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A homologous expression system for cloned zein genes

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Summary. Expression of the genes encoding the 10-, 15-, and 27-kDa zeins is maintained in suspension cultures derived from developing endosperm tissue of maize (*Zea mays* L.). Although expression of these genes is reduced in endosperm cultures as compared with that in endosperm tissue from developing kernels, it remains specific to the origin of explant, since no transcripts are detected in leaf tissue-derived suspension cultures. Transcript sizes are identical to those in developing seed endosperm tissue. Furthermore, accurate transcription initiation of the 10- and 27-kDa zein genes is observed by S1 nuclease mapping. Protoplasts isolated from endosperm cultures are capable of expressing foreign genes when transfected by electroporation. We demonstrate that the 5′ flanking sequences of the 10- and 27-kDa zein genes are capable of promoting chloramphenicol acetyltransferase (*CAT*) gene expression in these transfected protoplasts. Our observations show that these maize endosperm cultures can be used as an efficient homologous system to study transcriptional regulation of zein genes.

Key words: Maize – Endosperm culture – Electroporation – Tissue-specificity – Protoplasts

Introduction

Zeins are the alcohol-soluble fraction of storage proteins in maize (*Zea mays* L.). They constitute more than 50% of total endosperm proteins at seed maturity. Zeins consist of a group of heterologous hydrophobic proteins, which are classified according to their M_r on sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) into subclasses with M_r of 27, 22, 19, 16, 15, and 10 kDa (Gianazza et al. 1976; Lee et al. 1976). Based on structural similarities, they are also classified into α- (22 and 19 kDa), β- (15 kDa), γ- (16 and 27 kDa), and δ- (10 kDa) zeins (Esen 1986). Zeins are encoded by a complex multigene family of over 100 gene members (Wienand and Feix 1980; Hagen and Rubenstein 1981; Burr et al. 1982) and regulated in a tissue- and developmental stage-specific manner. Their expression is confined to triploid (3n) endosperm tissue and starts at a specific stage [around 12 days after pollination (DAP)] during endosperm development (Marks et al. 1985a). Furthermore, the onset of elevated zein gene expression coincides with the genome amplification process starting at this particular stage in endosperm development (Kowles and Phillips 1985).

The relatively long life cycle of maize plants and the lack of a readily attainable maize transformation method have rendered the study of zein gene regulation rather difficult. To circumvent these problems, zein gene regulation has been studied by stable or transient transformation experiments in heterologous systems including dicot plant species, mononuclear alga *Acetabularia mediterranea*, and yeast. Recently, protoplasts isolated from endosperm tissue at 10 DAP have been utilized as a homologous system in a transient expression study, and functional activity of a 19-kDa zein gene promoter was demonstrated (Schwall and Feix 1988). However, isolation of a large quantity of protoplasts was only feasible with the endosperm tissue at their developmental stages prior to 11 DAP.

On the other hand, maize endosperm cells have been successfully cultured, and unlike many other cultured plant cells, they remain differentiated. They maintain the syntheses of starch (Chu and Shannon 1975), an-

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thocyanins (Racchi 1985; Racchi and Manzocchi 1988; Saravitz and Boyer 1987), and zeins (Shimamoto et al. 1983; Lyznik and Tsai 1989), which are characteristic of developing endosperm cells. As for the zein synthesis, accumulation in protein bodies has been observed in cultured maize endosperm cells as in endosperm cells of developing kernels, indicating the similarity in cellular processes between the two systems (Shimamoto et al. 1983; Felker 1987). RNA slot-blot analysis confirmed transcription of the 27-kDa zein genes in short-term cultured endosperm cells (Manzocchi et al. 1989). These findings suggest that cultured maize endosperm cells might be used as a homologous system to study regulation of zein gene expression.

We have initially characterized such maize endosperm cultures for the expression of zein genes. We are particularly interested in regulation of γ- and δ-class zein genes. Unlike α-zeins, which are encoded by a large multigene family of 50–100 gene members (Hagen and Rubenstein 1981; Burr et al. 1982; Heidecker and Messing 1987), γ- and δ-zeins together with β-zein are encoded by genes present in few copies (Boronat et al. 1987; Das and Messing 1987; Wilson and Larkins 1984; Kirihara et al. 1988 b), which simplifies molecular analysis of their gene regulation. In the work presented here, we show tissue-specific expression of genes encoding 10-, 15-, and 27-kDa zeins in cultured maize endosperm cells. Accurate transcription initiation of the 10- and 27-kDa zein genes was observed. By developing a transient expression system with protoplasts isolated from these cultured endosperm cells, we have demonstrated the promoter function of the 5′ flanking sequences of the 10- and 27-kDa zein genes. Our findings indicate that these maize endosperm cells are an efficient homologous system for studying the regulation of zein genes.

