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Construction and evaluation of a maize (*Zea mays*) chimaeric promoter with activity in kernel endosperm and embryo

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Chimaeric promoters contain DNA sequences from different promoters. Chimaeric promoters are developed to increase the level of recombinant protein expression, to precisely control transgene activity or to combat homology-based gene silencing. Sets of chimaeric promoters, each containing different lengths of DNA from maize (*Zea mays*) 27zn (27 kDa γ-zein) endosperm-specific promoter and the Glb1 (Globulin-1) embryo-specific promoter were created and tested in a transient expression assay of GFP (green fluorescent protein). Promoter fragments with the highest activity were combined to create the chimaeric promoter A27znGlb1. In the context of the chimaeric promoter, the selected Glb1 promoter fragment was necessary and sufficient to activate expression in embryo tissue and was functionally equivalent to the native Glb1 promoter. Similarly, the selected 27zn promoter fragment in the chimaeric promoter was necessary and sufficient to activate expression in endosperm tissue and was functionally equivalent to the native 27zn promoter. Maize transgenic plants containing the A27znGlb1 chimaeric promoter fused to GFP were produced to characterize this promoter *in vivo*. Quantitative reverse-transcriptase PCR was used to determine that the promoter was active in the embryo, endosperm, pericarp and immature leaf tissues. GFP activity in plants containing the chimaeric promoter was not significantly different in endosperm than the activity of GFP fused to the full-length 27zn promoter, nor was it different in embryo from the activity of GFP fused to the full-length Glb1 promoter. Transgene copy numbers were shown to be between 4 and 12 copies in different events.

Introduction

The precise control of transgene activity is a major objective in plant biotechnology and is primarily achieved at the transcription level by promoter sequences. The choice of promoter is a key decision in biotechnology, as the promoter influences the temporal and spatial expression of the transgene [1]. Chimaeric promoters can be designed to effectively control transgene activity, increase transgene expression levels and combat homology-based gene silencing. In a previous study, two methods for creating a chimaeric promoter were compared: (1) *cis*-elements from one promoter can be replaced or combined with *cis*-elements from another promoter; or (2) cis-elements can be placed in a synthetic region of DNA to create a synthetic promoter [2].

Extensive characterization of seed storage proteins that includes their developmental and tissue-specific regulation has been reviewed [3–6]. Seed-storage-protein promoters are useful for producing foreign proteins in seeds because they are well characterized and very strong. In maize (*Zea mays*), the 27zn (27 kDa γ-zein), which has endosperm-specificity, has been used to drive production of valuable proteins [7,8]. Marzabal et al. [9] reported that 27zn transcription was controlled by a promoter *cis*-element in the 27zn promoter called the 'bifactorial endosperm box'. The bifactorial endosperm box is a *cis*-acting element that, in part, regulates 27zn transcription and consists of a 5′ pb (prolamin box) motif (TGT/CAAAG) and a 3′ GZM (GCN4 zein motif) (G/ATGAGTCAT/C) [9]. The pb motif is a conserved motif found in all classes of zein gene and is considered a general transcription enhancer [10]. In wheat (*Triticum aestivum*), the bifactorial endosperm box of the low-molecular-mass protein glutenin [11] was reported to require both motifs for endosperm-specific transcription to occur [12].

The embryo-preferred Glb1 (Globulin-1) promoter is another seed-storage-protein promoter that has been used to produce recombinant proteins in maize kernels [13]. Glb1

Key words: chimaeric (27 kDa γ-zein + Globulin-1) promoter, kernel embryo, kernel endosperm, maize (*Zea mays*), recombinant protein expression, transgene.

Abbreviations used: A27znGlb1 promoter, chimaeric [27zn (27 kDa γ-zein) + Glb1 (Globulin-1)] promoter; ABA, abscisic acid; ABRE, ABA response element; C_t, threshold-cycle value; DAP, days after pollination; EST, expressed sequence tag; GFP, green fluorescent protein; pb, prolamin box; QRT–PCR, quantitative reverse-transcriptase PCR; RFU, relative fluorescence units.

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The nucleotide sequence of the chimaeric promoter A27znGlb1 will appear in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number EF064989.

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accumulates to one-half of the total globulin protein content in the embryo, with low amounts of Glb1 being found in the endosperm [14]. Its regulation has been well characterized and transcription is known to respond to the plant hormone ABA (abscisic acid) [15]. ABA is a positive regulator of Glb1 expression that acts by a gene-regulatory pathway which involves ABREs (ABA response elements) located in the Glb1 promoter [16,17]. The Glb1 promoter ABREs are similar to the Em (transcription-factor-binding box) elements of wheat and have the same conserved consensus sequence (Em1a: ACGTGGCGA; Em1b: ACGTAGCCG; and Em2: CGAGCCAG) and are located in positions −118, −76 and −161 bp from the transcription start site [18]. Promoter deletions of the ABREs have been used to show that these elements are necessary for ABA-responsiveness and transcription initiation of Glb1 [19].

Given the use of cereal seeds such as maize, rice (*Oryza sativa*) and barley (*Hordeum vulgare*) to produce valuable recombinant proteins for industrial, food and feed, and biopharmaceutical applications, increasing the amount of recombinant protein in seed-tissues is important. Seeds have evolved specialized tissues that produce, aggregate and store protein in a compact space. One way to improve the value of grain for nutritional, industrial and biopharmaceutical applications would be by the implementation of biotechnological modifications of seed protein content so that valuable proteins are contained in the seed. Maize seed is ideal for valuable protein production because of high seed protein content, high biomass, relative ease of transformation and well-characterized promoters. In addition, maize, rice and barley seeds have already been successfully used as production platforms for valuable protein production for commercialization [20]. A limitation of using maize seeds as an expression platform is that recombinant proteins accumulate to low levels. One way to increase the level of recombinant-protein accumulation in seeds is to express a protein in multiple seed tissues, thereby creating more recombinant protein per kernel [1]. Glb1 transcription is highest in embryo, but does show some endosperm transcription as well. Since the Glb1 promoter is already active in the endosperm at a low level, enhancing the endosperm activity of the Glb1 promoter by adding zein promoter enhancer elements that increase transcriptional activity in endosperm may be a method to increase recombinant protein expression in the seed.

