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Functional analysis of the regulatory region of a zein gene in transiently transformed protoplasts

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Abstract

The transcription of zein genes in maize is tissue-specific and developmentally regulated. The 5′ regulatory region of many zein genes contains two promoters, P1 and P2, lying approximately 1000 bases apart. The promoter/enhancer activity of various fragments of the two promoter regions of the zein gene E19 have been analysed by means of transient expression experiments. The results indicate that the various regions differentially affect the expression of the GUS reporter gene activity in protoplasts from tobacco leaves, maize immature endosperms and *in vitro* endosperm cell cultures. In tobacco protoplasts only the proximal promoter region, P2, activates GUS expression, while in endosperm culture cells only the distant promoter, P1, gives significant activity. The P1 region, both in direct and opposite orientation, stimulates a low level of GUS expression in protoplasts from immature endosperms. =

Introduction

Zeins, the maize seed storage proteins, are encoded by a multigene family of related sequences, whose coordinated expression is highly regulated and restricted to specific stages of endosperm development [10, 27]. The analysis of cloned zein genes has led to the identification of a typical regulatory region, in which two promoters, P1 and P2, lying approximately 1000 bases apart, determine the appearance of long and short zein transcripts [13]. On the basis of sequence homology and nuclear protein-binding sites, it has been suggested that conserved nucleotide motifs play a specific role in their temporal and spatial expression [3, 14].

In the attempt to elucidate the specific function of sequences from the zein promoter region, dicotyledonous plants have been transformed *via* Agrobacterium with entire zein genes or promoter fragments [18, 22, 31, 33]. The effectiveness of various zein promoter regions in driving transcription of a reporter gene has also been tested by means of transient expression in protoplasts of different origin [4, 25].

We here report transient expression experiments of the reporter GUS gene driven by separated fragments from the regulatory region of a 19 kDa zein gene, in protoplasts from tobacco leaves and maize endosperm tissues; the results indicate that the activity and specificity of the various regions depend upon the fragment/protoplast system considered.

Materials and methods

Plant material and cell cultures

Nicotiana tabacum SRI plants were grown in axenic conditions on MS salts [19], 3% sucrose at 25 °C with a 16/8 h light/dark programme. Maize A69Y, from the collection of the Istituto Sperimentale per la Cerealicoltura, Sez. Bergamo, was employed for the preparation of protoplasts from developing endosperms and for the induction of endosperm suspension cultures [16, 17].

Protoplasts

Tobacco mesophyll protoplasts were prepared from young leaves, wounded with carborundum and incubated in enzyme solution (1% Cellulase Onozuka RS, 0.5% Macerozyme R-10, MS salts, 13 mM $CaCl_2$, 0.5 M mannitol pH 5.5) for 16 hours at 25 °C in the dark. Protoplasts were recovered by resuspending macerated leaves in K3 medium [20], filtering through a 85 μm nylon mesh and centrifuging at $100 \times g$ for 10 min. The floating protoplast band was diluted in EPT buffer (0.5 mM Hepes, 1 mM $CaCl_2$, 0.6 M mannitol pH 7.2) at 10^6 cells/ml. Protoplasts from maize endosperms (10 days after pollination) were prepared according to Schwall and Feix [25], and resuspended in EPE buffer (0.5 mM Hepes, 1 mM $CaCl_2$ pH 7.2, adjusted to 1500 mosm/kg H_2O with sorbitol) at 3×10^6 cells/ml. Protoplasts from maize endosperm cell suspensions were obtained as described [17] from cells five days after subculturing; after floating in K3 medium, they were resuspended in EPT at 10^6 cells/ml.

