JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

A *Vigna radiata* 8S Globulin α′ Promoter Drives Efficient Expression of GUS in *Arabidopsis* Cotyledonary Embryos

Mo-Xian Chen,[‡,§] Yue-Ning Yang,[†,§] Shu-Xiao Zheng,[‡] Chao Xu,[†] Ying Wang,[†] Jie-Sheng Liu,[†] Wei-Dong Yang,[†] Mee-Len Chye,[*,‡] and Hong-Ye Li[*,†]

[†]College of Life Science, Jinan University, Guangzhou 510632, China

[‡]School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China

ABSTRACT: Plants are proven effective bioreactors for the production of heterologous proteins including those desired by the biopharmaceutical industry. However, the potential of plants as bioreactors is limited by the availability of characterized plant promoters that can drive target gene expression in relatively distant plant species. Seeds are ideal for protein storage because seed proteins can be kept stably for several months. Hence, a strong promoter that can direct the expression and accumulation of target proteins within seeds represents a powerful tool in plant biotechnology. Toward this end, an effort was made to identify such a promoter from *Vigna radiata* (mung bean) to drive expression in dicot seeds. A 784-bp 5′-flanking sequence of the gene encoding the 8S globulin α′ subunit (8SGα′) of the *V. radiata* seed storage protein was isolated by genome walking. When the 5′-flanking region was analyzed with bioinformatics tools, numerous putative *cis*-elements were identified. The Green Fluorescent Protein (GFP) regulated by this promoter was observed to be transiently expressed in protoplasts derived from *V. radiata* cotyledons. Finally, transgenic *Arabidopsis* plants expressing the β-glucuronidase (GUS) reporter gene driven from the 8S globulin α′ promoter showed strong GUS expression in transgenic embryos in both histochemical and quantitative GUS assays, confirming high expression within seeds. Therefore, the *V. radiata* 8S α′ promoter has shown potential in directing expression in seeds for bioreactor applications.

KEYWORDS: *embryo, genome walking, protoplast, seed, Vigna radiata*

INTRODUCTION

Plants are attractive and promising low-cost bioreactors for the expression of heterologous proteins because they do not present risks in endogenous pathogen contamination.[1] Both nuclear[2] and plastid transformation[3,4] have been explored in using plants as bioreactors. Protein expression can be targeted to various organs, such as tubers and seeds, which naturally accumulate large amounts of proteins and confer stable storage.[5] Many valuable products such as polypeptide antibodies, biopharmaceuticals, and edible vaccines have been successfully expressed using this approach.[6−8] For example, insulin production could be directed to soybean seed storage vacuoles by the use of a *Coix lacryma-jobi* cotyledonary vacuolar signal peptide and the tissue-specific promoter of the gene encoding the sorghum seed storage protein, gamma-kafirin.[9] However, some problems have emerged when using plants as bioreactors, mainly related to low productivity and instability of the heterologous proteins.[10] Therefore, the identification, characterization, and development of strong promoters will be invaluable for more efficient transcription of heterologous genes, especially across monocots and dicots.

Although numerous plant promoters including several constitutive promoters and inducible promoters have been identified, the most common promoter used for ectopic expression in transgenic plants is, too often, the nonplant *35S* promoter from the Cauliflower Mosaic Virus. It is powerful in driving heterologous protein expression, but as a constitutive promoter, the product is expressed at every developmental stage in all cells throughout the life-cycle of the plant. Heterologous proteins have been expressed from the *35S* promoter in many plants including the model plant tobacco[11] and crops such as potato[12] and lettuce.[13] To avoid unnecessary heterologous gene expression and any potential adverse effects arising, for example, in the competition for nutrients during plant growth, it is deemed essential to identify new promoters, which can confer both strong and tissue-targeted expression.[14]

Seeds have been proven an ideal storage venue for target proteins, and the identification of promoters that display high expression within seeds, even if confined to embryos or endosperm, would be of great potential in biotechnology.[10] Reports have revealed that single-chain antibodies produced in seeds of rice and wheat can retain high biological activities even after several years of storage.[15] Furthermore, the trait was stably inherited in subsequent generations.[16] In our study[17] on the mung bean storage proteins, we had found that the 8S globulin could accumulate to high levels within the seed, implying that its gene promoter targets expression within seeds. Along this line of investigation, the 5′-flanking sequence in each of three 8S globulin subunits was identified.[17] Previously, the 5′-flanking sequence of a related promoter from the β-subunit of the 8S globulin had been described, and results from transient expression in mung bean cotyledonary cells had identified it to be a potential seed-specific promoter of moderate promoter strength.[17] Promoters of the other subunit genes including the α and α′ subunits of 8S globulin have not yet been characterized. In

Received: April 9, 2013
Revised: June 13, 2013
Accepted: June 13, 2013
Published: June 13, 2013

the current study, given our experience in the use of the green fluorescent protein (GFP)[18−23] and β-glucuronidase (GUS),[24−27] we have used these reporters to investigate gene regulation from the 5′-flanking region of the gene encoding the α′ subunit of the 8S globulin (8SGα′).

MATERIALS AND METHODS

Plant Materials. Mung bean (*Vigna radiata* L.) and *Arabidopsis thaliana* (ecotype Columbia-0) were grown in a growth chamber under 8 h dark (21 °C) and 16 h light (23 °C) cycles. *Arabidopsis* seeds were surface sterilized and germinated on MS (Murashige and Skoog) medium supplemented with 50 μg/mL kanamycin. They were cold stratified for 4 days at 4 °C in the dark and then incubated in a tissue culture room at 21 °C under continuous light for 2−3 weeks. Seedlings were transplanted to plastic pots containing steam-sterilized soil and raised in a growth chamber under a 23 °C 16 h light/21 °C 8 h dark regime. *Arabidopsis* was transformed using the floral dip method.[28] Tissues were harvested and subsequently stained using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, cyclohexylammonium salt (X-Gluc) (Invitrogen, Carlsbad, USA) in histochemical staining assays or subject to quantitative assays for GUS activity.[29]

Plant Protein Extraction and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Total proteins were extracted from developing mung bean cotyledons and separated using 10% SDS-PAGE, followed by Coomassie Brilliant Blue staining for protein visualization. Protein densitometer analysis was carried out using software Gelpro32 integrated in the Gel Documentation System WD-9413B (Liuyi, China). Protein identification was determined using a tandem mass spectrometer (MS/MS).