In the present study we tested the hypothesis that key promoter elements from the 27zn and Glb1 promoters can be combined to create a chimaeric promoter that has the additive activity of each parent promoter. We created and tested a series of chimaeric promoters fused to the reporter gene coding for GFP (green fluorescent protein). These promoters consisted of promoter elements originating from the Glb1 and 27zn promoters and were evaluated

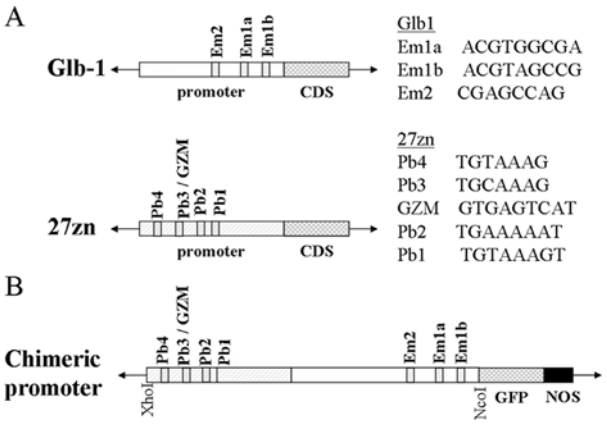

Figure 1 (A) Native Glb-1 and 27zn promoters with detailed transcription-factor-binding-site locations indicated in the promoter region and detailed transcription-factor-binding-site sequences indicated on the right, and (B) a chimaeric promoter construct containing full-length promoters of Glb1 and 27zn

(**A**) 'CDS' represents the native Glb1 and 27zn coding sequence. (**B**) The ATG translational start site is contained within the NcoI restriction site. 'GFP' is the GFP coding sequence, and 'NOS' represents the non-transcriptional terminator sequence.

using a transient expression system to quantify chimaeric promoter activity. We then tested a selected chimaeric promoter in stable maize transformants to determine its tissue-specificity. The chimaeric promoter had activity in embryo and endosperm tissue that was equivalent to the additive activity of its parent promoters.

Materials and methods

DNA manipulations

Plasmid pAct1IsGFP-1 [21] was used to prepare all constructs for transient and stable transformations. Plasmid pAct1IsGFP-1 contained the synthetic GFP (sGFPS65T) coding sequence [22] and nos terminator sequences. Maize promoter sequences through the ATG translational start codons were inserted in pActIsGFP-1 using restriction sites XhoI and NcoI so that the maize sequences were translationally fused to the GFP coding sequence (Figures 1A and 1B). Restriction sites were introduced into the maize promoter fragments using PCR amplification of genomic DNA with primers containing the desired restriction sites. PCR reactions contained 2 μl of maize genomic DNA from the inbred line Va26 (12 ng), 5 μl of GoTaq® Reaction Buffer, 0.2 μl of GoTaq® DNA Polymerase (Promega Corporation, Madison, WI, U.S.A.), 1 μl of dNTP (10 mM), 1 μl of each primer (10 pmol) and 16 μl of nuclease-free water. PCR was performed at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, repeated for 35 cycles, followed by a single cycle of 72 °C for 10 min. PCR products were visualized by agarose-gel

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Table 1 Primers used to create truncated Glb1 construct series (a) and to create the truncated 27zn construct series (b)

Underlined sequences indicate restriction-enzyme sites located within the promoter.

(a) Primers used to create truncated Glb1 construct series

Primer no.	GenBank® accession no.	Glb1 construct series 5 forward primers	Glb1 construct series reverse primers
1	EF064977	GCTAGCACAAGTTACGACCG	CCATGGGGGTTGGCTGTATGCAGAAG
2	EF064978	GCTAGCTGAGAGATTTAGGCC	CCATGGGGGTTGGCTGTATGCAGAAG
3	EF064979	GCTAGCTATTAGTCGTTAGCTTC	CCATGGGGGTTGGCTGTATGCAGAAG
4	EF064980	GCTAGCAAATTGTCCGCTGCC	CCATGGGGGTTGGCTGTATGCAGAAG
5	EF064981	GCTAGCCGGAGCCCGGATAAG	CCATGGGGGTTGGCTGTATGCAGAAG
6	EF064982	GCTAGCCTTCCTCCACGTAG	CCATGGGGGTTGGCTGTATGCAGAAG

(b) Primers used to create the truncated 27zn construct series

Primer no.	GenBank® accession no.	27zn construct series forward primers	27zn construct series reverse primers
6	EF064983	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCCACCGAGACGGCTG
7	EF064984	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCGAATTAGATTTAGCTTG
8	EF064985	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCAACGATTTTTGTCCTG
9	EF064986	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCTGTATCAGATG
10	EF064987	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCAATTTTGGTTGATG
11	EF064988	CGATCGTCCCGTCCGCGTCAATA	ACATGCATGCGTTGTTTCGTGTTCC

