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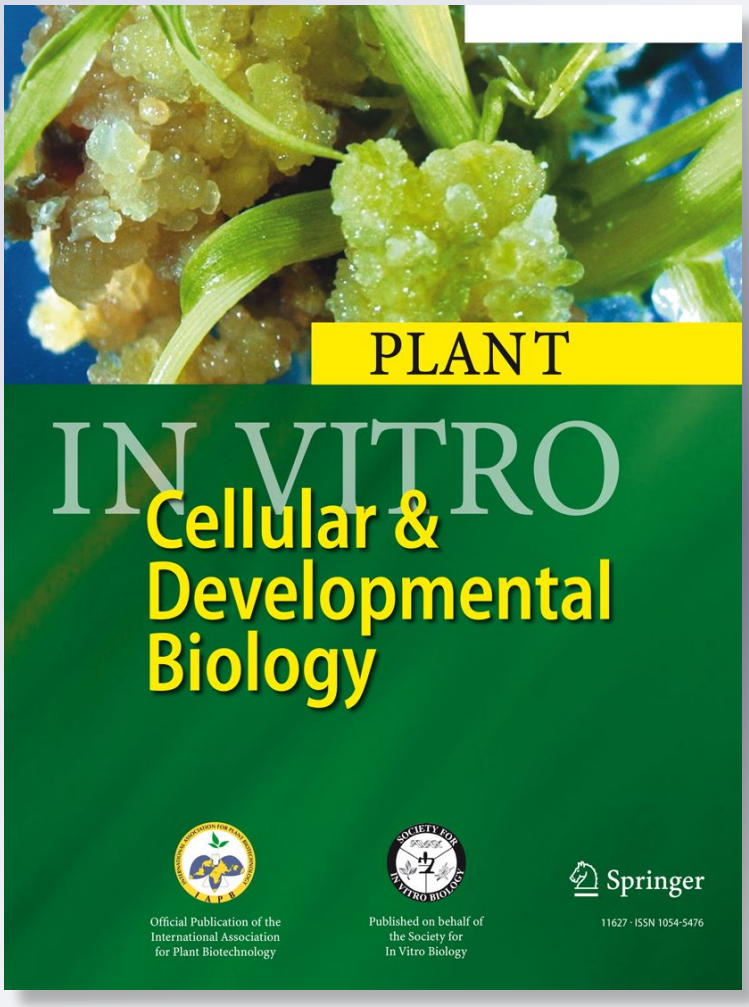

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Establishment and characterization of a maize Hi-II endosperm culture

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Abstract An *in vitro* continuous endosperm callus culture derived from developing endosperm of transformation-amenable maize Hi-II genotype was obtained. The endosperm callus was composed of cells that differentiated into aleurone-like and starchy endosperm-like cell types. This callus has been maintained for 4 yr. Endosperm callus cells transcribe and produce zein proteins at a level similar to developing endosperm tissue. Starchy endosperm cells of the endosperm callus displayed active starch biosynthetic activity. The dual cell physiology of this culture limited the utility of the cell line for promoter analysis and transient assays of gene expression in the current culture conditions. However, because such cell line can be readily initiated and easily maintained for a long period of time, it provides an alternative tool for analysis of transgene expression in endosperm callus derived from transgenic maize lines in Hi-II background.

Keywords Endosperm callus · Maize · Tissue culture · Transient analysis · Zeins

Introduction

The endosperm of corn is the main part of the kernel used for food, feed, and fuel. Because of its importance to humans, the endosperm is an attractive target for quality improvement and recombinant protein production. The endosperm of maize has been studied for several decades and has been the focus of recent biotechnological approaches to diversify its use by humans. Efforts have been made to alter endosperm protein composition (Crow and Kermicle 2002), starch quality, and digestive properties (Ao *et al*. 2007). Numerous reviews have summarized the potential use of maize as a bioreactor for production of recombinant proteins (Kusnadi *et al*. 1998; Hood *et al*. 1999; Streatfield *et al*. 2003; Ramessar *et al*. 2008) and biofuel (Dhugga 2007; Torney *et al*. 2007).

Seed development studies often rely on the availability of mutants (Walbot 1991) or on the generation of transgenic maize in which seed-specific promoters drive the expression or down-regulation of genes of interest in different compartments of seeds (Shen and Petolino 2006). Both processes are time-consuming and laborious due to the extended period needed from planting to endosperm filling and because of the requirement for extensive screening and characterization of mutant or transgenic lines. Difficulty in studying endosperm cells at the molecular level also arises from the nature of the endosperm tissue itself, which is less amenable to manipulations than other abundant tissues such as leaves and roots. Tissue culture of maize endosperm, therefore, has been the focus of extensive research for decades (Gruis *et al*. 2007), with reports of successful *in vitro* growth of maize endosperm as early as 1949 (La Rue 1949). Since then, endosperm cultures have been analyzed for their culture requirements (Straus and La Rue 1954; Shannon and Liu 1977), spontaneous changes *in vitro* (Straus 1958), ultrastructure (Felker 1987), anthocyanin production (Racchi and Manzocchi 1988), sugar uptake in suspension cultures (Felker and Goodwin 1988), zein expression (Shimamoto *et al*. 1983), and glycoprotein synthesis (Riedell and Miernyk 1988). *In vitro* endosperm suspension cultures were used for transient transformation

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analysis of zein gene regulation and chimeric gene expression (Quayle *et al*. 1991; Ueda and Messing 1991). Recently, endosperm callus culture generated from a transgenic maize line was used for study of the aleurone cell specification in the seed (Gruis *et al*. 2006).

The genotypes used routinely for endosperm cultures are currently limited. Very few lines have been analyzed thoroughly and most studies have been performed using cultures derived from inbred line A636 (Ueda and Messing 1991). The effect of inbred and hybrid genotypes on culture establishment (Shannon and Batey 1973) has been studied; not all genotypes of maize are amenable to endosperm callus culture. Genotype-dependent tissue culture responses for the generation of embryogenic maize tissues are well known (Armstrong *et al*. 1991).

The purpose of this work was to establish a continuous *in vitro* culture of maize endosperm that possesses the characteristics of developing endosperm. Here, we describe the generation and properties of a maize endosperm culture line derived from Hi-II endosperm at 11 d after pollination. The Hi-II genotype was selected for this work because it is a genotype amenable for maize transformation and is often used in the generation of stable transgenic corn plants. Endosperm cultures from transgenic maize plants could accelerate studies of seed-specific gene regulation by providing larger amounts of tissue than single seeds.

The endosperm cultures reported here have been maintained *in vitro* continuously for periods ranging from 1–5 yr. Biochemical and molecular characterization of these lines suggests that the Hi-II maize endosperm culture displays properties of immature endosperm tissues with the ability to synthesize starch and zein proteins and to exhibit differentiation into aleurone and starchy endosperm cells. This culture has potential as a tool to enhance knowledge of endosperm development and recombinant protein production.