Protoplast transformation and determination of GUS expression

The TA 750 transfection apparatus (Kruss GmbH, Hamburg, FRG) was used for electroporation; a special device was attached to the output electrode connections in order to monitor each pulse by means of a Philips oscilloscope (Model PM3302). Electroporation conditions: electric pulse (500–2000 V/cm), pulse length (0.05–0.3 ms), number of pulses (1–3), distance between electrodes (0.5–1 mm) and plasmid concentration (8–50 μg/test) were optimized for each type of protoplast in different buffers (pH 6.2–7.2). The most reproducible results were obtained with electroporation between concentric electrodes (1 mm gap), with a single pulse of 1500 V/cm for 0.1 ms, on 0.5 ml protoplast suspensions ($0.5–1.0 \times 10^6$ cells in EPT or EPE medium) with the addition of 35 μg of plasmid DNA and 20 μg of sonicated calf thymus DNA. After electroporation, the protoplasts were left on ice for 10 min and then diluted to 100 000 cells/ml in plating medium (K3 for SRI mesophyll protoplasts; conditioned BMS [17] for maize protoplasts). After 48 h incubation at 25 °C in the dark, cell survival (50–80%) was determined by fluoresceine diacetate staining [32]; β-glucuronidase activity was determined on cell extracts according to Jefferson [12]. Protein concentration was determined by the BioRad protein assay.

Preparation of nuclear extracts and gel-retardation assay

Crude nuclear extracts were prepared from frozen maize endosperms (10–15 days after pollination) according to the protocol developed by J. Clarke [7]. Grinding buffer (HB) and nuclei resuspension buffer (WB) were as described [9]; nuclear proteins were extracted according to Siebenlist *et al.* [26]. Protein was determined by the Bradford procedure [5].

For gel-retardation experiments, the binding reaction was carried out at 25 °C for 15 min in a total volume of 15 μl. Approximately 0.5 ng of end-labelled DNA fragment was incubated in binding buffer (20 mM Hepes pH 8.0, 1 mM DTT, 1 mM EDTA, 100 mM NaCl) in the presence of 4 μg of nuclear extract and 3 μg each of poly(dA-dT) and poly(dC-dG) as competitors. Electrophoresis was performed in 0.5 × TBE at 12 V/cm on an 8% polyacrylamide gel (50:1 cross-linking ratio); gels were then fixed (5% acetic acid, 20% methanol), dried and exposed to X-ray film.

Construction of the chimaeric genes

The GUS gene, together with the NOS terminator sequence, was obtained as an *Eco* RI/*Hin*d III fragment from pBI 101.1 [12] and inserted into the pUC8 vector. The upstream regulatory region of the zE19 gene [28], encompassing the −7 (*Rsa* I) up to the −1450 (*Hpa* I) nucleotides from the ATG start codon, and derived restriction fragments were inserted by blunt-end ligation in the single *Sma* I site in front of the GUS coding sequence (Fig. 1). In puE1GUS the fragment extends from the *Rsa* I (−7) to the *Mae* III site (−300), and contains the TATA, CAAT and 7-11-7 boxes; puE12GUS, starting from the same *Rsa* I site and extending up to the *Hpa* I site (−425), contains in addition the −300 box. The puE3 fragment extends between the proximal *Hpa* I (−425) and the distal *Hpa* I (−1450) sites and contains the putative P1 promoter. Nucleotide sequences of the P2 region [28] and of the P1 region are registred in the EMBL Nucleotide Data Library (Acc. no. X63667).

Two 35S CaMV promoter constructs were used as references. The *pCaMV* contains the 35S region of the pBI 121.1 [12] and *DP33* the *Dde* I-*Dde* I fragment of the CaMV sequence encompassing −289 to +131 with regard to the CAP site [21] thus containing the leader sequence of the longest CaMV transcripts.

Results and discussion

Analysis of the 5′ regulatory region of E19 gene and binding of endosperm nuclear factors

Figure 1 reports the constructs utilized in the present work, and shows the relative positions of the P1 and P2 promoters, and the boxes determining endosperm nuclear-factor-binding activity. The sequence of the P1 region of the zE19 gene was determined and fragment sequences relevant to the present work (Fig. 1) are compared to other zein genomic sequences [6, 11, 28].

