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Functional analysis of regulatory elements in a plant embryo-specific gene

(soybean/promoter/transgenic plants/seed proteins)

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ABSTRACT **Previously we demonstrated the expression of a plant embryo-specific gene encoding the α′ subunit of β-conglycinin, a seed storage protein of soybean (*Glycine max*), in transgenic petunia plants. To examine the regulatory elements that control the expression of this embryo-specific gene (Gmg17.1), a series of deletion mutants was made that contain the α′-subunit gene flanked in the 5′ direction from +14 nucleotides to −8.5 kilobases (kb) relative to the site of transcription initiation. Each of these deletion mutants was introduced into the genome of petunia cells with the help of Ti-plasmid-derived vectors. Petunia plants were regenerated from transformed cells and expression of the introduced soybean gene was examined. When the α′-subunit gene was flanked by 159 nucleotides upstream (Gmg17.1Δ−159), the gene was expressed at a low level in immature embryos. When the gene was flanked by 257 nucleotides upstream of the site of transcription initiation (Gmg17.1Δ−257), a high level of expression was obtained. An additional 8 kb of DNA sequence (which includes the sequence GTGGATAG at −560, which is identical to the core enhancer sequence of simian virus 40 and some animal genes) did not significantly increase the level of expression. The increase in expression level between the Δ−159 and Δ−257 mutants was at least 20-fold. Analysis of the nucleotides between Δ−159 and Δ−257 reveals four repeats of a 6-base-pair (G+C)-rich sequence (A$\overset{A}{\underset{C}{G}}$CCCA). The deletion Gmg17.1Δ−159 contains a single AACCCA sequence. We suggest that the (G+C)-rich repeats play a critical role in determining the level of expression of the α′-subunit gene in transgenic plants.**

Transcriptional control of eukaryotic protein-encoding genes plays a major role in determining the final level of the gene product. Transcription of most genes requires the presence of the "TATA box" and other upstream regulatory elements. The TATA element, a highly conserved sequence (consensus TATAA), is located about 30 nucleotides upstream of the site of transcription initiation. Deletion of the TATA element often leads to multiple sites of transcriptional initiation (1, 2). Other upstream DNA sequence elements, such as the "CAAT" sequence, are common elements in many eukaryotic gene promoters (3, 4). Elements referred to as enhancers, originally discovered in viral genomes, are *cis*-acting regulator sequences that can strongly stimulate transcription from promoters of nearby genes (5). Enhancer elements, by definition, have an effect that is independent of orientation and rather insensitive to position relative to the gene. Enhancers also control the expression of some cellular genes, frequently in a tissue-specific manner (6, 7). Transcriptional enhancement presumably involves the interaction of enhancer sequences with specific trans-acting factor(s) (4, 8).

In higher plants, there is a marked similarity with animal genes in the arrangement of functional elements in the promoter, such as the TATA box and upstream elements (9–12). Some promoters of plant genes also contain sequence(s) homologous to the animal core enhancer sequence GTGG$\overset{AAA}{TTT}$G (10–15). To date there have been a limited number of reports on the functional analysis of higher plant gene promoters (14–18), most of which deal with promoters that are regulated by light.

Synthesis of the legume seed storage proteins is confined to stages of cell enlargement and seed maturation in embryo development and represent excellent model systems for studies of plant gene expression. The β-conglycinins, major storage proteins of soybean, are multimeric proteins with sedimentation coefficients of 7 S to 9 S and contain subunits referred to as α′ (76 kDa), α (72 kDa), and β (53 kDa). The expression of genes encoding these subunits during soybean embryogenesis has been well documented (19, 20). We previously demonstrated the tissue-specific expression of a gene encoding the α′ subunit of β-conglycinin in transgenic petunia plants (21). We here report the functional analysis of regulatory elements that control embryo-specific and high-level expression of this gene. Our results indicate that a relatively small [49 base pairs (bp)] DNA sequence in the α′-subunit gene promoter plays an important role in controlling the expression of this embryo-specific gene.