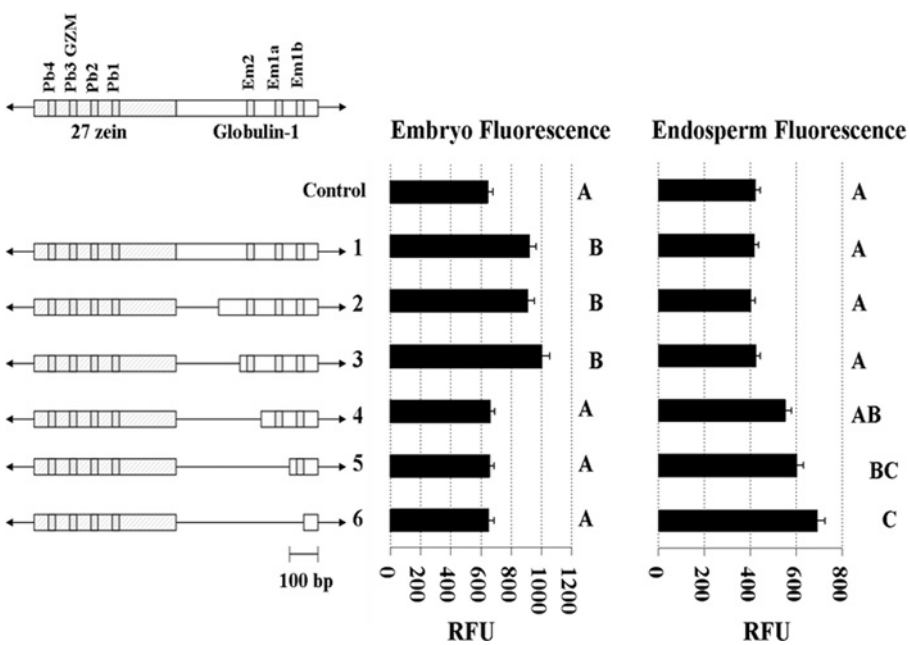

Figure 2 Fluorescence levels of GFP in transient expression assays of the truncated Glb1 construct series

On the left, the truncations of the Glb-1 promoter fused with the full-length 27zn promoter used in each assay are indicated. The control construct is pUC19. The bar graphs show fluorescence levels of each construct in embryo and endosperm tissue. Error bars show the S.E.M. Levels not connected by the same letter within the same tissue are significantly different at $P < 0.05$.

electrophoresis in the presence of ethidium bromide and then inserted in the pActIsGFP-1 vector. Primers used to perform PCR are shown in Tables 1a and 1b. Promoter A27znGlb1, which was used for plant transformation, contains the Glb1 promoter fragment **3** from Figure 2 and the 27zn promoter truncation fragment **10** from Figure 3, and its GenBank accession number is EF064989®.

Transient expression

Transient expression assays were used to test the transcriptional activity of promoters using the reporter gene coding for GFP. In our transient expression assay, maize kernels 13–17 DAP (days after pollination) of inbred line Va26 were harvested, sterilized and dissected to isolate the embryo and endosperm tissues. Up to 16 embryos or endosperms

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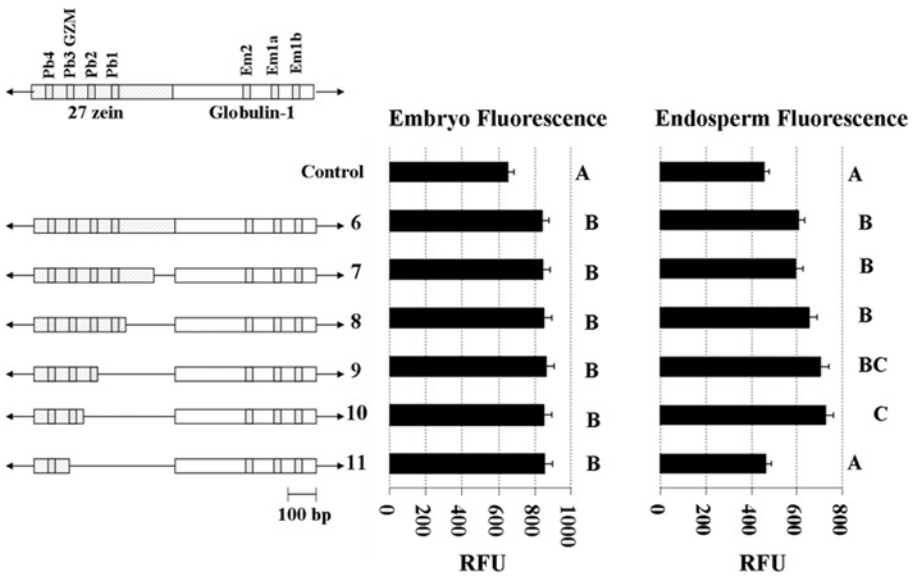

Figure 3 Fluorescence measurements of transient GFP expression assays of the truncated 27zn construct series

On the left, truncations of the 27zn promoter fused with the full-length Glb-1 promoter are shown. pUC19 is the control construct in this Figure. Bar graphs show fluorescence levels of each construct in embryo and endosperm tissue. Error bars show the S.E.M. Levels not connected by the same letter are significantly different at $P < 0.05$.

were placed in Petri dishes containing Murashigue and Skoog salt and vitamin mixture and phytagar (Gibco). Transient expression was accomplished by projectile bombardment of each Petri dish three times using 1.5 μg of plasmid DNA in each bombardment. Transformed tissues were incubated for 48 h at 27 °C in the dark. Each bombarded tissue fragment was then ground separately in 200 μl of GFP extraction buffer containing 30 mM Tris/HCl, 10 mM EDTA, 10 mM NaCl and 5 mM dithiothreitol. After centrifugation at 10 000 ***g*** for 10 min and collection of the supernatant, extracted GFP was quantified using a spectrofluorimeter (Tecan, Mannedorf/Zurich, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. ANOVA was performed on the results of each transient expression experiment to establish the significance of variation between treatments. Outliers, defined as those measurements with Studentized residuals from a generalized linear model greater than 3.5 or less than -3.5, were removed from the analysis. When significant treatment variation was found, means were compared using Student's *t* test at a $P < 0.05$ significance level.

Plant transformation and plant material

Maize plant transformation was performed at the Plant Transformation Facility at Iowa State University using microprojectile bombardment with the construct A27znGlb1 co-bombarded with a construct containing the *bar* phosphinothricin acetyltransferase gene that confers herbicide resistance [23]. Herbicide-resistant T0 callus cells were screened for the presence of the transgene by PCR (primers: forward CTTAACAACTCACAGAACATCAAC; reverse CGTCCAGCTCGACCAGGATG) that amplify a region containing the GFP coding sequence using GoTaq® Master Mix (Promega), and positive calli were regenerated to plants. Plants from each of these events were crossed with the non-transgenic maize inbred line B73 to produce F_1 seed. We visually screened transgenic seeds from 22 transformation events for endosperm and embryo fluorescence using a Dark Reader UV lamp and GFP filter (Clare Chemical Research, Dolores, CO, U.S.A.). Ears from six transformation events containing visually detectable GFP-expressing kernels were identified. Seeds from these ears were planted and the seeds from three of these ears did not germinate. F1 plants from the remaining three transformation events were grown and crossed to the inbred line B73, resulting in BC1F1 kernels. BC1F1 kernels were used for the analyses presented here.

