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Corresponding Author:	Kan Wang UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	kanwang@iastate.edu
Order of Authors:	Alicia Masters
	Minjeong Kang
	Morgan McCaw
	Jacob Zobrist
	William Gordon-Kamm
	Todd Jones
	Kan Wang
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TITLE:

Immature Embryo Transformation of Recalcitrant Maize Inbred Lines Using Morphogenic Genes and Mediated by *Agrobacterium*

AUTHORS AND AFFILIATIONS:

Alicia Masters[1,2], Minjeong Kang[1,3], Morgan McCaw[1,3], Jacob Zobrist[1,3], William Gordon-Kamm[2], Todd Jones[2], Kan Wang[1,2]

[1]Department of Agronomy, Iowa State University, Ames, Iowa, USA
[2]Department of Applied Science and Technology, Corteva Agriscience, Johnston, Iowa, USA
[3]Crop Bioengineering Center, Iowa State University, Ames, Iowa, USA

Corresponding Author:

Kan Wang (kanwang@iastate.edu)

Email Addresses of Co-Authors:

Alicia Masters (aliciad@iastate.edu, Alicia.Masters@corteva.com)
Minjeong Kang (mjkang@iastate.edu)
Morgan McCaw (mccawm@iastate.edu)
Jacob Zobrist (jzobrist@iastate.edu)
William Gordon-Kamm (William.Gordon-Kamm@corteva.com)
Todd Jones (Todd.j.jones@corteva.com)

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Agrobacterium tumefaciens, B73, *Bbm*, genetic transformation, Mo17, QuickCorn, transgenic, W22, *Wus2*, *Zea mays*

SUMMARY:

Plant morphogenic genes can be used to improve genetic transformation of recalcitrant genotypes. Described here is an *Agrobacterium*-mediated genetic transformation (QuickCorn) protocol for three important public maize inbred lines.

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.

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 but as well as crop improvement on a global scale to support an evergrowing 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, such that 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].

This protocol demonstrates the QuickCorn method based on Jones et al[11], which was improved from the method of 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 in 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

1.1. For exact growth medium recipes for this protocol, please refer to **Table 1**.

1.2. For preparing 1 L of media, place a 2 L beaker on a stir plate and place a stir bar inside.

1.3. Fill beaker with 900 mL of distilled water and turn on the stir plate. The stir bar should be spinning at a medium speed.

1.4. Weigh all powdered ingredients and dissolve in a beaker.

1.5. Measure out all liquid ingredients, if any, and add to the beaker.

1.6. Bring the final volume to 1 L using distilled water.

1.7. Measure the pH and adjust to recipe specifications.

1.8. 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.

1.9. If formulating a solid growth medium, after the pH is adjusted, add agar directly into a bottle or flask.

1.10. 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.

1.11. 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.

1.12. Autoclave at 121 °C for 25 min.

1.13. 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.

1.14. Once cooled, add all post sterilization additives (see **Table 1**) and mix thoroughly.

1.15. After all ingredients are added, pour the designated volume into the container of choice in a laminar flow hood.

1.16. 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.

1.17. Allow growth medium to cool and solidify.

1.18. 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

2.1. 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.

2.2. 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.

2.3. It usually takes about 70–90 days after seed germination for ears to emerge. As ear shoots emerge, cover them with a shoot bag as to prevent uncontrolled pollination from occurring.

2.4. 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.

2.5. 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.

2.6. The next morning, gently bend the plant over and tap the bag to encourage pollen to be released.

2.7. 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.

2.8. Use the pollen from the same plant (selfing) or from another plant of the same inbred (sibbing).

2.9. 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.

2.10. 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.

2.11. 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.

2.12. Using a scalpel, slice off the cap of a single kernel that appears similar to the majority of other kernels in size and color.

2.13. Use a spatula (with a ruler) to remove the embryo as described in step 4.7. 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 supply in the growth media. The primary utility of the auxotroph Agro strain is for biocontainment purposes. However, 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.

3.1. 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.

3.2. 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**).

3.3. 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.

3.4. 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 Agrobacteria are in the proper growth phase for infection.

3.5. 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.

3.6. 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

4.1. 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.

4.2. Remove the husks and silks. Insert a handle into the base of the ear. The handle can be a pair of forceps, screwdriver, etc.

4.3. 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 0.1% Tween 20.

4.4. 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.

4.4.1. 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.