MATERIALS AND METHODS

Construction of Gmg17.1 Promoter Deletion Mutants and Conjugation into *Agrobacterium tumefaciens*. The α′-subunit gene Gmg17.1 is on an 11.5-kilobase (kb) *Eco*RI fragment of soybean genomic DNA and has ≈8.5 kb of DNA sequence upstream from the transcription start site (12). A series of 5′ deletion mutants was made by restriction digestion and BAL-31 exonuclease digestion (22). The digested DNA molecules were ligated with *Bam*HI linkers and recloned into the plasmid pUC8. Mutants with end points at −904, −454, −257, −208, −159, −69, −42, and +14 relative to the transcription start site (confirmed by DNA sequence analysis) were selected for subsequent experiments.

The intermediate plasmid used in these experiments was pMON200 (see Fig. 1) (23). The 11.5-kb *Eco*RI fragment of Gmg17.1 was ligated in both orientations (Gmg17.1A and Gmg17.1B) to pMON200 restricted with *Eco*RI. The 5′ deletion mutants were excised from their pUC8 plasmids with *Bam*HI and *Eco*RI and were ligated to pMON200 restricted with *Eco*RI and *Bgl* II. The intermediate vector and its derivatives in *Escherichia coli* then were mobilized into an *A. tumefaciens* GV3111SE, carrying a disarmed Ti plasmid (pTiB6S3-SE) (23). Transformation of petunia (*Petunia hybrida*, VR) leaf discs and plant regeneration on medium

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Abbreviations: bp, base pair(s); kb, kilobase(s).

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containing kanamycin were carried out as described (24). Regenerated plants were tested for the production of nopaline (25).

Quantitation of Gene Copy Number in Transgenic Petunia Plants. Nuclear DNA, isolated from leaves of transgenic plants using a previously described method (26), was digested with either *Bgl* II or *Hin*dIII and *Eco*RI restriction enzymes. Restricted DNA was fractionated by electrophoresis in 0.8% agarose gels, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled DNA fragment. The probes used were either a *Bgl* II fragment containing part of the coding region of the α′-subunit gene (0.78 kb, see Fig. 1) or a *Hin*dIII and *Eco*RI fragment from the intermediate plasmid containing the α′-subunit gene (1.4 kb, see Fig. 3). The gene copy number was determined by comparing intensities of bands on x-ray films representing hybridization to reconstructions, consisting of 1–10 genome equivalences of the gene in the plasmid pMON200, with those from the genomic DNA. For these calculations, it was assumed the haploid petunia genome contained 1.55 pg of DNA (27).

Isolation and Analysis of RNA from Immature Seeds of Transgenic Petunia Plants. Total RNA was isolated from immature petunia seeds removed from seed capsules collected between 15 and 17 days after pollination (21). Total RNA was resolved by electrophoresis in 1.5% agarose gels containing formaldehyde, blotted to nitrocellulose (28), and hybridized to a purified DNA fragment containing α′-subunit gene encoding sequences (the *Bgl* II fragment indicated in Fig. 1) labeled with ^{32}P by nick-translation to a specific activity of 3×10^8 cpm/μg of DNA. The filters were hybridized in a solution containing 0.75 M NaCl/75 mM sodium citrate, pH 7.2, 40% formamide at 42°C overnight, washed at 42°C in 0.15 M NaCl/15 mM sodium citrate containing 0.1% $NaDodSO_4$ for 1 hr, and exposed to x-ray film. Autoradiograms were scanned with a Joyce–Loebl chromscan 3 densitometer.

Extraction and Analysis of Proteins from Mature Petunia Seeds. Seeds of each of the transgenic plants were collected separately; thus, each seed lot contains a mixture of seeds that are heterozygous and homozygous with respect to the introduced gene. Proteins were extracted and quantitated as described (21) and analyzed after fractionation in a 10% polyacrylamide gel containing $NaDodSO_4$ ($NaDodSO_4$/PAGE). Proteins were electrophoretically transferred to nitrocellulose and allowed to react with rabbit polyclonal antibodies against β-conglycinins as described (21). Antibody:antigen reactions were detected with ^{125}I-labeled donkey anti-rabbit antibody, followed by autoradiography.

RESULTS

Construction of Deletion Mutants of the Promoter of α′-Subunit Gene Gmg17.1. The complete sequence of the gene encoding the α′ subunit of β-conglycinin has been reported (12). The structural sequences in the genomic clone Gmg17.1 are flanked by about 8.5 kb of soybean DNA on the 5′ side and about 400 nucleotides on the 3′ side of the gene. The TATAA sequence is positioned at −30, and the CCAAT sequence is positioned at −65. The sequence GTGGATAG, at position −560, is identical to the consensus enhancer core sequence GTGG$\overset{AAA}{TTT}$G of some animal genes (5).

