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Method Article

Transient expression of fluorescently tagged proteins in developing maize aleurone cells

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A B S T R A C T

Expressing transgenes in the endosperm of cereals by developing stably transformed lines is an expensive and labor-intensive process. An alternative that is less expensive and faster is to express the transgenes transiently. We describe here a detailed protocol to express transiently genes in maize aleurone cells by biolistic bombardment of *in vitro* cultured developing endosperms. Maize endosperms are isolated from kernels at 6–8 days after pollination and placed on culture medium plates for 1–2 days. Afterwards, the endosperms can be transfected with either a single gene or multiple transgenes simultaneously. Microparticles coated with the selected plasmids are delivered into the aleurone cells by biolistic bombardment. As a demonstration, we co-expressed two transgenes simultaneously, one tagged by GFP and the other tagged by mCherry. Our transfection efficiency is comparable to that obtained with *Agrobacterium*-mediated transformation, but requires a shorter time for gene expression after transfection. We provide optimized conditions and parameters for key steps in this procedure.

- Small, non-binary plasmids can be used to drive expression of fluorescent proteins.
- Optimized distribution of DNA-coated microparticles maximizes transfection of *in vitro* grown maize endosperms while minimizing cellular damage.
- Transgene expression can be detected as early as one day after bombardment.

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A R T I C L E I N F O

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Specifications Table

Subject Area	Agricultural and Biological Sciences
More specific subject area	Transient gene expression in maize endosperm
Method name	Transient expression by biolistic bombardment in developing maize endosperm
Name and reference of original method	<p>Plant material, <i>In vitro</i> endosperm culture, and aleurone bombardment pressure:</p> <p>Gruis, D. F., Guo, H., Selinger, D., Tian, Q., & Olsen, O. A. (2006). Surface position, not signaling from surrounding maternal tissues, specifies aleurone epidermal cell fate in maize. <i>Plant Physiology</i>, 141(3), 898–909.</p> <p>Gold particle preparations and bombardment chamber setup:</p> <p>Wang, K., & Frame, B. (2009). Biolistic gun-mediated maize genetic transformation. In <i>Transgenic maize</i> (pp. 29–45). Humana Press, Totowa, NJ.</p> <p>Whitham, S. A., Lincoln, L. M., Chowda-Reddy, R. V., Dittman, J. D., O'Rourke, J. A., & Graham, M. A. (2016). Virus-induced gene silencing and transient gene expression in soybean (<i>Glycine max</i>) using Bean pod mottle virus infectious clones. <i>Current protocols in plant biology</i>, 1(2), 263–283.</p>
Resource availability	N.A.

Introduction

The endosperm in cereals consists of starchy endosperm cells, which accumulate storage proteins and starch, the peripheral (epidermal) aleurone cells, which mobilize these storage compounds during germination, and transfer cells, which are in contact with the plant vascular system [1]. The maize endosperm is an important model for studying growth, development, and nutrient accumulation of cereal endosperms. However, it is expensive and time-consuming to obtain genetically modified maize lines for cellular and molecular analyses. As a result, methods for gene delivery directly into maize endosperm cells have been developed, including polyethylene glycol (PEG)-mediated DNA delivery of endosperm protoplasts [2,3], *Agrobacterium tumefaciens*-mediated transformation of maize endosperm [4], and biolistic bombardment of maize aleurone cells [5]. For the study of maize aleurone cells, these methods present advantages and disadvantages. PEG-mediated gene delivery into aleurone protoplasts involves the time-consuming and technically challenging process of dissecting aleurone tissue, optimizing protoplast culture conditions, and purifying aleurone protoplasts from contaminating starchy endosperm protoplasts. In addition, once removed from their cell wall and tissue context, the aleurone protoplasts may not faithfully follow the behavior of aleurone cells grown *in planta*. *Agrobacterium*-mediated transformation and biolistic bombardment rely on the *in vitro* culture of developing endosperms (6–8 days after pollination or DAP). Under the *in vitro* culture condition, both starchy endosperm and aleurone cells differentiate normally as their counterparts grown in intact kernels [4–6], which is a critical advantage to study cellular processes with a relevant developmental context. Once the endosperms are established on culture plates, gene delivery can be achieved by co-cultivation with *Agrobacterium* or bombardment with DNA-coated microparticles. *Agrobacterium*-mediated transformation is an unexpensive method since it does not require a specialized biolistic bombardment system or microparticles; however, the transgene must be cloned into a binary vector.