DNA Extraction and Genome Walking. DNA from mung bean was extracted from leaves as previously described.[30] The plant tissues were ground in grinding buffer from a DNA extraction kit (Takara, Japan) according to the manufacturer's manual. The concentration of the DNA was determined by spectrophotometry and agarose gel analysis.

The 5′-flanking sequence of the gene encoding the mung bean 8S globulin α′ subunit (8SGα′) was amplified by Polymerase Chain Reaction (PCR)-based genome walking using reverse primers: P4 (5′-TGAGTTGGGTTGGGGTATTAGAGTA-3′), P5 (5′-ACGATGCC-GAAGGAGACAGAAAGTG-3′), and P6 (5′-CTGAATAGAGTGCG-GAACCACCTGT-3′) according to the 8SGα′ cDNA sequence (GenBank accession EF990626), and degenerated "adaptor primer (AP)" primers provided in the Genome Walking Kit (Takara, China). Amplification procedures were as follows: first PCR, 94 °C 1 min, 98 °C 40 s, 94 °C 30 s, 65 °C 1 min, 72 °C 2 min, 5 cycles; 94 °C 30 s, 25 °C 3 min, 72 °C 2 min, 94 °C 30 s, 65 °C 1 min, 72 °C 2 min, 94 °C 30 s, 65 °C 1 min, 72 °C 2 min, 15 cycles; 94 °C 30 s, 44 °C 1 min, 72 °C 2 min, 72 °C 10 min. Second and third PCR were carried out as follows: 94 °C 30 s, 65 °C 1 min, 72 °C 2 min, 94 °C 30 s, 65 °C 1 min, 72 °C 2 min, 94 °C 30 s, 44 °C 1 min, 72 °C 2 min, 15 cycles, 72 °C 10 min. The PCR product was cloned in vector pMD18-T (Takara) and confirmed by DNA sequence analysis.

Promoter Analysis. The 5′-sequence flanking 8SGα′ was subject to a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). The transcription start site and TATA box were predicted with software Softberry (http://www.softberry.com). Transcription elements were predicted using software PLACE (http://www.dna.affrc.go.jp/database/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Transient Expression of *8SGα′::GFP* in *V. radiata* Cotyledonary Protoplasts. Generation of cotyledonary protoplasts transiently expressing fusion *p8SGα′::GFP* consisting of *GFP* driven from the 8SGα′ promoter was carried out by polyethylene glycol-calcium transfection[31] using plasmid *p8SGα′::GFP*, a pBI121 (Clontech) derivative, obtained by replacing *35S::GUS* with *8SGα′::GFP*. The control used in transient expression studies was a pBI121 derivative in which *GUS* was replaced with *GFP*. The overnight protoplast culture was selected and observed under an LSM 510 META laser-scanning confocal microscope (Zeiss, Germany).

Generation of Transgenic *Arabidopsis* Expressing *8SGα′::GUS*. *Agrobacterium tumefaciens* strain LBA4404 was transformed by electroporation with plasmid *p8SGα′::GUS*. Plasmids pBI121 (Clontech) and pBI101 (Clontech) were used as positive and negative controls, respectively. These constructs were introduced into *A. thaliana* by *Agrobacterium*-mediated "floral dip" transformation.[28] The primary transformants (T_0) were grown in a growth chamber until seed set. Seeds were collected, surface-sterilized, and then germinated on MS-medium supplemented with kanamycin (50 μg/mL). Putative T_1 transformants showing dark-green true leaves on kanamycin-resistant medium were confirmed by PCR using 8SGα′ promoter-specific primers. These PCR-verified seedlings were then planted in soil for self-fertilization to obtain the T_2 generation. In the T_2 generation, an average of three to five independent lines per construct, all harboring single-copy inserts that displayed a simple Mendelian 3:1 segregation ratio to kanamycin, were selected. Seeds from each line were collected, and seedlings from the T_3 population that were 100% kanamycin-resistant were deemed homozygous and used for subsequent analysis.

Histochemical and Fluorometric Assays of GUS Activity. GUS histochemical staining using *Arabidopsis* tissues was carried out as previously described.[29] Plant material was harvested and incubated in GUS histochemical X-Gluc standard buffer (100 mM sodium phosphate, pH 7.5, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 0.1% (v/v) Triton X-100, 1 mg/mL X-Gluc). The sample was vacuum infiltrated in GUS staining solution for 1 h, then kept at 37 °C for 2 h to overnight, depending on the intensity of the staining desired. Tissue chlorophyll was removed with several changes of 70% ethanol and incubation at 65 °C. Stained samples were analyzed and photographed.

Arabidopsis mature seeds were collected for analysis of GUS activity by using 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as the reaction substrate.[29] GUS activity was calculated as picomoles of hydrolyzed 4-MUG per mg protein per minute. Three biological replicates were tested for each line, and two independent experiments were performed.

RESULTS

SDS-PAGE Analysis of Mung Bean Protein. Total protein from developing mung bean cotyledons was resolved on 10% SDS-PAGE. A predominant band of 48 kD was observed (Figure 1), accounting for approximately 70% of the total soluble protein according to densitometer analysis. This major band was excised, and the peptides were determined by MS/MS. The results identified the band as mung bean 8S globulin.