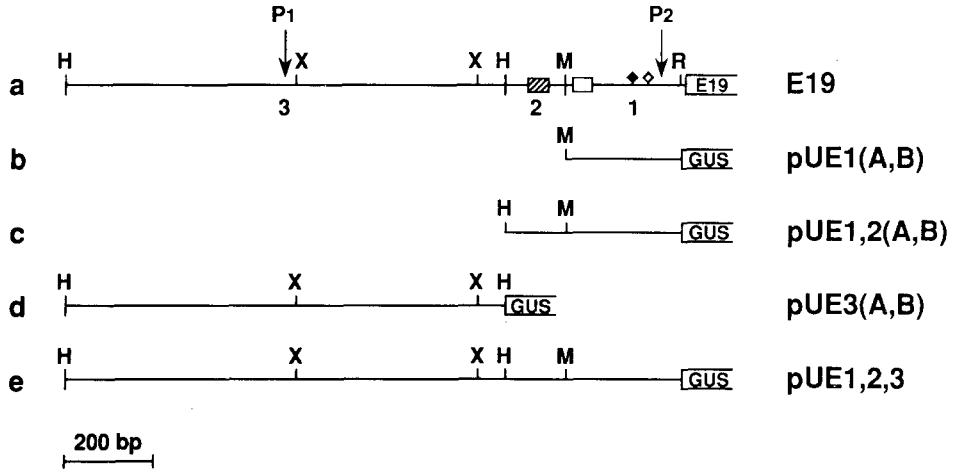
The results obtained from gel retardation experiments (Fig. 2) show a sequence-specific interaction of nuclear extract and the *Xba* I-*Rsa* I promoter fragments of Fig. 1. The method used for the preparation of nuclear extracts from maize endosperm allows the detection of strong interactions, as in the case of the gene pMS1 [15], but not of weaker interactions around the start of transcription. As shown in Fig. 2, only fragment b (which contains the −300 element) and fragment e (containing the 7-11-7 sequence) were retarded. That the interaction detected on fragment b actually involves the −300 sequence is confirmed by the fact that fragments c and d, obtained from digestion of fragment b at the *Mae* III site present in the −300 element, are devoid of any binding activity (Fig. 2).

The analysis of the zE19 gene by S1 mapping [8], by sequence comparisons and by gel-retardation experiments has allowed the identification in the P2 region of the transcription start point, CAAT/TATA boxes and the two sequence motifs with specific endosperm nuclear-factor-binding activity. In the P1 region, sequence comparison also reveals the presence, as in the P2 region, of the CAAT/TATA boxes, situated 190 bp and 210 bp, respectively, downstream of a degenerate −300 element (Fig. 1). Both the −300 elements of the P1 and P2 regions contain, however, the core sequence (GTAAAGG) common to most light-chain zein genes and other cereal storage protein genes [3, 29]. The 7-11-7 element is present, however, only in the P2 region.

Promoter activity in transiently transformed protoplasts

In order to assign a specific function to the putative regulatory regions (7-11-7; −300) we constructed the puE1GUS and puE12GUS plasmids. The puE123GUS and puE3GUS constructs were made in order to verify the promoter and/or enhancer function of fragment 3. Plasmids were constructed so as to have fragments in both orientations, direct (A) and opposite (B) (Fig. 1).