In this study, we present a protocol for gene delivery into developing maize aleurone cells by biolistic bombardment that incorporates optimized steps and modifications to earlier published methods [5,7,8]. We describe step-by-step how to a) set up maize endosperms for *in vitro* culture, b) prepare microparticles coated with plasmid DNA, and c) set up conditions for bombarding DNA-coated microparticles into maize aleurone cells. To validate our method, we co-expressed two endoplasmic reticulum (ER) markers, one tagged by mCherry and one tagged by GFP, in developing maize aleurone cells. We confirmed the expression of mCherry and GFP by fluorescence spectral analysis and calculated transfection efficiency. The transfection efficiency of our method is comparable to that

of *Agrobacterium*-mediated methods [4], but it requires a shorter period of time for detectable gene expression (1 day for our methods, 4 days for *Agrobacterium*-mediated transformation).

Method details

Plant growth and in vitro culture of maize endosperm

Materials

1. Maize Hi II F1 seeds.
2. 100 mm x 15 mm sterile Petri dishes.
3. Murashige and Skoog (MS) Basal Salt Mixture (MS), powder (MilliporeSigma, M5524–50 L).
4. Murashige Skoog (MS) powdered vitamin mixture (Fisher Scientific, 50,213,490).
5. 6-Benzylaminopurine (MilliporeSigma, B3408).
6. Thiamine hydrochloride (MilliporeSigma, T1270).
7. L-Asparagine (MilliporeSigma, A4159).
8. Carbenicillin, Disodium Salt (Dot Scientific, DSC46000).
9. Gelrite (RPI, G35020).
10. Dimethyl sulfoxide (MilliporeSigma, D8418).
11. Sucrose.
12. Sterile and non-sterile Milli-Q water.
13. 1 M potassium hydroxide (made in Milli-Q water).
14. Stainless-steel scalpel.
15. Fine-tipped tweezers.
16. 70% ethanol.

Procedure

Maize Hi-II [9] F1 seeds can be grown and pollinated in a greenhouse all year long. The maize ears used for the demonstration in this study were collected from plants grown in the greenhouse of the Wisconsin Crop Innovation Center (University of Wisconsin, Madison) under a 16 h light/8 h darkness photoperiod, with supplemental lighting provided at an intensity of $330 \mu\text{mol m}^{-2} \text{s}^{-1}$ at ~1.7 m below the lights, and average temperatures of 28 °C during the light period and 21 °C during the dark period. The plants were self- or sib- pollinated after flowering. The maize ears were collected at 6–8 day DAP. Kernels at this developmental stage appear white and are ~3–5 mm in diameter (Fig. 1A-D).

Culture plates should be prepared prior to kernel collection. The medium for endosperm *in vitro* culture system (EICS; [5,6]) consists of 4.3 g/l Murashige and Skoog Basal Salt, 0.5% v/v Murashige Skoog vitamins stock solution, 5 mg/l thiamine HCl, 400 mg/l Asn, 10 $\mu\text{g/L}$ 6-benzylaminopurine, 15% sucrose with pH adjusted to 5.8 and 3 g/l gelrite for solid medium. We also supplemented the EICS medium with 500 $\mu\text{g/ml}$ carbenicillin [4] to prevent bacterial contamination. We recommend making all the stock solutions as described in Table 1 and store them at –20 °C. Then, 250 ml of liquid and 250 ml solid media can be made as follows:

1. Dissolve 2.15 g MS basal salt and 75 g sucrose in 400 ml Milli-Q water, add 1 ml of the 6-benzylaminopurine stock solution, and adjust the pH of the solution to 5.8 by slowly adding 1 M potassium hydroxide.
2. Adjust the total volume of the solution to 500 ml and mix the solution well. To prepare solid medium, add 0.75 g gelrite and 250 ml of the well-mixed solution into a 500 mL glass bottle. Pour the rest 250 ml of the solution into another glass bottle, and autoclave for 20–30 min.
3. Let the solutions cool down to approximately 50 °C and add the rest of the stock solutions listed in Table 1 to both bottles. Mix well the solid medium and pour it into sterile disposable plates (approximately 25 ml per plate). Store solid medium plates and the liquid medium in the dark at 4 °C.

