Video Article

Agrobacterium-Mediated Immature Embryo Transformation of Recalcitrant Maize Inbred Lines Using Morphogenic Genes

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URL: https://www.jove.com/video/60782
DOI: doi:10.3791/60782

Keywords: Engineering, Issue 156, *Agrobacterium tumefaciens*, B73, *Bbm*, genetic transformation, Mo17, QuickCorn, transgenic, W22, *Wus2*, *Zea mays*

Date Published: 2/14/2020

Citation: Masters, A., Kang, M., McCaw, M., Zobrist, J.D., Gordon-Kamm, W., Jones, T., Wang, K. *Agrobacterium*-Mediated Immature Embryo Transformation of Recalcitrant Maize Inbred Lines Using Morphogenic Genes. *J. Vis. Exp.* (156), e60782, doi:10.3791/60782 (2020).

Abstract

Demonstrated here is a detailed protocol for *Agrobacterium*-mediated genetic transformation of maize inbred lines using morphogenic genes *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*). *Bbm* is regulated by the maize phospholipid transferase gene (*Pltp*) promoter, and *Wus2* is under the control of a maize auxin-inducible (*Axig1*) promoter. An *Agrobacterium* strain carrying these morphogenic genes on transfer DNA (T-DNA) and extra copies of *Agrobacterium* virulence (*vir*) genes are used to infect maize immature embryo explants. Somatic embryos form on the scutella of infected embryos and can be selected by herbicide resistance and germinated into plants. A heat-activated *cre/loxP* recombination system built into the DNA construct allows for removal of morphogenic genes from the maize genome during an early stage of the transformation process. Transformation frequencies of approximately 14%, 4%, and 4% (numbers of independent transgenic events per 100 infected embryos) can be achieved for W22, B73, and Mo17, respectively, using this protocol.

Video Link

The video component of this article can be found at https://www.jove.com/video/60782/

Introduction

Transformation is a basic tool for evaluating foreign gene expression in maize and producing genetically modified corn lines for both research and commercial purposes. Access to high throughput transformation can facilitate the increased need for maize molecular and cellular biology studies[1]. The ability to genetically transform crop species is vital to both public and private laboratories. This allows for both fundamental understanding of gene regulation mechanisms as well as crop improvement on a global scale to support an ever-growing population.

The discovery that immature embryos from maize could be used for the production of regenerable callus originated in 1975[2]. Since this revelation, most scalable maize transformation protocols have required callus formation and selection prior to regeneration[3]. During the process of genetic transformation, *Agrobacterium*-infected or biolistic-bombarded immature embryos are cultured on media for embryogenic callus induction. Induced calli are then cultured on selective media (e.g., containing an herbicide) so that only transformed callus pieces are able to survive. These herbicide-resistant putative transgenic calli are bulked up and regenerated into plants. While this method is effective, the process is long and labor-intensive, and it can take upwards of 3 months to complete[4]. More importantly, conventional maize transformation protocols possess a much larger limitation, that is, only a limited number of maize genotypes can be transformed[5,6].

Lowe et al.[7,8] previously reported a "QuickCorn" transformation method that not only greatly reduced the duration of the transformation process but also expanded the list of transformable genotypes. The QuickCorn method utilizes maize orthologs (*Zm-Bbm* and *Zm-Wus2*) of the *Arabidopsis* transcription factors *BABY BOOM (BBM)*[9] and *WUSCHEL (WUS)*[10]. When incorporated in the transformation vector system, these genes work synergistically to stimulate embryogenic growth[7].

The QuickCorn protocol described in this work was based on the protocol in Jones et al[11], which was a further improvement of the method reported by Lowe et al[7,8]. In the present study, an *Agrobacterium* strain LBA4404(Thy-) harboring a binary vector construct PHP81430 (**Figure 1**) and accessory plasmid PHP71539[12] are used for transformation. The T-DNA of PHP81430 contains the following molecular components. (1) The transformation selective marker gene *Hra* expression cassette. The maize *Hra* (*Zm-Hra*) gene is a modified acetolactase synthase (ALS) gene that is tolerant to ALS-inhibiting herbicides such as sulfonylureas and imidazolinones[13,14]. The *Zm-Hra* gene is regulated by the sorghum ALS promoter[8] and potato proteinase inhibitor II (*pin*II) terminator[15]. The T-DNA also contains (2) an expression cassette possessing

the transformation screenable marker gene *ZsGreen*. This green fluorescent protein gene *ZsGreen* from *Zoanthus* sp. reef coral[16] is regulated by a sorghum ubiquitin promoter/intron and rice ubiquitin terminator.

Additionally, the T-DNA contains (3) the morphogenic gene *Bbm* expression cassette. *Bbm* is a transcription factor associated with embryo development[9,17]. *Bbm* is regulated by the maize phospholipid transferase protein (*Pltp*) promoter[8] and rice *T28* terminator[18]. *Zm-Pltp* is a gene with strong expression in the embryo scutellar epithelium, silk hairs, and leaf subsidiary cells (flanking the guard cells), low expression in reproductive organs, and no expression in roots[8]. It also contains (4) the morphogenic gene *Wus2* expression cassette. *Wus2* is another transcription factor associated with the maintenance of the apical meristem[19]. *Zm-Wus2* is under the control of a maize auxin-inducible promoter (*Zm-Axig1*)[20] and maize *In2-1* terminator[21]. Finally, the T-DNA contains (5) the *cre-loxP* recombination system. The *cre* recombinase gene[22] is under the control of maize heat shock protein 17.7 (*Hsp17.7*)[23] promoter and potato *pin*II terminator. Two *loxP* sites (in the same orientation)[24] flank four gene expression cassettes including *ZsGreen*, *cre*, *Bbm* and *Wus2*.