Genome Walking To Obtain the 5′-Sequence Flanking *8SGα′*. As shown in Figure 2, in the third round of genome

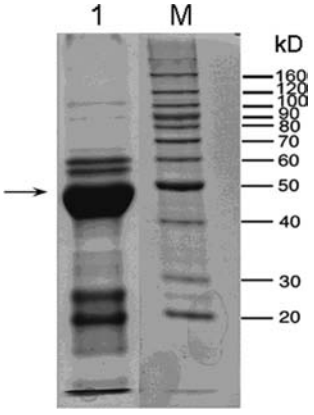

Figure 1. Gel electrophoresis of total protein from developing mung bean cotyledons. Lane 1, total protein from developing mung bean cotyledons was separated by SDS-PAGE and stained with Coomassie Brilliant Blue; lane M, low-range SDS-PAGE molecular weight standards (Bio-Rad). Arrow indicates the 8S globulin band of 48 kD.

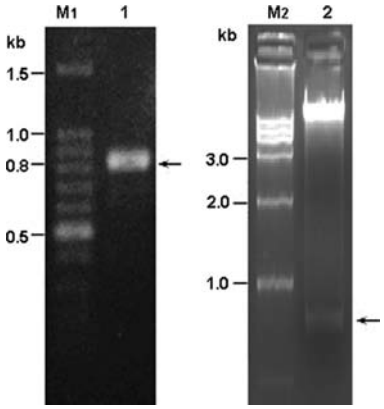

Figure 2. The *8SGα′* 5′-flanking sequence cloned from genome walking and *Hin*dIII-*Bam*HI restriction endonuclease analysis of construct *p8SGα′::GFP*. Left panel, agarose gel electrophoresis following PCR on 1% agarose gel. Lane M1: DNA marker "100-bp ladder". Lane 1: The PCR band corresponding to 0.8 kb from the third round of genome walking is shown (arrowhead denotes the putative *8SGα′* fragment). Lane M2: DNA marker, "1-kb plus". Lane 2: Plasmid *p8SGα′::GFP* digested with *Hin*dIII+*Bam*HI showing the insert containing the 0.8-kb *8SGα′* fragment (denoted by an arrow) in the right panel.

walking, the putative *8SGα′* 5′-flanking sequence was amplified from the mung bean DNA template and appeared as a 0.7-kb band on 1% agarose gel electrophoresis. The PCR product, subsequently cloned into the pMD18-T vector, was confirmed by DNA sequence analysis.

The *8SGα′* 5′-flanking sequence consists of a 784-bp region upstream of the start codon. This sequence has been submitted to GenBank (accession no. HQ214071). A BLAST search (GenBank) revealed that the 245-bp portion at its 3′-end shares 85% similarity to two other 5′-flanking sequences of genes from *Phaseolus vulgaris* encoding *α*- and *β*-phaseolin (GenBank accession nos. X52626 and J01263, respectively). These three sequences, including their predicted "start codons" (ATG), are aligned in Figure 3. Their sequences adjacent to the start codon show highest similarity.

The *8SGα′* 5′-flanking sequence contains many A/T repeats, which account for more than 58.5% of the total nucleotides within the 784-bp region examined. It has been suggested that AT-rich sequences correspond to the regions where transcription factors bind.[32] Putative *cis*-elements were identified and analyzed using several promoter analysis software including PlantCARE, PLACE, and Softberry. As shown in Figure 4, this fragment contains common core promoter elements such as the TATA box and seven putative CAAT boxes. Besides these, it also possesses *cis*-elements usually represented in the 5′-flanking regions of genes encoding seed proteins, such as the RY-element, which binds to a family of transcription factors with a B3 domain,[33] endosperm-specific *cis*-regulatory elements such as the Skn-1 motif, which is required for high expression in the endosperm in cooperative interaction with other motifs,[34] the CGTCA-motif responsive to methyl jasmonate, and circadian *cis*-regulatory elements[35] such as the G-Box, as well as Box I involved in light responsiveness.[36,37] This isolated fragment flanking the mung bean *8SGα′* should be subject to future tests for promoter strength, spatial expression, and inducibility to verify the potential function of each putative *cis*-element.

Spatial Expression of *8SGα′*. Expression vectors, *p8SGα′::GFP* and *p8SGα′::GUS*, were constructed (Figure 5) to investigate the spatial expression of *8SGα′*. We were interested to test if the *8SGα′* 5′-flanking region can be used to direct the