To prepare developing maize endosperms for *in vitro* culture, we usually place 10–15 excised endosperms on each EICS solid medium plate. We recommend preparing two or three plates for each

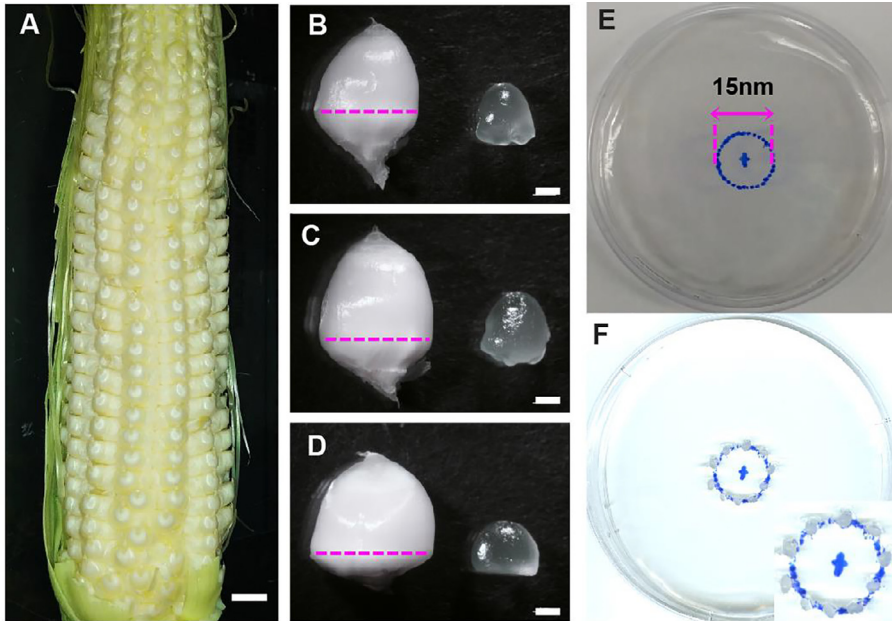


Fig. 1. Endosperm dissection from 8 DAP maize Hi-II ears and culture on solid medium. A) 8 DAP maize Hi-II ear. B-D) Maize kernels and endosperms collected from the top, middle, and bottom regions of the maize ear shown in A. E, F) Maize endosperms on solid medium for *in vitro* culture. Scale bar in A is 5 mm. Scale bars in B, C, D are 1 mm.

Table 1

Stock solutions for making the EICS culture medium supplemented with Carbenicillin.

Chemical	Concentration	Solvent	Sterilization	Amount to add to 250ml EICS medium after autoclave
MS powdered vitamin	1000x (as directed by the manufacturer)	Milli-Q water	filtration	1.25 ml
6-benzylaminopurine	5 µg/ml	Dimethyl sulfoxide	Not required	\
Thiamine HCl	25 mg/ml	Milli-Q water	filtration	50 µl
L-Asparagine (Asn)	40 mg/ml	Milli-Q water	filtration	2.5 ml
Carbenicillin, Disodium Salt	250 mg/ml	Milli-Q water	filtration	500 µl

transgene or combination of transgenes to be delivered. The dissection of endosperms from Hi-II ears at 6–8 DAP should be performed in a sterile laminar flow hood as follows:

1. Take the EICS liquid medium and solid medium plates out of the fridge. Draw with a permanent marker a circle 15 mm in diameter at the center of each plate bottom part (Fig. 1E).
2. Remove kernels from the ear with a scalpel. Sterilize kernels by submersion in 70% ethanol for approximately 2 min and rinse them with sterilized Milli-Q water. For the remaining the steps, all tools and culture medium need to be kept sterile. The scalpel and tweezers can be sterilized by spraying with 70% ethanol. Kernels should be dissected while submerged in sterile Milli-Q water to avoid dehydration.
3. With the scalpel, cut away the bottom part of the kernels and expose the endosperm as shown in Fig. 1B-D. Use the fine-tipped tweezers to carefully remove the pericarp and release the endosperm. Endosperms at 6–8 DAP are small (~2–3 mm in diameter, Fig. 1B-D), semi-translucent, and fragile. It is critical to handle the dissected endosperms with care and avoid

dehydration. Pour some sterilized liquid EICS medium in a sterile plastic petri dish and submerge the endosperms into the liquid medium right after dissection and before placing them onto the EICS solid plates.

4. Place approximately 10–15 endosperms on each EICS solid medium plate with the apical part of the endosperm facing up and the basal part in contact with the medium (Fig. 1F). We recommend making small, shallow dents on the solid medium along the circle drawn on each plate bottom to keep the endosperms stably in position. Alternatively, endosperms can be placed in two concentric rings at each side of the drawn circle, to increase the number of transfected endosperms.
5. After placing the endosperms onto EICS plates, place 100 μ l sterilized Milli-Q water on top of them. Wrap the plates with parafilm and stack them vertically. Wrap the plate stack with aluminum foil and place it in a 28 °C growth chamber (70% humidity) for 1–2 days before bombardment.

