Development of maize (Zea mays) endosperms can be excised from the maternal tissues and undergo tissue/cell-type differentiation under in vitro conditions. We have developed a method to transform in vitro-grown endosperms using Agrobacterium tumefaciens and standard binary vectors. We show that both aleurone and starchy endosperm cells can be successfully transformed using a short cocultivation with A. tumefaciens cells. The highest transformation rates were obtained with the A. tumefaciens EHA101 strain and the pTF101.1 binary vector. The percentage of aleurone cells transformed following this method varied between 10% and 22% whereas up to the eighth layer of starchy endosperm cells underneath the aleurone layer showed transformed cells. Cultured endosperms undergo normal cell type (aleurone and starchy endosperm) differentiation and storage protein accumulation, making them suitable for cell biology and biochemical studies. In addition, transgenic cultured endosperms are able to express and accumulate epitope-tagged storage proteins that can be isolated for biochemical assays or used for immunolabeling techniques.

The endosperm is a unique plant tissue that arises from a second fertilization event between a male gamete and the central cell. Its main function is to provide nutrients to the embryo during seed development or during germination. In cereals, the endosperm consists of three main cell types: the starchy endosperm cells, which constitute the bulk of the endosperm and accumulate large quantities of storage proteins and starch; the epidermal aleurone cells; and the transfer cells, which are in contact with the maternal vascular tissues (Olsen, 2004). The cereal endosperm is important as a model system to study plant development, cell differentiation, programmed cell death, and synthesis, trafficking, and accumulation of storage compounds. In addition, it is a major source of carbohydrate and proteins for human and animal nutrition.

In spite of its importance, cell biology studies on the cereal endosperm using modern imaging approaches such as expression of fluorescent subcellular markers are very scarce because: (1) the endosperm is deeply immersed in maternal tissues and therefore, not readily available for imaging analysis and (2) the long time required for transformation and regeneration of stable transgenic plants. Although several approaches for culturing maize (Zea mays) endosperm in vitro have been reported in the past years (Shimamoto et al., 1983), only recently a novel method developed by Odd-Arne Olsen and colleagues (Gruis et al., 2006) has proven to be successful in retaining endosperm tissue and cell type identity in in vitro conditions. Cultures derived from transgenic maize lines in which endosperm cell types are identified by the activity of specific promoters have shown that aleurone and starchy endosperm cell identity continues to be established in vitro (Gruis et al., 2006).

Although Agrobacterium tumefaciens is not a natural pathogen of most monocots (Cleene, 1985; Binns and Thomashow, 1988), it has been successfully used to transform many cereals, including maize, wheat (Triticum aestivum), Sorghum, barley (Hordeum vulgare), and rice (Oryza sativa; Grimsley et al., 1989; Gould et al., 1991; Chan et al., 1993; Ishida et al., 1996, 2007; Gurel et al., 2009; Harwood et al., 2009; Hensel et al., 2009). In the case of maize, stable transgenic plants can be obtained by A. tumefaciens-mediated transformation using either super-binary or standard-binary vectors (Frame et al., 2002; Mohanty et al., 2009a, 2009b). However, transformation of isolated maize endosperms has been only possible using transient transformation approaches such as biolistic methods (Torrent et al., 1997; Gruis et al., 2006) and protoplast transfection (Gallie and Young, 1994). Unfortunately, these two methods are not always ideal for cell biology studies. On one hand, biolistic methods often result
in high-copy number transgenic events and on the other, protoplasts are usually highly stressed cells not suitable for detailed protein localization studies. *A. tumefaciens*-mediated transformation methods circumvent these disadvantages by resulting in a low-copy number of transgenes in intact tissues.

We have developed a method to transform in vitro-grown endosperms using a brief incubation time with *A. tumefaciens* cells carrying standard binary vectors. We present here a detailed explanation of the method and quantitative information on the transformation efficiency using different *A. tumefaciens* strains, culture density, and incubation time. We also provide evidence that the in vitro-differentiated aleurone and starchy endosperm cells are comparable to the corresponding cell types differentiated in planta and therefore, suitable for cell biology studies. In addition, we show that transgenic cultured endosperms are able to express and accumulate epitope-tagged storage proteins that can be isolated for biochemical assays or used for immunolabeling imaging techniques.