Because the presence of the morphogenic genes is not desired for plant maturity and subsequent progeny, the heat-induced *cre-loxP* recombination system was built into the T-DNA to remove morphogenic genes from the maize genome to allow normal callus regeneration and plant development. Upon heat treatment, the expression of CRE protein removes all transgenes except for the *Hra* selection gene. Successful transformants should be herbicide-resistant but *ZsGreen*-negative. To further enhance transformation frequency, the *Agrobacterium* strain also harbors an additional accessory plasmid (PHP71539) that has extra copies of *Agrobacterium* virulence (*vir*) genes[12].

The QuickCorn method is different from conventional maize transformation protocols, as it does not involve a callus induction step during transformation. During the first week after infection with *Agrobacterium*, somatic embryos develop on the scutellar epithelium of the infected immature embryos. The embryos are then transferred to a medium with hormones that encourage embryo maturation and shoot formation. Rapidly transferring the somatic embryos onto maturation/shoot formation medium skips the traditional callus stage previously used for maize transformation and permits direct generation of T0 plants[8]. Compared to previously published maize transformation methods[6], the QuickCorn method is faster, more efficient, and less genotype-dependent. Using this method, rooted plants are typically ready to transfer to soil in just 5-7 weeks, rather than the three or more months required by traditional protocols. The purpose of this article is to provide an in-depth description and demonstration of the method, allowing for easier replication in a laboratory setting typically found in most academic institutions.

Protocol

1. Growth media preparation

1. For exact growth medium recipes for this protocol, please refer to **Table 1**.
2. For preparing 1 L of media, place a 2 L beaker on a stir plate and place a stir bar inside.
3. Fill beaker with 900 mL of distilled water and turn on the stir plate. The stir bar should be spinning at a medium speed.
4. Weigh all powdered ingredients and dissolve in a beaker.
5. Measure out all liquid ingredients, if any, and add to the beaker.
6. Bring the final volume to 1 L using distilled water.
7. Measure the pH and adjust to recipe specifications.
8. If formulating a liquid growth medium, no agar is added. Attach a filter sterilizer to a vacuum pump and pour the liquid growth medium through the filter. Turn on the pump and wait until all liquid is pulled through. Place a cap on the container and attach a label.
9. If formulating a solid growth medium, after the pH is adjusted, add agar directly into a bottle or flask.
10. Pour the 1 L liquid growth medium into a 2 L Erlenmeyer flask, or divide it into two 1 L autoclavable bottles (500 mL each). If two bottles are used, divide the agar and add directly to the bottles.
11. Cover flask with a breathable cover, such as two layers of aluminum foil, to allow steam to escape. If using a bottle, loosely cap the screw lid on the top.
12. Autoclave at 121 °C for 25 min.
13. After autoclaving, remove the growth medium from the autoclave and cool to 55-60 °C (a water bath set to 55 °C can make this easier). Keep the growth medium in a liquid state for a few hours until it is convenient to pour plates.
14. Once cooled, add all post sterilization additives (see **Table 1**) and mix thoroughly.
15. After all ingredients are added, pour the designated volume into the container of choice in a laminar flow hood.
16. Growth medium can be poured into desired Petri dishes manually or using a liquid dispensing apparatus. When pouring manually, it is recommended to transfer a large volume of autoclaved medium into a smaller sterile beaker (500 mL) for ease of handling.
17. Allow growth medium to cool and solidify.
18. The growth medium will be available for use once becoming solid and is best used the following day after drying slightly overnight in a sterile flow hood as stacks of lidded plates. After overnight drying, transfer the plates into plastic sleeves, fold over the loose end, and keep this in place with a small bit of tape. This prevents excessive drying. Medium can be stored in a cool, dark, and clean environment (4-16 °C) for up to 1 month.

2. Growing donor plants and harvesting immature ears

1. Grow any publicly available maize inbred (i.e., B73, Mo17, or W22) in a greenhouse in 1.5 gallon (5.9 L) pots containing a soilless substrate. Use a 16/8 (day/night) photo period, with average temperatures of 25.5 °C during the day and 20 °C at night.
2. Plants are watered as needed and fertilized with a controlled release fertilizer (N-P-K of 15-9-12), which can either be incorporated into the soil mix or added to the surface after planting.
3. It usually takes about 70-90 days after seed germination for ears to emerge. As ear shoots emerge, cover them with a shoot bag to prevent uncontrolled pollination from occurring.