Zein genes are strictly developmentally regulated and tissue-specifically transcribed by a com-

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          ////////////////////
zE19 TTCAGTGGTTGAGAAAAAGGTAAAGGTGTGTCCACACTCACGTGCTCGGCTGCCTCCGCCGAGACGAGGCATGGCGTTGCAAGGTGAGGC -1135
pMS1   A                                    *C  GC AC   AA       T
abl1                                        **CG GC *CC                          G

zE19 ATGGCATGGTGTAGCACGATAGCAGTCATGGTCGTGGCACAGTGTAGCATTGCTGTGGATAGATAGATAAACTATTGTTGTAGTTAGAGT -1045
pMS1           C A      C                            T                              T   C
abl1           C        C                          C  T

zE19 GCATGAGTTTTTGAATGCAACACTAGAATTTTCACAATTTAACTTAATTAAGAAGTTATATCTTGATGACATAGAGTTACCTGACCATTG - 955
pMS1
abl1                 G

                          ////////////////////
zE19 .- -610 bp- -.TGTGGCTATCGTTACACATGTGTAAAGGTATTGCATCACACTATTGTCACCCATGTATTTGGACAATACCGAGAGG  -270
pMS1                                          G     AC                   A            C
abl1                                                 C

                                                                  ........................
zE19 AAAAACCACTTATTTATTGTATTTTATCAAGTTTGTCTTGCTTACGTATAAATTATAACCCAACAAAGTAATCACTAAATGTCAAAACCA  -180
pMS1                             T                                                    T
abl1                             *

zE19 ACTAGATACCATGTCATCTCTACCTTATCTTACTAATATTCTTTTTGCAAAATCCAAAATTAATCTTGCACAAGCACAAGGACTGAGATG   -90
pMS1                                      *
abl1

zE19 TGTATAAATATCTCTTAAATTAGTAGCTAATATATCGCACATATTATTTAGACCAACTAGCAACATAG*AAGCACAATAGTGTACCAACAATG +3
pMS1                 G                                G                  A
abl1                                                  G
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Fig. 1. Schematic presentation of the chimaeric genes containing the fragments of the zE19 promoter and the sequences of relevant elements in the P1 and P2 regions. In the upper panel, a is the zE19 gene promoter region with restriction sites relevant for the constructions. 1, 2 and 3 identify the fragments of the various constructs; arrows indicate the P1 and P2 regions; open and dashed boxes the regions with nuclear protein-binding activity; open and closed diamonds represent the TATA and CAAT boxes, respectively. b, c, d and e show the plasmid constructs containing the three fragments or the entire regulatory region fused to the GUS reporter gene. A and B indicate the direct (A) or inverted (B) orientation of each fragment with respect to the GUS gene (see Table 1). Restriction sites: R, *Rsa* I; M, *Mae* III; H, *Hpa* I; X, *Xba* I. In the lower panel the sequences of the P1 and P2 regions of the E19 gene is compared to other zein genes [6, 11] of the same subfamily. Nucleotides are numbered relative to ATG start codon. Only nucleotide differences of pMS1 and gz19ab11 respect to zE19 are indicated. Stars indicate gaps introduced to maximize homology. Potential CAAT and TATA boxes are overlined; arrows indicate transcription starts. Slashes indicate the

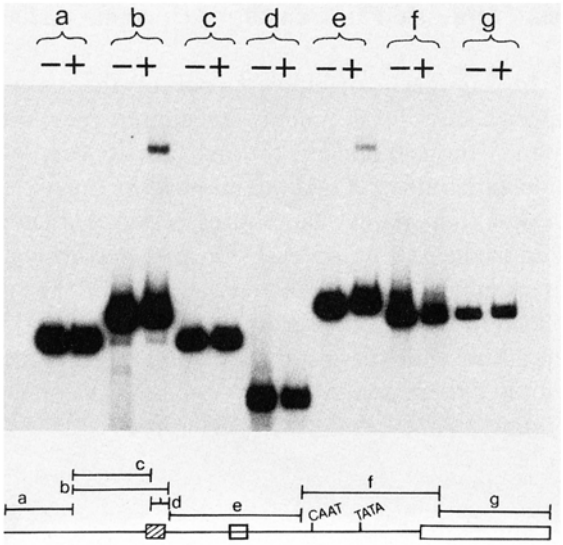

Fig. 2. Gel-retardation experiment. The end-labelled fragments of the P2 region in presence (+) or absence (−) of endosperm-nuclear proteins were separated on polyacrylamide gels. The normal or shifted migration of each fragment has been visualized by autoradiographs on X-ray film.