4.5. Prepare a 2 mL microcentrifuge tube filled with 700A liquid medium. This tube will be used to collect the immature embryos.

4.6. 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**).

4.7. 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).

4.8. 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.

4.9. 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.

4.10. 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 tipping the plate to about a 45° angle 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.

4.11. 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.

4.12. 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 °C in the dark.

4.13. Incubation 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

5.1. 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 °C dark incubator for 1–2 h.

5.1.1. 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.

5.2. 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 using fine tip forceps or surgical scissors, if present.

5.3. Place 10–15 embryos per plate to avoid overcrowding. Keep the embryos in this medium for 2 weeks in the 26 °C dark incubator.

5.4. 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.

5.5. 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.

5.6. As the plant becomes more vigorous, remove the plant from rooting medium and rinse the roots with tap water to remove agar.

5.7. Then transplant individual plant into a 3 in^2 (~19 cm^2) pot 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

6.1. 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.

6.2. 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.

6.3. 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.

6.4. 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.

6.5. 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.

6.6. For a majority of maize genotypes, the optimal time for pollination is 2–3 days after tassel or silk emergence.

6.7. 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).

6.8. 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.

6.9. Follow steps 2.5–2.10 for pollination details.

6.10. 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.

6.11. 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 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 AND TABLE LEGENDS:

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].

Figure 2: Starting materials. B73 ears harvested 12 days post-pollination (**A**). Immature embryos of B73 (**B**), Mo17 (**C**), and W22 (**D**).

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**).

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.

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**).

Table 1: Media compositions for maize transformation.

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.

ACKNOWLEDGMENTS:

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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 Morgan McCaw, Minjeong Kang, Jacob Zobrist, and Kan Wang have nothing to disclose.

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Figure 1

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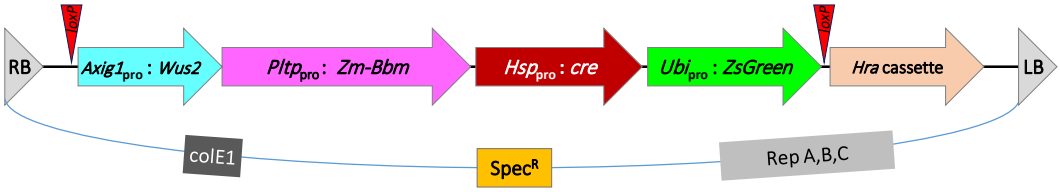

PHP81430 (27,666 bp)
T-DNA (19,788 bp)
Vector backbone (7,878 bp)