Materials and Methods

Plant materials and tissue culture media. Maize plants were grown in the greenhouse of the Agronomy Department at Iowa State University in Ames, IA under previously published conditions (Frame *et al*. 2006). Plants were sib- or self-pollinated and ears were harvested 11 d after pollination (DAP). Genotypes B73, B104 and Hi-II (Armstrong *et al*. 1991) were used to test for callus induction from isolated endosperm. Hi-II parent B pollen was used to pollinate Hi-II parent A ears to obtain F_1 Hi-II seeds. Plants germinated from F_1 seeds were either self- or sib-pollinated to generate F_2 immature ears, which were used for endosperm culture initiation.

Full-strength, solid Murashige and Skoog (1962) medium as modified by Ueda and Messing (1991) was used, except that 0.25% Gelrite (Research Products International Corp, Mount Prospect, IL) was used in place of Bacto-agar. This medium is hereafter referred to as MSA.

Endosperm isolation, culture initiation, and maintenance. F_2 Hi-II ears harvested at 11 DAP were surface sterilized according to Frame *et al*. (2000). A sterile scalpel blade was used to remove the caps of the kernels, one row at a time, and the endosperms were isolated using a sterile spatula for placement on fresh MSA medium, carefully avoiding the embryo or any maternal tissue. Plates were sealed with parafilm and incubated in the dark at 28°C. One to 3 mo after initial plating, endosperm callus was transferred onto new MSA plates and subsequently subcultured every 2–3 wk.

Tissue preparation for microscopy. Tissue for microscopy (11 DAP maize kernels and endosperm callus) was prepared as described previously (Chikwamba *et al*. 2003) with the following modifications. Tissue blocks were incubated in a gradually increasing concentration of white London Resin (LR white, Structure Probe, Inc, West Chester, PA), starting with 1:3, 1:1, 3:1 (vol:vol) LR white to ethanol for 8–12 h each time, and finally with 100% LR white overnight. The tissue blocks were cast in gelatin capsules for 48–72 h in a cold room (8–12°C), under UV light to polymerize the resin. Semi-thin sections (1 μm) were cut using an ultramicrotome with glass knives and mounted onto Probe-On Plus (Fisher Scientific, Norcross, GA) slides for staining.

Sections were stained using toluidine blue and I_2/KI solutions for 10 min in the dark. Slides were washed 3× with distilled water and air-dried. Cover slides were applied on the sections after addition of a drop of xylene and Permount (Fisher Scientific). Brightfield and phase contrast images were taken at the Microscopy and Nanoimaging facility at Iowa State University, and images were processed using the AxioVision software (Carl Zeiss Microscopy, LLC, Peabody, MA).

RNA extraction and cDNA synthesis. RNA was extracted from 11 DAP Hi-II endosperms, Hi-II endosperm callus and Hi-II embryo callus, using a Qiagen RNeasy kit (QIAGEN Inc, Valencia, CA) following manufacturer's instructions. Invitrogen's SuperScript III First-Strand Synthesis Super Mix (Invitrogen, Carlsbad, CA) was used for first-strand cDNA synthesis from quantified RNA, following supplier's instructions.

Semi-quantitative PCR. Semi-quantitative polymerase chain reaction (PCR) was done using 0.5 μL of cDNA

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as template for all PCR reactions. Primer sequences are listed in Table 1. Biolase DNA polymerase (Bioline USA Inc, Tauton, MA) was used following supplier's specifications. PCR products were analyzed in a 1% agarose gel stained with ethidium bromide and imaged on a UV transilluminator.

Protein extraction. One hundred milligrams of tissue (11 DAP endosperm, endosperm callus and embryo callus) were homogenized and extracted with 600 μL of an aqueous extraction buffer (Chikwamba *et al*. 2002) or an ethanol-based extraction buffer (Et; 75% ethanol, 5% β-mercaptoethanol). Samples were incubated at 24°C in a vortex shaker for at least 30 min and were pelleted by centrifuging at 14,000 rpm for 10 min in a tabletop microcentrifuge. Supernatants were transferred to fresh microfuge tubes and used directly for denaturing polyacrylamide gel electrophoresis, or freeze dried and resuspended in 200 μL of 2× Laemmli SDS-sample buffer (Laemmli 1970) for the same purpose. Gels were electrophoresed at 110 V for 20 min and 130 V for 50 min and subsequently washed in water. Protein gels were then stained with Coomassie blue for total protein or transferred onto membranes for Western blot analysis.

Western blots. The contents of the gels were transferred to a nitrocellulose membrane for Western blots using a semi-dry transfer apparatus (BioRad Laboratories, Inc., Hercules, CA) at 10 V for 30 min. Membranes were washed with water and then with phosphate-buffered saline with Tween-20 (PBST; 0.01 M Na_2HP0_4, 0.003 M KH_2PO_4, 0.1 M NaCl, 0.05% Tween-20, pH 7.2) for 5 min. Membranes were blocked with 5% dry milk in phosphate-buffered saline with Tween-20 (PBST) for 1 h at 24°C, followed by one wash with PBST. Incubations with primary and secondary antibodies diluted in 1% dry milk in PBST were carried on for 1 h at 24°C, followed by two washes with PBST and two washes with PBS (5 min each). Primary antibodies (P. Scott, USDA-ARS, Ames, IA) were used as follows: rabbit anti-alpha zein (1:2,000) and rabbit anti-gamma zein (1:3,000). Secondary antibody, goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma-Aldrich Co. LLC, St. Louis, MO), was added at 1:5,000 dilution. Membranes were washed with alkaline phosphatase substrate buffer for 1 min before adding 20 mL of alkaline phosphatase substrate (BioRad). Membranes were then incubated for 5–10 min until the signal was visible, and the reaction was then stopped by adding water. The membrane was air-dried overnight and photographed using a Fuji digital camera.

Zymograms. A 100–1,000 mg sample was homogenized using a handheld microfuge pestle with or without freezing with liquid nitrogen. Samples were extracted with Buffer 1 [25 mM sodium phosphate buffer, pH 6.6, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 μg/mL Leupeptin, 0.5 mM Pefabloc (Sigma-Aldrich Co. LLC, St. Louis, MO)], or Buffer 2 (50 mM Tris–acetate, pH 7.5, 10 mM DTT). The protein (15 μg) was mixed with native loading buffers (25% glycerol, 0.1% bromophenol blue; or 30%

Table 1. Primer sequences used for semi-quantitative PCR

Protein	Sequence		
Actin	F, 5′	ATTCAGGTGATGGTGTGAGCCACAC	3′
	R, 5′	GCCACCGATCCAGACACTGTACTTCC	3′
Luminal binding protein, cBiPe3	F, 5′	CGTTCCTGCTCGGCGTCCTGCT	3′
	R, 5′	CCACCCTTCTTGTCCAAACCATAGGC	3′
19kD alpha zein (D1, D2)	F, 5′	ATTCCACAATGCTCACAACAATACC	3′
	R, 5′	CAATAAGGTGGTAGGATTCGTCAAAGC	3′
22kD alpha zein (1, 3, 4, 5)	F, 5′	TCATTATTCCACARTGCTCACTTGCTC	3′
	R, 5′	CGCTGTTGTAGGTACGCAGCAGAGT	3′
18kD delta zein	F, 5′	ATGGCAGCCAAGATGTTTG	3′
	R, 5′	GCACTGTCATCATGTTTGGC	3′
10kD delta zein	F, 5′	ATGGCAGCCAAGATGCTT	3′
	R, 5′	CGCAGTGACATTGTGGCA	3′
16kD gamma zein	F, 5′	ATGAAGGTGCTGATCGTTGC	3′
	R, 5′	GCAGGACCACACCGTATGTC	3′
27kD gamma zein	F, 5′	ATGAGGGTGTTGCTCGTTGC	3′
	R, 5′	ATGCCTCAGGAACTCGACGC	3′
50kD gamma zein	F, 5′	ATGAAGCTGGTGCTTGTGG	3′
	R, 5′	TGACCCTGGAATTGTTGTTG	3′