**RESULTS AND DISCUSSION**

**In Vitro-Cultured Endosperms Show Normal Cell Structural Features and Storage Protein Accumulation Patterns**

Endosperms from maize inbred lines with good culture growth properties, such as A636, are able to proliferate very well under in vitro conditions. Moreover, endosperms grown according to the endosperm in vitro culture system (EICS) developed by Gruis et al.

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**Figure 1.** Structural features of aleurone and starchy endosperm cells. A to D, Overviews and cross sections of cultured endosperms at different stages of development. Developing endosperms were excised at 6 DAP and kept in culture for 1 (A), 4 (B), 6 (C), and 8 (D) d. Note the differentiating aleurone (Al) and starchy endosperm (St E) cells in 6 + 6 (6 DAP + 6 DIC) and 6 + 8 endosperms. E, Longitudinal section of an A636 maize kernel at 23 DAP and the corresponding endosperm cross section showing the aleurone and starchy endosperm cells. E, Embryo; End, endosperm. F, Accumulation of endogenous 22-kD α, 15-kD β, and 27-kD γ zeins in developing kernels (K) at 6, 9, 12, 16, 23, and 30 DAP and in cultured endosperms (E) at the corresponding developmental stages (6 + 0, 6 + 3, 6 + 6, 6 + 10, 6 + 17, 6 + 24). G to L, Ultrastructural features of aleurone and starchy endosperm cells from in vitro- and in planta-grown endosperms. Protein storage vacuoles (PSV) and lipid bodies (LB) developed in aleurone cells of both systems (G and J). Based on the results from immunolabeling experiments with specific antibodies, the deposition patterns of the 22-kD α (H and K) and 27-kD γ zeins (I and L) in protein bodies of starchy endosperm cells are also identical in both systems. Bars = 0.5 mm in A, B, C, D, and E; 50 μm in A’, B’, C’, D’, and E’; 1 μm in G and J; and 200 nm in H, I, K, and L.
Reyes et al.

(2006) are able not only to proliferate but also to establish aleurone and starchy endosperm cell identity. This has been shown by monitoring the activation of starchy endosperm- and aleurone-specific promoters. Endosperms are excised from the kernels 6 d after pollination (DAP) and kept on agar medium containing Murashige and Skoog basal medium, vitamins, amino acids, cytokinin, and 15% Suc. In vitro-grown endosperms do not reach the same final size as endosperms developed in planta (Fig. 1); in addition, tissue differentiation is accelerated in in vitro compared to in planta-grown endosperms. We observed that the epidermal layer started to acquire structural features of aleurone cells around 6 d in culture (DIC), that is 6 DAP plus 6 DIC (or 6 + 6 endosperms; Fig. 1C [C']). By the 6 + 8 stage, aleurone cells and starchy endosperm cells were clearly differentiated, the surface of the cultured endosperms had acquired a yellowish color due to the accumulation of lipid bodies in the aleurone cells, and the starchy endosperm cells contained large starch granules (Fig. 1D [D']). In terms of endosperm differentiation, the 6 + 8 endosperm stage was equivalent to approximately 22 to 25 DAP endosperms developed in planta (Fig. 1E [E']).

In addition, we also compared the steady-state levels of the 22-kD α, 15-kD β, and 27-kD γ zeins between in vitro- and in planta-grown endosperms (Fig. 1F). Developing kernels at 6, 9, 12, 16, 23, and 30 DAP and cultured endosperms at the equivalent time points (6 DAP + 0 DIC or 6 + 0, 6 + 3, 6 + 6, 6 + 10, 6 + 17, 6 + 24) were collected and assayed for storage protein expression. Western-blot analysis of the 27-kD γ zein showed that the earliest detectable expression starts at 9 DAP (6 + 3 stage for cultured endosperms) with steadily increasing expression through 30 DAP (6 + 24 stage for cultured endosperms) both in cultured and in planta-grown endosperms. Twenty-seven kilodalton γ zein accumulation appears higher in in vitro-grown endosperm compared to developing kernels at the equivalent developmental stage, which is consistent with the zein transcript profiles reported previously by Gruis et al. (2006) and our own observations that cultured endosperm show accelerated differentiation (Fig. 1, D and E [D' and E']). Comparable expression patterns were also observed for other endosperm storage proteins, such as 22-kD α and 15-kD β zeins (Fig. 1F). Thus, the endogenous storage proteins follow similar expression patterns in cultured and in planta-developed endosperms.