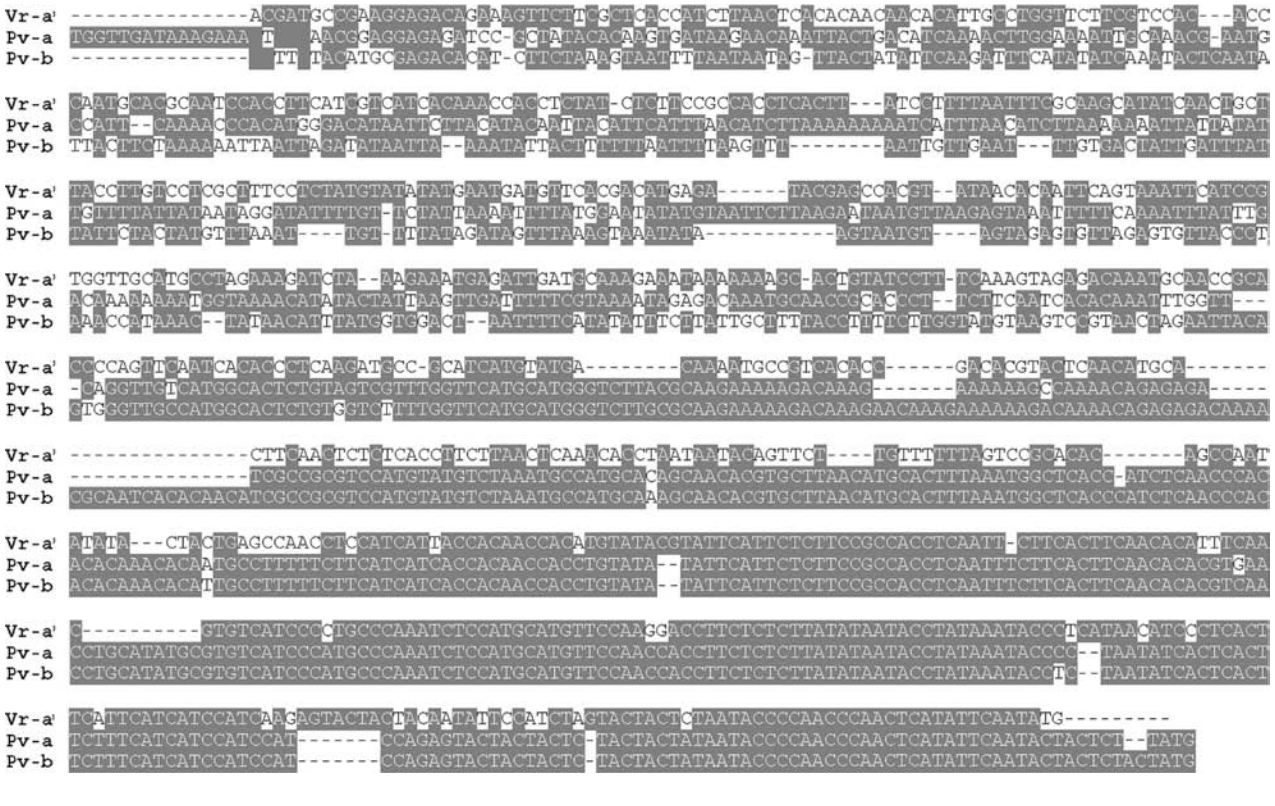

Figure 3. Alignment of the 5′-sequences flanking mung bean *8SGα′* and the genes encoding *Phaseolus vulgaris α*- and *β*-phaseolin. Vr-a′, *8SGα′*; Pv-a, *P. vulgaris α*-phaseolin; Pv-b, *P. vulgaris β*-phaseolin. The last "ATG" shown in each sequence marks the start codon for each peptide.

dx.doi.org/10.1021/jf401537q | *J. Agric. Food Chem.* 2013, 61, 6423−6429

```
-706    ACGATGCCGA AGGAGACAGA  AAGTTCTTCG CTCACCATCT TAACTCACAC AACAACACAT TGCCTGGTTC
-636    TTCGTCCACA CCCAATGCAC GCAATCCACC TTCATCGTCA TCACAAACCA CCTCTATCTC TTCCGCCACC
                              CAAT-box       Skn-1-motif  CGTCA-motif
-566    TCACTTATCC TTTTAATTTC GCAAGCATAT CAACTGCTTA CCTTGTCCTC GCTTTCCTCT ATGTATATAT
                                 CRE      MBS
-496    GAATGATGTT CACGACATGA GATACGAGCC ACGTATAACA CAATTCAGTA AATTCATCCG TGGTTGCATG
                                        G-Box        CAAT-box
-426    CCTAGAAAGA TCTAAAGAAA TGAGATTGAT GCAAAGAAAT AAAAAAAGCA CTGTATCCTT TCAAAGTAGA
                                                                  Box I
-356    GACAAATGCA ACCGCACCCC AGTTCAATCA CACCCTCAAG ATGCCGCATC ATGTATGACA AAATGCCGTC
                CAAT-box                    CAAT-box                    CGTCA-motif
-286    ACACCGACAC GTACTCAACA TGCACTTCAA CTCTCTCACC TTCTTAACTC AAACACCTAA TAATACAGTT
               G-Box
-216    CTTGTTTTTT AGTCCGCACA CAGCCAATAT ATACTACTGA GCCAACCTCC ATCATTACCA CAACCACATG
                                  CAAT-box                                  CANNTG
-146    TATACGTATT CATTCTCTTC CGCCACCTCA ATTCTTCACT TCAACACATT TCAACGTGTC ATCCCCTGCC
                                        CAAT-box              ABRE  Skn-1-motif
-76     CAAATCTCCA TGCATGTTCC AAGGACCTTC TCTCTTATAT AATACCTATA AATACCCTCA TAACATCCCT
        CAAT-box  RY-element                          TATA-box
-6      CACTTCATTC ATCATCCATC AAGAGTACTA CTACAATATT CCATCTAGTA  CTACTCTAAT  ACCCCAACCC
              +1 Transcription start
65       AACTCATATT CAATATG
                        Start codon
```

Figure 4. Putative *cis*-elements of the 5′-flanking sequence of the gene encoding mung bean *8SGα′*. The putative *cis*-elements are boxed (CAAT-box, Skn-1-motif, MBS, G-box, Box I, CGTA-motif, CANNTG, RY-element, TATA-box). The transcriptional start site is indicated with +1, and the last "ATG" (underlined) denotes the start codon.

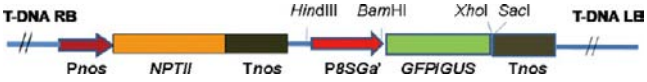

Figure 5. Schematic map of expression vectors *p8SGα′::GFP* and *p8SGα′::GUS*. The constructs were derived from pBI121. Elements and restriction sites between the T-DNA RB (right border) and LB (left border) region are shown. P*nos*, T*nos*, promoter and terminator, respectively, of the gene encoding nopaline synthase (*nos*); *P8SGα′*, promoter of *8SGα′*; *NPTII*, the gene encoding neomycin phosphotransferase; *GFP*, the gene encoding the green fluorescent protein; *GUS*, the gene encoding *β*-glucuronidase. Unique restriction sites including *Hin*dIII, *Bam*HI, *Xho*I, and *Sac*I are marked.