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glycerol, 0.25% bromophenol blue) and loaded onto a native polyacrylamide gel containing 0.3% corn or potato starch. Electrophoresis was run using 1× Tris–Glycine native running buffer with or without 2 mM DTT. Gels were run for 2 h at 100 V, incubated in renaturation buffer (100 mM Tris–Cl, pH 7.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT) overnight at 24°C. Analyses for starch enzyme activities in large (16 cm×20 cm×0.15 cm) native gels were performed as described previously (Dinges *et al*. 2001). Finally, gels were stained with I_2/KI solution (1 g/10 g/l) until bands became visible. Gels were imaged using a FujiPix digital camera and a white light box.

DNA constructs. Construct A, pAHC25 (Christensen and Quail 1996), was a gift of Dr. P. Quail (University of California—Berkeley/USDA Plant Gene Expression Center, Berkeley, CA). Construct B, Vp1P-Gus (Cao *et al*. 2007), was a gift from Dr. P. Becraft (Iowa State University, Ames, IA). Construct C, 22αzP-GUS, was generated by Gateway® recombination using entry vectors containing the maize 22 kD α-zein promoter (a gift from Dr. D. Jackson, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY), the GUS gene and the nos terminator (Christensen and Quail 1996). Construct D, 35S P-GFP (Mankin *et al*. 2003), was a gift from Dr. W. Thompson (North Carolina State University, Raleigh, NC). Construct E, 27γz P-GFP (Shepherd *et al*. 2008), was a gift from Dr. P. Scott (USDA, Ames, IA).

Transient bombardments and gene expression analysis. DNA (1–4 μg) was coated onto gold particles and introduced into target tissues as described previously (Frame *et al*. 2000). Tissue was bombarded at 1,100 psi, with a 6 cm distance from the macrocarriers. After bombardment, plates were wrapped and incubated in the dark at 28°C for 3 d. For GUS histochemical analysis (Jefferson 1987), tissue was immersed in 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-Gluc) staining solution containing 1.5 mM X-Gluc (Biosynth International, Inc, Itasca, IL), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 50 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, and 1% DMSO at 37°C in dark for 16 hr. Reactions were stopped by replacing X-Gluc solution with 95% ethanol. For GFP and GUS analysis, samples were visualized and imaged using an Olympus SZH10 stereoscope (Leeds Precision Instruments, Inc., Minneapolis, MN) coupled to a SPOT RT color CCD camera (Diagnostic Instrument Inc., Sterling Heights, MI). Images were taken under brightfield for GUS-stained samples or using a band pass filter at 460–490 nm with emission filter at 510–550 nm for GFP detection, and acquired using SPOT Advanced software (Diagnostic Instrument Inc.).

Results

Establishment of a continuous endosperm culture. Ears of B73, B104 and Hi-II genotypes were tested for callus initiation from isolated endosperms. Three mo after culture initiation, a few endosperms of Hi-II genotype started to develop callus (Fig. 1*A*, arrow). Proliferative calli were obtained only on media containing asparagine (MSA). The frequency of endosperm callus (ENC) culture initiation was 0% for B73 and B104 genotypes, and 3% (3 of 100 isolated endosperms develop callus) for Hi-II.

When comparing the ENC to Hi-II embryogenic callus (EMC) cultures derived from immature embryos under the stereomicroscope, ENC did not have the friability that characterizes the Hi-II embryogenic type II callus or the firmness that characterizes type I callus. Callus growing on isolated endosperms was subcultured onto fresh MSA medium and allowed to grow for 1–2 mo. Growth was slow under these conditions, and more frequent subculture of the callus every 10–14 d seemed to accelerate callus growth. Our oldest culture has been growing for 4–5 yr under the conditions described. Several cultures have been initiated and maintained since, proving that the procedure is reproducible.

Hi-II maize endosperm callus cells differentiate into aleurone and starchy endosperm cells. To examine the nature of the endosperm callus culture compared to fresh kernels, tissue was fixed, embedded and sectioned for light microscopy (Fig. 1*D*, *E*). A wild-type Hi-II kernel (Fig. 1*D*) dissected at 11 DAP showed the traditional staining of a maize kernel, with the pericarp (p), aleurone (a), and starchy endosperm (se) cell layers stained by toluidine blue. The aleurone cells showed a densely stained cytoplasm representing a tightly packed single layer between the pericarp and starchy endosperm. On the other hand, starchy endosperm cells showed a lightly stained cytoplasm, with numerous starch granules developing in the cells (Fig. 1*E*). The ENC sections (Fig. 1*E*) showed cells with a densely stained cytoplasm (c) and nucleus (n). Most of these cells were also highly vacuolated (v) cells. Initial analysis of the ENC indicated that the cells were morphologically more similar to aleurone than to starchy endosperm.

To further study the physiology of ENC cells, freshly isolated endosperm tissue and ENC sections were stained using iodine to determine the presence of starch (Fig. 1*F–I*). When whole pieces of callus were stained with iodine solution, an EMC culture stained brown (Fig. 1*C*), while the ENC culture stained brown and dark purple (Fig. 1*B*). Wild-type 11 DAP Hi-II kernel sections showed purple I_2/KI-starch complexes in the granules (Fig. 1*F*, arrows) when viewed under brightfield; the localization of starch granules

