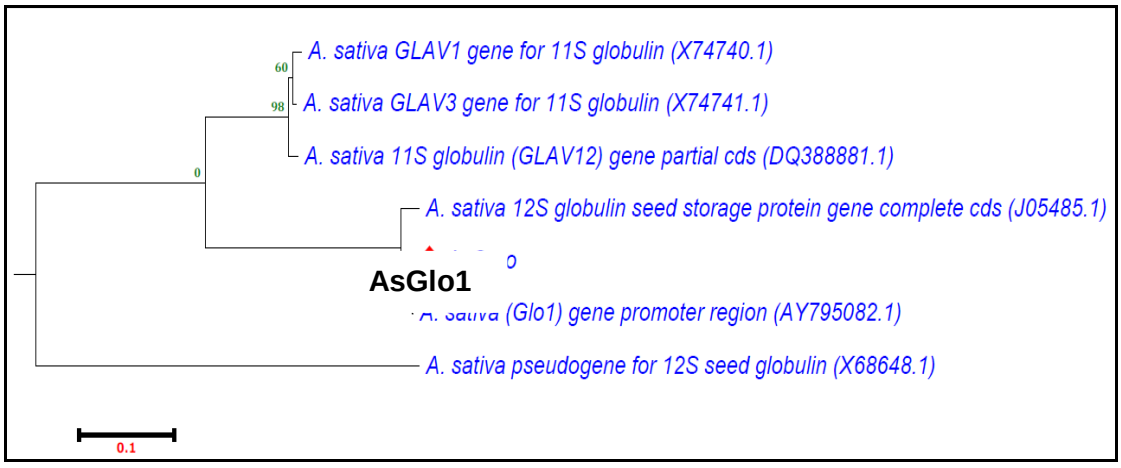


Figure 4 Evolutionary relationship of AsGlo1 fragment and six other taxa

From Table 2, the pairwise distance alignment shows AsGlo1 promoter is a closest relative to the *A. sativa* α-globulin (AsGlo1) gene promoter region (Gene Bank Accession Number: AY795082.1) with a pairwise distance of 0.003. The sequences were divided into two broad groups, 11S and 12S globulin families. This confirms that *A. sativa* α-globulin (AsGlo1) gene promoter (AY795082.1) region is the source of the targeted promoter and shows that our present studies successfully managed to clone the right fragment of AsGlo1 promoter. The alignment also confirms the AsGlo1 promoter belongs to the 12S

globulin protein family, which is the closest relative of the AsGlo1. The second relative of the α-globulin promoter is *Avena sativa* 12S α-globulin seed storage protein gene complete cds (Gene Bank Accession Number: J05485.1) with a pairwise distance of 0.034. This further confirms the targeted AsGlo1 fragment formed one cluster, indicating their close relationship.

Table 2 The distance pairwise alignment of AsGlo1 and the six relatives

	1	2	3	4	5	6	7
A.sativa GLAVI gene for 11S globulin (X74740.1)		0.007	0.005	1.586	0.661	0.662	0.686
A.sativa (GLAVI2) gene partial cds (DQ388881.1)	0.026		0.005	1.547	0.693	0.694	0.718
A.sativa GLAV3 gene for 11S globulin (X74740.1)	0.013	0.015		1.573	0.667	0.668	0.692
A.sativa pseudogene for 12S seed globulin (X68648.1)	0.687	0.676	0.683		1.837	1.841	1.909
AsGpro	0.314	0.325	0.320	0.797		0.002	0.009
A.sativa (Glo1) gene promoter region (AY795082.1)	0.316	0.325	0.321	0.795	0.003		0.009
A.sativa 12S globulin seed storage protein gene complete cds (J05485.1)	0.317	0.326	0.323	0.816	0.034	0.031	

■4.0 CONCLUSION

The new recombinant plasmid, pCAMGpro (~12,870 bp) together with any gene of interest can be used for high expression of any protein of interest in the endosperm of cereal crops. Endosperm is the most valuable plant-based production system for the production of recombinant proteins over other tissues because it is more cost-effective, it is easier to scale-up agricultural yield, provides a larger storage ability and safe long-term storage.

Acknowledgement

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