Notes:

- a. After autoclaving, gelrite tend to crystalize quickly when under 50 °C. We recommend making EICS fresh solid medium for each use and pour into plates right after preparation.
- b. The center of the circle drawn on the plate bottom will later line up with the center of the gene gun. We do not place endosperm in the center of this circle because this is the area where the microparticles travel at highest speed and can cause excessive tissue damage.

Microparticle Preparation for Coating

Materials

1. 1.0 μ m Gold Microparticles (Bio-rad, #1,652,263) or equivalent product (e.g., tungsten microparticles of similar size).
2. Ethanol.
3. Sterile Milli-Q water.
4. Ultrasonic water bath.
5. Tabletop centrifuge.
6. Vortex mixer.
7. Sterile 1.5 ml Eppendorf tubes.

Procedure

The purpose of this step is to sterilize the microparticles and aliquot them into small volumes. Tungsten particles are less expensive and more heterogenous in size and shape compared to gold particles. The disadvantage of using tungsten particles is that tungsten can degrade DNA over time and may have adverse effect on certain cells [10]. The disadvantage of using gold microparticles is that they tend to agglomerate in water and in high humidity condition. This problem can be solved by using an ultrasonic water bath during the preparation and coating of the microparticles. The procedure described below is adapted from [7] and [8]:

1. Weigh 60 mg of 1.0 μ m gold microparticles in a sterile 1.5 ml Eppendorf tube.
2. In a sterile laminar flow hood, add 1 ml 100% ethanol at –20 °C into the tube with the particles. Place the tube in an ultrasonic water bath for 15 s. Tap the closed tube on the bench to gather all droplets to the tube bottom and let the tube sit until most of the gold particles have settled down to the bottom of the tube. This takes approximately 10–30 min at room temperature.
3. Spin the tube in a tabletop centrifuge for 60 s at 5000x g and remove the ethanolic supernatant by pipetting. Make sure to keep the teardrop-shaped gold pellet undisturbed while pipetting.
4. To rinse the gold microparticles, add 1 ml ice-cold, sterile Milli-Q water by dribbling the water down the side of the microcentrifuge tube. Resuspend the pellet by gently tapping the tube with a finger and let the gold particles settle down again.
5. Spin the particles inside the tube in a tabletop centrifuge for 60 s at 5000x g and remove the water by pipetting. Repeat this rinsing step two more times.
6. After removing the water from the final wash, suspend the gold particles in 1 ml sterile Milli-Q water.

7. Place the tube in an ultrasonic water bath for 15 s. Immediately after, place the tube on a vortex mixer to keep the gold particle suspended in water during the subsequent aliquoting process.
8. Pipette 50 μ l (i.e., 3 mg) of suspended gold microparticles into sterile 1.5 ml tubes. This amount of microparticles is enough for six bombardments where \sim 8 μ l or 480 μ g of gold microparticles are used for each bombardment.
9. Store gold microparticles aliquots at -20 °C.

Coating Gold Microparticles with Plasmid DNA

Materials

1. Prepared gold microparticles or equivalent products (e.g., tungsten microparticles of similar size).
2. Milli-Q water (sterile).
3. 1.5 ml Eppendorf tubes (sterile).
4. 15 ml falcon tubes.
5. Plasmid(s) with selected transgene(s).
6. Spermidine Free Base (RPI, S92150).
7. Glycerol.
8. Calcium chloride dihydrate.
9. 100% and 70% isopropanol.
10. Anhydrous calcium chloride pellets.
11. Tabletop centrifuge.
12. Vortex mixer.
13. Ultrasonic water bath.
14. Macrocarriers (Bio-rad, 1,652,335).
15. Stopping screens (Bio-rad, 1,652,336).

Procedure

The following steps explain how to coat the gold microparticles with plasmid DNA for gene delivery. First, the gold microparticles will be coated with plasmid(s) to be used for transformation by mixing plasmid(s), prepared microparticles, and a spermidine master mix. Then, the coated microparticles will be washed and spread onto sterilized macrocarriers to dry in a drying chamber. When spreading the coated microcarriers onto macrocarriers, instead of spreading the particles evenly in the center of the macrocarriers as described in many protocols, we spread them in a circle around the edge of the central circle of the macrocarrier corresponding to the arrangement of the endosperms on the EICS plates (Fig. 2A-B). The procedure described below is adapted from [5,7], and [8]. As mentioned in the previous section, tungsten microparticles can be used to replace the gold microparticles but have a few potential drawbacks that should be taken into consideration (see Note 1 at the end of this section).