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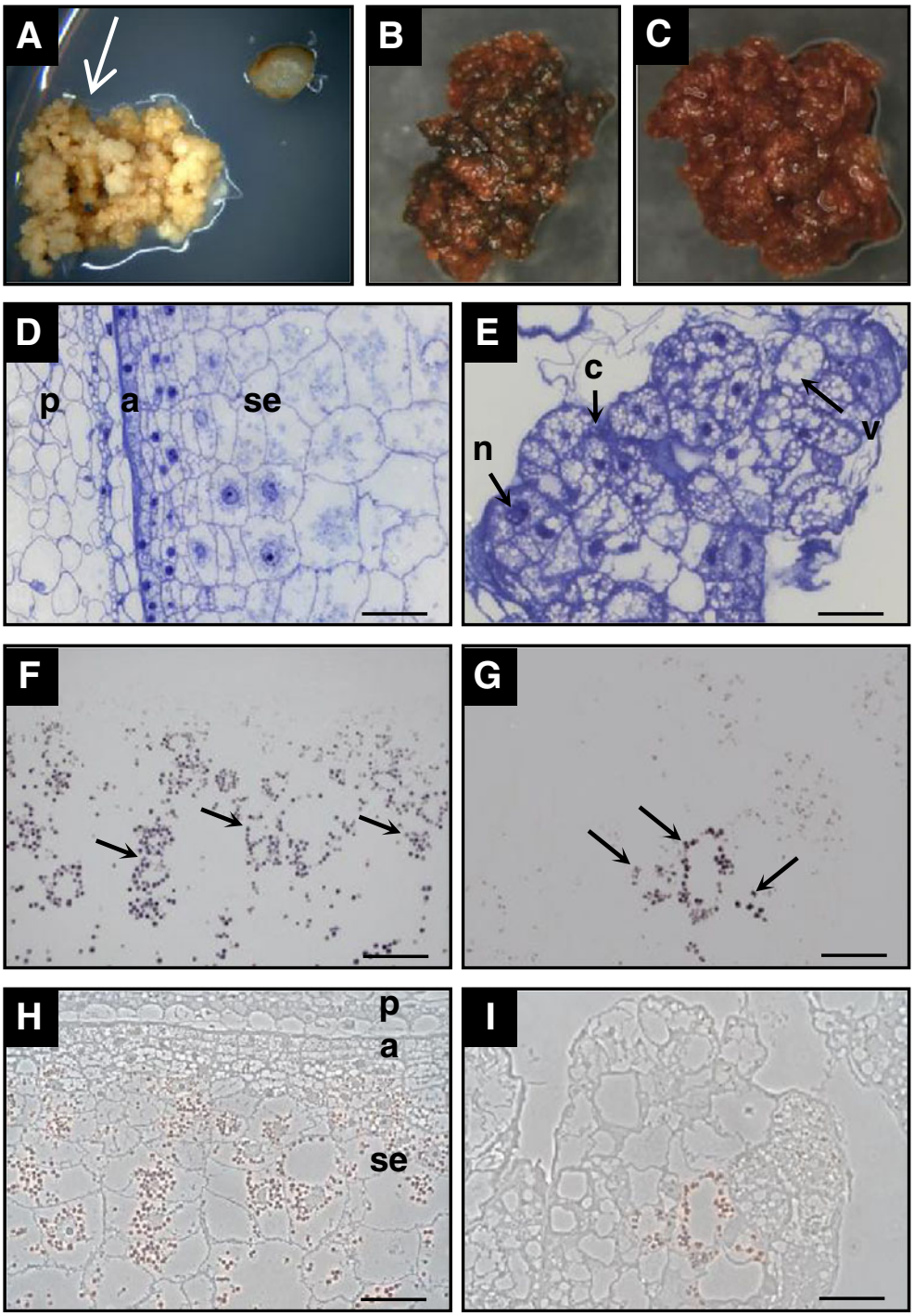

Figure 1. Maize endosperm callus culture derived from Hi-II developing endosperm. (*A*) Developing Hi-II maize endosperm dissected at 11 DAP were cultured on MSA medium and callus proliferated from some endosperms (*arrow*). (*B*) Hi-II maize endosperm callus stained with I_2/KI. (*C*) Hi-II maize embryo-derived callus stained with I_2/KI. (*D–I*) Semi-thin sections (1 μm) of developing Hi-II maize endosperm (*D*, *F*, *H*) and maize endosperm callus (*E*, *G*, *I*) stained with toluidine blue (*D*, *E*) and I_2/KI (*F–I*). Stained sections were viewed and imaged under brightfield microscopy (*D–G*) and phase contrast microscopy (*H*, *I*). *Bars* 20 μm. *DE* developing maize endosperm (11 DAP), *ENC* maize endosperm callus culture, *EMC* maize embryo callus culture, *p* pericarp, *a* aleurone, *se* starchy endosperm, *c* cytosol, *n* nucleus. *Black arrows* in *F* and *G*, I_2/KI-stained starch granules.

corresponded to the starchy endosperm (Fig. 1*H*) when viewed under phase contrast. ENC sections also showed some starch granule staining (Fig. 1*G*, arrows), indicating that the ENC cells had characteristics of both aleurone and starchy endosperm.

Maize endosperm storage protein transcripts were detectable in Hi-II maize endosperm callus tissue. We further investigated if the starchy endosperm cells present in the ENC culture retained starchy endosperm characteristics regarding transcriptional activity. The endosperm tissue is not only the major organ for starch deposition in maize but also serves as a protein storage reservoir. To determine the presence or absence of transcripts of storage proteins in the maize endosperm tissue, we carried out semi-quantitative PCR (sqPCR) on cDNAs derived from maize 11 DAP developing endosperms (DE), ENC and EMC total RNAs (Fig. 2*A*). Zein storage proteins that express in starchy

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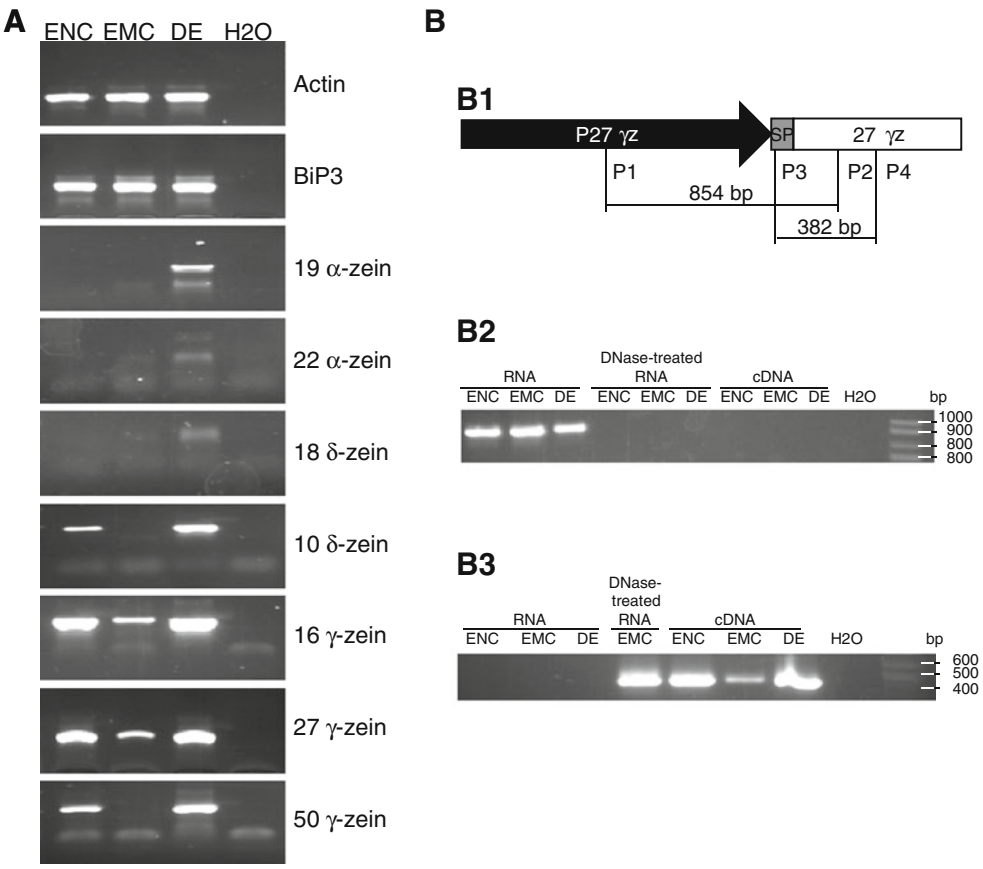