Materials and methods

Maize cell cultures

Endosperm tissue cultures were established from developing endosperm tissue (13 DAP) from maize inbred line A636. Callus cultures were initiated from the excised endosperm tissues on a semisolid medium consisting of Murashige and Skoog (1962) (MS) salts supplemented with 0.15 g/l L-asparagine, 0.5 mg/l thiamine HCl, 3% (w/v) sucrose, and 0.8% (w/v) Bacto agar, pH 5.8. One to two months after the culture initiation, calli proliferating on the surface of the explants were transferred into a liquid medium (the same medium as above, except that agar is omitted). Endosperm cell cultures were maintained thereafter in liquid suspension for more than 1 year, while being subcultured routinely every 7 days. Suspension cultures derived from immature leaf tissues of germinating seedlings of maize inbred line Black Mexican Sweet (BMS) were obtained from Dr. P. Anderson (Plant Science Research, Inc., Minnetonka/MN). They were maintained in a liquid medium consisting of MS salts supplemented with 0.25 mg/l thiamine HCl, 2% (w/v) sucrose, and 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), pH 5.8, and routinely subcultured every 3–4 days. All tissue cultures were kept in the dark at 26 °C in a growth room. A636 endosperm and BMS suspension cultures were shaken on horizontal shakers at 160 and 250 rpm, respectively.

RNA blot analyses

Total RNA was isolated from maize plant tissues and cultured cells according to the procedure described by Das et al. (1990). Northern and slot-blot analyses were performed according to the procedures described by Cruz-Alvarez et al. (1991). The amounts of RNA loaded are described in the figure legends. The 10-kDa zein gene probe used was a 450-bp NcoI-XbaI fragment of cDNA clone p10kz-1 from inbred line W22 (Kirihara et al. 1988 a). The 15-kDa zein gene probe used was a 932-bp EcoRI-BamHI fragment of genomic subclone pGEMZ14 (obtained from Dr. B. Larkins) from inbred line W64A (Pedersen et al. 1986). The 27-kDa zein gene probe used was a 1.2-kb SphI-SalI fragment of a genomic subclone from inbred line W22 (Geraghty 1985). The 17S rDNA probe used was a 1.5-kb SstI fragment of the M13 clone 6L-1 (Messing et al. 1984). All probes were labeled with ^{32}P-dCTP by Nick-translation (Rigby et al. 1977). Hybridization intensities on the autoradiograms for the slot blots were quantitated densitometrically with a Joyce-Loebl Chromoscan-3 densitometer at 530 nm.

Determination of the 5′ ends of the 10- and 27-kDa zein mRNA by S1 nuclease mapping

Transcription initiation sites were determined for the 10- and 27-kDa zein genes in both developing (16 DAP) endosperm tissue from inbred line A636 and cultured endosperm cells, by S1 nuclease mapping (Sharp et al. 1980). Probes for the two zein genes were prepared from genomic subclones by isolating DNA fragments which extend from the 5′ coding regions of the genes to the 5′ flanking sequences preceding them (Fig. 3 A). The probe for the 10-kDa zein mRNA was prepared from a genomic subclone derived from inbred line BSSS53. A 1,238-bp HindIII-BanI fragment was initially isolated, and the terminal phosphates were removed with calf intestine alkaline phosphatase (CIP, Boehringer Mannheim). It was 5′ end-labeled with γ-^{32}P-ATP by T4 polynucleotide kinase. Subsequently, the radiolabeled fragment was digested with AvaII and a 188-bp 5′ end-labeled AvaII-BanI fragment was purified by electrophoresis in an 8% polyacrylamide gel. The probe for the 27-kDa zein mRNA was prepared from a genomic subclone derived from inbred line W22. A 474-bp HpaII fragment was isolated and 5′ end-labeled with γ-^{32}P-ATP by T4 polynucleotide kinase. Subsequently, it was digested with RsaI and a 343-bp fragment purified by electrophoresis in an 8% polyacrylamide gel.