1. Prepare 50% glycerol (v/v), 2.5 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1 M spermidine using Milli-Q water. Sterilize the glycerol and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solutions by autoclaving and sterilize the spermidine solution by filtration. Aliquot the solutions in sterile 1.5 ml Eppendorf tubes, and store them at -20 °C.
2. Prepare 70% isopropanol (v/v) in Milli-Q water. Store 10 ml 70% and 10 ml 100% isopropanol in two separate 15 mL falcon tubes at -20 °C.
3. Place one macrocarrier into each macrocarrier holder to be used for bombardment using a tweezer (Fig. 2A-B); avoid touching macrocarriers with bare hands. Wrap the assembled macrocarrier holders and stopping screens in aluminum foil and autoclave them.
4. Add anhydrous calcium chloride pellets to sterile plastic petri dishes to cover the bottom of the plate and place a piece of filter paper on top before putting the lid back on (Fig. 2A). This is to create a drying chamber with low humidity for the coated microparticles to dry without agglomerating after being spread on the macrocarriers. Sterilize these drying chambers by placing them under an ultraviolet lamp for \sim 10 min.
5. Isolate plasmids and resuspend them in Milli-Q water to reach a final concentration of 500 – 1000 ng/ μ l. For example, we use 1.5 μ g the of the pRTL2 plasmids [11] per bombardment. The remaining steps should be carried out in a sterile laminar flow hood.

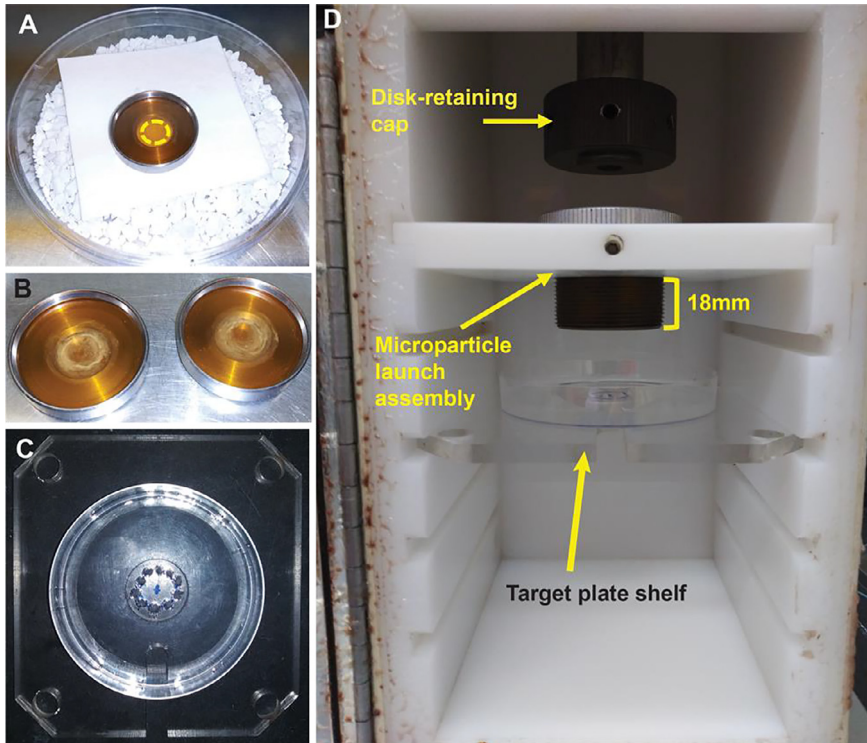


Fig. 2. Loading of coated gold particles onto macrocarriers for bombardment in a PDS-1000/He Biolistic Particle Delivery System. A) Macrocarrier with coated gold microparticles in a drying chamber. The yellow line shows the area where the coated particles should be applied. B) Macrocarriers with dried DNA-coated microparticles. C) Position of endospore culture plate on the target plate shelf of the biolistic bombardment system. D) setup of the bombardment chamber.

6. To make the spermidine master mix, first add 90 μl of sterile Milli-Q water to a tube containing 10 μl of 1 M spermidine to make 0.1 M spermidine. Then, to a new sterile Eppendorf tube add the following per each plasmid or plasmid combination for bombardment:
 - 50 μl of 50% glycerol.
 - 25 μl of 2.5 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.
 - 10 μl 0.1 M spermidine.
7. Always prepare enough spermidine master mix for one extra bombardment than needed. Mix the master mix by pipetting the solution up and down and place the tube on ice until use. Discard any remaining spermidine.
8. The resulting 85 μl of spermidine master mix can be used to coat enough amount of gold microparticles for two to three bombardments. We added 6 μg of plasmid DNA (3 μg of each plasmid encoding different fluorescent markers for two bombardments) into one sterile Eppendorf tube and added sterile Milli-Q water to make the total volume to be 10 μl .
9. Thaw the prepared gold microparticle aliquots. Keep the tubes containing the plasmid DNA, gold microparticles, and spermidine master mix on ice.
10. Place the tube containing the plasmid DNA on a vortex mixer at medium speed. Place the tube containing gold microparticles in an ultrasonic water bath for 10 s. Using a wide-bore 200 ml tip, pipette gold microparticles up and down to mix well and add 16 μl (for two bombardments) or 24 μl (for three bombardments) of gold microparticles to each tube containing plasmids. Continue vortexing for 2 min.
11. Add 85 μl of the spermidine master mix to each tube, close tube lids, and then continue vortexing for another 10 min.