Figure 2. Semi-quantitative PCR on cDNA from maize endopserm callus culture, maize embryo callus culture and maize kernels 11 DAP. (*A*) Maize storage protein transcripts. (*B*) Analysis of 27 kD γ-zein (*B1*) Expression using primers to determine possible template contamination in embryo callus samples. (*B2*) Amplification with primers spanning the promoter region and coding sequence. (*B3*) Amplification with primers spanning the coding region only. *DE* developing maize endosperms (11 DAP), *ENC* maize endosperm callus culture, *EMC* maize embryo callus culture.

endosperm were chosen as main marker proteins. Also tested were constitutive protein transcripts such as actin and luminal binding protein as controls. Genes tested and primers used are listed in Table 1.

Semi-quantitative PCR (sqPCR) of control transcripts indicated that the transcripts for maize actin and maize luminal binding protein (BiP3) showed equal intensities in all three tissues tested (Fig. 2*A*). However, the transcript levels for most of the zein genes tested were differentially expressed in ENC, EMC, and DE. Transcripts were detected for 19 kD α-, 22 kD α-, and 18 kD δ-zeins in DE, but in ENC and EMC, transcript levels were very low or undetectable. Transcript levels of 19 kD α- and 22 kD α-zeins typically peak at 17 DAP (Dolfini *et al*. 1992; Woo *et al*. 2001). At 10 DAP, α-zein transcripts are of low abundance and restricted in localization to certain areas of the endosperm. Since we do not know which areas of the dissected endosperms were more prone to form callus, this could explain why α-zeins levels were much lower in ENC than in DE.

On the contrary, transcripts were detected in both ENC and DE samples for 10 kD δ-, 16 kD γ-, 27 kD γ-, and 50 kD γ- zein. It has been shown that 16 kD γ- and 27 kD γ-zeins were expressed strongly and uniformly throughout the starchy endosperm (Woo *et al*. 2001) and could be a consequence of their role in protein body assembly. The 10 kD δ-zein, however, occurs usually at lower levels compared to the other zeins (Woo *et al*. 2001). The accumulation of 10 kD δ-zein transcript is restricted to a small group of cells of the abgerminal side of the endosperm (Woo *et al*. 2001). This observation may be helpful to understand the origin of the ENC culture.

Interestingly, transcripts for 16 kD γ- and 27 kD γ-zein were also detected in EMC. To determine whether the presence of these transcripts in EMC were the products of sample contamination or contamination with DNA template, validation experiments were conducted (Fig. 2*B1*). Primer set P1–P2 was to detect an 854-bp fragment spanning the promoter into the coding region of the 27 kD γ-zein gene, which should only give an amplification product in the presence of DNA template, since transcripts carry only coding sequences. Similarly, primer set P3–P4 was designed to amplify a 382-bp fragment in the coding sequence of 27 kD γ-zein (Fig. 2*B1*). Both primer sets were tested in total RNA, DNase I-treated and cDNA samples. Using total RNA template resulted in the presence of amplification bands of the sizes predicted (Fig. 2*B2* and *B3*), which confirmed that the primer pair was functional. With DNase-treated RNA samples, amplification products were only recovered with primers P3–P4, which proved that samples used for cDNA synthesis were free of contaminating DNA. cDNA samples gave no PCR

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product for P1–P2 primers, ruling out the possibility of DNA contamination in the EMC. That the same cDNA samples were used for all sqPCR reactions also rules out contamination with ENC or kernel RNA, in which the same patterns of amplification would be observed for all genes tested.

The sqPCR results indicated that the ENC culture possessed the ability to transcribe storage protein genes that are commonly expressed in the starchy endosperm of developing maize kernels. While the morphological analysis showed mixed populations of cells, the presence of zein transcript confirmed that ENC has functional starchy endosperm cells as well, capable of transcribing maize storage protein genes.

Maize endosperm storage proteins were expressed and accumulated in Hi-II maize endosperm callus tissue. To confirm if ENC derived from Hi-II genotype also accumulated certain zein proteins to detectable levels, protein extracts from DE, ENC, and EMC cultures were evaluated (Fig 3*A*). Zeins were readily extracted in ethanol-based buffers (Fig. 3*B* and *C*). These blots showed that ENC, unlike EMC, accumulated zein storage proteins, α- and γ-zeins to degrees comparable to those found in fresh endosperm tissues.

Similar findings had been reported for endosperm cultures of the inbred line A636 in which zeins were also detected (Shimamoto *et al*. 1983). However, two of the main classes of zeins were specifically identified here (Fig. 3), compared to the generic "zein" antibody used previously (Shimamoto *et al*. 1983). More than one class of zein was accumulated (γ-zeins in Fig. 3*B* and α-zeins in Fig. 3*C*). The ability of the Hi-II ENC culture to transcribe, translate and accumulate zein storage proteins was maintained for 4 yr on solid media.

Characterization of starch metabolism enzyme activity. Due to the nature of the endosperm tissue, accumulation and activity of starch biosynthetic enzymes was also expected. To determine whether starch enzymes were active in ENC maintained for 3 yr, zymogram analysis was performed (Dinges *et al*. 2001). Proteins from ENC, EMC, and DE were separated by native-PAGE on a starch containing gel and visualized by iodine staining (Fig. 4). Iodine would form an insoluble purple precipitate in presence of starch. Upon hydrolysis, highly branched regions would stain red, while regions with less branching would stain blue. Complete starch hydrolysis could be observed as white bands in the purple background, where the iodide could not bind.

Figure 4 displays activities of several starch metabolism enzymes. Gel was compared to the published results (Colleoni *et al*. 2003) in which starch-metabolizing activities were characterized. It is notable that a similar set of enzymes is present and active in EMC, ENC and DE samples. ENC extracts showed activities of branching enzyme I, pullulanase, and α-amylase. However, branching enzyme II and isoamylase activities were difficult to discern. Identifying active starch metabolism and the associated enzymes opens possibilities to further study starch synthesis in ENC.