QRT–PCR (quantitative reverse-transcriptase PCR) to determine promoter tissue-specificity in stably transformed plants

To determine which tissues contained GFP mRNA, we performed a real-time QRT–PCR on the transgenic plants. Transgenic maize tissue samples were frozen in liquid nitrogen, ground to a fine consistency and total RNA was isolated using a Total RNA kit (Ambion, Austin, TX, U.S.A.). QRT–PCR was performed on 250 ng of total mRNA in

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a reaction mixture containing 12 μl of Brilliant® SYBR® Green Master Mix (Stratagene, La Jolla, CA, U.S.A.), 12 μl of doubly distilled water, 0.05 μl of Stratascript™ RT/RNase Block (Stratagene) and 1 μl of each primer (0.5 μM final concn.). Reaction conditions were 55 °C for 30 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 60 s and 72 °C for 30 s. This was followed by a temperature ramp from 55 °C to 94 °C to characterize the PCR product. Quantitative measurements were performed using the MX3000P real-time PCR system (Stratagene). PCR reactions were evaluated by comparing the dissociation curve for each product with that of the expected PCR product. Reactions without RT were run to determine whether DNA was present in the sample. If DNA was detected, it was destroyed with successive applications of DNase and the experiment was repeated. Reactions were run in duplicate. The average C_t (threshold-cycle value) for amplification of transgene in each tissue was subtracted from the average C_t of the transgene in embryo tissue to get a ΔC_t value. The relative mRNA concentration for each tissue was calculated using the equation:

$$\text{Concentration} = 2^{C_t}$$

The target in the QRT–PCR was the coding sequence of the GFP transgene (primers: forward CTGAAGTTCATCTGCACCACCG: reverse GTGGTTGTCGGGCAGCAGC).

Evaluation of transgene copy number

To estimate the transgene copy number, quantitative RT-PCR analyses were performed using the MX3000P real-time PCR system. A PCR reaction containing 12 μl of Brilliant® SYBR® Green Master Mix, 12 μl of doubly distilled water, 1 μl of each primer (0.5 μM final concn.) and 1 μl of template DNA (6 ng of total DNA) isolated from leaf tissue using the Master Pure™ Plant Leaf DNA Purification Kit (Epicentre, Madison, WI, U.S.A.) was carried out under conditions of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 59 °C for 60 s and 72 °C for 30 s. Reactions were run in triplicate and the results were averaged for further analysis.

The relative quantification method that compares a target gene with an endogenous gene of known concentration [24] was used to quantify transgene copy number resulting from each transformation event. The coding sequence of the endogenous gene, namely that coding for Globulin-1, was used in this experiment and is present in one copy in the genome [18] (primers: forward CACTGTGGAACACGACAAAGTCTG; reverse CTCACCATGCTGTAGTGTCACTGTGAT). The target gene in this experiment was the GFP transgene (primers: forward CCTCGTGACCACCTTCACCTA; reverse ACCATGTGATCGCGCTTCT). Standard curves were created by making a 5-fold serial dilution series of the template DNA and plotting the dilution factor versus the threshold amplification cycle. These standard curves were used to determine the PCR efficiencies for amplification of the GFP transgene and the endogenous Glb1 gene [25]. Threshold cycles were compared between the Glb1 dilution series and the transgene dilution series to determine the copy number of the transgene.

Fluorescence quantification in seed tissues of transgenic plants

Eight transgenic BC1F1 kernels from each event that showed visual fluorescence were selected and mature embryo and endosperm tissues were manually separated from the kernels and ground with a mortar and pestle into a fine consistency for determination of fluorescence levels. A portion (28 mg) of each tissue sample was placed into a well of a black 96-well flat-bottomed assay plate (Corning Inc., Corning, NY, U.S.A.). The fluorescence levels of the dry ground samples were measured in triplicate using the Tecan spectrofluorimeter at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Average fluorescence intensities were calculated for each tissue in each event and the non-transgenic control inbred line B73.

Results

Chimaeric-promoter design

The objective of the present study was to design a promoter with high transcriptional activity in both endosperm and embryo seed tissues and minimal transcriptional activity in other tissues. Our approach was to combine elements of two promoters, one with strong endosperm activity and one with strong embryo activity, into a single chimaeric promoter. To develop this promoter, we fused regions known to be important for transcriptional activity of the 27zn and Glb1 promoters (Figure 1A). The chimaeric promoter was oriented so that the 27zn promoter elements are 5′ of the Glb1 promoter elements. The reason for fusing the promoters in this order was that the known Glb1 promoter elements are 300 bp upstream of the TATA box in the native Glb1 promoter, whereas the known 27zn promoter elements are 700 bp upstream of the TATA box (Figure 1B). Thus, in the chimaeric promoter, the relative positions of the *cis*-elements were approximately maintained relative to the locations of these elements in the native promoters. The chimaeric promoters were then fused to the coding sequence of the GFP gene *sGFP(S65T)* [22]. To identify regions necessary for transcription in the chimaeric promoter, two truncation series were made using site-directed mutagenesis to create restriction sites, which were used to remove regions of the promoter. The first truncation series lacked fragments of the Glb1 promoter of different

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lengths starting at the 5′ end, with several promoters in the series lacking known transcription-factor-binding boxes such as Em1a, Em2a or Em2 (Figure 2). The second truncation series lacked fragments of different lengths starting from the 3′ end of the 27zn promoter, with several members of the series lacking the known transcription-factor-binding sites Pb1, Pb2 or Pb3/GZM (Figure 3).