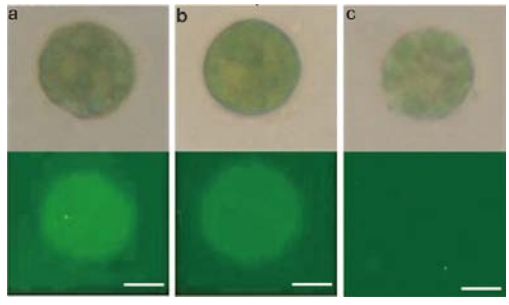

Figure 6. Transient GFP expression driven by the *8SGα′* promoter in mung bean cotyledonary protoplasts. Images were captured by fluorescent microscopy. (a) Protoplast transfected with *8SGα′::GFP*; (b) protoplast transfected with *35S::GFP*; and (c) untransfected control. Top panel shows bright view, and bottom panel represents the green fluorescence channel. Bar = 10 *μ*m.

expression of foreign proteins in seeds. Transient expression in mung bean cotyledon protoplasts (Figure 6) revealed that GFP expression from the *8SGα′* promoter was comparable to the *35S* promoter, indicating its effective promoter activity.

To further examine the spatial expression of the *8SGα′* promoter, *p8SGα′::GUS* transgenic *Arabidopsis* plants were obtained. GUS histochemical assays showed that GUS was abundantly expressed in both developing and mature embryos, but not in the mature seed coat (endosperm and testa layer) (Figure 7a–d). GUS was also detected in other plant tissues such as seedling roots (Figure 7e and f), leaf vasculature (Figure 7g), stems (Figure 7h), flowers (Figure 7i), and silliques (Figure 7j), albeit at lower levels. Taken together, these results indicate that, although other parts of the plant showed some GUS expression, the expression of the *8SGα′* promoter was predominantly directed to the embryo during seed maturation.

To further confirm strong transcriptional activity of the *8SGα′* promoter within seeds, both GUS histochemical staining and enzyme activity quantification were performed on freshly harvested *Arabidopsis* seeds (Figure 8). The *8SGα′::GUS* expressing plants showed high GUS activity in histochemical staining (Figure 8a) and quantitative assays (Figure 8b). Vector-transformed seeds were used as a negative control, while *35S::GUS* transgenic seeds served as a positive control (Figure 8a). In comparison to the constitutive *35S* promoter, the *8SGα′* promoter displayed slightly lower activity than *35S::GUS*, whereas the pBI101 (negative vector control) transgenic plant seeds exhibited near zero activity (Figure 8b).

dx.doi.org/10.1021/jf401537q | *J. Agric. Food Chem.* 2013, 61, 6423–6429

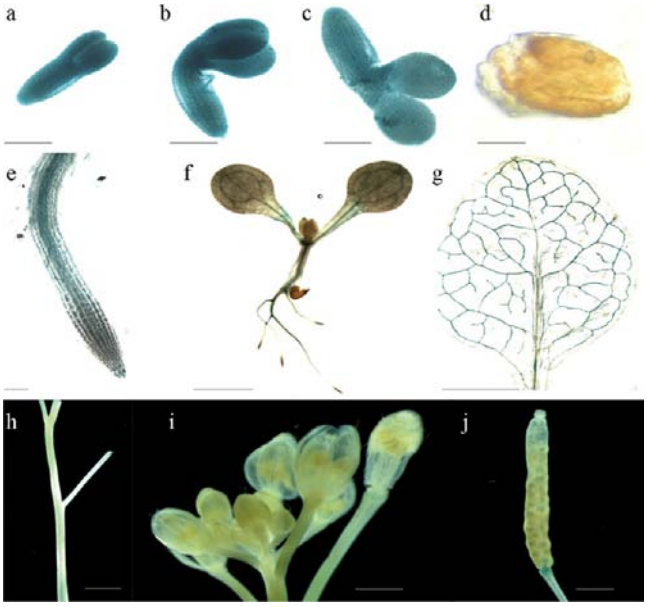

Figure 7. Expression analysis of *8SGα′::GUS* in transgenic *Arabidopsis*. Plant tissues were subject to GUS staining for 2 h and then photographed. (a) Embryo in midtorpedo stage. (b) Embryo in bent-cotyledonary stage. (c) Mature embryo. (d) Seed coat of mature embryo. (e) Root tip. (f) 7-day-old seedling. (g) 4-week-old rosette leaf. (h) Stems. (i) Flowers. (j) Siliques. Scale bar = 20 mm (a)−(e), 5 mm (f), 1 cm (g), 1 mm (h)−(j).

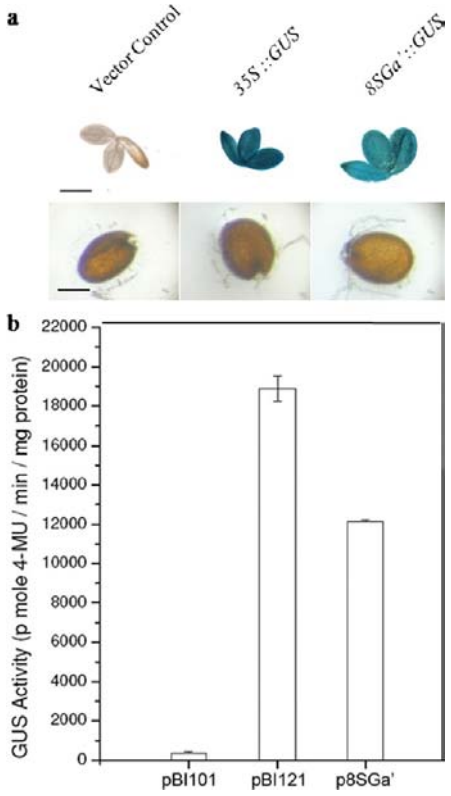

Figure 8. GUS staining and activity measurement of freshly harvested seeds. (a) Freshly harvested seeds of transgenic *Arabidosis* plants were separated into two parts, the embryo and seed coat, and subsequently stained for 12 h. Scale bar = 20 mm. (b) Mature seeds (20 mg) of transgenic *Arabidopsis* including vector control (pBI101), *35S::GUS* (pBI121), and *8SGα′::GUS* (p8SGα′) lines were used in GUS quantification. The bars in histogram are means (±SD) of three biological replicates.