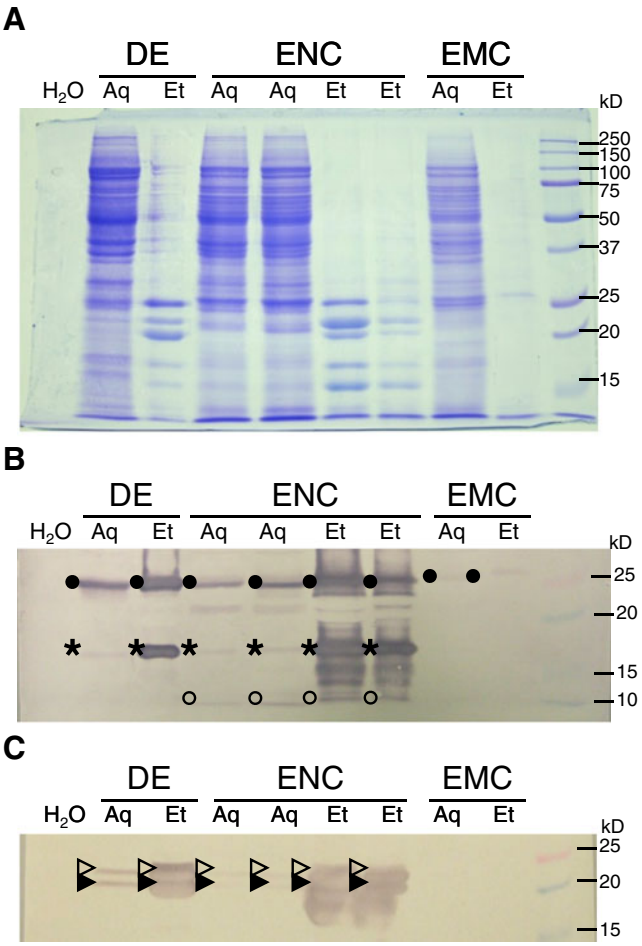

Figure 3. Proteins from fresh endosperm (11 DAP), endosperm callus and embryo callus. Total proteins extracted with aqueous (Aq) and ethanol (Et) buffers were separated by SDS-PAGE and stained for total proteins with Coomassie stain (*A*). Separated proteins were transferred onto a nitrocellulose membrane and probed using anti γ-zein (*B*) or anti α-zein (*C*) antibodies. *DE* developing maize endosperm (11 DAP). *ENC* maize endosperm callus culture, *EMC* maize embryo callus culture, *closed circle* 27 kD γ-zein, *Asterisks* 16 kD γ-zein, *open circle* 10 kD γ-zein, *open arrowhead* 22 kD α-zein, *closed arrowhead* 19 kD α-zein.

Contrary to α-amylase, β-amylase activity was only detectable in EMC and ENC, but not in DE (Fig. 4). This observation, together with the fact that α- and β-amylase activities seem to be stronger in both in EMC and ENC, may suggest a potential response to tissue culture conditions such as high sucrose in the culture media. Identification of starch metabolism activities in ENC may

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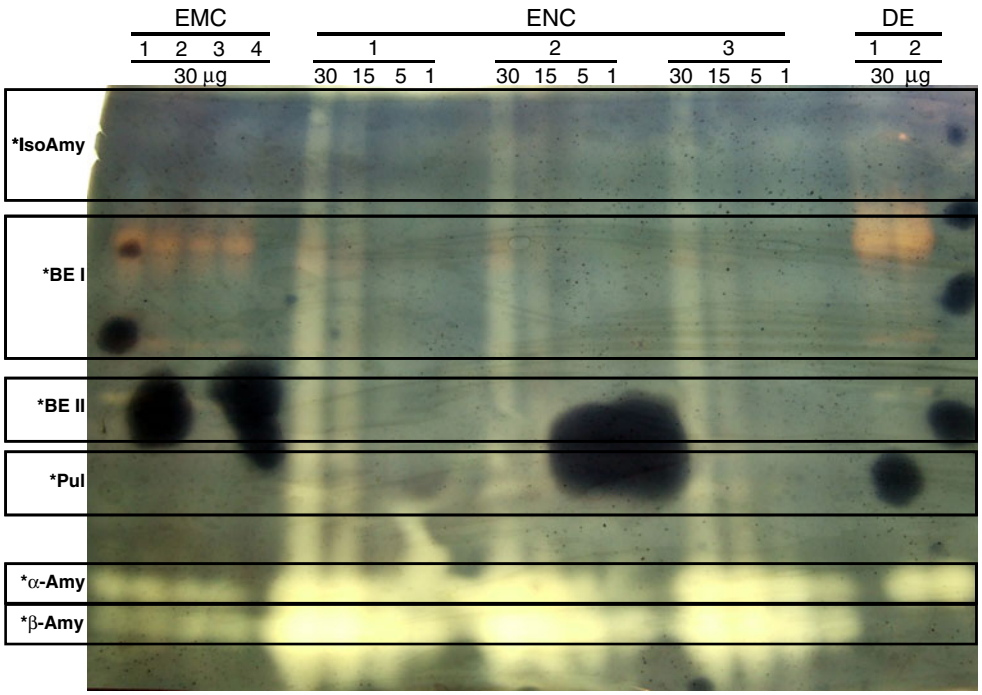

Figure 4. Zymogram analysis of endosperm callus culture, embryo callus culture and developing maize endosperm for detection of starch enzyme activities. Aqueous protein extracts were separated on Native-PAGE gels containing maize starch. Activity bands were revealed by incubation of the gel in I_2/KI solution and washing in water. *White* and *red bands* indicate regions in which enzymes have degraded the starch substrate. *DE* developing maize endosperm (11 DAP), *ENC* maize endosperm callus culture, *EMC* maize embryo callus culture, *E* empty lanes. * Putative starch metabolism enzymes based on (Colleoni *et al*. 2003). *Dark spots* are due to unsolubilized starch in the gel stained purple with iodide. *IsoAmy* isoamylase, *BEI* branching enzyme I, *BEII* branching enzyme II, *PuI* pullulanase I, *α-Amy* α-Amylase, *β-Amy* β-Amylase.

permit the characterization and comparative analysis of starch, starch properties of ENC with kernel counterparts and starch metabolism enzyme response to *in vitro* culture.

Endosperm callus culture for transient analysis of gene expression. One of the motivations for establishing an ENC is to use it for rapid gene testing, avoiding the lengthy process required for developing stable transgenic maize plants or requirement to isolate fresh endosperm material. To test if our ENC is suitable for transient gene expression analysis, we used particle bombardment to deliver a series of constructs including reporter genes GUS and GFP under control of different promoters. Marker genes driven by the constitutive maize Ubiquitin 1 promoter (PUbi) and the CaMV 35S promoter (P35S) resulted in detectable expression of both GUS enzyme (Fig. 5*A*) and GFP protein (Fig. 5*B*). Use of the aleurone-specific promoter Viviparous 1 (PVp1) also resulted in detectable accumulation of GUS enzyme (Fig. 5*C*).

For starchy endosperm-specific promoter evaluation, GFP expression could be easily detected in ENC bombarded with the construct carrying 27 kD γ-zein promoter (Fig. 5*D*). Interestingly, no expression was observed in ENC tissue bombarded with GUS constructs regulated by the 22 kD α-zein promoter (Fig. 5*E*) or the 27 kD γ-zein promoter (data not shown).