As a first step toward identification of the DNA sequences required for expression of the α′-subunit gene, a series of promoter deletion mutants was created. Nomenclature for the mutants with indications of the extent of the deletions is given in Fig. 1. The Gmg17.1Δ+14 mutant does not contain any soybean 5′-flanking sequences. Gmg17.1Δ−42 contains a TATA sequence. Gmg17.1Δ−69, Gmg17.1Δ−159, Gmg17.1Δ−208, Gmg17.1Δ−257, and Gmg17.1Δ−454 contain the TATAA and CCAAT sequences but do not have the GTGGATAG sequence. Gmg17.1Δ−904 includes all of the elements (Fig. 1). Each deletion mutant was cloned into pMON200 (23), which was subsequently cointegrated with a disarmed Ti plasmid in *A. tumefaciens*. The modified *A. tumefaciens* was used to transform petunia leaf tissues, and plants were regenerated from transformed cells selected on medium containing kanamycin. The plants that were kanamycin resistant and that produced nopaline were grown in the greenhouse. Total RNA from immature seeds and proteins from mature seeds were examined for expression of the α′-subunit gene.

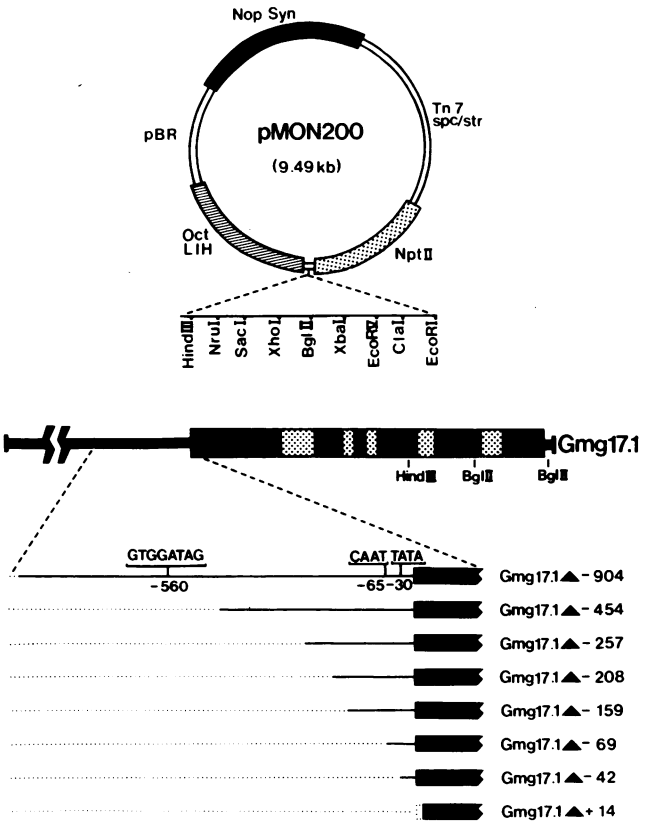

FIG. 1. Construction of Gmg17.1 deletion mutants. The α′-subunit gene Gmg17.1 is encoded on an 11.5-kb *Eco*RI fragment of DNA that has 8.5 kb of sequence upstream from the transcription start site (boxed area indicates the α′-subunit gene encoding sequence). The 5′ deletion mutants were made by digestion with BAL-31 and mutants with end points at +14, −42, −69, −159, −208, −257, −454, and −904 were selected and ligated to *Bam*HI linkers. pMON200 was linearized with *Eco*RI or *Eco*RI and *Bgl* II and ligated with Gmg17.1 or Gmg17.1 promoter deletion mutants.

From 3 to 11 different, independent transformants were produced for each of the deletion mutants. The data presented here represent an average of the levels of gene expression in these plants.

Quantitation of Gene Copy Numbers in Transgenic Plants. Quantitation of α′-subunit gene copy numbers in 42 different transgenic plants showed that 75% of the plants contain a single copy of the α′-subunit gene, whereas the remainder contains two to five copies of the gene (for examples, see Fig. 2*A* and Fig. 3*A*). The α′-subunit gene is inserted at different sites of the petunia genome in different transformants (data not shown). Transgenic plants with a single gene copy were chosen for the following experiments unless otherwise indicated.