Transient expression analysis

To determine the relative strength of the promoters in each chimaeric-promoter truncation series, transient expression analyses of the two series described above were performed in 13–17 DAP immature embryo and endosperm tissues from the maize inbred line Va26. Truncations from the 5′ end of the Glb1 promoter had little effect on transient GFP expression in embryo tissue until the removal of the Em2 transcription-factor-binding box that decreased fluorescence to the baseline level (Figure 2). A previous report by Liu et al. [19] showed that removing the Em2 and Em1a elements from the Glb1 promoter inactivated the promoter, as did changing the Em1a element using site-directed mutagenesis. The 27zn promoter was not modified in this truncation series; however, GFP fluorescence increased in endosperm tissue as larger regions of the Glb1 promoter were removed. This increase may be due to the change in position of the 27zn promoter relative to the TATA box. Removal of portions of the Glb1 promoter decreased the distance of the 27zn promoter elements from the TATA box.

The truncation series outlined in Figure 3 lacks sequences of the 27zn promoter of different lengths starting from the 3′ end. The remaining parts of the 27zn promoter were fused to a full-length Glb1 promoter. As before, the Glb1 promoter was on the 3′ end of the chimaeric promoters near the GFP coding sequence. Embryo fluorescence was not significantly different among members of the truncation series. Modification of the 27zn sequences 5′ of the Glb1 promoter had little effect on Glb1 promoter function. This result was consistent, as the Glb1 promoter remained full-length and in an identical position relative to the TATA box in all members used in the experiment. Fluorescence in the endosperm, however, increased as more DNA was removed from the 3′ end of the 27zn promoter, until the last construct in the series was reached, when promoter activity decreased. The highest level of transient GFP fluorescence occurred in the 27zn promoter truncation fragment that was the smallest length but contained the Pb4 and the Pb3/GZM boxes. This observation is consistent with the results of the first truncation series: moving the key 27zn promoter elements closer to the TATA box increased endosperm transcription levels. Removal of the Pb1 and Pb2 boxes increased the level of transient expression relative to construct **6**; however, this increase was potentially due to the change in proximity of the remaining promoter elements to the TATA box. Removal of the Pb3/GZM box decreased GFP transient expression to the no-GFP control level in endosperm, illustrating the importance of this sequence for endosperm transcription. This result is consistent with previous reports that show the importance of both the Pb3 and GZM boxes in the transcriptional activity of the 27zn promoter [10]. The data from this truncation series support the hypothesis that the proximity of the 27zn Pb3/GZM box to the TATA box is an important determinant of endosperm promoter activity.

The two transient expression experiments detailed in Figure 2 and Figure 3 allowed us to design a chimaeric promoter containing the parts of the 27zn and Glb1 promoters that gave the best expression in endosperm and embryo respectively. The truncated promoter with the highest embryo activity from the Figure 2 truncation series was construct **3**, and the promoter with the highest endosperm activity in Figure 3 was construct **10**. Therefore, a construct consisting of the section of the Glb1 promoter from construct **3** and the section of the 27zn from construct **10** was constructed for further testing. This promoter was designated A27znGlb1 (Figure 4).

The A27znGlb1 promoter was compared with the parent truncated promoter (**3**), the full-length Glb1 and 27zn promoters and a control construct (pUC19) in a transient expression analysis in immature embryo (Figure 4). The GFP transient expression fluorescence level of A27znGlb1 was not significantly different than the parent promoter (promoter **3**) from Figure 2, nor was it different from the full-length Glb1 promoter in embryo. The A27znGlb1 promoter was also tested by transient expression analysis in immature endosperm (Figure 5). The activity of the A27znGlb1 chimaeric promoter was not significantly different from that of the full-length promoter, nor from that of the parent truncation construct (promoter **10**) from Figure 3. Thus, in transient-expression experiments, the activity of the A27znGlb1 chimaeric promoter was not significantly different than that of the full-length Glb1 promoter in embryo, nor was it different from that of the full-length 27zn promoter in endosperm. The A27znGlb1 promoter was therefore a good candidate to test in stable-transformation experiments.

Stable transformation of a chimaeric promoter

The transient-expression results suggested that the A27znGlb1 chimaeric promoter should be active in both embryo and endosperm tissue. To determine whether this was the case, we evaluated the A27znGlb1 in plants stably transformed using microprojectile bombardment [23]. Selection of positive events was based on visual evaluation and confirmation of GFP fluorescence. Seeds from 22 transformation events were received from the Plant Transformation Facility at Iowa State University. These were screened for fluorescence by visual evaluation of the F_1

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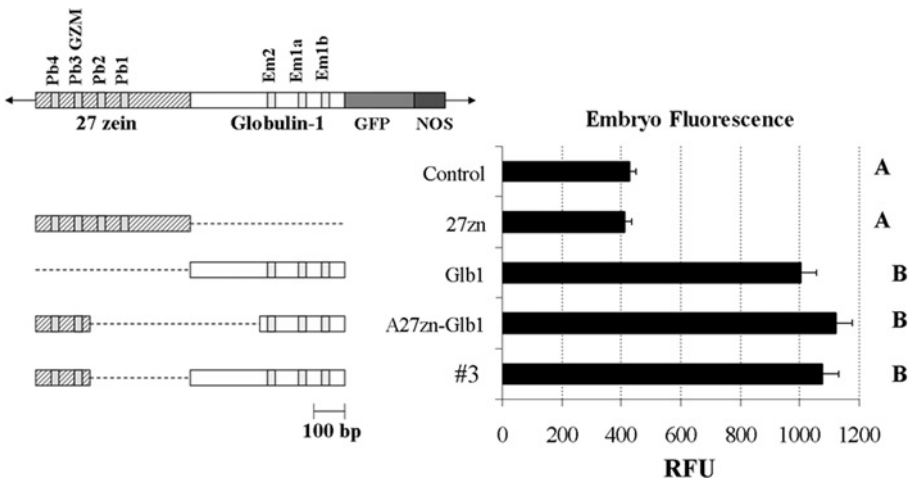

Figure 4 Fluorescence measurements in transient GFP expression to evaluate chimaeric promoter in immature embryo seed tissue

On the left are chimaeric promoters comprising regions of the 27zn and Glb1 promoters. The bar graph shows fluorescence measurements of each construct in embryo seed tissue (15 DAP). Error bars show the S.E.M. for the group mean. Levels not connected by the same letter are significantly different at $P < 0.05$.