Compared to a typical biolistic-mediated transient gene expression assay using GUS or GFP as marker in maize embryo-derived callus tissue, the ENC transient results were not very informative because of low and sporadic expression. Expression of the constitutive PUbi and P35S, as well as PVp1, suggests the possible presence of aleurone-like cells in the culture, a property that has been used to study the position effect on aleurone cell differentiation (Gruis *et al*. 2006). We cannot explain why we did not see higher levels of marker gene expression under these endosperm-specific promoters in the ENC culture. However, this same phenomenon occurred when bombarding endosperm slices of developing endosperms (data not shown) and has been reported for other endosperm suspension cultures (Quayle *et al*. 1991). When stable transgenic maize lines carrying the P27γz -GUS and the P35S -GUS were analyzed, strong GUS expression was observed in the seed endosperm for the P27γz (Fig. 5*G*) but predominantly in seed embryo and aleurone layer for the 35S P (Fig. 5*H*).

The absence of expression with a P22αz-driven GUS was unexpected, considering that α-zein proteins were

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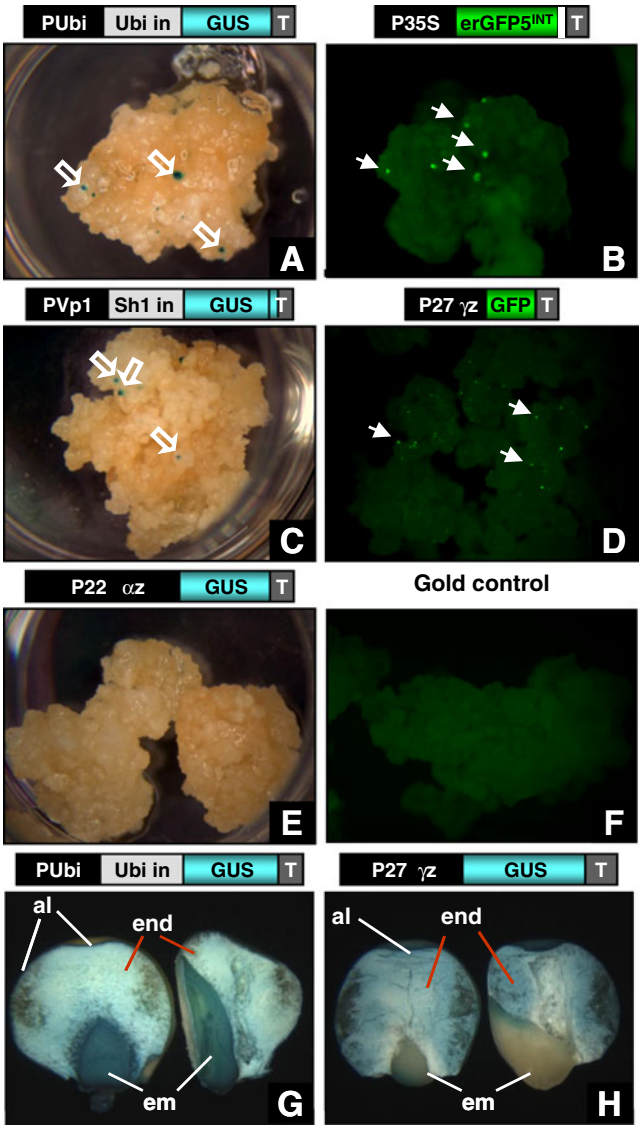

Figure 5. Analysis of transient gene expression in bombarded endosperm callus. Maize endosperm callus was bombarded using the indicated constructs. GUS and GFP expression were assessed visually. (*A*) PUbi1-GUS (Christensen and Quail 1996). (*B*) P35S-GFP (Mankin and Thompson 2001). (*C*) PVp1-GUS (Cao *et al*. 2007). (*D*) P27γz-GFP (Shepherd et al. 2008). (*E*) P22αz-GUS. (*F*) Gold control. (*G*) Transgenic maize kernel expressing pUbi1-GUS in embryo tissue and aleurone. (*H*) Transgenic maize kernel expressing p27γz-GUS (Chikwamba, unpublished) in the starchy endosperm. *Block arrows* GUS foci, *arrows* GFP foci, *GUS* β-glucuronidase, *GFP* green fluorescent protein, *PUbi1* maize ubiquitin 1 promoter, *P35S* cauliflower mosaic virus 35S promoter. *PVp1* maize viviparous 1 promoter, *P27γz* maize 27 kD γ-zein promoter, *P22αz* maize 22 kD α-zein promoter, *Ubi in* ubiquitin intron, *Sh1 in* shrunken 1 intron, *erGFP5INT* GFP targeted to the endoplasmic reticulum.

detected in ENC (Fig. 3*C*). Additional factors may be required for transient gene expression when using the α-zein promoter. Maize Opaque 2 (O2; Schmidt *et al*. 1992) and prolamin box binding factor (PBF) (Vicente-Carbajosa *et al*. 1997) proteins act as transcription factors for α-zein protein expression. When maize O2 and PBF were co-bombarded with the GUS gene driven by rice seed storage protein glutelin promoter (*Gt1*), transient GUS expression was enhanced three- to sixfold compared to the *Gt1*-GUS control in rice immature endosperm cells (Hwang *et al*. 2004). In addition, the 22 kD α-zein may not express strongly at this particular stage of endosperm development. Our sqPCR results showed that 22 kD α-zein transcripts were very low (Fig. 2). Future experiments co-delivering the transcription factors could help elucidate if the α-zeins in the ENC could be increased by providing O2 and PBF.

Discussion

This work describes the establishment and maintenance of a continuous *in vitro* callus culture derived from 11 DAP freshly isolated endosperm of genotype Hi-II over a period of 4 yr. Initiation of the ENC for Hi-II was relatively straightforward following the published medium used to establish ENC for inbred A636 (Ueda and Messing 1991), although the frequency was low (avg 3%). We were unable to initiate ENC lines for inbreds B73 and B104. Even though line A636 has been extensively used (Felker 1987; Felker and Goodwin 1988; Riedell and Miernyk 1988; Ueda and Messing 1991), current genotypes of interest for transformation include Hi-II, B73, and B104. Hi-II is preferred because of its excellent tissue culture response and transformation competency. B73 and B104 are inbreds widely used in the community, and the genome of B73 has been sequenced.

Gruis *et al.* (2006) used ENC from stable transgenic Hi-II lines expressing marker proteins under various tissue-specific promoters to study endosperm cell fate. The main differences in culture media formulations between that work and the present study were their use of 15% sucrose (compared to 3% in this work), 400 mg/L asparagine (compared to 2 g/L in this work) and 5 mg/L thiamine (0.5 mg/L in this work). Gruis *et al.* (2006) also added 10 μg/L 6-benzylaminopurine (Gruis *et al*. 2006). For the initiation of the ENC, fresh endosperm at 6 DAP was used in Gruis' work instead of 11 DAP used in this work.