DISCUSSION

Mung bean is a legume, and its seeds are particularly rich in proteins. The accumulation of 8S globulin was predicted at 70% of total soluble protein fraction in developing cotyledons (Figure 1), confirming its temporal and spatial expression of 8S globulin in seeds. Transient *GFP* expression and transgenic *GUS* expression demonstrated that the mung bean *8SGα′* promoter could drive the expression of heterologous genes (*GFP* and *GUS*) within the embryo of a dicot such as *Arabidopsis*. Also, quantification of GUS activity in transgenic *Arabidopsis* seeds provided evidence that the *8SGα′* promoter is a strong promoter that is comparable to the constitutive CaMV *35S*. Fu et al.[38] introduced a synthetic promoter to efficiently confine expression in plant embryos. Here, we suggest that the naturally occurring mung bean *8SGα′* promoter is a potentially useful promoter for directing expression of biopharmaceuticals in embryos.

A strong and selectively activated promoter has very significant applications in bioengineering, especially in enhancing the efficiency of a bioreactor. The promoter of *8SGα′*, a gene encoding a seed storage protein, can greatly satisfy the bioengineering needs in heterologous protein expression in embryos. Major advantages of such an expression system include reduction not only in production cost but also in problems arising from cold storage, transportation, end-product purification, and aseptic delivery associated with conventional vaccines.[39]

Few dicot seed promoters have been studied in comparison to monocots. One of the most successful examples among dicot promoters characterized is from the bean seed storage protein gene, *β*-phaseolin (*phas*).[40] It is exclusively expressed in seed tissues in beans[40] and is able to drive expression in transgenic tobacco and *Arabidopsis* seeds.[41] By using this well-characterized promoter fragment together with proper sorting signals, it was possible to drive high-level expression of heterogeneous protein up to 36.5% of total soluble proteins in transgenic *Arabidopsis* seeds.[7]

A comparison among the mung bean *8SGα′* promoter and seed promoters from other species showed high nucleotide sequence similarity among them, especially within the last 300-bp proximal region including the transcription start site and the 5′-UTR sequences (Figure 3), suggesting some universality. According to the alignment results, the 5′-flanking sequence (−140/+80) of the mung bean *8SGα′* shares over 85% sequence similarity with the −180/+78 region of the *β*-phaseolin promoter *phas*. Within the proximal 295-bp region of the *phas* promoter, over 20 motifs have been verified to possess binding activity through dimethyl sulfate in vivo footprinting.[42] Among the *cis*-elements, some conferred spatial expression in embryos.[41] Moreover, two CANNTG motifs had been demonstrated to significantly influence *phas* promoter activity either positively (−163/−158, CACCTG) or negatively (−100/−95, CATATG).[43] Similar sequences could be located in the −536/−531 and −152/−147 regions of the mung bean *8SGα′* promoter (Figure 4).

Spatial and temporal expression studies showed that *8SGα′* is strongly expressed in seed embryonic tissues. In comparison to *35S::GFP*, the *8SGα′::GFP* construct showed relatively high expression of GFP in mung bean cotyledonary protoplasts (Figures 5 and 6). The *8SGα′* promoter was expressed in early torpedo staged embryos to mature embryos (Figure 7a−c). Despite GUS staining in other tissues (Figure 7e−j), GUS

dx.doi.org/10.1021/jf401537q | J. Agric. Food Chem. 2013, 61, 6423−6429

transcriptional activity in *Arabidopsis* seeds from the promoters of the mung bean *8SGα′* and the bean *β*-phaseolin (*phas*) was comparable to the *35S* promoter (Figure 8a and b). Likely, conservation in homology within their 5′-flanking sequence was attributed to high expression in seed embryos.

Besides the 300-bp proximal fragment, sequence analysis of the 5′-flanking region of *8SGα′* also revealed the presence of other putative upstream elements such as the G-box and Box I, which are related to light signaling.[44] These motifs generally bind phytochrome interacting factors, which can efficiently upregulate expression under dark conditions.[45] Therefore, it is likely that the promoter is induced by shaded light (refers to light that consists of a ratio between red light and far red light of less than 1).[46] The conserved RY-element was also noted, and it has been previously identified to play a critical role in activating transcription of the gene encoding the *Arabidopsis* seed-specific oilbody protein, oleosin.[47] Moreover, the presence of the W-box and the ABRE box implies that defense signals such as salicylic acid, jasmonic acid, or abscisic aicd may regulate gene expression as in the case of *Arabidopsis AtTRXh5*[48] and *AtRD29A*.[49] Such possibilities in its inducible expression will greatly enhance its practical value when applied to plant bioreactors. However, further analysis is essential to address the detailed functions and precise regulatory mechanisms of the *8SGα′* promoter. These results are expected to provide valuable strategies in the use of this promoter for plant biotechnology.

To conclude, the identification of the *8SGα′* promoter offers a first step toward its potential use for the expression of foreign proteins in plant bioreactors. This will greatly differ from the conventional strategy in using the soybean seed expression system, which depends on the use of targeting sequences to localize the protein into a specific compartment, for example, the storage vacuole to achieve accumulation of the heterologous protein. Instead, a promoter that is expressed in seed provides a more cost-effective way in enhancing temporal and spatial expression of a protein.

AUTHOR INFORMATION

Corresponding Author

*Tel.: 86-20-85228470 (H.-Y.L.); 852-22990319 (M.-L.C.). E-mail: thyli@jnu.edu.cn(H.-Y.L.); mlchye@hkucc.hku.hk (M.-L.C.).

Author Contributions

§These authors contributed equally.

Funding

We thank the Guangdong Natural Science Foundation (No.20094401120010), SRFDP (No. 9151063101000001), and the Wilson and Amelia Wong Endowment Fund for supporting this project. M.-X.C. and S.-X.Z. received research postgraduate studentships from the University of Hong Kong.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Sala, F.; Manuela Rigano, M.; Barbante, A.; Basso, B.; Walmsley, A. M.; Castiglione, S. Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. *Vaccine* **2003**, *21*, 803−808.