Strand-specific probes (20,000 cpm) were precipitated with ethanol together with total RNA isolated from developing (16 DAP) endosperm tissue (25 µg) or from cultured endosperm cells (50–75 µg). They were resuspended in 30 µl of hybridization buffer consisting of 0.4 *M* NaCl, 40 m*M* PIPES (pH 6.4), 1 m*M* EDTA, and 80% (v/v) formamide. They were then heat-denatured at 70 °C for 10 min and allowed to anneal for 12 h at 46–50 °C. After hybridization, the reaction mixtures were incubated with 300 µl of S1 nuclease (500 units/ml) in 0.25 *M* NaCl, 50 m*M* sodium acetate (pH 4.6), 4.5 m*M* $ZnSO_4$, and 20 µg/ml salmon sperm DNA as a carrier, at 37 °C for 30 min. The S1 nuclease reaction was terminated and the nucleic acids were precipitated by the addition of 10 µl of 500 m*M* EDTA, 50 µl of 4 *M* ammonium acetate, 1 µl of tRNA (10 mg/ml), and 1 ml of ethanol. The products of the S1 nuclease reactions were analyzed together with Maxam and Gilbert (1980) sequencing reactions of the probes on 6% polyacrylamide gels containing 8 *M* urea.

Construction of chimeric genes

Plasmid pFFCAT was used for construction of chimeric genes containing 5′ flanking sequences of the 10- and 27-kDa zein genes. pFFCAT, provided by Dr. R. Dewey, contains a 777-bp TaqI fragment of the *CAT* coding sequence (from −30 to +747 with respect to the ATG initiation codon) cloned into the SalI site of pFF19 (Timmermans et al. 1990) (Fig. 4A). The 5′ flanking sequences of the 10-kDa zein gene were isolated from genomic subclones pG10BH7 (Kirihara et al. 1988b) as a 1,118-bp HindIII-BamHI fragment spanning from −1,076 to +42 with respect to the cap site, ATC, as described in the Results section. The BamHI site has been created by converting the nucleotides AG to TC (located at +43 and +44 with respect to the cap site) by site-directed mutagensis. Subsequently, it was cloned into the HindIII/BamHI sites of pFFCAT, thus replacing the CaMV35S promoter. It was designated as pZ10(−1076/+42)CAT (Fig. 4A). For the 27-kDa zein gene, a 1,103-bp PvuI fragment spanning from −1042 to +61 (with respect to the cap site, ATC, as described in the Results section) was isolated from a genomic subclone. The fragment was blunt-ended with T4 DNA polymerase and subcloned into the HincII site of pUC119 plasmid. Subsequently, the 5′ flanking sequence was isolated by digesting the plasmid at the polylinker with HindIII and XbaI, and cloned into the HindIII/XbaI sites of pFFCAT, replacing the CaMV35S promoter. It was designated as pZ27(−1042/+61)CAT (Fig. 4A). As a negative control in the transient expression experiments, a promoter-less *CAT* construction (ϕ-CAT, Fig. 4A) was prepared by digesting pFFCAT with HindIII and SmaI. The protruding HindIII end was made blunt by Klenow reaction and the plasmid was religated.

Protoplast isolation

Protoplasts were isolated enzymatically from endosperm suspension cells. Approximately 20–30 g (fresh weight) of suspension cells was collected in sterile, 50-ml disposable tubes and washed once with CPW solution (Evans and Cocking 1977) containing 0.65 *M* D-mannitol. They were incubated in a total volume of 100 ml of enzyme mixture consisting of 3% (w/v) cellulysin (Calbiochem), 1% (w/v) macerase (Calbiochem), and 0.25% (w/v) cellulase (Worthington) in CPW solution with 0.65 *M* D-mannitol (pH 5.4), with gentle shaking (50 rpm) on a horizontal shaker for 10–12 h at room temperature in the dark. Isolated protoplasts were purified by passing the digestion mixture successively through a 140- and a 74-μm stainless steel sieves. When required, protoplasts were further purified by the sucrose [21–23% (w/v)] flotation method (Evans and Cocking 1977). Purified protoplasts were washed twice with CPW solution with 0.65 *M* D-mannitol and the protoplast density was determined with a hemacytometer.