Similar to Gruis *et al.* (2006), the morphological characterization of the ENC in our work confirmed the dual nature of the culture, revealing the presence of both aleurone-like and starchy endosperm-like cells. Many reported studies using ENC cultures were in the form of suspension culture (Shannon and Liu 1977; Felker 1987; Felker and Goodwin 1988; Riedell and Miernyk 1988; Quayle *et al*. 1991; Ueda and Messing 1991). The ENC established in our work is a continuous callus culture, maintained on solid media in Petri dishes, that remains

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viable after 4 yr *in vitro*. Compared to suspension culture, use of callus cultures is easier to manage, requiring less frequent transfer, less media and space for storage, and no need for dedicated equipment such as a shaker incubator. On the other hand, because cultures grow slower on solid media than in liquid media, ENC grown on plates may have different aging processes than those grown in suspension culture and possess less homogenous cell proliferation and development. It would therefore be of interest to compare gene expression profiles and other characteristics of ENC from both suspension and solid cultures that were initiated and maintained at the same time.

In our study, transcript analysis of members of the α-, δ-, and γ-zeins were similar to those reported previously (Ueda and Messing 1991). Identification of transcripts for the 10 kD δ-, 16 kD γ-, 27 kD γ-, and 50 kD γ-zeins in a long-term culture derived from endosperms (11 DAP) corresponded with the patterns of expression for these types of zeins (Woo *et al*. 2001). Very low to undetectable levels of transcripts of the 19 kD α-, 22 kD α-, and 18 kD δ-zeins in ENC cultures correlated well with what has been observed previously for developing endosperms (Woo *et al*. 2001) in which 10 DAP transcripts were not only of low abundance but limited to specific regions of the endosperm tissue. Our results were comparable with transcript analysis of 10 kD δ-, 15 kD β-, and 27 kD γ-zeins in endosperm suspension cultures of line A636 started at 13 DAP reported previously (Ueda and Messing 1991), specifically for 10 kD δ- and 27 kD γ-zeins. Ueda and Messing (1991) did relative quantification of the transcripts and reported that transcripts for 10 kD δ-, 15 kD β- and 27 kD γ-zeins showed reduced levels by 199-, 22-, and 46-fold, respectively, when compared to developing endosperms (16 DAP). Our results on semi-quantitative PCR confirmed their results for 10 kD δ- and 27 kD γ-zeins and suggest that the same is especially true for α-zeins. Massively parallel signature sequencing was used to compare the steady-state levels of endosperm marker transcripts between *in vitro*- and *in planta*-grown endosperms (Gruis *et al*. 2006). Genes analyzed included the 27 kD γ- and 16 kD γ-zeins and two highly aleurone-specific genes. Their results indicated that when cultured *in vitro*, endosperm 27 kD γ- and 16 kD γ-zein transcript levels initially increased, but markedly decreased after a period in culture. No long-term culture data was reported (Gruis *et al*. 2006). Interesting prospects include future studies of the induction or repression of these genes *in vitro* and the significance that this may have biologically.

At the protein level, several groups had probed to detect zeins (Shimamoto *et al*. 1983; Racchi and Manzocchi 1988). Maize 19 and 22 kD α-zeins were detected in ethanol extracts and protein bodies recovered from A636 endosperm suspension cultures; zein levels from A636 ENC were substantially lower than from 20 DAP kernels (Shimamoto *et al*. 1983). Zeins were also detected in pigmented and unpigmented long-term cultures of maize hybrid K55/W23 at 10 DAP, even though they were not detected in freshly isolated developing endosperms of the same age. Both of the aforementioned studies used antibodies that reacted to 19 and 22 kD zein bands, likely the α-zeins of these molecular weights. We have used two antibodies that react specifically to α- and γ-zeins. Our Western blots show that the accumulation of γ-zeins corresponded to transcript level accumulation. However, for α-zeins, the presence of proteins was somewhat unexpected, since transcript levels were very low. This could be the result of the endosperms "aging" *in vitro* to later stages of development, as has been suggested previously (Shimamoto *et al*. 1983). Also, protein accumulation could be better tolerated by the culture than transcript accumulation.

The presence of 16 and 27 kD γ-zeins in EMC culture was not expected. Low expression of these zein genes may occur in embryo tissues at the early stages of seed development. We have observed previously some "leaky" expression of a color marker gene in stable transgenic embryo culture while using the 27 kD γ-zein promoter (K. Wang, unpublished). Such observations also correlated with what has been observed more recently in accumulation of GFP expression in maize embryogenic callus driven by the 27 kD γ-zein promoter (Wu and Messing 2009). In this last study, accumulation of both GFP and 27 kD γ-zein proteins were observed in callus derived from maize immature embryos.

Morphological analysis of ENC of genotype A636 has shown that suspension cultures can accumulate starch (Felker 1987). In this work, we confirmed that Hi-II ENC cultured in plates also accumulated starch, as evidenced by dark purple staining with iodide. We also presented evidence that starch metabolism enzymes were active as shown by starch zymogram analysis. The patterns of enzyme activities of EMC and DE were similar to ENC and to each other. However, EMC and ENC showed strong activities of putative α- and β- amylases compared to DE. Future work will help establish if this is the result of *in vitro* culture and what role these enzymes are playing in these cells.

Our initial interest in establishing an ENC was to perform transient expression analysis of transgenes driven by starchy endosperm-specific promoters to bypass the need to generate stable transgenic lines; as well as preparing fresh developing endosperm materials. However, this work suggests that bombardment of ENC for transient analysis using reporter genes as we typically do for a similar transient assay in the EMC system may not be the best approach at this stage. The successful expression of

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reporter genes driven by constitutive promoters in ENC reflected the presence of aleurone-like cells in the culture. However, the level of expression obtained was much less than that obtained by similar analysis using other systems such as EMC, immature zygotic embryos or even leaves. Bombardment of reporter genes under control of endosperm-specific zein genes failed to generate any transient expression, with the exception of P27γz-GFP in our work. This observation had been reported previously in A636 ENC system (Quayle *et al*. 1991). These authors reported failure to express zein promoter-driven constructs and used an attenuated 35S promoter adding a "−300 box" from zeins, which specifically enhanced transient expression in ENC but not in EMC. In our hands, freshly isolated developing endosperm performed similarly to ENC cultures in transient analysis (data not shown). *Agrobacterium*-mediated transformation of developing maize endosperms gave strong transient expression when using 17 DAP endosperms (Requesens *et al*. 2010); in the future, it would be interesting to test this new approach with established endosperm cultures as well.

Because of the ease of ENC establishment for a transformation-amenable genotype and longevity of the culture, it should be possible to establish endosperm callus cultures for transgenic tissues as well. We have been able to successfully initiate ENC cultures from transgenic maize endosperm expressing GFP driven by the P27γz promoter (data not shown; transgenic line described in Moeller *et al*. [2009]). In a similar fashion, Gruis *et al.* (2006) established an ENC from 6 DAP kernels of transgenic Hi-II maize carrying endosperm-specific expressing fluorescent proteins (RFP and YFP). The low sugar and high asparagine in our media, compared to high sugar and low asparagine used in Gruis *et al.* (2006), might have some effect in the long-term survival of our cultures.

While an ENC culture cannot be solely used for the interpretation of gene function and regulation, it can provide a tool for testing hypotheses related to mechanisms underlying endosperm development and protein storage deposition in a fast, flexible, and cost-effective way, as long as proper controls are included to rule out any *in vitro* tissue culture-induced variations.

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