Figure 2

Click here to access/download;Figure;Figure 2_600dpi.tif

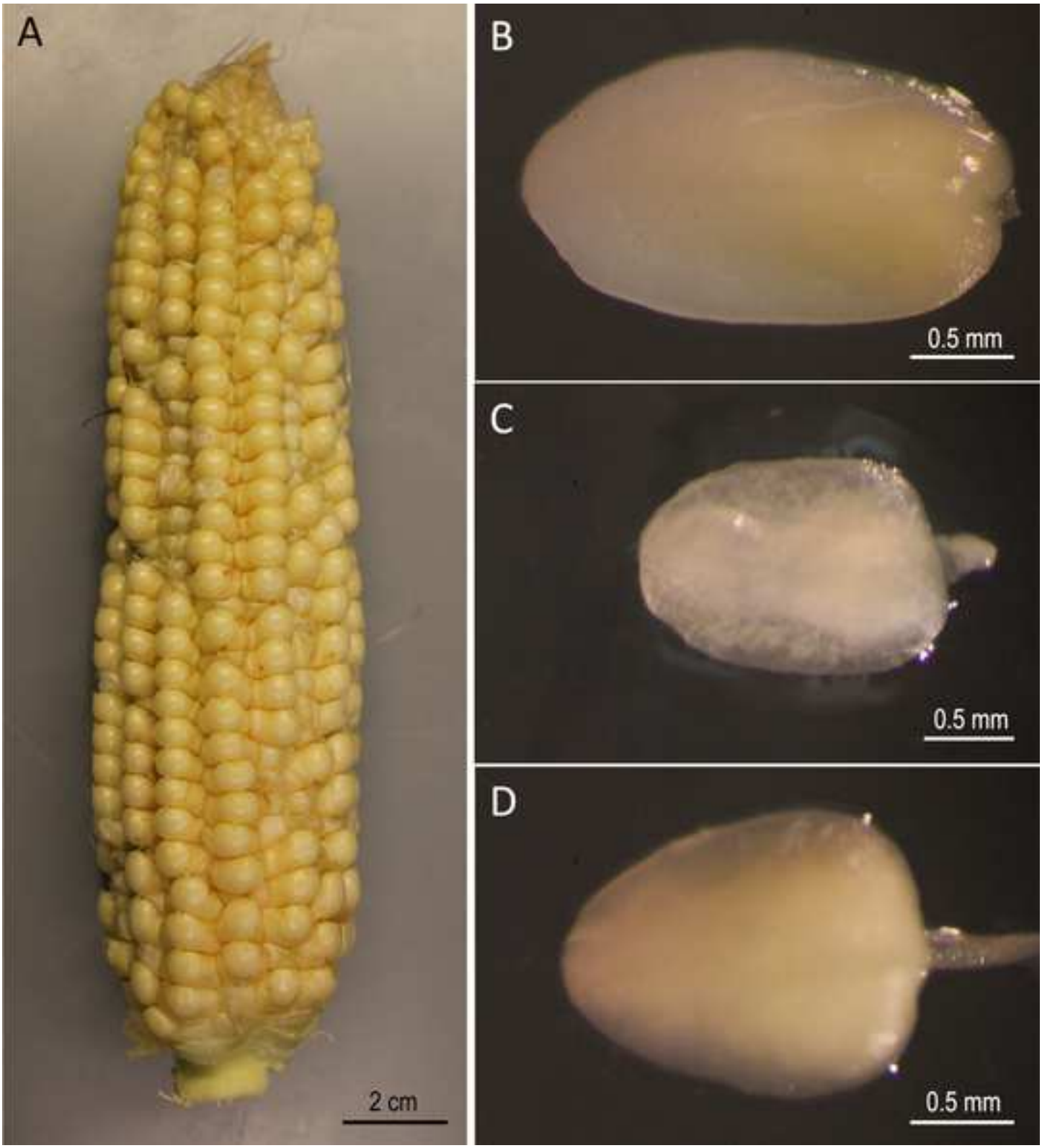