4. About 2-3 days after silks have emerged and if pollen will be available the following day, cut the silks using scissors that have been sterilized in 70% ethanol. Cut the silks and husk roughly 2.5 cm below the end of the husk leaves, where the silks emerge. Pollination can be performed the next day. Be sure to resterilize scissors between each ear.
5. Once anthers emerge from a tassel, cover the tassel with a tassel bag and non-skid paper clip at the base of the bag around the stalk.
6. The next morning, gently bend the plant over and tap the bag to encourage pollen to be released.
7. Remove the tassel bag and fold the top of the bag over to prevent pollen from escaping. It is generally best to bag the tassel 1 day before it will be used (to avoid build-up of dead pollen and shed anthers). Fresh pollen may be collected from tassels for about 3-5 days. When anthers emerge from the inner florets at the base of the tassel, that tassel will likely not produce viable pollen the next day.
8. Use the pollen from the same plant (selfing) or from another plant of the same inbred (sibbing).
9. Remove the ear bag or cut the end of the bag to expose the silks, then quickly pour pollen from the tassel bag onto the silks.
10. Cover the ear with the tassel bag immediately and staple the base of the bag around the stalk to secure it. It may be helpful to physically isolate the plant from flowering plants of different genotypes during pollination to help prevent cross-pollination. Leave the tassel bag on the ear until the immature ear is ready to harvest.
11. 9-12 days after pollination, screen ears for embryo size. Slide the pollination bag up the stalk to expose the ear. Gently pull the husk down to expose kernels on about one-third to one-fourth of the circumference of the ear and about one-third of the distance down the ear. Kernels near the tip will not be representative of the average embryo size.
12. Using a scalpel, slice off the cap of a single kernel that appears similar to the majority of other kernels in size and color.
13. Use a spatula (with a ruler) to remove the embryo as described in step 4.6. Measure the length of the embryo using a built-in ruler on the spatula or a digital caliper. If the embryo is between 1.5-2.0 mm, harvest the ear. If it is ~1.3 mm, the ear may be ready to harvest later in the day and can be checked again in about 7-8 h.

3. Preparing *Agrobacterium* suspension culture for infection

NOTE: The *Agrobacterium* strain LBA4404(Thy-) containing PHP81430 (**Figure 1**) and PHP71539[12] is stored as a glycerol stock at -80 °C. These materials can be obtained from Corteva Agriscience through a Material Transfer Agreement. LBA4404(Thy-) is an auxotrophic strain that needs thymidine supplied in the growth media. The primary utility of the auxotroph Agro strain is for biocontainment purposes. It has the additional benefit of reducing Agro overgrowth. The auxotrophic Agro strain does not grow without supplemental thymidine. Nevertheless, thymidine can (presumably) be supplied by dying plant tissue in the culture. Therefore, there is still a need to provide an antibiotic in the medium to completely control the auxotrophic Agro. However, it will be easier to control due to compromised growth of the auxotrophic strain in the absence of thymidine.

1. Four days before the date of infection, initiate a "mother" plate from the glycerol stock by streaking the bacteria on a YP plate with 50 mg/L thymidine, 50 mg/L gentamicin, and 50 mg/L spectinomycin (**Table 1**). Incubate the "mother" plate in a 20 °C incubator for 3 days.
2. One day before the infection experiment, prepare a "working" plate by selecting one to five colonies from the "mother" plate and streaking the bacteria from the "mother" plate to a new YP plate (with thymidine, gentamycin, and spectinomycin; **Table 1**).
3. Streak the daily "working" plate in sequential quadrants and run the loop 1x through the just-streaked area into the successive quadrant, repeating to form quadrants that have been serially diluted. Incubate the "working" plate overnight in a 27 °C incubator.
4. After completing embryo dissection (step 4.8), use a loop or similar tool to collect *Agrobacterium* from a region of the "working" plate where bacterial growth is visible as thin streaks of colonies.
   NOTE: Avoid areas of the plate with a dense lawn of bacterial growth. The *Agrobacterium* growth has likely already started to decline in dense areas, while in the areas with visible colonies the bacteria are in the proper growth phase for infection.
5. Suspend the collected bacteria in a 50 mL tube containing 10 mL of 700A liquid medium (**Table 1**). Vortex to suspend the bacteria culture completely.
6. Measure the optical density at a wavelength of 550 nm. Adjust the volume until the OD is between 0.35-0.45, with 0.4 being the optimal value.
   NOTE: If the OD is higher than 0.45, add more 700A liquid medium. If the OD is lower than 0.35, inoculate more Agro colonies in the suspension culture.

4. Embryo dissection, infection, and co-cultivation

1. Select suitable ears for transformation experiments; these should have a good seed set and have embryos that range in size from 1.5-2.0 mm. They are typically harvested between 9-12 days after pollination. Harvested ears can be used fresh or stored for 1-4 days at 4 °C, though quality of response will likely degrade progressively with prolonged storage beyond the first day.
2. Remove the husks and silks. Insert a handle into either the base or the top of the ear. The handle can be a pair of forceps, screwdriver, etc.
3. Place ears in a large container (e.g., 2 L beaker with the handle upwards, fill the container with disinfection solution. Disinfection solution is 1.8 L of 20% commercial bleach (1.65% sodium hypochlorite) and a couple of drops of surfactant Tween 20.
4. Sterilize the ears inside a laminar flow bench. After 20 min, empty the bleach solution and rinse the ears 3x (5 min each) using a generous amount of sterile distilled water. Remove the water and allow ears to dry for several minutes.
   NOTE: It is important that the ears be completely submerged in the bleach solution for 20 min. Move the ears carefully around in the bleach solution occasionally to dislodge air bubbles.
5. Prepare a 2 mL microcentrifuge tube filled with 700A liquid medium. This tube will be used to collect the immature embryos.
6. Take the ear and using a sterile scalpel, remove the top 1-2 mm of the kernel crowns to expose the endosperm. Use a micro spatula to remove the immature zygotic embryo (IZE). The IZE will be located within the kernel, on the side facing the tip of the ear, and near the attachment to the cob. Using the spatula, insert it into the endosperm in the pericarp furthest away from the embryo, then gently twist upward to dislodge the endosperm and allow for removal of the embryo (**Figure 2**).
7. Using the spatula, transfer the embryo into the tube containing the 700A liquid medium. Continue doing this until up to 100 embryos have been collected. Multiple tubes may be filled (~100 embryos/tube) before proceeding to the next step. At this point, the *Agrobacterium* suspension should be prepared (see step 3.5).