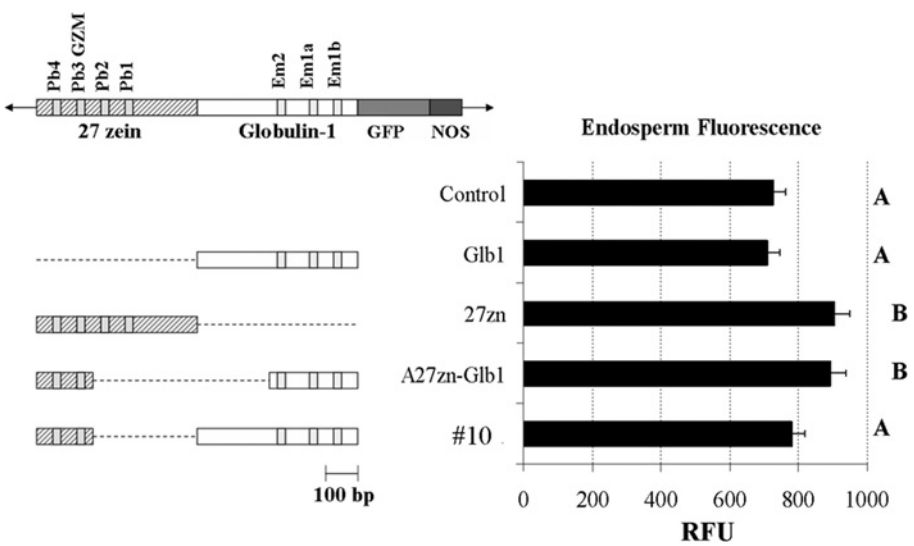

Figure 5 Fluorescence measurements of transient GFP expression to evaluate chimaeric promoters in immature endosperm seed tissue

On the left are chimaeric promoters comprising regions of the 27zn and Glb1 promoters. On the right are fluorescence measurements of each construct in endosperm seed tissue (15 DAP). Error bars show the S.E.M. for the group mean. Levels not connected by the same letter are significantly different at $P < 0.05$.

seeds. Six positive events with fluorescence in both embryo and endosperm tissues were selected, and seeds from these events were planted. Of these six GFP-positive events, seeds from three events failed to germinate, leaving three GFP-positive events. Two of the three non-viable events had small kernels and pericarp GFP fluorescence without embryo or endosperm fluorescence. The three viable GFP-positive events were crossed with the inbred line B73 and the resulting BC1F1 seeds were evaluated visually for GFP fluorescence. As in the F_1 generation, these seeds were found to display fluorescence in both the embryo and endosperm seed tissues, whereas no GFP fluorescence was visible in B73 non-transgenic control kernels. Cross-sections of these seeds are shown in Figure 6.

Transgene copy number

Transgene copy number can influence expression of transgenes in plants. To determine the copy number of the A27znGlb1-GFP transgene, we performed a relative transgene-copy-number analysis by quantitative PCR using genomic DNA from F_1 plants of the three events characterized above as template. The C_ts of the endogenous single-copy Globulin-1 gene was compared with that of the transgene to estimate transgene copy number. All PCR efficiencies

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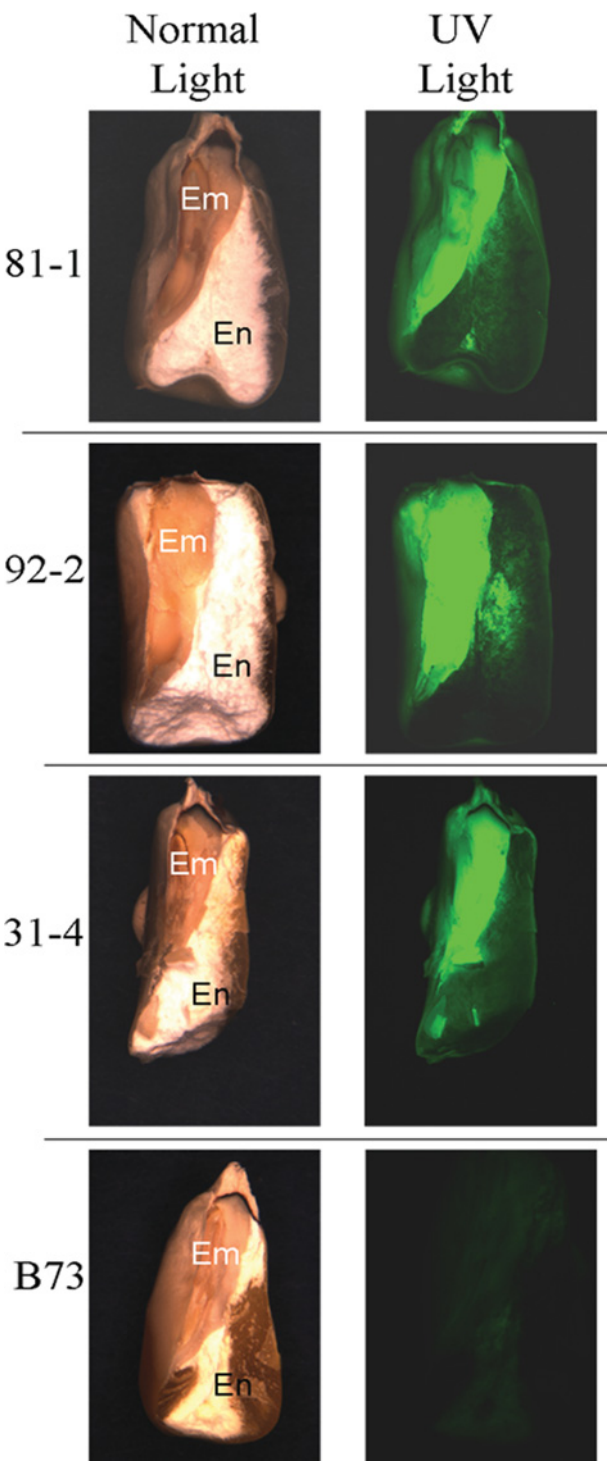

Figure 6 Visual evaluation of seed cross-sections for GFP fluorescence

'En' indicates endosperm tissues, and 'Em' indicates embryo tissue. The same photographic parameters were used for each picture.

were over 90%. Transgene copy numbers ranged from 4 to 12 (Table 2) which is typical for particle-bombardment-mediated transformation.