At the ultrastructural level, aleurone and starchy endosperm cells from in vitro- and in planta-developed endosperms were similar. Protein storage vacuoles and lipid bodies formed in aleurone cells (Fig. 1, G and J) and protein bodies and starch granules filled the starchy endosperm cells. Using specific antibodies (Woo et al., 2001; Holding et al., 2007), we also checked the deposition patterns of the 22-kD α and the 27-kD γ zeins in protein bodies of starchy endosperm cells from 6 + 8 endosperms and 23-DAP kernels and found that they were identical, with 27-kD γ zein located in the outer zone and 22-kD α zein occupying the central core of the protein bodies (Fig. 1, H, I, K, and L).

Previous analysis on cell-type-specific promoter activity and transcript profiles (Gruis et al., 2006) together with our structural and immunolabeling analysis indicate that EICS is a suitable system for studying cell biology aspects of endosperm development and differentiation in maize.

A. tumefaciens-Mediated Endosperm Transformation Using Standard Binary Vectors

To analyze membrane dynamics or any other trafficking process it is often necessary to introduce transgenes, such as subcellular fluorescent markers, into the cell/tissues under study. We developed a protocol to transform in vitro-grown maize endosperms using A. tumefaciens-mediated transformation. This method allows for the incorporation of a low-copy number of transgenes in intact endosperm tissues. We used the A. tumefaciens strain EHA101 (Hood et al., 1986) and a pTF101.1 binary vector (Frame et al., 2002) containing GFP coding sequence fused to the endoplasmic reticulum (ER) retention signal KDEL under the control of the rice actin 1 promoter, OsAct1prom (McElroy et al., 1991). This promoter has been shown to be active in both aleurone and starchy endosperm cells of cereals (Cho et al., 2002).

Figure 2. Confocal images of in vitro-grown endosperms (6 + 8 developmental stage) transformed with an ER-targeted GFP construct. Endosperm tissues were stained with propidium iodide to visualize cell walls. A, Paradermal overview of the aleurone layer showing aleurone cells expressing ER-targeted GFP (asterisks). B, Detail of an aleurone cell expressing ER-targeted GFP. C, Overview of an endosperm cross section showing transformed starchy endosperm cells (asterisks). D, Detail of a starchy endosperm cell expressing ER-targeted GFP. Bars = 50 μm in A and C; 5 μm in B; 20 μm in D. Al, Aleurone layer; St E, starchy endosperm.
For transformation, 6 DAP excised developing endosperms were cocultivated for 3 min with different culture densities of *A. tumefaciens* and placed on solid EICS culture medium supplemented with 500 mg/mL carbenicillin.

EICS Transformation Efficiency Is Highly Dependent on *A. tumefaciens* Culture Density

Transformation efficiency was evaluated using two parameters: (1) the percentage of transformed epidermal/aleurone cells and (2) the number of cell layers underneath the epidermal/aleurone layer containing transformed cells (Fig. 2). Since autofluorescence is a common phenomenon in the maize endosperm, only those cells that showed a fluorescence ER pattern and emission spectra corresponding to GFP (measured with the meta detection system of the Zeiss 510 LSM) were scored as transformed cells (Fig. 2). The number of transformed cells was analyzed at 4, 6, and 8 d after cocultivation (Fig. 3A).

We found that the transformation efficiency in the epidermal/aleurone layer was directly correlated to the density of *A. tumefaciens* cultures used to transform the endosperms. With the lowest *A. tumefaciens* concentration we tested (optical density at 600 nm [OD$_{600}$] = 0.2) the percentage of transformed epidermal/aleurone cells varied between 5% and 15%, whereas with the highest concentration (OD$_{600}$ = 0.8), the transformation efficiency in the epidermal/aleurone layer varied between 10% and 22% (Fig. 3A). No major changes were found in the percentage of transformed cells at the different time points checked in this study (Fig. 3A).