8. Remove the 700A liquid medium from the embryo tube with a 1 mL pipette. Add fresh 700A medium to wash the embryos, then remove that media as well.
9. Add 1 mL of the *Agrobacterium* suspension and vortex on a low setting (3/10) for 30 s or invert tube 12x-15x to mix. Allow this tube to rest horizontally on bench for 5 min.
10. After 5 min, transfer the entire tube of embryos and *Agrobacterium* suspension onto a plate of 562V co-cultivation medium (**Table 1**). This can be achieved by placing the plate on the bench and quickly pouring the tube contents onto the plate. Gently swirl the plate to distribute the embryos and remove the *Agrobacterium* suspension using a 1 mL pipette.
11. Make sure that the embryos are placed with the scutellum (round) side facing upwards. Use a magnifying glass or a dissecting scope, if needed. Place plates in plastic boxes (19 cm x 28 cm x 5.1 cm) and incubate the plates overnight 16-18 h at 21 °C in the dark. No individual plate wrapping using paraffin film or vent tape is necessary.
12. After overnight co-cultivation, move the infected embryos, scutellum side up, onto resting medium 605T (**Table 1**). Place around 30 embryos per plate. Incubate the plates at 26-28 °C in the dark.
13. Incubate for 4-10 days (7 days is preferred). At this time, the development of somatic embryos can be observed on the surface of the zygotic scutellum (**Figure 3**).

5. Selection, heat treatment, and regeneration

1. After the resting period, heat shock the embryos. Place the box containing the plates of embryos in a 45 °C incubator with 70% relative humidity for 2 h. Then, remove the box from the 45 °C incubator and place in the 26-28 °C dark incubator for 1-2 h.
   NOTE: If unable to attain 70% humidity in an incubator, add a double layer of autoclaved paper towels to the bottom of the plate box and soak with autoclaved water to maintain humidity within the box. Return the plates to the box on top of the paper towels and seal the lid before placing at 45 °C. Use a small digital hygrometer/thermometer to monitor the temperature and humidity.
2. Transfer the heat-treated IZEs from the resting medium to the shoot formation medium (13329A) containing 0.05 mg/L imazapyr as a selective agent (**Table 1**). When transferring, remove coleoptiles, if present, using fine tip forceps or surgical scissors.
3. Place 10-15 embryos per plate to avoid overcrowding. Keep the embryos in this medium for 2 weeks in the 26 °C dark incubator.
4. Transfer the embryos to rooting medium (13158; **Table 1**) for 1-2 weeks. Place around eight pieces per plate and incubate in a light room or light chamber (16 day/8 night, 20-150 µmol/m^2/s) at 27 °C.
5. As plantlets develop, place stronger plantlets containing both shoots and vigorous roots onto new plates of rooting medium, place one plant per plate. This will allow for stronger plantlet growth. Place the plates in the light room or light chamber for another 7-14 days.
   NOTE: Carefully remove any callus pieces associated with the plantlet to ensure it is in good contact with the medium.
6. As the plant becomes more vigorous, remove the plant from rooting medium and rinse the roots with tap water to remove agar.
7. Transplant individual plants into 3 in^2 (~19 cm^2) pots containing a pre-wetted soilless substrate. Place the pots in a tray (27 cm x 54 cm) with drain holes and cover the flat with a plastic humidity dome. This acclimation step can be achieved either in growth chamber or in greenhouse with growth conditions described in section 2 (step 2.1) above.

6. Transplanting to the greenhouse and the production of T1 seeds

1. Check the plants 2x per day. Water as needed. Ensure that the plants are neither dried out nor overwatered. Maintaining a slightly dry substrate encourages root growth.
   NOTE: The humidity dome can be removed 4-7 days after transplanting. Plants should be grown in these small pots until they have visibly recovered from the stress of transplant to soil. This should take about 9-14 days.
2. Transplant the entire soilless plug and plantlet into a 1.5 gal (5.9 L) pot. Maintain in the greenhouse and water when soil feels dry to the touch.
3. Add a controlled release fertilizer with N-P-K of 15-9-12 to the pot, which can be either incorporated into the substrate mix or applied to the surface.
4. When ear shoots begin to emerge from the plant, use a shoot bag to cover the ear shoots. Be sure to use a bag that is semi-transparent so that the emerging silks can be observed without removal of the bag. The shoot bag allows for controlled pollination to occur. It is important to always bag transgenic tassels.
5. After the silks emerge (1-2 days), trim the emerged silks to a uniform length. This will be about 2.5 cm below the top of the husk leaves. Use a pair of clean scissors that have been sterilized in 70% ethanol. By trimming the silks, a uniform tuft develops the following day for pollination to occur.
6. For a majority of maize genotypes, the optimal time for pollination is 2-3 days after tassel or silk emergence.
7. Collect the pollen from either the same plant (if being self-pollinated) or from a wild-type of the same inbred (if outcrossing or incrossing).
8. Collect the pollen in a tassel bag and apply it to the tuft of silk of the T0 plant. If the pollen is from a wild-type (non-transgenic) plant, place the pollen in a plain brown tassel bag. If the pollen is from a transgenic plant, place the pollen in a green striped bag to indicate that the pollen is transgenic.
9. Follow steps 2.5-2.10 for pollination details.
10. About 2 weeks after pollination, remove the tassel bags from ears, and allow for dry-down to begin. To help dry down, stop watering the plant 21-25 days after pollination. You can also pull back the husk leaves to expose the seed. This practice also helps prevent mold.
11. About 45 days after pollination, harvest seeds and store in cold storage at 4-12 °C.