(2) Li, H. Y.; Ramalingam, S.; Chye, M. L. Accumulation of recombinant SARS-CoV spike protein in plant cytosol and chloroplasts indicate potential for development of plant-derived oral vaccines. *Exp. Biol. Med.* **2006**, *231*, 1346−1352.

(3) Lee, M. Y. T.; Zhou, Y. X.; Lung, R. W. M.; Chye, M. L.; Yip, W. K.; Zee, S. Y.; Lam, E. Expression of viral capsid protein antigen against Epstein-Barr virus in plastids of *Nicotiana tabacum* cv. SR1. *Biotechnol. Bioeng.* **2006**, *94*, 1129−1137.

(4) Zhou, Y. X.; Lee, M. Y. T.; Ng, J. M. H.; Chye, M. L.; Yip, W. K.; Zee, S. Y.; Lam, E. A truncated hepatitis E virus ORF2 protein expressed in tobacco plastids is immunogenic in mice. *World J. Gastroenterol.* **2006**, *12*, 306−312.

(5) Vianna, G. R.; Cunha, N. B.; Murad, A. M.; Rech, E. L. Soybeans as bioreactors for biopharmaceuticals and industrial proteins. *Genet. Mol. Res.* **2011**, *10*, 1733−1752.

(6) Daniell, H.; Streatfield, S. J.; Wycoff, K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci.* **2001**, *6*, 219−226.

(7) De Jaeger, G.; Scheffer, S.; Jacobs, A.; Zambre, M.; Zobell, O.; Goossens, A.; Depicker, A.; Angenon, G. Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nat. Biotechnol.* **2002**, *20*, 1265−1268.

(8) Ma, J. K.; Chikwamba, R.; Sparrow, P.; Fischer, R.; Mahoney, R.; Twyman, R. M. Plant-derived pharmaceuticals − the road forward. *Trends Plant Sci.* **2005**, *10*, 580−585.

(9) Cunha, N. B.; Araujo, A. C.; Leite, A.; Murad, A. M.; Vianna, G. R.; Rech, E. L. Correct targeting of proinsulin in protein storage vacuoles of transgenic soybean seeds. *Genet. Mol. Res.* **2010**, *9*, 1163−1170.

(10) Li, H. Y.; Jiang, L. W. Seeds as bioreactors for producing biopharmaceuticals. In *Floriculture, Ornamental and Plant Biotechnology*, 1st ed.; Teixeira da Silva, J. A., Ed.; Global Science Books, Ltd.: London, UK, 2006; Vol. *IV*, pp 30−34.

(11) Fung, K. L.; Zhao, K. J.; He, Z. M.; Chye, M. L. Tobacco-expressed *Brassica juncea* Chitinase BjCHI1 shows antifungal activity in vitro. *Plant Mol. Biol.* **2002**, *50*, 283−294.

(12) Chye, M. L.; Zhao, K. J.; He, Z. M.; Ramalingam, S.; Fung, K. L. An agglutinating Chitinase with two chitin-binding domains confers fungal protection in transgenic potato. *Planta* **2005**, *220*, 717−730.

(13) Xu, Z. F.; Qi, W. Q.; Ouyang, X. Z.; Yeung, E.; Chye, M. L. A proteinase inhibitor II of *Solanum americanum* is expressed in phloem. *Plant Mol. Biol.* **2001**, *47*, 727−738.

(14) Romero, C.; Belles, J. M.; Vaya, J. L.; Serrano, R.; Culianez-Macia, F. A. Expression of the yeast *trehalose-6-phosphate >synthase* gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta* **1997**, *201*, 293−297.

(15) Stoger, E.; Ma, J. K.; Fischer, R.; Christou, P. Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotechnol.* **2005**, *16*, 167−173.

(16) BäUmlein, H.; Boerjan, W.; Nagy, I.; Bassfüner, R.; Van Montagu, M.; Inzé, D.; Wobus, U. A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants. *Mol. Gen. Genet.* **1991**, *225*, 459−467.

(17) Yang, Y. N.; Niu, Y. F.; Xu, C.; Wang, Y.; Liu, J. S.; Yang, W. D.; Li, H. Y. Characterization of upstream sequences from the 8S globulin gene of *Vigna radiata*. *Afr. J. Biotechnol.* **2011**, *10*, 11477−11482.

(18) Li, H. Y.; Chye, M. L. Membrane localization of *Arabidopsis* acyl-CoA binding protein ACBP2. *Plant Mol. Biol.* **2003**, *51*, 483−492.

(19) Li, H. Y.; Chye, M. L. *Arabidopsis* Acyl-CoA-binding protein ACBP2 interacts with an ethylene-responsive element-binding protein, AtEBP, via its ankyrin repeats. *Plant Mol. Biol.* **2004**, *54*, 233−243.

(20) Leung, K. C.; Li, H. Y.; Xiao, S.; Tse, M. H.; Chye, M. L. *Arabidopsis* ACBP3 is an extracellularly targeted acyl-CoA-binding protein. *Planta* **2006**, *223*, 871−881.

(21) Chen, Q. F.; Xiao, S.; Chye, M. L. Overexpression of the Arabidopsis 10-kilodalton acyl-coenzyme A-binding protein ACBP6 enhances freezing tolerance. *Plant Physiol.* **2008**, *148*, 304−315.

(22) Gao, W.; Xiao, S.; Li, H. Y.; Tsao, S. W.; Chye, M. L. *Arabidopsis thaliana* acyl-CoA-binding protein ACBP2 interacts with heavy-metal-binding farnesylated protein AtFP6. *New Phytol.* **2009**, *181*, 89−102.