We also analyzed the number of transformed starchy endosperm cells in endosperm cross sections. Transformed cells were detected from the first layer (the starchy endosperm cells right beneath the aleurone layer) up to the eighth layer of starchy endosperm cells (Table I).

Active periclinal (parallel to the surface of the epidermal layer) divisions have been reported to occur in in vitro-grown endosperms (Gruis et al., 2006). Therefore, at least some transformed starchy endosperm cells found in deeper areas of the endosperms are likely to be derived from transformed epidermal/aleurone cells.

**Transformation Efficiency Is Higher with the *A. tumefaciens* Strain EHA101**

To evaluate the ability of a different *A. tumefaciens* strain to transform maize endosperms, the same binary vector containing the GFP reporter was introduced into *A. tumefaciens* GV3101 cells. We used both *A. tumefaciens* EHA101 and GV3101 strains for side-by-side endosperm transformation at a concentration of 0.8 OD$_{600}$ and during 3- or 10-min cocultivation time (Fig. 3B). We analyzed the transformation efficiency in the aleurone layer 8 d after cocultivation. We found that the transformation efficiency was much higher when the EHA101 strain was used. In fact, no transformed cells were detected after the 3-min incubation treatment with the GV3101 strain (Fig. 3B).

| OD$_{600}$ | No. of Starchy Endosperm Cell Layers with Transformed Cells |
|------------|----------------------------------------------------------|
|            | Day 4 | Day 6 | Day 8 |
| 0.2        | 3 ± 0 | 2 ± 1 | 3 ± 1 |
| 0.4        | 3 ± 1 | 3 ± 0 | 3 ± 0 |
| 0.6        | 3 ± 1 | 4 ± 1 | 4 ± 1 |
| 0.8        | 5 ± 1 | 4 ± 2 | 5 ± 3 |

**Figure 3.** Transformation efficiency of epidermal/aleurone cells in in vitro-grown endosperms. Endosperms were transformed with *A. tumefaciens* cells carrying a pTF101.1-derived binary vector containing an ER-targeted GFP under the control of the *OsAct1* promoter. A, Transformation efficiencies recorded at different time points after 3 min cocultivation with different cell culture densities of *A. tumefaciens* EHA101. B, Transformation efficiencies using two *A. tumefaciens* strains (EHA101 and GV3101) and different cocultivation times. Transformation efficiency was measured in the epidermal/aleurone layer 8 d after cocultivation. The data depicted in both graphs correspond to the average of three independent experiments with three biological replicates in each. ND, Not detected.
Transgenic in Vitro-Cultured Endosperms Express and Accumulate Tagged Storage Proteins

To assess the accumulation levels of proteins encoded by transgenes transformed into in vitro-grown endosperms, we expressed the 22-kD α zein, 15-kD β zein, and the 27-kD γ zein constructs containing also the CZ19B1prom:DsRed reporter gene. Light (A–C) and fluorescence images (A’–C’) of transformed endosperms. D, Immunoblot analysis of storage protein expression in in vitro-grown endosperms using epitope tag antibodies and antibodies against the native storage proteins.

Figure 4. Expression of epitope-tagged storage proteins in in vitro-grown endosperms. Endosperms were transformed with C-terminal HSV-tagged 22-kD α zein (A and A’), FLAG-tagged 15-kD β zein (B and B’), or HA-tagged 27-kD γ zein constructs containing also the CZ19B1prom:DsRed reporter gene. Light (A–C) and fluorescence images (A’–C’) of transformed endosperms. D, Immunoblot analysis of storage protein expression in in vitro-grown endosperms using epitope tag antibodies and antibodies against the native storage proteins.