Accumulation of α′-Subunit RNA in Transgenic Petunia Plants. As shown in Fig. 2*B*, α′-subunit mRNA was not detected in embryos of plants containing deletion Δ+14, Δ−42, or Δ−69. A small amount of α′-subunit mRNA was detected when the gene was flanked by 159 nucleotides

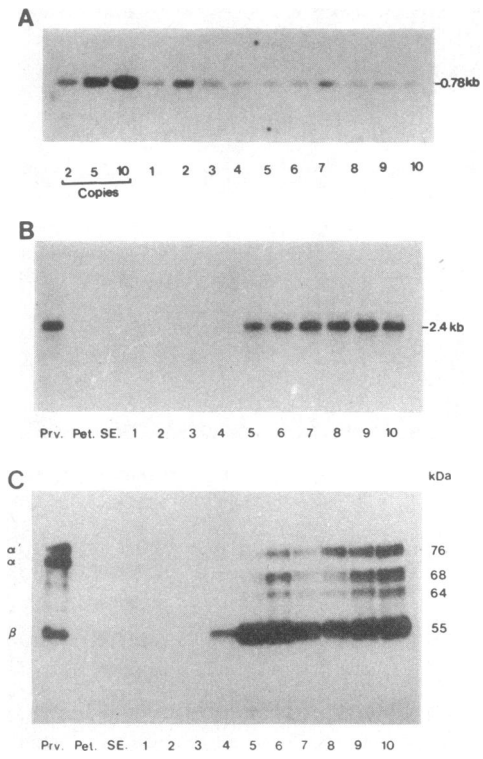

FIG. 2. (*A*) Quantitation of the numbers of α'-subunit genes in the transgenic petunia plants. DNA isolated from petunia leaves was digested with *Bgl* II, fractionated on 0.8% agarose gels, transferred to nitrocellulose paper, and probed with a ^{32}P-labeled fragment from the encoding sequence of Gmg17.1. The first three lanes on the left show copy number reconstructions of 2, 5, and 10 copies. Lanes 1–8 represent examples of transformants with deletion mutants Gmg17.1-Δ+14, Δ−42, Δ−69, Δ−159, Δ−208, Δ−257, Δ−454, and Δ−904, respectively. Lane 9 represents DNA from a plant with Gmg17.1 in one orientation; lane 10 represents DNA from a plant with Gmg17.1 in the opposite orientation (relative to the NPT II gene; see Fig. 1). (*B*) Transcripts of the α'-subunit gene in immature seeds. Lanes indicated as Prv., Pet., and SE. refer to the total RNA isolated from the Provar cultivar of soybean, petunia, and petunia transformed with the pMON200 vector, respectively. Lane Prv. contained 1 μg of soybean RNA; the remaining lanes contained 25 μg of petunia RNA. RNAs were subjected to electrophoresis in agarose gels containing formaldehyde, blotted to nitrocellulose, and hybridized to a ^{32}P-labeled fragment from the α'-subunit gene Gmg17.1. The size of the transcript in soybean and transgenic plants is about 2.4 kb. Lanes are as in *A*. (*C*) Immunoblot analysis of proteins from seeds of transgenic petunia plants. Lanes 1–10, Pet., and SE. contain 20 μg of total seed proteins from plants as described in *A* and *B*. Lane Prv. contains 0.8 μg of protein from Provar soybean seeds. Molecular masses of the α' subunit (76 kDa) and related, breakdown products of the α' subunit (1) are indicated.

(Δ−159, lane 4, Fig. 2*B*), and increasing amounts of RNA accumulated in embryos of plants containing deletions Δ−208 and Δ−257. However, additional nucleotides to ≈8.5 kb 5′ of the gene did not increase the level of expression of the α'-subunit gene. The results of these RNA blot hybridization experiments were quantitated by scanning autoradiograms on a densitometer. As shown in Table 1, the amount of RNA that accumulates in different plants containing the gene flanked by at least 257 nucleotides differed by no more than 2-fold.

In contrast, expression of the deletion Gmg17.1Δ−159 is considerably lower than deletions Δ−208 to Δ−8.5 kb (Fig. 2*B*, lane 4). Although the size of the RNA is not changed, densitometric scanning indicates that the level of accumulation of the α'-subunit mRNA in plants containing Δ−159 is only 5% of the level of RNA in Gmg17.1 expression (Table 1).