Representative Results

Demonstrated here is a step-by-step protocol for *Agrobacterium*-mediated genetic transformation of three public maize inbred lines (B73, Mo17, and W22) that have been significant in the field of maize genetics. Transformation of the three inbred lines could not be achieved using conventional maize transformation protocols[5]. **Figure 1** and **Figure 2** show the construct and starting materials, respectively, used here. Ears are generally harvested 9-12 days after pollination. IZEs with lengths ranging between 1.5-2.0 mm are the best explants for transformation for this protocol (**Figure 2**).

Eight days after infection, *ZsGreen*-expressing somatic embryos were visualized under the GFP channel of a fluorescent microscope (**Figure 3**). Infected IZEs were subjected to heat treatment 8 days after infection (steps 5.1 and 5.2). This treatment induced the expression of CRE recombinase that excised the *Bbm*, *Wus2*, *cre*, and *ZsGreen* expression cassettes flanked between the two *loxP* sites (**Figure 1**). The heat-treated tissues were then cultured on shoot formation medium containing the herbicide imazapyr for selection of transformed tissue after morphogenic gene removal.

Proliferating tissues with maturing embryos or shoot buds that were resistant to imazapyr were observed around 3-4 weeks after infection (**Figure 4**). Some imazapyr-resistant tissues were negative for ZsGreen, suggesting that *cre*-mediated excision likely occurred in these tissues (**Figure 4**). After the tissues were moved to rooting medium and light incubation, shoots started to develop (**Figure 5**). Healthy and vigorous growing shoots with well-developed roots were harvested (**Figure 5**). Some tissues appeared to have multiple shoots (**Figure 5E,F,G**). This type of "grassy" regenerant may be due to clonal plants having identical transgene integration patterns. Molecular biological analysis is required to genotype these plants.

All three public inbred lines responded well using this protocol as well as the construct used in this work. W22 produced the highest frequency of imazapyr-resistant shoots, with a frequency of approximately 14% (about 14 transgenic shoots per 100 infected immature embryos). Both B73 and Mo17 produced about 4% transgenic shoots. These frequencies indicate all transgenic shoots, including both plants carrying the morphogenic genes and plants with the morphogenic gene removed by the CRE-mediated excision.

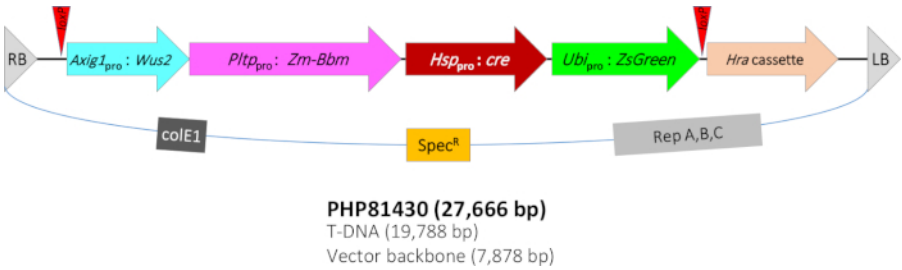

Figure 1: Schematic representation of the T-DNA region of the binary plasmid PHP81430. RB = right T-DNA border; *loxP* = CRE recombinase target site; *Axig1*$_{pro}$:*Wus2* = maize auxin-inducible promoter (*Zm-Axig1*) + *Zm-Wus2* + maize *In2-1* terminator; *Pltp*$_{pro}$:*Zm-Bbm* = maize phospholipid transferase protein (*Zm-Pltp*) promoter + *Zm-Bbm* + rice *T28* terminator (*Os-T28*); *Hsp*$_{pro}$:*cre* = maize heat shock protein 17.7 promoter (*Zm-Hsp17.7*) + *cre* recombinase gene + potato proteinase inhibitor II (*pinII*) terminator; *Ubi*$_{pro}$:*ZsGreen* = sorghum ubiquitin promoter/intron (*Sb-Ubi*) + green fluorescent protein *ZsGreen* gene + rice ubiquitin terminator (*Os-Ubi*); *Hra* cassette = sorghum acetolactase synthase (*Sb-Als*) promoter + maize Hra (*Zm-Hra*) gene + *pinII* terminator; LB = left T-DNA border; colE1, replication origin of plasmid ColE1[25]; Spec^R = spectinomycin resistant gene *aadA1* from Tn21 for bacterium selection[26]; Rep A,B,C = replication origin from pRiA4 of *Agrobacterium rhizogenes*[27]. Please click here to view a larger version of this figure.