Figure 3

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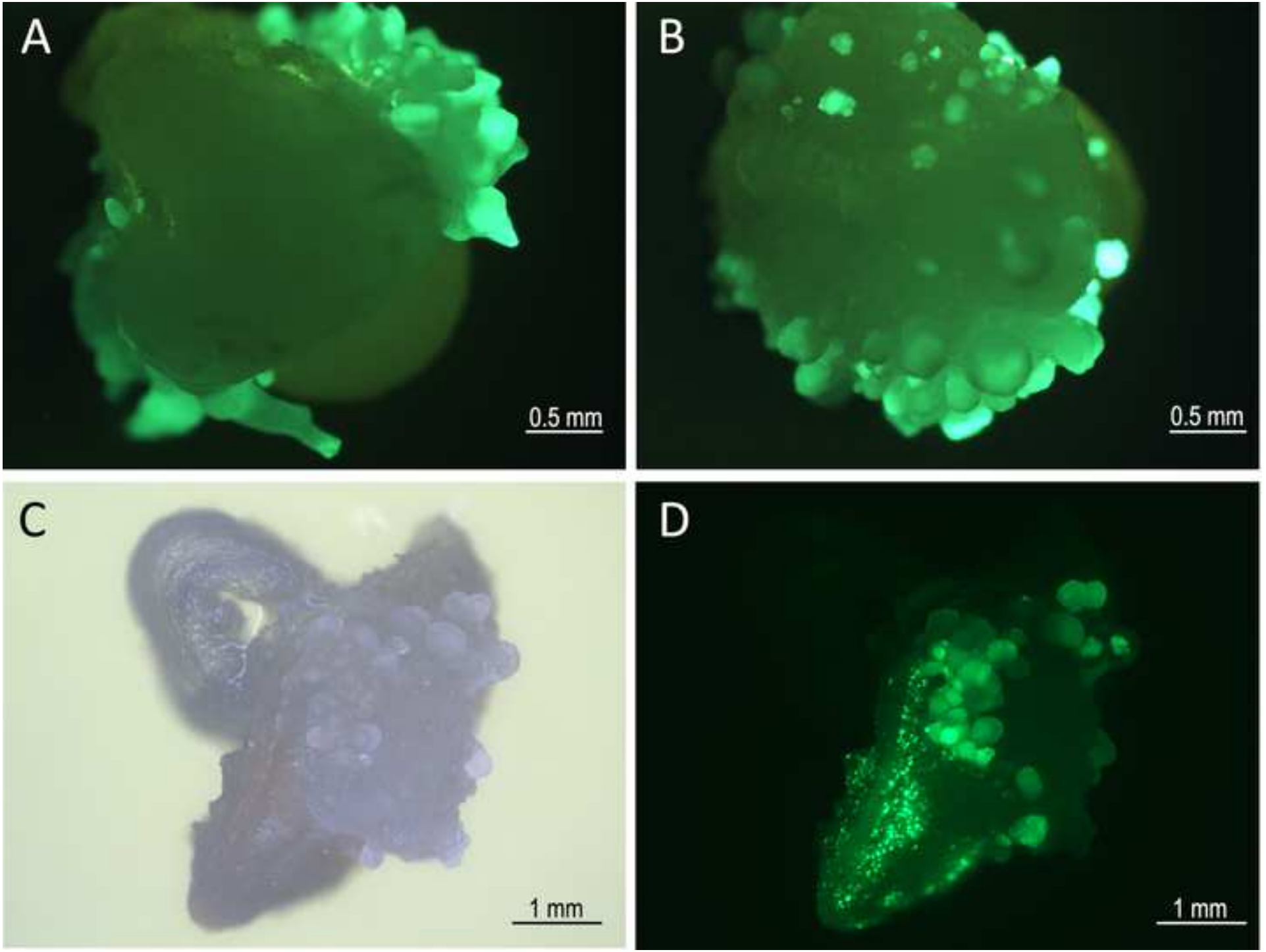