(23) Xiao, S.; Gao, W.; Chen, Q. F.; Chan, S. W.; Zheng, S. X.; Ma, J.; Wang, M.; Welti, R.; Chye, M. L. Overexpression of *Arabidopsis* acyl-CoA binding protein ACBP3 promotes starvation-induced and age-dependent leaf senescence. *Plant Cell* **2010**, *22*, 1463−1482.

(24) Rawat, R.; Xu, Z. F.; Yao, K. M.; Chye, M. L. Identification of *cis*-elements for ethylene and circadian regulation of the *Solanum melongena* gene encoding cysteine proteinase. *Plant Mol. Biol.* **2005**, *57*, 629−643.
(25) Xiao, S.; Chye, M. L. Overexpression of Arabidopsis ACBP3 enhances NPR1-dependent plant resistance to *Pseudomonas syringe* pv *tomato* DC3000. *Plant Physiol.* **2011**, *156*, 2069−2081.
(26) Du, Z. Y.; Chen, M. X.; Chen, Q. F.; Xiao, S.; Chye, M. L. Overexpression of Arabidopsis acyl-CoA-binding protein ACBP2 enhances drought tolerance. *Plant Cell Environ.* **2013**, *36*, 300−314.
(27) Du, Z. Y.; Chen, M. X.; Chen, Q. F.; Xiao, S.; Chye, M. L. Arabidopsis acyl-CoA-binding protein ACBP1 participates in the regulation of seed germination and seedling development. *Plant J.* **2013**, *74*, 294−309.
(28) Clough, S. J.; Bent, A. F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **1998**, *16*, 735−743.
(29) Jefferson, R. A.; Kavanagh, T. A.; Bevan, M. W. Gus fusions: *β*-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **1987**, *6*, 3901−3907.
(30) Dellaporta, S. L.; Wood, J.; Hicks, J. B. A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* **1983**, *1*, 19−21.
(31) Yoo, S. D.; Cho, Y. H. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2007**, *2*, 1565−1572.
(32) Joazeiro, C. A.; Kassavetis, G. A.; Geiduschek, E. P. Alternative outcomes in assembly of promoter complexes: the roles of TBP and a flexible linker in placing TFIIIB on tRNA genes. *Genes Dev.* **1996**, *10*, 725−739.
(33) Fujiwara, T.; Beachy, R. N. Tissue-specific and temporal regulation of a beta-conglycinin gene: roles of the RY repeat and other *cis*-acting elements. *Plant Mol. Biol.* **1994**, *24*, 261−272.
(34) Rupert, P. B.; Daughdrill, G. W.; Bowerman, B.; Matthews, B. W. A new DNA-binding motif in the Skn-1 binding domain-DNA complex. *Nat. Struct. Biol.* **1998**, *5*, 484−491.
(35) Munoz, E.; Baler, R. The circadian E-box: when perfect is not good enough. *Chronobiol. Int.* **2003**, *20*, 371−388.
(36) Donald, R. G. K.; Cashmore, A. R. Mutation of Either G-Box or I-Box Sequences Profoundly Affects Expression from the *Arabidopsis rbcS-1A* Promoter. *EMBO J.* **1990**, *9*, 1717−1726.
(37) Xu, Z. F.; Chye, M. L.; Li, H. Y.; Xu, F. X.; Yao, K. M. G-box binding coincides with increased *Solanum melongena* cysteine proteinase expression in senescent fruits and circadian-regulated leaves. *Plant Mol. Biol.* **2003**, *51*, 9−19.
(38) Fu, H. H.; Brown, J. A.; Francis, K.; Song, H. S. Expression cassettes for embryo-specific expression in plants. WIPO Patent 2011067712, 2011.
(39) Daniell, H.; Singh, N. D.; Mason, H.; Streatfield, S. J. Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci.* **2009**, *14*, 669−679.
(40) Burow, M. D.; Sen, P.; Chlan, C. A.; Murai, N. Developmental control of the *β*-phaseolin gene requires positive, negative, and temporal seed-specific transcriptional regulatory elements and a negative element for stem and root expression. *Plant J.* **1992**, *2*, 537−548.
(41) Chandrasekharan, M. B.; Bishop, K. J.; Hall, T. C. Module-specific regulation of the *β*-phaseolin promoter during embryogenesis. *Plant J.* **2003**, *33*, 853−866.
(42) Li, G.; Hall, T. C. Footprinting in vivo reveals changing profiles of multiple factor interactions with the *β*-phaseolin promoter during embryogenesis. *Plant J.* **1999**, *18*, 633−641.
(43) Kawagoe, Y.; Campell, B. R.; Murai, N. Synergism between CACGTG (G-box) and CACCTG *cis*-elements is required for activation of the bean seed storage protein *β*-phaseolin gene. *Plant J.* **1994**, *5*, 885−890.
(44) Castillon, A.; Shen, H.; Huq, E. Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. *Trends Plant Sci.* **2007**, *12*, 514−521.
(45) Ni, M.; Tepperman, J. M.; Quail, P. H. PIF3, a phytochrome-interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix-loop-helix protein. *Cell* **1998**, *95*, 657−667.
(46) Jaillais, Y.; Chory, J. Unraveling the paradoxes of plant hormone signaling integration. *Nat. Struct. Mol. Biol.* **2010**, *17*, 642−645.
(47) Che, N.; Yang, Y.; Li, Y.; Wang, L.; Huang, P.; Gao, Y.; An, C. Efficient LEC2 activation of OLEOSIN expression requires two neighboring RY elements on its promoter. *Sci. China, Ser. C: Life Sci.* **2009**, *52*, 854−863.
(48) Laloi, C.; Mestres-Ortega, D.; Marco, Y.; Meyer, Y.; Reichheld, J. P. The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. *Plant Physiol.* **2004**, *134*, 1006−1016.
(49) Narusaka, Y.; Nakashima, K.; Shinwari, Z. K.; Sakuma, Y.; Furihata, T.; Abe, H.; Narusaka, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high-salinity stresses. *Plant J.* **2003**, *34*, 137−148.