Electroporation of endosperm protoplasts

The isolated endosperm protoplasts were washed once with phosphate buffer saline (PBS) containing 0.65 *M* D-mannitol and resuspended in the same buffer at a final density of 2–3×10^6 protoplasts/ml. They were kept on ice until electroporation. The covalently closed circular plasmid DNA containing the chimeric gene construct was suspended in 500 μl of PBS containing 0.65 *M* D-mannitol in a 2.9-ml disposable spectrophotometer cuvette (Ultra-VU cuvettes-micro, Fisher). Five hundred microliters of endosperm protoplast suspension was added to the cuvette and mixed thoroughly with the plasmid DNA suspension. While keeping the cuvette on ice, electroporation was carried out at 250 V, 600 μF using a stainless steel electrode with a gap width of 4.5 mm (PDS, Inc.). The electroporated protoplasts were cultured in 10 ml of the endosperm suspension culture medium supplemented with 0.65 *M* D-mannitol in a plastic petri dish. The dish was sealed with parafilm and incubated at 25 °C in the dark. To standardize the electroporation efficiency for different constructions, 10 μg of pFFGUS plasmid DNA (Timmermans et al. 1990), containing the β-glucuronidase reporter gene fused to the CaMV35S gene promoter (with a duplicated enhancer) and terminator, was cotransfected in each electroporation experiment.

Chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) enzyme assays

At the end of a 44- to 48-h culture period, transfected protoplasts were collected in a 15-ml conical disposable tube. Subsequently, they were resuspended in 400 μl of 250 m*M* TRIS (pH 7.0), 10 m*M* EDTA in a 1.5-ml microfuge tube and homogenized with a disposable pellet pestle (Kontes Scientific Glassware/Instruments). The homogenate was spun down in a microcentrifuge for 10 min at 4 °C, and the protein concentration in the supernatant was determined using a BioRad Protein Assay Kit. *CAT* and *GUS* assays were carried out according to the procedures described by Malmberg et al. (1985) and Jefferson et al. (1987), respectively. The *CAT* activity was quantitated by measuring in a scintillation counter the radioactivity of the silica gel spots containing the ^{14}C-labeled chloramphenicol and acetylated forms. *GUS* activity was determined fluorimetrically, using 4-methyl umbelliferyl glucuronide (MUG) as a substrate. Fluorescence was measured with a Perkin-Elmer Fluorescence Spectrometer (model LSD-3B), with excitation at 365 nm and emission at 455 nm.

Results

Tissue-specific expression of the 10-, 15-, and 27-kDa zein genes in cultured endosperm cells

RNA blot analysis of total RNA isolated from different tissues of maize plants showed endosperm-specific expression of the genes encoding the 10-, 15-, and 27-kDa zeins (Fig. 1). Transcripts of these three zein genes were also detected in total RNA isolated from suspension culture cells derived from developing endosperm tissue. To rule out the possibility that the observed zein gene expression in cultured cells had arisen from aberrant gene regulation during tissue culture manipulation, we examined the expression of these zein genes in BMS suspension culture cells derived from leaf tissue. Transcripts of the three zein genes could not be detected in the BMS cells (Fig. 1). No gross rearrangement of these gene loci was detected in the genomic DNA isolated from cultured endosperm cells by Southern blot analysis (data not shown). These observations indicate that the expression of the 10-, 15-, and 27-kDa zein genes in these maize endosperm cultures is regulated in a tissue-specific manner.

However, in cultured endosperm cells the expression levels were reduced for all three zein genes as compared with those in the developing endosperm tissue (16 DAP). For better quantitation of the reduction in zein gene expression levels, RNA was further analyzed by slot blots (Fig. 2). When hybridization to the three zein gene

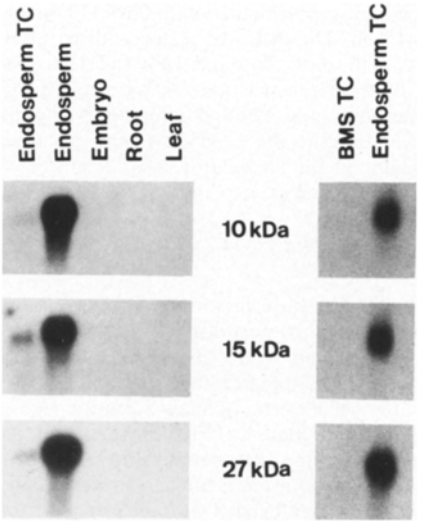

Fig. 1. Northern blot analysis of RNA isolated from maize plant tissues and tissue cultures. Total RNA was isolated from endosperm (16 DAP), embryo (16 DAP), root and leaf tissues of a A636 maize plant, as well as from A636 endosperm tissue culture and BMS leaf tissue culture. RNA samples were fractionated in a formaldehyde-agarose gel, transferred onto a filter, and hybridized to the zein specific probes as indicated: 5 μg of total RNA from each maize tissue was analyzed (*left*); 20 μg of total RNA from maize tissue cultures was analyzed (*right*)