Figure 5. Chart showing the main steps and timeline of the protocol to transform in vitro-grown endosperms using A. tumefaciens EHA101 cells carrying pTF101.1-derived binary vectors.
Materials and Methods

Isolation and in Vitro Growth of Endosperms

Maize (Zea mays; inbred line A636) were grown in a greenhouse under a 14-h light/10-h dark photoperiod, supplemental lighting (700 μmol m⁻² s⁻¹), and average temperature of 28°C during the day and 21°C during the night. Endosperms were isolated and cultured in vitro as described by Gruis et al. (2006). Briefly 6 DAP ears were harvested and surface sterilized. Using a scalpel the kernels were dissected, the maternal tissue removed, and the fertilized embryo sacs isolated to obtain clean endosperms. Developing endosperms were immediately placed on liquid EICS culture medium until cocultivation with Agrobacterium tumefaciens. After the cocultivation, the isolated endosperms were placed on solid EICS culture medium (4.3 g/L Murashige and Skoog media; 0.5% w/v Murashige Skoog vitamins stock solution; 5 mg/L thiamine HCl; 400 mg/L Asu; 10 μg/L 6-benzylaminopurine; 15% Suc; and 3 g/L Gelrite, pH to 5.8) supplemented with 500 μg/mL carbenicillin and kept on dark at 25°C. Isolated endosperm not exposed to A. tumefaciens cultures were used as a control.

Plasmids

A DNA fragment containing OsActin1promGFP-KDEL was cloned into the pTiT101.1 vector using a two-step cloning strategy. The GFP-KDEL sequence was amplified from the CD3-955 vector (Nelson et al., 2007) using the forward primer 5'-TCTAGAATGAAGGTACAGGAGGGT-3' and the reverse primer 5'-CCCCGCTTACAATTCTGATG-3' containing a HindIII restriction site and reverse 5'-TCTAGAATGAAGGTACAGGAGGGT-3' containing an XbaI restriction site. The vector pDM302 carrying the OsActin1 promoter was kindly donated by Ayaj Garg (Cornell University) and the GFP coding region fused to the ER retention signal KDEL (CD3-955; Nelson et al., 2007) was obtained from the Arabidopsis Biological Resource Center at Ohio State University. The resulting plasmid was introduced into competent A. tumefaciens cells by freeze-thaw transformation (Chen et al., 1994). Additional binary vectors used in this study contained CZ1981prom (U.S. patent 6225529) fused to DsRed (CLONTECH), F2prom23Kd a zein fused to the HSV tag (QPELAPEDPED), 27Kdzein53Kd β zein gene fused to FLAG (DYKDDDDK), and the 27Kdzein53Kd β zein gene fused to HA (YPYDVPDYA). Sequence data for the zein genes used in this article can be found in the GenBank/EMBL data libraries under accession numbers AF371261, AF371264, and AF371274.

A. tumefaciens-Mediated Transformation

A. tumefaciens EHA101 or GV3101 strains carrying the different constructs were grown at 25°C for 2 d in Luria-Bertani medium supplemented with the appropriate antibiotics for strain (100 μg/mL kanamycin for EHA101 or 100 μg/mL gentamicin plus 10 μg/mL rifampicin for GV3101) and plasmid selection (100 μg/mL spectinomycin for pTiT101.1). After 2 d of growth, the cultures were centrifuged and washed twice with the infiltration media (EICS culture media supplemented with 100 μg acetylsyringone). Finally the bacterial suspension was diluted with infiltration media to adjust the inoculum concentration to the final OD₆₀₀₅₀ value.

Transformation was performed by cocultivating the isolated endosperm with the bacterial suspensions under gentle agitation. After cocultivation with A. tumefaciens cultures, the endosperms were washed three times with EICS culture medium supplemented with carbenicillin, plated, and kept in the dark.

Confocal Imaging of Fluorescent Proteins

Cross or paradermal sections of the transformed endosperms were imaged using a 510 Zeiss laser-scanning confocal microscope. The transformed tissues were excited with 488 nm and the GFP emission was detected using a 500 to 530 band-pass filter. The GFP emission spectrum was collected for every image using the spectral meta detector. Only those cells that showed a positive signal for GFP were scored as transformed cells.

The percentage of transformed cells was calculated by determining the number of transformed cells over the total of cells per field. The number of total cells per field ranged from 45 to 160 in the different images obtained. At least three fields were analyzed for each section and a total of three independent experiments were analyzed.

For determining the number of starchy endosperm layers (layers underneath the epidermal/aleurone layer) containing transformed cells, we used transverse sections of the endosperms.