Figure 4

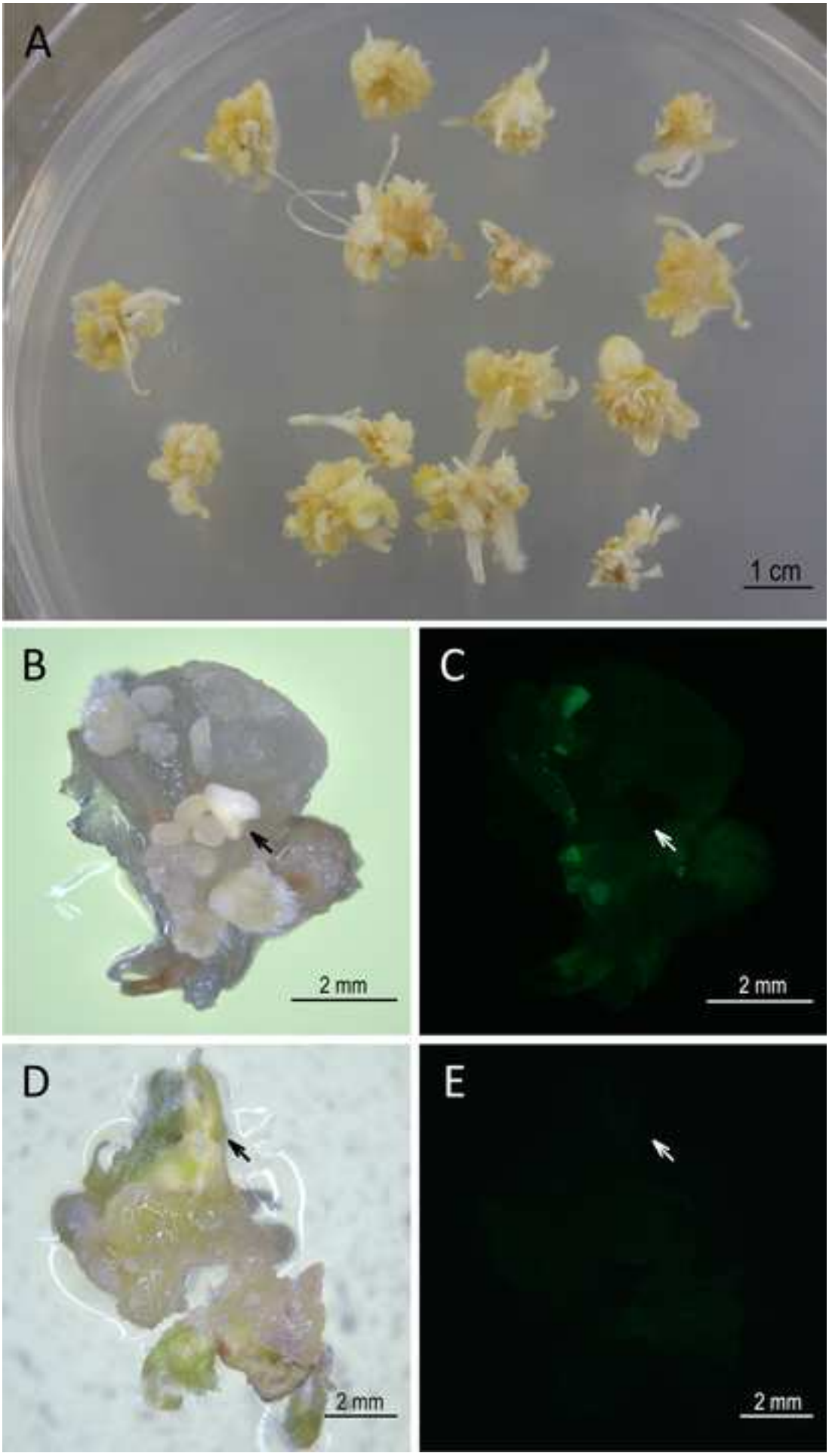

Figure 5

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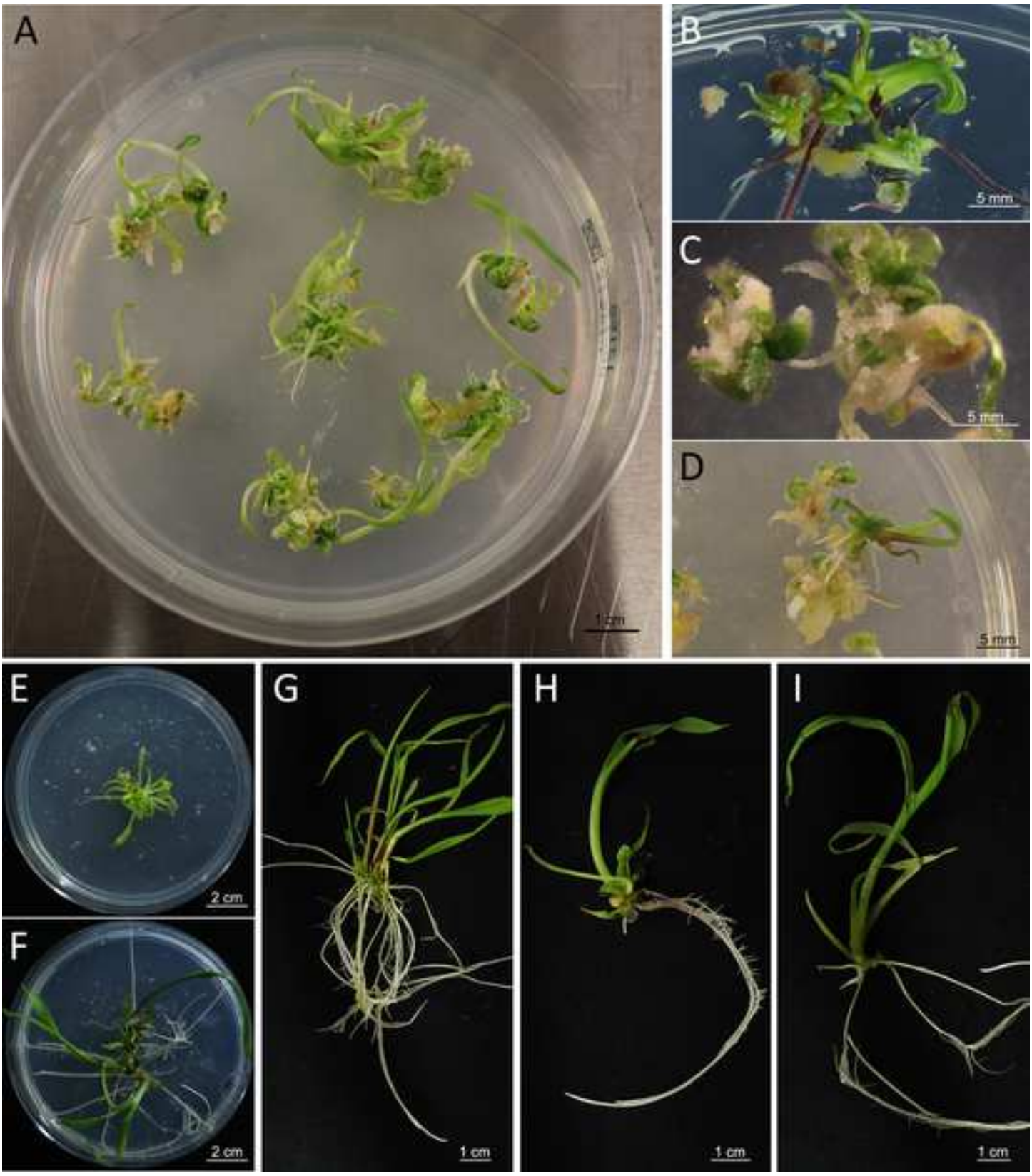