Accumulation of Soybean Protein in Seeds of Transgenic Petunia Plants. Mature seeds of the transgenic plants accumulate, in addition to the 76-kDa α' subunit, several antigenically related polypeptides with apparent molecular masses of 68 kDa, 64 kDa, and 55 kDa (Fig. 2*C*). The 55-kDa polypeptide is slightly larger than the β subunit (53 kDa). As presented earlier, the smaller polypeptides probably resulted from specific proteolytic cleavage of the 76-kDa α' subunit (21).

The level of α'-subunit mRNA reflects the amount of soybean protein that accumulates in the transgenic petunia plants. Fig. 2*C* and Table 1 show that mature seeds of petunia plants containing the deletion mutants Gmg17.1Δ−208, Δ−257, Δ−454, and Δ−904 accumulate approximately the same level of protein as in plants containing Gmg17.1. The amount of protein accumulated in plants containing these deletions does not vary by more than 2-fold. As expected from mRNA measurements, the amount of protein produced in the Δ−159 deletion was less than that produced in plants containing Gmg17.1 by a factor of 15–20 (Table 1).

Plant-to-Plant Variation in the Expression of α'-Subunit Gene. To determine whether or not there was marked variability in the amount of RNA or in the amount of accumulated soybean protein in independently isolated transgenic plants, we compared the level of mRNA and protein in embryos of a number of different transgenic petunia plants. Fig. 3 shows the number of gene copies and the amount of accumulated RNA and protein in eight transgenic plants containing Gmg17.1. Table 2 compiles the results of these experiments as well as those of plants containing three different promoter deletion mutations. The results demonstrate that the variation between different plants is no more than 2-fold. It also demonstrates that the deletion mutations did not cause an increase in the variation. These results contrast with the expression of other foreign genes introduced into tobacco and petunia plants, which can be as great as 25- to 50-fold (29). The low degree of variability of α'-subunit gene expression in transgenic plants indicates that this is an excellent system in which to assess promoter activity.

Tissue-Specific Expression of α'-Subunit Gene. The expression of the α'-subunit gene remains under stringent developmental control regardless of the extent of the promoter deletions. We were unable to detect α'-subunit mRNA in leaves of any of the plants containing the deletion mutants even when the level of sensitivity was increased 100-fold by using high levels of poly(A)$^+$ RNA from leaves in hybridization reactions (data not shown). We also did not find the α'-subunit protein in the leaves of transgenic petunia plants.

DISCUSSION

We here describe the functional analysis of the DNA sequences that control the expression of a plant embryo-specific gene. A soybean seed storage protein gene encoding the α' subunit of β-conglycinin and a series of deletion mutants made in the sequence 5′ of the gene were introduced into petunia cells by means of a modified Ti plasmid, and expression of the α'-subunit gene was examined. The expression pattern of this embryo-specific gene in transgenic plants was very similar to that in soybean plants—i.e., the expression of the α'-subunit gene occurs in a tissue-specific manner and is developmentally regulated. Our analyses indicated that the transcript level in immature petunia seeds is at least 100 times higher than in petunia leaves. This result is similar to that of studies of expression of this gene in soybean plants (20). We reported previously that the soybean proteins that accumulated in the petunia plant seeds assemble into multimers of 7 S to 9 S, similar to that of β-conglycinins isolated from soybean seeds (21).

Table 1. Relative levels of expression of α′-subunit gene Gmg17.1 and promoter deletion mutants in transgenic petunia plants

		Promoter deletion mutant							
	Gmg17.1	Δ+14	Δ−42	Δ−69	Δ−159	Δ−208	Δ−257	Δ−457	Δ−904
I	4.8	0	0	0	0.2	3.3	4.4	4.3	4.3
R, %	100	0	0	0	5	69	92	90	89
I	14.1	0	0	0	0.6	9.2	11.4	10.4	11.1
P, %	100	0	0	0	4	65	80	73	78

I, relative optical density of bands on x-ray films caused by hybridization of RNA with a ^{32}P-labeled probe (Fig. 2*B*) or ^{125}I-labeled donkey anti-rabbit antibody (Fig. 2*C*). Each RNA or protein blot was exposed to x-ray film for different lengths of time. Films on which the emulsion was not overexposed were scanned with a Joyce–Loebl chromscan 3 densitometer. Integration of peak areas is given as relative units. R, relative amount of RNA, given as percentage of the amount of RNA that accumulated in seeds expressing Gmg17.1. P, relative amount of protein, given as percentage of the amount of protein accumulating in mature seeds expressing Gmg17.1. For these calculations the relative amounts of all of the polypeptides (55 kDa to 76 kDa) were scanned.