Table 2 Transgene copy number

Event	Copy no.	S.D. ($n = 3$)
31-4	6	0.41
81-1	10	0.12
92-2	4	0.43

Tissue-specificity of stably expressed A27znGlb1

The objective of the present study was to develop a promoter with high activity in seed tissues and minimal activity in other tissues. To determine whether the A27znGlb1 promoter met this objective, we performed a tissue survey of A27znGlb1 transcript levels in stably transformed maize plants using QRT–PCR and compared transcript levels with those of the parent promoters, 27zn and Glb1. We tested field- and greenhouse-grown F_1 plants from one event (number 92-2) for the presence of transgene mRNA in different tissues. Our results showed GFP mRNA was located in immature leaf, embryo, pericarp and endosperm tissue (Table 3). Immature leaf was harvested at 4 days after germination, and embryo, pericarp and endosperm tissues were taken 18 DAP. The tissue-specificities of the 27zn promoter [26] and the Glb1 promoter [27,19,29] are given for comparison.

Fluorescence ratios in different transformation events

The position in which a transgene inserts into the genome can affect the level of expression of the transgene [28]. However, little information is available about whether the genomic context of a transgene influences expression levels differently in different tissues. The transgenic plants bearing the chimaeric promoter–GFP construct are an ideal tool with which to address this question, because they have strong, easily measurable activity in two tissues. To address this issue, we examined the ratio of embryo to endosperm fluorescence in kernels from the different transformation events containing the chimaeric promoter. We compared these fluorescence ratios among each transgene positive event. Embryo fluorescence was higher than endosperm overall, with embryo to endosperm fluorescence ratios of 2.9:1, 3.3:1 and 2.8:1 for events 31-4, 81-1 and 92-2 respectively. The relatively small variation in these ratios does not support the hypothesis that the genomic context of this transgene influences expression ratios differently in different tissues.

Quantification of GFP levels in embryo and endosperm tissues of stably transformed plants

To determine the level of activity of the chimaeric promoter A27znGlb1 in transgenic plants, we compared three transgenic events (92-2, 31-4 and 81-1) that each contained the

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Table 3 mRNA levels of A27znGlb1-GFP in different tissues of transgenic plants compared with the 27 zn and Glb1 mRNA levels

Construct	Immature leaf	Cob	Root	Embryo	Silk	Leaf	Pericarp/aleurone	Tassel	Immature root	Endosperm
A27znGlb1–GFP[a]	0.017	–	–	1.000	–	–	0.002	–	–	0.005
27zn[b]	–	n.t.[c]	–	–	n.t.	–	–	n.t.	n.t.	+
Glb1[b]	+	n.t.	n.t.	+	n.t.	+	+	+	n.t.	+

[a] Values in this row are levels of A27znGlb1-GFP mRNA relative to that in embryo tissue and were determined by QRT–PCR.
[b] The 27zn and Glb1 results are taken from the literature as cited in the text; – and + indicate the absence or presence of the mRNA respectively.
[c] n.t., not tested.

chimaeric promoter with the activity of the native 27zn and Glb1 promoters (Figures 7A and 7B). The native 27zn and Glb1 promoters were fused to GFP and expressed in transgenic plants created in a previous study [27]. The events chosen for comparison were those with the highest fluorescence level among three events for native Glb1 promoter and two events for native 27zn promoter. Comparison of the A27znGlb1 promoter with the 27zn and Glb1 promoter indicated that two of the three transgenic events containing the A27znGlb1 promoter had activity that was not significantly different than the Glb1 promoter in embryo tissue, with an average relative fluorescence value of 32 000 RFU (relative fluorescence units) between events as compared with that of the Glb1 transgenic line, which was about 40 000 RFU. A27znGlb1 and the native Glb1 promoter gave significantly higher values than the 27zn transgenic line and the B73 non-transgenic control in embryo, which both showed values of about 10 000 RFU and were not significantly different from each other. In endosperm tissue, the A27znGlb1 promoter activity gave an average value of 11 000 RFU between events and was not significantly different than the native 27zn transgenic line that had activity of about 13 000 RFU. The A27znGlb1 promoter gave a significantly higher value than the Glb1 transgenic line, with 5000 RFU, and the B73 inbred line control, with about 3000 RFU. We therefore conclude that the chimaeric promoter A27znGlb1 approximately retains the promoter activity of the native Glb1 promoter in embryo tissue and the native 27zn promoter in endosperm tissue.

Discussion

The objective of the present study was to develop a promoter that was transcriptionally active in both endosperm and embryo and had minimal activity in other tissues of the plant. This objective was based on the hypothesis that key promoter elements from the 27zn and Glb1 promoters can be combined to create a chimaeric promoter that has the additive activity of each parent promoter.

To accomplish this objective, we combined elements of the 27zn and Glb1 promoters to determine whether we could produce additive tissue specificity (embryo plus endosperm) in a single promoter. We considered several methods of combining the 27zn and Glb1 promoters. We could have simply combined the promoter elements known to be necessary for transcription from the 27zn and Glb1 promoters into a single promoter. However, this requires that we know exactly what promoter elements are required for transcriptional activity. The 27zn and the Glb1 promoter regions are well characterized, yet there is uncertainty regarding which regions are critical for activity, especially in the context of a chimaeric promoter. We therefore decided to develop two truncation series to determine what regions of each promoter were necessary for activity in the context of a chimaeric promoter [2]. We tested the members of each series in quantitative transient expression assays. The transient expression studies suggested which sequences should be included in an optimal chimaeric promoter. This chimaeric promoter was constructed and called A27znGlb1 and was compared with the native 27zn promoter, the Glb1 promoter and the parental promoters from the first round of testing in transient GFP expression assays. The activity of the A27znGlb1 was not statistically different from the promoter activity of 27zn and Glb1 promoters. The effectiveness of this promoter may be explained by the fact that the positions relative to the TATA box of the promoter elements known to be important in the native promoters were similar in the A27znGlb1 promoter to the positions of these elements in the native promoters.