Table 1. GUS activity in transient expression experiments (pmol 4-MU/min per mg protein). Data are the average of two experiments, one in duplicate and one in triplicate samples. The standard error is indicated in parenthesis.

Promoter	Protoplast source		
	tobacco leaves	endosperm culture	endosperm tissue
None*	6.05 (± 0.25)	19.90 (± 0.1)	2.80 (± 0.8)
pCaMV	32.80 (± 2.08)	83.00 (± 5.4)	6.90 (± 0.9)
DP33	78.80 (± 3.7)	234.00 (± 118)	7.60 (± 0.1)
puE123	18.25 (± 1.65)	17.00 (± 0.4)	4.90 (± 0.1)
puE12A	17.85 (± 2.05)	15.90 (± 2.3)	4.05 (± 1.45)
puE12B	6.30 (± 0.1)	10.75 (± 0.85)	4.75 (± 0.75)
puE1A	6.60 (± 0.1)	10.10 (± 0.2)	4.75 (± 1.25)
puE1B	n.d.	11.60 (± 1.7)	6.50 (± 0.5)
puE3A	6.70 (± 1.6)	31.50 (± 1.7)	7.75 (± 0.75)
puE3B	6.45 (± 3.4)	15.30 (± 5.4)	8.25 (± 1.75)

* Protoplasts electroporated without plasmid DNA.
n.d.: not determined.

plex system of *trans*-acting factors and *cis*-regulatory sequences [2, 16]. One of the aims of the present investigation was to define the functional role of E19 promoter regions in transient expression and to correlate it with the data on their permanent expression already obtained in transgenic petunia plants, where developmental and endosperm specific expression were found [22].

The GUS activity of the E19 constructs and of *pCaGUS* and *DP33GUS* as controls, were analysed in transient expression experiments in *N. tabacum* leaf protoplasts (as a control cell system) and in protoplasts from maize endosperm suspension cultures and from native endosperms ten days after pollination, as monocot tissue-specific cells. Endosperm cultures have been already characterized for the occurrence of zein proteins and transcripts [16, 17]. The results are summarized in Table 1.

In tobacco leaf protoplasts the reporter gene activity is significant for the entire E19 promoter fragment (P1 + P2 regions) and for the shortened version (puE12A, −425 bp) in which both the 7-11-7 and the −300 elements are present. When the inverted versions of the −425 bp fragment or only the distal fragment 3 are used the activity is completely lost. Roussel *et al.* [24] and Boston *et al.* [4], have obtained in carrot protoplasts comparable results with the promoter fragment (P2 region) of a zein gene of the same subfamily, containing the elements present in the 12A construct. These results and our data may suggest that both regulatory elements are necessary to activate transcription.

However, in a recent paper Thompson *et al.* [30] reported significant GUS activity from a zein promoter sequence from which the 7-11-7 element was deleted. Since the puE1A construct, containing only the 7-11-7 element, fails to give any GUS activity, this points to the enhancer function of the −300 element present in frag-

sequences of the '−300 element' present in both P1 and P2 regions showing the core GTAAAGG and corresponding to the slashed box of the upper panel and of Fig. 2. The dotted sequence, showing the 7-11-7 element, corresponds to the open box of the upper panel and of Fig. 2.

ment 2 and may suggest an endosperm-specific function for the 7-11-7 motif. Accordingly, Quayle *et al.* [23] have recently reported the enhancement of the 35S CaMV promoter by the −300 box in protoplasts from an endosperm culture.

In our endosperm culture protoplasts, GUS activity is driven only by the construct containing the fragment 3, in direct orientation (Table 1). We remind that these cultured cells do express zein, but at a very low level [16], and that maximal zein accumulation occurs in the stationary phase of growth [17]; we have not possibly employed this cell system in a stage of culture with optimal zein transcription activity.