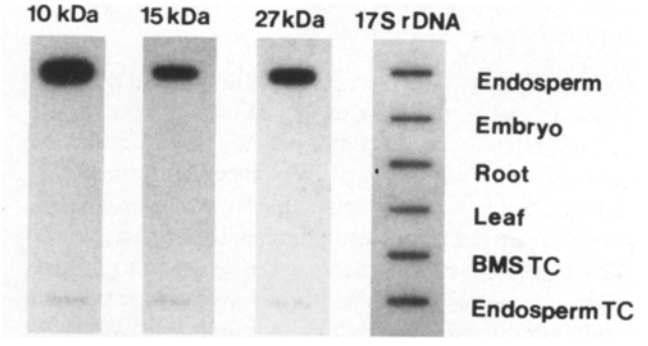

Fig. 2. Quantitative comparison of zein message levels in endosperm culture. Of total RNA isolated from the maize tissues indicated, 2 μg was blotted on a filter in a slot-blot apparatus. The filter was hybridized to probes specific for the 10-, 15-, and 27-kDa zein genes and for 17S rDNA. Hybridization intensity on the autoradiogram was quantitated by densitometry

probes was standardized by hybridization to the 17S rDNA probe, we found that the reductions of RNA levels for the 10-, 15-, and 27-kDa zein genes were about 199-, 22-, and 46-fold, respectively. Nevertheless, expression of these zein genes in the cultured cells is significant, since no transcripts were detected in the embryos (16 DAP) and roots or leaves of young plants when the same amounts of total RNA from these tissues were used for the analysis (Fig. 1).

Accurate transcription initiation of the 10- and 27-kDa zein genes in cultured endosperm cells

In the RNA blot analysis described previously (Fig. 1), a discrete major transcript was identified for the 10-, 15-, and 27-kDa zein genes in both cultured endosperm cells and developing endosperm tissues. The sizes of these transcripts appeared to be identical in both systems, suggesting accuracy in transcription of zein genes in the cultured endosperm cells. To further analyze accuracy of transcription, transcription initiation sites were determined for the 10- and 27-kDa zein genes by S1 nuclease mapping. Since genomic subclones and DNA sequence data are not available for the 10- and 27-kDa zein genes in inbred line A636, the probes for S1 nuclease mapping experiments were prepared by utilizing the genomic subclones derived from inbred lines BSSS53 and W22 for the 10- and 27-kDa zein genes, respectively (Fig. 3A). Thus, total RNA isolated from developing endosperm tissue (16–18 DAP) of inbred lines BSSS53 and W22 were also used in the experiments as controls for the 10- and 27-kDa zein gene probes, respectively.

As shown in Fig. 3B, 2–4 major bands were detected in the S1 protection assays for both 10- and 27-kDa zein transcripts with total RNA derived from developing endosperm tissue as well as from cultured endosperm cells of inbred line A636. The sizes of these bands are identical for the two systems, demonstrating accurate transcription initiation for the two zein genes in cultured endosperm cells. Their sizes are also identical to those for the control RNAs, indicating that the DNA sequences around the 5′ coding and flanking regions of the 10- and 27-kDa zein genes are conserved between inbred lines BSSS53 and A636, and between W22 and A636, respectively. Comparison of the protected bands with the Maxam-Gilbert (1980) sequencing reactions of the corresponding probes mapped the transcription initiation sites to positions -61/-60 and -71/-70/-69/-68, with respect to the ATG initiation codons on the genomic clones, for the 10- and 27-kDa zein genes, respectively. These transcription initiation sites are marked by the consensus trinucleotide, ATC, where a majority of transcripts initiate at nucleotide A. Transcription initiation at or around this consensus trinucleotide has also been observed for the α-zein (Messing 1987) and β-zein genes (Marks et al. 1985b; Boston and Larkins 1986), indicating the conservation of transcription initiation sites for many zein genes.