The current studies showed that having only the first 69 bp upstream of the transcription initiation site is not sufficient for expression of the α′-subunit gene. We could not detect expression in embryos or leaves of transgenic plants with Gmg17.1Δ+14 (six plants tested), Δ−42 (four plants tested), and Δ−69 (six plants tested). Within the first 69 nucleotides, there are several elements that have been considered important sequences in regulating plant gene expression, including the TATA box and CAAT sequences. It has been previously shown that TATA and CAAT sequences are essential for expression of the nopaline synthase promoter (30) and the cauliflower mosaic virus 35S promoter (31). However, several examples of promoters of other animal (32) and plant (16) genes have shown that the TATA but not the CAAT sequence plays an important role in regulating gene expression. The observation that mRNA of the α′-subunit gene is not detected in plants containing the Δ−42 and Δ−69 deletion mutations may be due to the gene actually being "off" or "on" at a level below our detection limits.

Maximal transcription of the α′-subunit gene requires about 260 nucleotides upstream from the transcription start site. The deletion mutant Gmg17.1Δ−257 gives essentially the same level of mRNA and protein as the complete α′-subunit gene clone Gmg17.1 (Table 2). Other deletion mutants (Δ−454 and Δ−904) provided comparable levels of gene expression. Interestingly, the sequence GTGGATAG (at −560 relative to the α′-subunit gene), which is homologous to the consensus core enhancer sequence of some

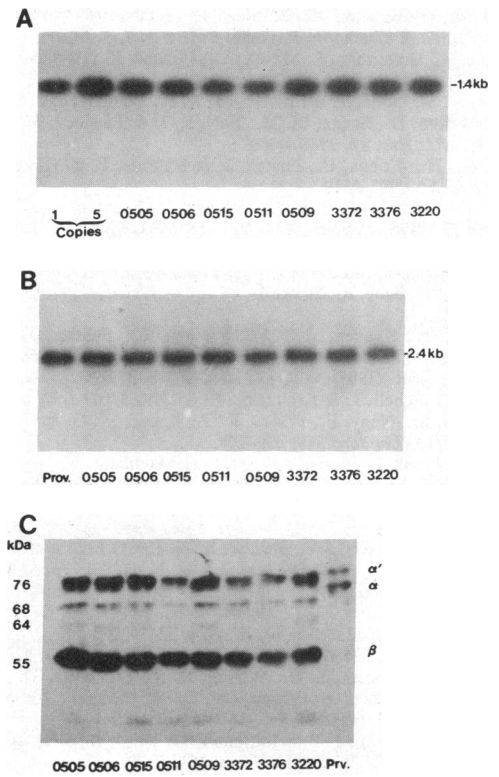

FIG. 3. Comparison of the level of expression of Gmg17.1 in embryos of eight independent transgenic petunia plants. (*A*) Gene copy number determinations in each of the transgenic plants. Genomic DNA was digested with *Hin*dIII and *Eco*RI and analyzed as in Fig. 2*A*. Copy number reconstructions are given. (*B*) Comparison of the amount of α′-subunit mRNA in embryos of transgenic plants 15–17 days after pollination. Each lane, except lane Prov., contains 25 μg of petunia embryo RNA. Lane Prov. contains 1 μg of RNA from immature soybean embryos. Analysis was carried out as in Fig. 2. (*C*) Quantitative comparison of the amount of α′ subunit and related polypeptides in mature seeds collected from transgenic plants. Each lane, except lane Prv., contains 20 μg of total seed protein. Lane Prv. contains 0.5 μg of soybean protein. Analysis was carried out as in Fig. 2.