The protoplasts from endosperm 10 days after pollination, characterized by undetectable levels of zein transcripts and zein polypeptides [2, 22], show low levels of GUS activity for all the constructs tested. In a similar experiment Schwall and Feix [25] also report a low level of CAT activity driven by the 1.5 kb upstream region of pMS1, a E19 related zein gene (Fig. 1). However, the data of table 1 for fragment 3 (A and B orientations) and for fragment 1B show levels of activity higher than the background and similar to those of constitutive promoters.

The published data and the results of the present work can be interpreted in terms that the promoter and/or enhancer functions recovered in various fragments of the 1.5 kb upstream region depends on the protoplast/construct system employed. It should be noted that none of the plant cells utilized represents a proper system (i.e. a developmental stage specific endosperm cell or tissue). Thus, the capacity of a sequence to drive the expression of a reporter gene, is probably related to the presence or to the absence (and also to the concentration) in the various cell systems, of different transcriptional factors that recognize specific motifs in each fragment. This interpretation is in agreement with the results and suggestions of Benfey *et al.* [1] on the combinatorial role of *cis*-regulatory elements and transcriptional factors in determining tissue- and cell-specific expression. If this is correct, it is quite hard to achieve a correlation between these transient expression data and those obtained with permanent expression in transformed petunia tissues [22].

In summary, definitive proof of the tissue-specific and developmental regulatory functions of the various fragments through transient expression experiments might be obtained by the use of *in vitro* endosperm cell cultures better characterized at the molecular level (for presence/absence of specific transcriptional factors) and by the application of the bombardment method to native immature endosperms of proper maize genotypes mutated in the zein regulatory genes *opaque-2* and *opaque-7* [27].

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References

1. Benfey PN, Ren L, Chua NH: Combinatorial and synergistic properties of CaMV 3S enhancer subdomains. EMBO J 9: 1685–1696 (1990).
2. Bianchi MW, Viotti A: DNA methylation and tissue-specific transcription of the storage protein genes of maize. Plant Mol Biol 11: 203–214 (1988).
3. Boronat A, Martinez MC, Reina M, Puigdomenech P, Palau J: Isolation and sequencing of a 28 kD glutelin-2 gene from maize. Common elements in the 5′ flanking regions among zein and glutelin genes. Plant Sci 47: 95–102 (1986).
4. Boston RS, Beckwar MR, Ryan RD, Goldsbrough PB, Larkins BA, Hodges TK: Expression from heterologous promoters in electroporated carrot protoplasts. Plant Physiol 83: 742–746 (1987).
5. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254 (1976).
6. Brown JWS, Wandelt C, Feix G, Neuhaus G, Schweiger HG: The upstream regions of zein genes: sequence analysis and expression in the unicellular green alga *Acetabularia*. Eur J Cell Biol 42: 161–170 (1986).
7. Clarke J: Ph.D. thesis, Rothamsted Experimental Station (1990).