12. Remove the tubes from the vortex mixer and let them sit on ice for 5 min. Centrifuge the tubes for 15 s at 5000x g, room temperature, to pellet the gold. Remove the supernatant by pipetting without disturbing the gold pellet.
13. Add 85 μ l of 70% isopropanol at -20 °C to wash the pellet by pipetting up and down a few times. Note that it is normal for the gold microparticles to agglomerate at this step.
14. Centrifuge the tubes for 15 s at 5000x g, room temperature, to pellet down the gold microparticles. Remove the supernatant by pipetting.
15. Add 85 μ l of 100% isopropanol at -20 °C to the pellet. Do not mix the pellet and the isopropanol.
16. Centrifuge the tubes for 15 s at 5000x g, room temperature, to pellet the gold microparticles. Remove the supernatant by pipetting.
17. Resuspend the pellets in 18 μ l (for two bombardments) or 26 μ l (for three bombardments) of 100% isopropanol by pipetting up and down a few times and close the tubes immediately to avoid evaporation. Place the tubes in an ultrasonic water bath for 10 s.
18. Spray tweezer with 70% ethanol and left it on a sterile surface to air dry. Use the tweezer to place one sterilized macrocarrier holder into a drying chamber.
19. Pipette up and down to mix well the gold microparticles using wide-bore tips, and spread 8.5 μ l of the gold suspension around the edge of the central circle of the macrocarrier (Fig. 2A). Close the petri dish immediately to avoid agglomeration of gold microparticles and wait until the isopropanol evaporates completely (Fig. 2B).
20. Use the tweezer to place the macrocarrier holders with dry, coated gold microparticles into a labeled (plasmid name) sterile plastic petri dish. The macrocarriers should be used within 2 h after coating.

Notes:

1. Tungsten microparticles are an alternative to gold microparticles due to their lower cost and comparable performance under most transformation scenarios. However, several potential problems with Tungsten microparticles have been reported, including toxicity in some tissues, nicking and degradation of DNA adhered to microparticles, acidification of aqueous environments, and microparticles oxidation overtime which potentially reduces DNA binding capacity [10,12].
2. To spread the coated gold microparticles evenly on the macrocarrier (Fig. 2A, 2B), it is best to move the wide-bore pipette tip in a circular motion around the center of the macrocarrier (Fig. 2A) while slowly dispensing the microparticles.

Endosperm Bombardment

Materials

1. Maize Hi-II endosperms after 1 to 2 days *in vitro* culture.
2. Assembled macrocarrier holders with DNA-coated microparticles.
3. Tweezer.
4. PDS-1000/He Biolistic Particle Delivery System (Bio-rad, 1,652,257).
5. Sterilized stopping screens (Bio-rad, 1,652,336).
6. 650 psi rupture disks (Bio-rad, 1,652,327) or 1100 psi rupture disks (Bio-rad, 1,652,329).
7. 70% isopropanol.
8. 70% ethanol.
9. Paper wipes.

Procedure

We use a PDS-1000/He Biolistic Particle Delivery System [13] from Bio-Rad for gene delivery. Detailed instructions for the use of this system can be found in the corresponding manual (<https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000070900.pdf>, Bio-Rad document number 10,000,070,900). Here we will focus key settings and parameters used for the bombardment of developing maize endosperms:

1. Adjust the brass nest until it is 18 mm below the microparticle launch assembly (Fig. 2D).

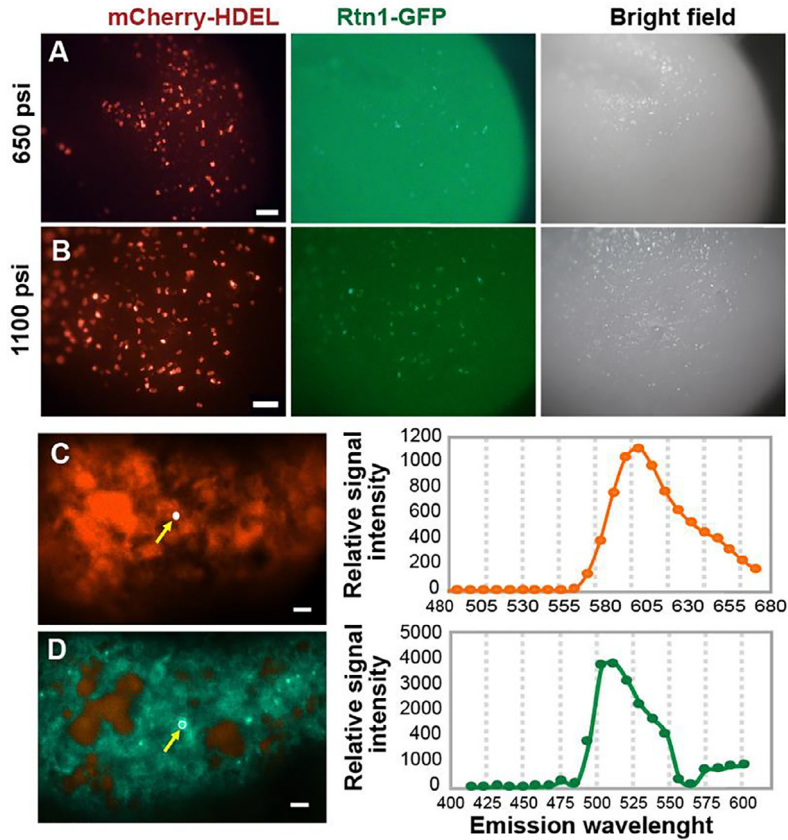


Fig. 3. Imaging of endosperms transfected with *35S::HDEL-mCherry* and *35S::Rtn1-GFP*. A, B) Endosperms bombarded using either 650 psi (A) or 1100 psi (B) rupture disks and imaged under a fluorescence dissecting microscope. Rupture disk excited by light generated by a mercury lamp passing a DS-Red filter and a GFP filter, respectively. C,D) Confocal imaging and fluorescence spectral analysis of an endosperm epidermal/developing aleurone cell with positive mCherry-HDEL (C) and Rtn1-GFP (D) signals. The fluorescence spectral data was collected from the area indicated by yellow arrow.

2. Clean the inside of the bombardment chamber by spraying with 70% ethanol. Dry the chamber with paper wipes.
3. Open the helium tank valve, turn on the vacuum pump, and turn on the PDS-1000/He system.
4. Use the tweezers to pick up a rupture disk, dip it briefly in 70% isopropanol, and place it in the disk-retaining cap (Fig. 2D). Screw the disk-retaining cap tightly to the end of gas acceleration tube. Use the tweezers to load the macrocarrier holder and stopping screen into the microparticle launch assembly. Place the microparticle launch assembly in the chamber (Fig. 2D).
5. Remove the lid and place the plate with *in vitro* cultured endosperms in the center of the target plate shelf (Fig. 2C). Place the target plate shelf into the L2 slot (i.e., 6 cm from the microparticle launch assembly) as shown in Fig. 2D.
6. Close the chamber door. Evacuate chamber and hold vacuum at 23–25 in Hg (11.3–12.3 psi). Press the fire button until the rupture disk is bursted.
7. Release vacuum from chamber, remove the plate, and put the lid back onto the plate.
8. Unload macrocarrier holder, stopping screens, and rupture disk and repeat steps 4–7 for another bombardment.

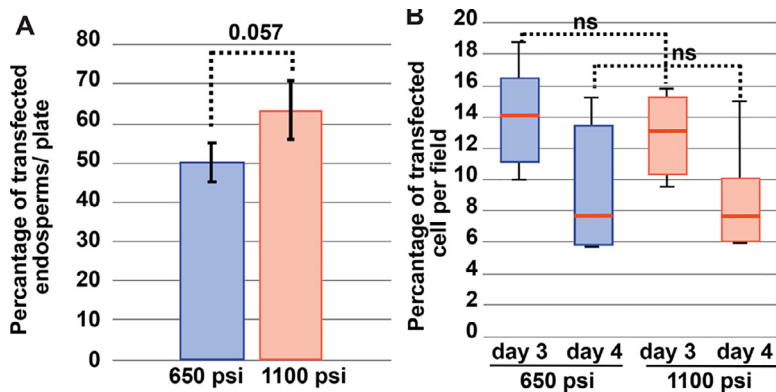


Fig. 4. Transfection efficiency. A) Percentage of endosperms expressing mCherry-HDEL per plate at 1 day after bombardment. Three plates with 10 endosperms each were bombarded using either 650 psi and 1100 psi rupture disks. B) Percentage of endosperm epidermal/ aleurone cells expressing mCherry-HDEL per field as imaged in a confocal laser scanning microscope with a 40x objective. Five to seven images of similar magnification as size were collected from two samples of four different endosperms; 66 – 160 cells were captured per field. Data were compared using a student's *t*-test.