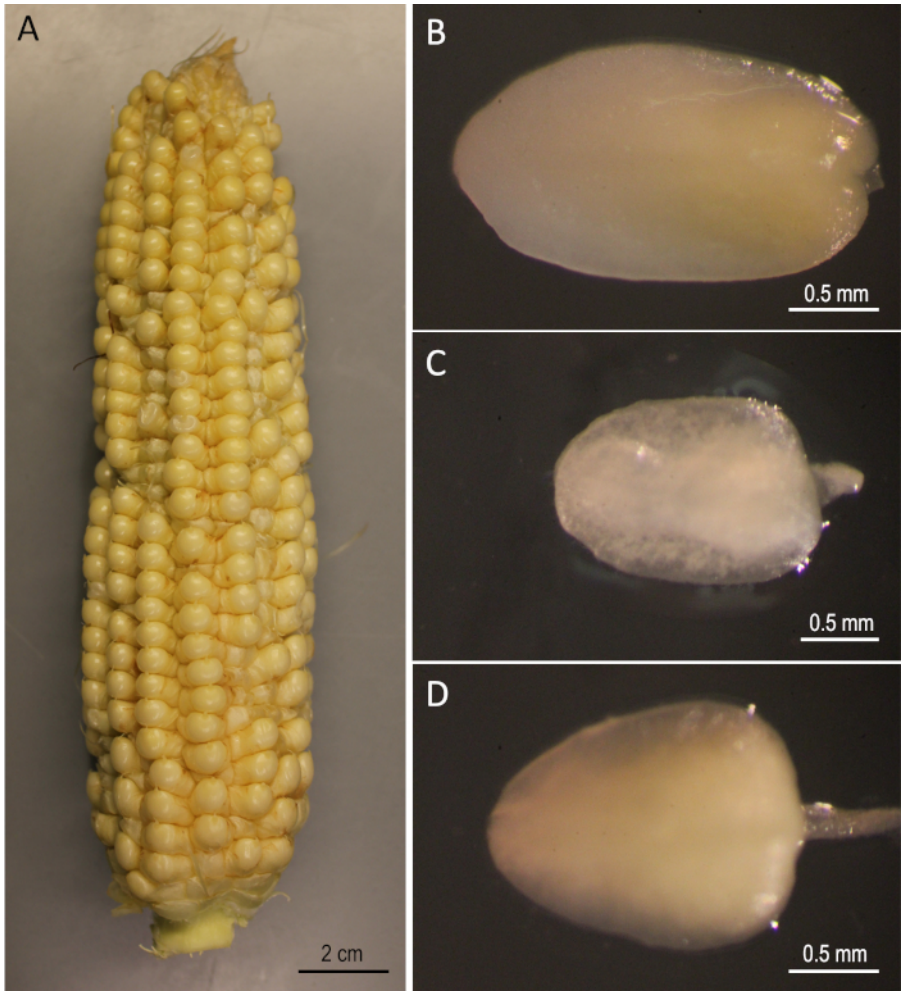

Figure 2: Starting materials. B73 ears harvested 12 days post-pollination (**A**). Immature embryos of B73 (**B**), Mo17 (**C**), and W22 (**D**). Please click here to view a larger version of this figure.

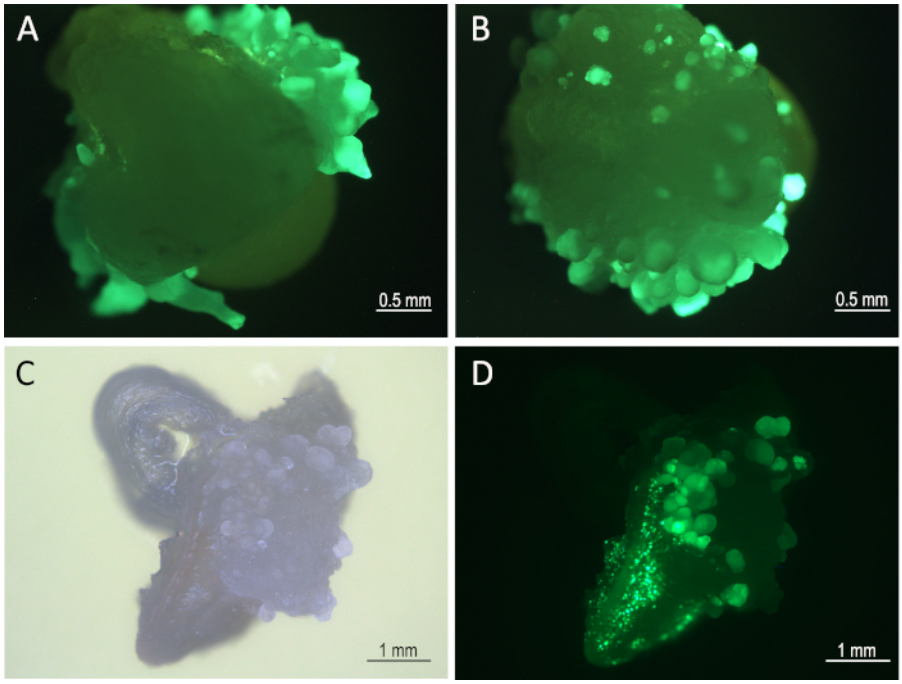

Figure 3: Tissue development on resting medium 1 week post-infection. Embryos (8 days post-infection) under a florescence microscope (GFP filter) showing GFP expressing somatic embryos of Mo17 (**A**) and W22 (**B**). Developing tissue (B73) under bright-field (**C**) and GFP filter (**D**). Please click here to view a larger version of this figure.