8. Coraggio I, Compagno C, Martegani E, Ranzi BM, Sala E, Alberghina L, Viotti A: Transcription and expression of zein sequences in yeast under natural plant or yeast promoters. EMBO J 5: 459–465 (1986).
9. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl Acids Res 11: 1475–1489 (1983).
10. Heideker G, Messing J: Structural analysis of plant genes. Annu Rev Plant Physiol 37: 439–466 (1986).
11. Kriz AL, Boston RS, Larkins BA: Structural and transcriptional analysis of DNA sequences flanking genes that encode 19 kilodalton zeins. Mol Gen Genet 207: 90–98 (1987).
12. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907 (1987).
13. Langridge P, Feix G: A zein gene of maize is transcribed from two widely separated promoter regions. Cell 34: 1015–1022 (1983).
14. Maier UG, Brown JWS, Schmitz LM, Schwall M, Dietrich G, Feix G: Mapping of tissue-dependent and independent protein binding sites to the 5′ upstream region of a zein gene. Mol Gen Genet 212: 241–245 (1988).
15. Maier UG, Grasser KD, Haass MM, Feix G: Multiple proteins bind to the P2 promoter region of the zein gene pMS1 of maize. Mol Gen Genet 221: 164–170 (1990).
16. Manzocchi LA, Bianchi MW, Viotti A: Expression of zein in long term cultures of wild type and opaque-2 maize endosperm. Plant Cell Rep 7: 639–643 (1989).
17. Manzocchi LA: Stable endosperm cell suspension cultures from wild-type and *opaque-2* maize. Plant Cell Rep 9: 555–558 (1991).
18. Matzke AJM, Stoger EM, Schernthaner JP, Matzke MA: Deletion analysis of a zein promoter in transgenic tobacco plants. Plant Mol Biol 14: 323–332 (1990).
19. Murashige T, Skoog F: A revised medium for rapid growth and bioassay with tobacco tissue cultures. Plant Physiol 15: 473–497 (1962).
20. Nagy JI, Maliga P: Callus induction and plant regeneration from mesophyll protoplasts of N. Silvestris. Z Pflanzenphysiol 78: 453–455 (1976).
21. Pierce DA, Mettler IJ, Lachmansingh AR, Pomeroy LM, Weck EA, Mascarenhas D: Effect of 35S leader modifications on promoter activity. In: Plant Gene Systems and Their Biology, pp. 301–310 Alan R. Liss, New York. (1987).
22. Quattrocchio F, Tolk AM, Coraggio I, Mol JNM, Viotti A, Koes RE: The maize zein gene zE19 contains two distinct promoters which are independently activated in endosperm and anthers of transgenic *Petunia* plants. Plant Mol Biol 15: 81–94 (1990).
23. Quayle TJA, Hetz W, Feix G: Characterization of a maize endosperm culture expressing zein genes and its use in transient transformation assays. Plant Cell Rep 9: 544–548 (1991).
24. Roussel DL, Boston RS, Goldsbrough PB, Larkins BA: Deletion of DNA sequences flanking an Mr 19000 zein gene reduces its transcriptional activity in heterologous plant tissues. Mol Gen Genet 211: 202–209 (1988).
25. Schwall M, Feix G: Zein promoter activity in transiently transformed protoplasts from maize. Plant Sci 56: 161–166 (1988).
26. Siebenlist U, Henninghausen L, Battey J, Leder P: Chromatin structure and protein binding in putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell 37: 381–391 (1984).
27. Soave C, Salamini F: Organization and regulation of zein genes in maize endosperm. Phil Trans R Soc Lond Ser B 304: 341–347 (1984).
28. Spena A, Viotti A, Pirrotta V: Two adjacent genomic zein sequences: structure, organization and tissue-specific restriction pattern. J Mol Biol 163: 799–811 (1983).
29. Thomas MS, Flavell RB: Identification of an enhancer element for the endosperm-specific expression of high molecular weight glutenin. Plant Cell 2: 1171–1180 (1990).
30. Thompson GA, Boston RS, Lyznik LA, Hodges TK, Larkins BA: Analysis of promoter activity from an α-zein gene 5′ flanking sequence in transient expression assays. Plant Mol Biol 15: 755–764 (1990).
31. Ueng P, Galili G, Sapanara V, Goldsbrough PB, Dube P, Beachy RN, Larkins BA: Expression of a maize storage protein gene in Petunia plants is not restricted to seeds. Plant Physiol 86: 1281–1285 (1988).
32. Widholm JM: The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol 47: 189–194 (1972).
33. Williamson JD, Galili G, Larkins BA, Gelvin SB: The synthesis of a 19 Kilodalton zein protein in transgenic petunia plants. Plant Physiol 88: 1002–1007 (1988).