When the A27znGlb1 promoter was tested in stable transformants, only six out of 22 events were selected on the basis of visual evaluation of GFP to continue in our maize breeding programme, three of which were viable. Two of the three non-viable events were very small kernels and unexpectedly had high levels of GFP fluorescence in pericarp. We attempted to grow all of the kernels that showed this phenotype. However, none of them germinated. The reason for this is unknown, but may be related to the unusual pericarp expression pattern or the small-kernel phenotype.

We performed QRT–PCR on the plants that were stably transformed with A27znGlb1 to determine transgene copy numbers. Our positive selections have a high gene

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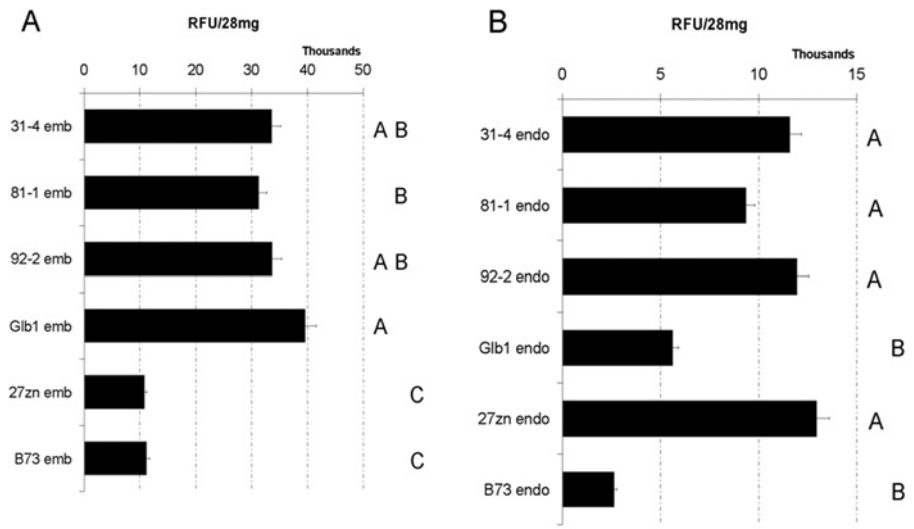

Figure 7 GFP fluorescence in RFU for embryo (A) and endosperm (B) tissue from each chimaeric promoter transgenic event and from transgenic events that contained the native Glb1 or 27zn promoter

B73 is the non-transgenic inbred line control. Error bars show the S.E.M. for the group mean. Levels not connected by same letter are significantly different at $P < 0.05$.

copy number relative to the endogenous control gene; this may be due to our final selection based on visual evaluation of GFP fluorescence, i.e. low copy number events may have a level of GFP fluorescence that was too low to detect visually. Our data does not support this conclusion, however, because the event with the highest copy number (81-1) had the lowest level of GFP expression in stably transformed plants.

The main objective of the present work was to produce a promoter with high activity in seed tissues and minimal activity in other tissues. To determine success, GFP fluorescence was evaluated visually in A27znGlb1 stable transgenic and non-transgenic B73 inbred line kernel cross-sections. The A27znGlb1 chimaeric promoter activated GFP expression in both endosperm and embryo seed tissue (Figure 6). We also compared GFP expression levels of the A27znGlb1 promoter with those of the native promoters Glb1 and 27zn in transgenic plants. The relative fluorescence of GFP endosperm and embryo on a per-mass basis indicated that the A27znGlb1 chimaeric promoter is similar in activity to the Glb1 in embryo tissue and to the 27zn promoter in endosperm tissue (Figure 7A and 7B). The results suggest we achieved additive promoter activity in seed tissues by combining the Glb1 and 27zn promoter elements in a spatial arrangement within the promoter for optimum activity. We conclude that the selected Glb1 promoter fragment in the chimaeric promoter was necessary and sufficient to activate expression in embryo tissue and was functionally equivalent to the native parent promoter, and that the selected 27zn promoter fragment in the chimaeric promoter was necessary and sufficient to activate expression in endosperm tissue and was functionally equivalent to the native parent promoter.

It was important to determine the tissue-specificity of the chimaeric promoter to verify that unexpected transcriptional activities were not introduced by the construction of the chimaeric promoter. To this end, we carried out QRT–PCR on transgenic plant tissues and compared the results with the known promoter tissue specificities of the 27zn [9] and Glb1 [14] promoters. Our results showed that GFP mRNA was located in the endosperm, embryo and pericarp seed tissues. GFP transcripts were not found in other tissues of the plant that were tested. Tassel (male flower) tissue did not contain measurable levels of GFP mRNA in the chimaeric transgenic line, even though EST (expressed sequence tag) frequency data suggested that the Glb1 promoter activated expression in that tissue [27]. This may be due to gene silencing of the GFP transgene in the tassel tissue or genotype-specific differences in the expression pattern of the Glb1 allele used for constructing the chimaeric promoter. GFP mRNA was also found in immature leaf at 8 days after germination. To our knowledge, zein expression in immature leaf has not been reported. Russell and Fromm [26] reported that the 27zn promoter activates expression only in the endosperm tissue of maize. Shepherd et al. [27] reported the finding of Globulin-1 ESTs in immature leaf and tassel using a digital Northern approach; however, the tassel EST level was low. From this we can conclude that the mRNA found in immature leaf in plants transformed with the chimaeric promoter is likely to be due to the activity of the Glb1 promoter portion of the chimaeric promoter in that tissue. In light of the results, the A27znGlb1

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chimaeric promoter meets the objective of the present study and supports the hypothesis that different promoter fragments can be combined to create a chimaeric promoter with the desired activities of the parent promoters.

The embryo-to-endosperm fluorescence ratio in the plant transformed with the chimaeric promoter was relatively constant at approx. 3:1 in each event, suggesting that there was not a tissue-specific effect on transgene expression in the three events examined. This ratio also remained constant even though the transgene copy number fluctuated from 4 to 10, suggesting that transgene copy number did not have a tissue-specific effect on embryo and endosperm fluorescence levels.

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