5′ Flanking sequences of the 10- and 27-kDa zein genes promote chimeric gene expression in cultured endosperm cells

The observations of tissue-specific expression and accurate transcription initiation of the 10- and 27-kDa zein genes in cultured endosperm cells have led us to test

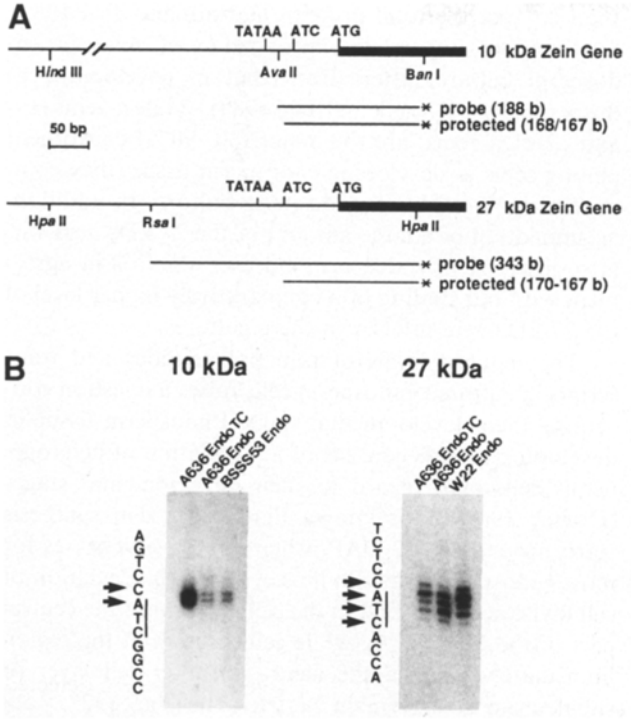

Fig. 3 A and B. S1 nuclease mapping of the 10- and 27-kDa zein genes. **A** Diagrammatic representation of the 5′ coding regions of the 10- and 27-kDa zein genes. The coding sequences of the two zein genes are indicated by *solid boxes*. The probes used for the 10- and 27-kDa zein genes are the 188-bp AvaII-BanI fragment and the 343-bp RsaI-HpaII fragment, respectively. The 5′ ends of the noncoding strands of these fragments were radiolabeled with γ-^{32}P-ATP. The sizes of the protected bands are also shown. The trinucleotide ATC found at the transcription initiation site for the two zein genes is indicated together with TATAA sequences and the ATG initiation codon. **B** S1 nuclease reaction productes were fractionated on 6% polyacrylamide gels containing 8 *M* urea. Maxam and Gilbert (1980) sequencing reactions of the probes were run along with the S1 nuclease reaction products (not shown). Higher counts of the S1 nuclease reaction products for the RNA samples from endosperm culture were loaded in the gels for the comparison. The nucleotide sequences of the coding strands in the region of the protected bands and the location of the bands (*arrows*) are shown at the *left margin* of each figure