Table 2. Quantitation of plant-to-plant variability in the level of protein accumulated in transgenic petunia plants carrying α′-subunit gene Gmg17.1 or the deletion mutants

P no.	I	P, %
Gmg17.1		
0505	2.5	151
0506	2.1	129
0515	1.8	109
0511	1.4	87
3372	1.1	67
3376	1.4	83
3220	1.2	80
Δ−904		
3645	1.1	68
3643	2.0	121
3641	1.7	107
Δ−257		
0516	1.9	116
0517	1.8	119
0519	1.1	69
3360	1.1	70
3661	1.5	94
Δ−208		
3639	0.9	56
3785	0.9	57
3787	0.9	54
3640	0.7	44
3771	0.8	49

P no., identification number given to different transgenic plants. I, as in Table 1. P, relative amount of protein accumulated, taken as a percentage of 1.63, the average of protein in seeds expressing Gmg17.1.

animal genes, does not cause a strong enhancement of α′-subunit gene expression in petunia plants. It has been shown that sequences homologous to GTGG$^{AAA}_{TTT}$G in the 5′-flanking regions of several plant genes are important for enhancing their expression in transgenic plants (14, 15, 31). Other plant embryo-specific genes also contain sequences homologous to the core enhancer element (10–12), but the function(s) of those sequences has not been identified.

A low level (5% of maximum) expression of the α′-subunit gene was observed when the first 159 nucleotides were present 5′ to the gene (Fig. 2, Table 1). An additional 49 nucleotides (Δ−208) raised the level of expression by 16-fold, whereas increasing the flanking DNA to −257 (Δ−257) resulted in the maximal level of expression (Table 1). However, since there is as much as a 2-fold variation in the expression of Gmg17.1 in different transgenic plants (Table 2), we are hesitant to conclude that the differences between the expression of Δ−208 and Gmg17.1, Δ−904, Δ−454, and Δ−257 are significant. Additional transgenic plants must be produced to further examine this question.

Because of the increase in the level of expression of the α′-subunit gene in plants containing the deletion Δ−257 compared to the Δ−159 deletion, we examined the DNA sequence in this region (Fig. 4). This sequence contains two imperfect direct repeats (underlined) of 28 nucleotides and five, 6-bp (G+C)-rich repeats (A$^{A}_{C}$GCCCA), four of which are located within the large repeats. The second and third repeats are separated by 16 bp and thus may be located on approximately the same face of the DNA helix. The fourth and fifth repeats are also separated by 16 bp. The mutant Gmg17.1Δ−159 (low level of expression) contains a single repeat, whereas the mutant Gmg17.1Δ−208 (with high level expression) contains four repeats. This suggests that the sequences A$^{A}_{C}$GCCCA may play an important role in the regulation of this embryo-specific gene.

Other sequenced plant embryo-specific genes also contain sequences homologous to the repeats (A$^{G}_{A}$CCCA). The β-subunit gene of phaseolin contains two such repeats in positions similar to those in the α′-subunit gene (AACCCA at −207 and CACCCA at −218) (12). These repeats also exist in the sequences 5′ of the gene encoding the β-subunit of β-conglycinin (S. J. Barker, J. J. Harada, and R. B. Goldberg, personal communication).

The (G+C)-rich sequence GGGCGG or its inverted form, CCGCCC, located around 100 nucleotides upstream of the start site of transcription, plays an important role in the regulation of some eukaryotic genes expressed *in vivo* and *in vitro* (3, 4). Furthermore, the transcription factor Sp1, a sequence-specific DNA binding protein isolated from HeLa cells, can enhance transcription by RNA polymerase II by 10- to 50-fold from a number of promoters, including those associated with the simian virus 40 early genes and the herpes simplex virus thymidine kinase gene, each of which contains at least one properly positioned (G+C)-rich box (4). We suggest that the five (G+C)-rich repeats in the α′-subunit gene promoter may be involved in transcriptional regulation and enhance the expression of the α′-subunit gene in transgenic petunia plants. Further experimentation is necessary to investigate the functions of those (G+C)-rich repeats by inserting the repeats in different promoters of plant genes and testing the effects, if any, on gene expression from these promoters in transgenic cells.

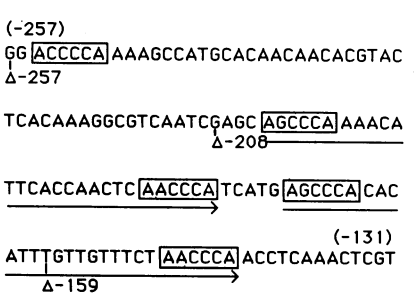

FIG. 4. The 127-nucleotide sequence (−131 to −257) in the 5′ region of the α′-subunit gene. Underlined sequences mark the two imperfect direct repeats. Boxed sequences highlight the five (G+C)-rich direct repeats.

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