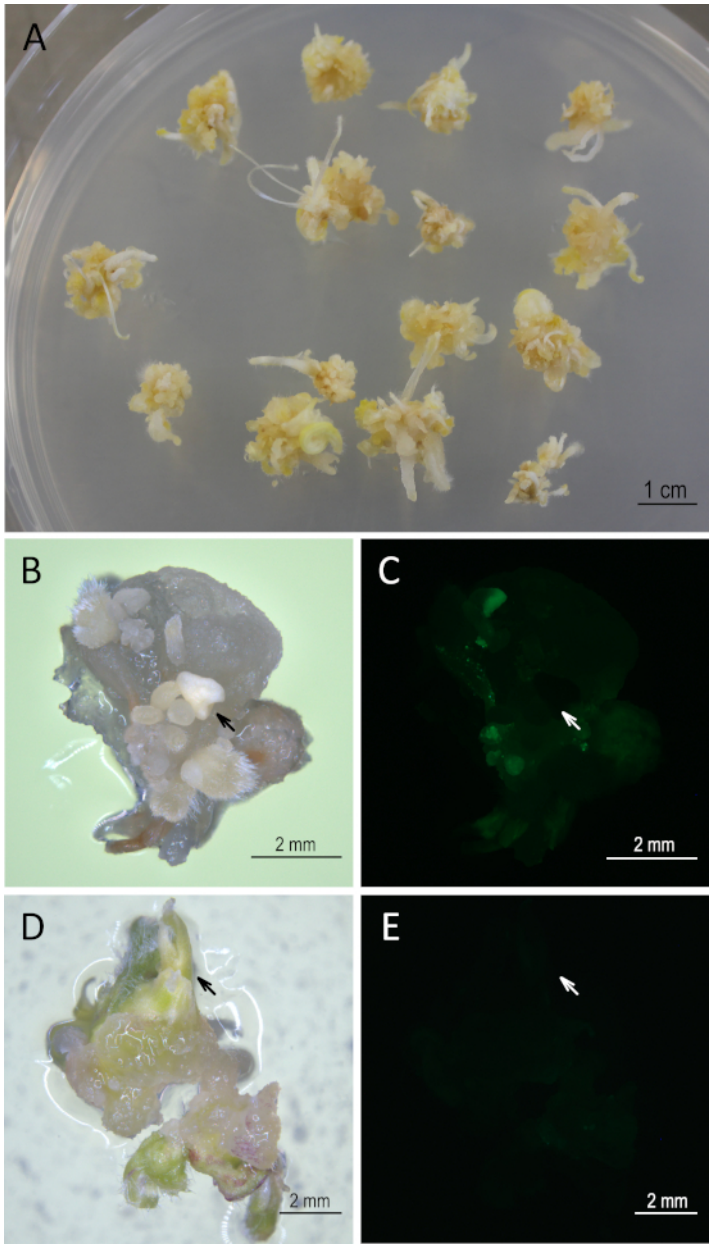

Figure 4: Tissue development on maturation medium with selection. A W22 maturation plate (**A**). Developing tissue (Mo17, 15 days post-infection) under bright-field (**B**) and GFP filter (**C**). Developing tissue (Mo17, 28 days post-infection) under bright-field (**D**) and GFP filter (**E**). Arrows point to regenerating tissues that are lacking GFP expression, suggesting the excision of *ZsGreen* gene between the *loxP* sites after heat-induced CRE protein activity. Please click here to view a larger version of this figure.

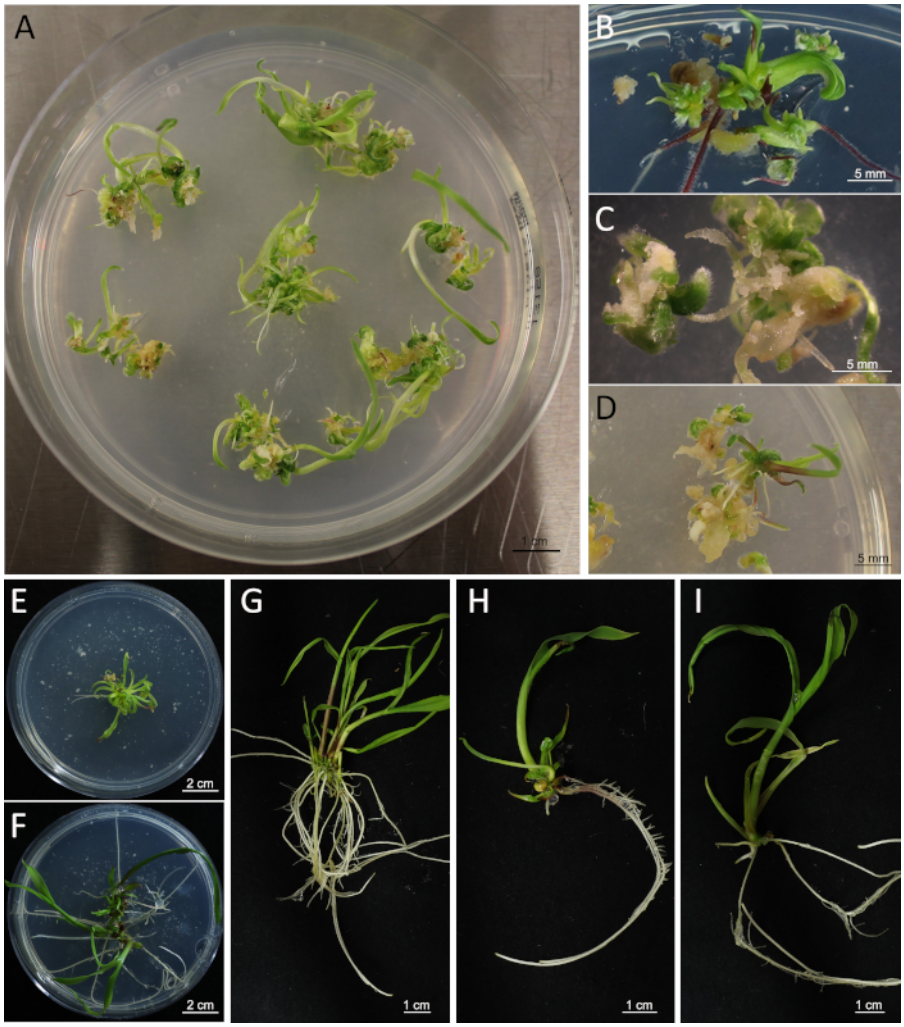

Figure 5: Tissue development on rooting media. Shoots of W22 (**A**), B73 (**B**), and Mo17 (**C,D**). Event with multiple shoots (grassy regenerants) of B73 (**E**) and W22 (**F,G**). Shoots with roots of B73 (**H**) and W22 (**I**). Please click here to view a larger version of this figure.