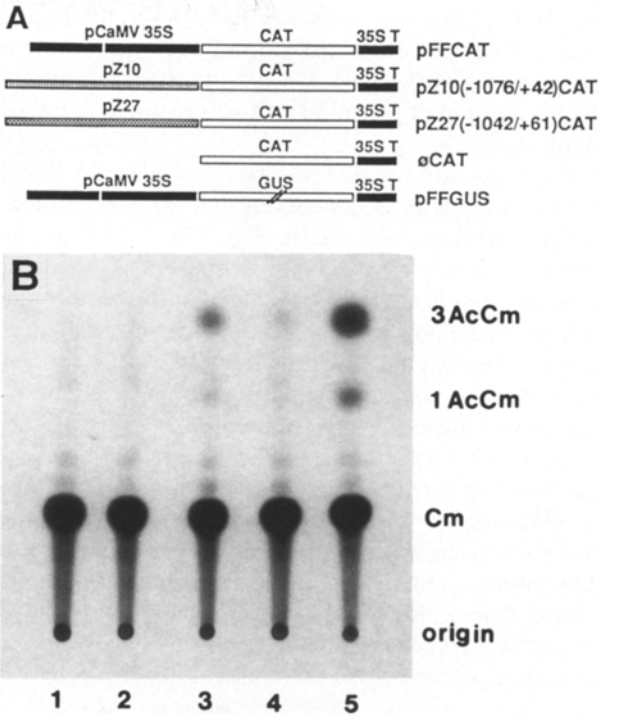

Fig. 4 A and B. Transient expression of chimeric genes in endosperm protoplasts. **A** Diagrammatic representation of the chimeric constructs used in the electroporation experiments. pFFCAT contains the *CAT* gene coding sequences fused to the CaMV35S promoter with a duplicated enhancer and the CaMV35S terminator in pFF19 (Timmermans et al. 1990). The pZ10(−1076/+42)CAT and pZ27(−1042/+61)contain 1.1-kb 5′ flanking sequences of the 10- and 27-kDa zein genes, respectively. End points with respect to the cap sites are designated in *parentheses*. Promoter-less *CAT* construct, ϕ-CAT, was used as a negative control. pFFGUS (Timmermans et al. 1990), containing the *GUS* coding sequences fused to the CaMV35S gene promoter and terminator, was cotransfected with each *CAT* construct to serve as an internal standard for electroporation. **B** *CAT* enzyme assay of transfected endosperm protoplasts. *Lane 1* no plasmid DNA added; *lane 2* 150 µg of promoter-less ϕ-CAT added; *lane* 3 150 µg of pZ10(−1076/+42)CAT added; *lane 4* 150 µg of pZ27(−1042/+61)CAT added; *lane 5* 25 µg pFFCAT added. For each assay, 100 µg of protein extract was used

whether or not these cultured cells could be used to study transcriptional regulation of zein genes. We have established an efficient method for isolating a large quantity ($0.5-2\times10^8$) of viable protoplasts from endosperm suspension cultures by enzymatic digestion of cell walls. Although isolated protoplasts seldom undergo active cell division, they remain viable for more than 2 weeks in culture. Thus, we have developed a transient gene expression system, by introducing chimeric gene constructions into these protoplasts by electroporation.

For the construction of chimeric genes, 5′ flanking DNA sequences of approximately 1.1 kb in size were isolated from genomic clones of the 10- and 27-kDa zein genes derived from inbred lines BSSS53 and W22, respectively. They were fused to the coding sequence of the *E. coli CAT* gene fused to the 3′ terminator sequences derived from the CaMV35S gene (Fig. 4A). Promoter-less *CAT* gene construct, ϕ-CAT, and pFFCAT, containing the CaMV35S promoter with a duplicated enhancer element (Timmermans et al. 1990), were used as negative and positive controls, respectively, for the transient gene expression experiments (Fig. 4A).

As shown in Fig. 4B, the CaMV35S gene promoter yielded a high level of *CAT* gene expression in transfected endosperm protoplasts at an input plasmid amount of 25 µg when assayed 48 h after transfection. On the other hand, levels of *CAT* gene expression promoted by the 5′ flanking sequences of the 10- and 27-kDa zein genes at

this input plasmid level were much lower (data not shown). To enhance the detection of *CAT* gene expression produced by the 5′ flanking sequences of zein genes, a higher amount of plasmid DNA was required per electroporation.

Electroporation with 150–200 μg of plasmid DNA resulted in easily detectable levels of *CAT* gene expression in transfected protoplasts (Fig. 4B). *CAT* gene expression driven by the 5′ flanking sequences of these zein genes was significant when compared with the negative controls, where no plasmid DNA or the same amount of promoter-less ϕ-CAT construction was electroporated (Fig. 4B). The promoter activity of the 5′ flanking sequences was higher, by six- to sevenfold, for the 10-kDa zein gene than for the 27-kDa zein gene, as determined by standardizing the *CAT* activity yielded by these 5′ flanking sequences to the GUS activity derived from the CaMV35S promoter in cotransfected pFFGUS plasmid. These observations have demonstrated that protoplasts isolated from cultured endosperm cells are capable of transiently expressing chimeric genes driven by the 5′ flanking sequences of the 10- and 27-kDa zein genes.

Discussion

Cultured maize endosperm cells are unique in that they remain differentiated rather than becoming "dedifferentiated" as do most cultured plant cells. An endosperm tissue-specific characteristic maintained in these cultured endosperm cells is the synthesis of zein proteins (Shimamoto et al. 1983; Lyznik and Tsai 1989). Biochemical and cellular processes involved in zein synthesis and accumulation in protein bodies in cultured endosperm cells follow, to some extent, those taking place in developing endosperm tissue (Shimamoto et al. 1983, Felker 1987). Our RNA analysis has revealed that genes encoding the 10-, 15-, and 27-kDa zeins are expressed in endosperm cultures of maize inbred line A636. Expression of these zein genes represents the maintenance of the differentiated state of explants rather than their reactivation during tissue culture manipulation, since these genes are not expressed in leaf tissue-derived BMS cultures. Furthermore, maintenance of accurate transcription of zein genes in endosperm cultures can be inferred from the following findings: (1) synthesis of a discrete major transcript for each zein gene, (2) sizes of the zein transcripts, which are identical to those in developing endosperm tissue, and (3) accurate transcription initiation sites (for the 10- and 27-kDa zein genes).