9. In a sterile laminar flow hood, apply 100 μ l of sterilized Milli-Q water onto the endosperms to keep them moist. Wrap the plates with parafilm and stack them vertically. Wrap the stack with aluminum foil and place them in an incubator at 28 $^{\circ}$ C (70% humidity). Gene expression can be detected after culturing the bombarded endosperms overnight.

Compared to a previous protocol for gene delivery *in vitro* grown endosperms [5], our protocol has some key advantages:

1. Instead of using large binary plasmids [5], we drive gene expression from pRTL2, a small (~4 kb), high-copy vector, using the *CaMV 35S* promoter.
2. We have used a larger amount of plasmid DNA (3 μ g) than in previous protocols (200 ng, [5]), achieving a relatively high transfection efficiency with fluorescent signal detectable from day 1 to day 4 after bombardment (results discussed in detail in the next section).
3. We have found that 650–1100 psi pressure range is adequate for endosperm bombardment. The use of 1100 psi rupture disks results in slightly higher although not statistically significant transfection efficiency compared to 650 psi rupture disks used previously [5], without causing excessive cell lesions (results discussed in detail in the next section). Having a wider range of bombardment pressures provides more flexibility in designing biolistic bombardment strategies for *in vitro*-cultured endosperms.

Method validation

To demonstrate the efficiency of our optimized protocol for gene delivery to maize Hi-II endosperm cells, we co-expressed HDEL-mCherry [14], a soluble ER lumen marker, and Rtn1-GFP [14], an ER membrane protein (Fig. 3A-B). We collected maize Hi-II ears at 8 DAP, cultured excised endosperms *in vitro* for 2 days, and bombarded them with gold microparticles coated with pRTL2 plasmids containing the *35S::HDEL-mCherry* and *35S::Rtn1-GFP* expression cassettes and using 650 psi and 1100 psi rupture disks. We monitored the expression of the two fluorescent reporters from day 1 to day 4 after bombardment. To measure transfection efficiency using either 650 psi or 1100 psi rupture disks, we bombarded six plates with 10 endosperms each. When coating the gold microparticles, we mixed 6 μ g of DNA (3 μ g of each *35S::HDEL-mCherry* and *35S::Rtn1-GFP* plasmids), 16 μ l (*i.e.*, 960 μ g) of gold microparticles, and 85 μ l of the spermidine master mix for two bombardments. We did the coating for a total of 3 times to obtain 6 macrocarriers with coated microparticles for bombardment. Three

EICS plates were bombarded using 650 psi rupture disks while the other three using 1100 psi rupture disks.

We were able to find developing aleurone cells co-expressing HDEL-mCherry and Rtn1-GFP in endosperms bombarded with 650 psi and 1100 psi rupture disks, respectively, without causing excessive tissue lesion or cell death (Fig. 3A-B). Since endosperm cells are highly autofluorescent, we confirmed the expression of HDEL-mCherry and Rtn1-GFP by confocal laser scanning microscopy and fluorescence spectral analysis (Fig. 3C-D). We observed fluorescent signal corresponding to the transfected reporters as early as 1 day and up to 4 days after bombardment. We then calculated the transfection efficiency as a) the percentage of endosperms with more than 20 transfected aleurone cells and b) the percentage of aleurone cells transformed per field of view using. There was a slight but not statistically significant increase in transfection efficiency with 1100 psi compared to 650 psi rupture disks (50–80% transfected endosperms per plate with 1100 psi compared to 40–60% with 650 psi rupture disks, p -value = 0.057; Fig. 4A). To calculate the percentage of transfected cells expressing the fluorescent reporters, we prepared fresh thin slices of endosperm containing aleurone cells and imaged them using a 40x objective under a confocal laser scanning microscope (Fig. 4B). We found that for both conditions (650 psi and 1100 psi rupture disks), the transfection efficiency was similar, reaching between 12 and 15% of aleurone cells 3 days after bombardment with a slight decline to 7–12% at 4 days after bombardment. This transfection rates are comparable to those reported for *Agrobacterium*-mediated transformation, where approximately 15% of aleurone cells expressed fluorescent reporters by 4 days after co-cultivation [4]. Therefore, we concluded that using a range of 650 to 1100 psi is adequate for biolistic bombardment of *in vitro*-cultured endosperm.

Declaration of Competing Interest

[MANDATORY – Delete as appropriate]

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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