The images were analyzed using the LSM image browser (www.zeiss.com/lsm) and edited using Adobe Photoshop CS4.

CONCLUSION

We have developed a protocol to transform endosperm tissue grown in vitro. Since in vitro-cultured endosperms undergo normal differentiation of aleurone and starchy endosperm cells, the possibility to introduce transgenes such as transgenes, allowing for easy imaging access to different endosperm cell types that are usually deeply immersed within other tissues.

The ability to introduce transgenes into in vitro-cultured developing endosperms provides biochemical and molecular means to study cereal endosperm cell fate differentiation and early endosperm development. The possibility of imaging fluorescent subcellular markers in both aleurone and starchy endosperm cells following a very simple and short protocol (Fig. 5) represents an important technical advance in cell biology studies of differentiating cereal endosperm cells. In addition, this system offers the possibility of expressing and isolating tagged/modified endosperm storage proteins that cannot be successfully expressed in other tissues/systems.
Structural Characterization and Gold Immunolabeling

In vitro-grown endosperms at different stages of development (6 + 1, 6 + 3, 6 + 6, and 6 + 8) and thin slices of endosperm tissue from developing kernels were processed by high-pressure freezing/freeze substitution. Substitution was performed in 2% OsO4 in anhydrous acetone at −80°C for 72 h, and followed by slow warming to room temperature over a period of 2 d. After several acetone rinses, samples were removed from the holders and infiltrated in Epon resin (Ted Pella Inc.) according to the following schedule: 5% resin in acetone (4 h), 10% resin (12 h), 25% resin (12 h), 50%, 75%, and 100% (24 h each concentration). Polymerization was carried out at 60°C. Sections were stained with 2% (uninverted acetocarmine) for 10 min followed by Reynold’s lead citrate (2.6% lead nitrate and 3.5% sodium citrate, pH 12) and observed in a FEI CM12 electron microscope.

For gold immunolabeling, high-pressure frozen samples were substituted in 0.2% uranyl acetate (Electron Microscopy Sciences) plus 0.2% glutaraldehyde (Electron Microscopy Sciences) in acetone at −80°C for 72 h, and warmed to −50°C for 24 h. After several acetone rinses these samples were infiltrated with Lowicryl HM20 (Electron Microscopy Sciences) for 72 h and polymerized at −50°C under UV light for 48 h. Sections were mounted on formvar-coated nickel grids and blocked for 20 min with a 10% (w/v) solution of nonfat milk in phosphate-buffered saline (PBS) containing 0.1%. Sections were incubated with the primary antibodies (1:10 in PBS-Tween 20) for 1 h, rinsed in PBS containing 0.5% Tween 20, and then transferred to the secondary antibody (anti-rabbit IgG 1:100 conjugated to 15-nm gold particles) for 1 h. Controls omitted the primary antibodies. The antibodies against 22-kD α zeins and 27-kD y zeins have been characterized elsewhere (Woo et al., 2001; Holding et al., 2007).

Immunoblot Analysis

In vitro-grown endosperms were ground with a plastic pestle in extraction buffer (50 mM Tris-HCl, 5 mM EDTA, and 2% SDS; pH 8.0) containing a protease inhibitor cocktail (Sigma-Aldrich) at a 1:2 (w/v) ratio. Cellular debris was removed by centrifugation at 140,000 rpm for 10 min. Protein extracts were diluted 1:2 (v/v) in sample buffer and boiled for 5 min before separation through a 4% to 12% (w/v) SDS polyacrylamide gel (Invitrogen) and then transferred to a cellulose membrane in a submerged blotting system (Mini-Trans Blot; Invitrogen). Membranes were blocked for 1 h with Tris-buffered saline containing 0.1% Tween 20. The sections were incubated with the primary antibodies (1:10 in PBS-Tween 20) for 1 h, rinsed in PBS containing 0.5% Tween 20, and then transferred to the secondary antibody (anti-rabbit IgG 1:100 conjugated to 15-nm gold particles) for 1 h. Controls omitted the primary antibodies. The antibodies against 22-kD α zeins and 27-kD y zeins have been characterized elsewhere (Woo et al., 2001; Holding et al., 2007).

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