Despite tissue-specific expression of the zein genes, their mRNA levels are drastically reduced in the maize endosperm culture. Reduction in the content of zein polypeptides has also been shown in maize endosperm cultures, where the alcohol-soluble protein fraction is less than 20% of the total proteins (Lyznik and Tsai 1989). Furthermore, the relative proportion of zeins in endosperm culture differs from that in developing endosperm tissue (Lyznik and Tsai 1989). While α-zeins (19- and 22-kDa zeins) are the major (80–90%) constituent among zeins in developing endosperm tissue, they comprise only 10–15% in endosperm culture. In addition, accumulation of a large amount of the 27-kDa zein was also observed in endosperm culture, which is in agreement with our finding of a comparatively higher level of the 27-kDa zein mRNA in these cultures.

The reduced levels of zein polypeptides and transcripts in cultured endosperm cells raises a question concerning their developmental stage. Endosperm tissue in developing kernels consists of a population of heterogeneous cells with regard to their developmental stages (Duvick 1961). It is known that active zein synthesis starts around 10–12 DAP, when cell division ceases for most endosperm cells (Phillips et al. 1985). Cessation of cell division occurs first in the cells present in the central part of the endosperm, while cells present at the region immediately beneath the aleurone (outer cell layer or subaleurone layer) remain meristematic (Fisk 1927; Randolph 1936). The fact that the cultured endosperm cells, derived from endosperm tissue harvested at 13 DAP, remain active in cell division suggests that they are enriched in meristematic cells, typically present at the outer cell layer of the endosperm. There is predominant synthesis of β- and γ-zeins in these cells, while α-zeins are synthesized at very low levels in the outer cell layer (Lending and Larkins 1989). Previous observation that the 27-kDa zein polypeptides are relatively abundant in cultured endosperm cells (Lyznik and Tsai 1989) supports the notion that these cultured cells represent the meristematic cells of the outer cell layer. We have observed that the 22-kDa zein gene expression in the endosperm culture was much more severely reduced than the 15- and 27-kDa zein gene expression (data not shown), which is also in agreement with the above notion.

Based on the observations of tissue-specific expression and accurate transcription of zein genes in endosperm cultures, we have established an efficient transient expression system by utilizing these cultures. A large quantity of protoplasts can be isolated from cultured endosperm cells and transfected with foreign genes by electroporation. We have shown that the 1.1-kb 5′ flanking sequences of the 10- and 27-kDa zein genes can promote expression of the *CAT* reporter gene in transfected endosperm culture protoplasts. The levels of *CAT* gene expression promoted by the 5′ flanking sequences of these two zein genes are much lower than that driven by the CaMV35S promoter with a duplicated enhancer element. A large amount of plasmid DNA harboring these chimeric gene constructs is required in electroporation to obtain detectable levels of *CAT* activity, suggesting weak

promoter activities of the 5′ flanking sequences of these zein genes. Weak promoter activity of the 5′ flanking sequences of the Z4 zein (α-zein) gene has also been reported previously in transgenic tobacco plants (Schernthaner et al. 1988). It is known that the endosperm cells that have ceased cell divisions continue an active DNA replication, leading to the increase in their C value up to 90 C by 16 DAP (Knowles and Phillips 1985). Observations of weak promoter activity of the zein gene 5′ flanking sequences, therefore, are not surprising if we consider that overexpression of zein genes in developing endosperm tissue may rely on the genome amplification process taking place during endosperm development. It is also interesting to note that the 5′ flanking sequences of the 10-kDa zein gene have a higher promoter activity than those of the 27-kDa zein gene, which is converse to the levels of these two zein transcripts in cultured endosperm cells.

In summary, maize endosperm cultures offer the following advantages as a homologous model system to study endosperm-specific gene regulation. Firstly, these cultures can be maintained in a laboratory throughout the year under a defined environmental condition, which circumvents a long waiting period and a large field or greenhouse space required for obtaining endosperm tissues from maize plants. Secondly, a large quantity of protoplasts can be easily isolated from these cultures for transfection with chimeric gene constructions. In the absence of a readily attainable transformation method for maize plants at present, although a recent breakthrough (Gordon-Kamm et al. 1990) promises the use of such an approach in the future, these maize endosperm cultures provide a valuable system for studying endosperm-specific gene regulation.

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