Table 1: Media compositions for maize transformation. Please click here to view this table (Right click to download).

Discussion

Traditional protocols for maize transformation follow the paradigm of isolating immature zygotic embryos to produce transgenic callus tissue, which is regenerated into fertile plants[4,6]. While this is effective, callus-based protocols can be time-consuming, and it often takes up to 3 months for the tissue culture process to produce plants. What makes the method presented here significant is that it is callus-free, efficient, quick, and allows for the regeneration of T0 plants in roughly half the timeframe. It also appears to be less genotype-dependent and can thus be effective for most publicly available inbreds[8,11].

While all steps should be effectively followed, correct growth media preparation is imperative. Growth media components need to be added at the correct stages, both pre- and post- autoclave, to ensure that the plant material receives the proper concentration of chemicals. This will ensure that sensitive compounds like antibiotics do not break down. It is also important that plant material is placed on the correct growth medium at each stage, as indicated in the protocol. Not placing material on the proper growth medium can result in material death. In addition, placing too many embryos or developing tissues on plates should be avoided. While placing twice as many tissue pieces may save the cost of chemicals and Petri dishes (and even incubator space), the growth of tissue in overcrowded plates can be seriously inhibited. While performing the infection, it should be ensured that the optical density of the *Agrobacterium* suspension is appropriate. If the bacterial suspension density is too low, proper infection may not occur.

The quality of starting materials is essential for success in transformation protocols. Ears used for embryo dissection must be healthy, meaning that the plant that produces them is healthy. They also must possess an adequate seed set and be pest- and disease-free. Also, old *Agrobacterium* should not be used. The "mother" plate should be no more than 2 weeks old. After this point, a new "mother" plate should be streaked to begin new experiments.

While this method has been shown to be less genotype-dependent, it cannot be assumed that all lines will be equally successful. There can still be variation amongst lines as well as differences in success based upon the construct being used. Ear-to-ear variability is also unavoidable when

working with immature embryos, so ideally experiments should use multiple ears to account for this. In this work, inbred W22 performed the best, with over ~14% transformation frequency, followed by B73 and Mo17 (~4% each). Lowe et al.[8] reported using the QuickCorn protocol for B73 and Mo17 transformation. In this work, the transformation frequencies ranged from 9%-50% for B73 and 15%-35% for Mo17.

One possibility for the lower transformation frequencies for B73 and Mo17 observed in this work may be attributed to seasonal ear quality fluctuation. Another difference between this work and that of Lowe et al.[8] is that different vector constructs were used here. In Lowe's work, morphogenic genes were not removed from the transformed plants but rather developmentally silenced in the later stages. In this work, the morphogenic genes were removed 8 days after the infection. It is possible that B73 and Mo17 may need a longer presence of *Bbm/Wus2* for the development of somatic embryos.

Using this method, there is a possibility of obtaining non-transgenic escape plants, multimeric insertions, and unexcised transgenes. These plants will not have a noticeably different phenotype, so detection by PCR is required to determine whether a plant is transgenic. To accomplish this, PCR primers within the excised region and primers flanking the excised region can be employed. Multiple independent transformations can also produce plants from the same immature embryo, making determination of total independent transformant recovery rate difficult. Our standard has been to calculate a transformation rate based on sampling one plant from each immature embryo that produced plants and dividing this by the number of embryos infected. This method almost certainly underestimates the actual number of independent events recovered as plantlets. Discrimination between independent events from the same embryo requires sequencing border regions around transgenes, and this will be prohibitively expensive and time-consuming for most applications; though, there may be cases in which these data are useful.

This method of tissue culture transformation has proven to be very effective, but problems can still occur. If plant material is not responding, it is possible that there is an issue with the particular inbred line, suggesting that variables such as growth media composition and timing of subculturing require adjustments. Another variable is proper vector design and accurate vector construction, if the original vector is altered. There can also be issues with imazapyr sensitivity, as some lines are more sensitive than others, and the concentration of imazapyr may need to be adjusted to achieve successfully transformed plants.

Over the last 30 years, maize tissue culture and transformation protocols have changed and progressed; and it is believed that this shortened protocol will further this progression. This method is effective for academic settings because it is less time-consuming than traditional methods. In addition, it does not demand highly trained operators, making it more amenable to widespread distribution when compared to traditional methods. In the future, this method can be combined with new technologies such as genome engineering.

Disclosures

Alicia Masters, William Gordon-Kamm, and Todd Jones are employees of Corteva Agriscience that supplied the protocol and maize ears of B73, Mo17, and W22 used in this article. The authors Minjeong Kang, Morgan McCaw, Jacob Zobrist, and Kan Wang have nothing to disclose.

Acknowledgments

We thank the Corteva greenhouse team for providing maize immature ears, the Corteva media prep lab for providing assistance in making media, Ning Wang from Corteva for help with the *Agrobacterium* construct, and Keunsub Lee from Iowa State University for assistance. This project was partially supported by National Science Foundation Plant Genome Research Program Grant 1725122 and 1917138 to K.W., by Predictive Plant Phenomics Research Traineeship Program (National Science Foundation Grant DGE-1545453) to J.Z., by the USDA NIFA Hatch project #IOW04341, by State of Iowa funds, and by Crop Bioengineering Center of Iowa State University.

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