Table 1

Click here to access/download;Table;3-Table 1 Media_2019-10-21.xlsx

YP (+Thy) Grow Agro strain	700A Infection	562V Co-cultivation	605T[1] Resting	13329A Shoot Formation	13158 Rooting
Peptone, 10 g/L Yeast Extract, 5 g/L NaCl, 5 g/L	MS Basal Medium, 4.4 g/L 2,4-D, 1.5 mg/L Sucrose, 68.5 g/L Glucose, 36 g/L	N6 Basal Salt Mixture, 4.0 g/L 2,4-D, 2.0 mg/L Sucrose, 30 g/L	MS Basal Salt Mixture (1x), 4.3 g/L N6 Macro Salts (0.6x), 60 mL/L B5 Micro Salts (0.6x), 0.6 mL/L Eriksson's Vitamins (0.4x), 0.4 mL/L S&H Vitamins (0.6x), 0.6 g/L Ferrous Sodium stock (0.6x), 6 mL/L KNO_3, 1.68 g/L Thiamine HCl, 0.2 mg/L Casein Hydrolysate, 0.3 g/L 2,4-D, 0.8 mg/L L-proline, 2 g/L Sucrose, 20 g/L Glucose, 0.6 g/L	MS Basal Medium, 4.4 g/L Zeatin, 0.5 mg/L Sucrose, 60 g/L	MS Basal Medium, 4.4 g/L Sucrose, 40 g/L
pH to 6.8 with NaOH Add Bactoagar, 15 g/L	pH to 5.8 with NaOH	pH to 5.8 with NaOH, then add Agar, 8 g/L			
Autoclave, cool to 55 °C and add					
Thymidine, 50 mg/L Spectinomycin, 50 mg/L Gentamicin, 50 mg/L	Thymidine, 50 mg/L	Thymidine, 50 mg/L Silver Nitrate, 1 mg/L Acetosyringone, 100 µM	Dicamba, 1.2 mg/L Cefotaxime, 100 mg/L Timentin, 150 mg/L Silver Nitrate, 3.4 mg/L	Thidiazuron, 0.1 mg/L BAP, 1 mg/L Carbenicillin, 100 mg/L Imazapyr, 0.05 mg/L	Imazapyr, 0.05 mg/L Benomyl, 100 mg/L (optional)
15x100 Petri dishes 20 mL/plate	Liquid	25x100 Petri dishes 35 mL/plate			

Stock	Concentration	Solvent	Diluent (solvent:diluent)	Sterilization	Storage
2,4-D[2]	1 mg/mL	1N KOH	dH_2O (1:100)	Filter sterilization	4 ˚C, up to 1 year
Acetosyringone	100 mM	DMSO	-	No sterilization	-20 ˚C, up to 2 year
BAP	1 mg/mL	1N NaOH	dH_2O (1:50)	Filter sterilization	-20 ˚C, up to 2 year
B5 Minor Salt stock (1000X)	Boric Acid, 3 g/L $MnSO_4$ • H_2O, 10 g/L Na_2MoO_4 • $2H_2O$, 0.25 g/L KI, 0.75 g/L	dH_2O	-	Autoclave	Room temp, up to 1 year
Benomyl	50 mg/mL	Acetone	-	No steilization	4 ˚C, up to 1 year
Carbenicillin	100 mg/mL	dH_2O	-	Filter sterilization	-20 ˚C, up to 2 year
Cefotaxime	100 mg/mL	dH_2O	-	Filter sterilization	-20 ˚C, up to 2 year
Dicamba[2]	2 mg/mL	1N KOH	dH_2O (1:100)	Filter sterilization	4 ˚C, up to 1 year
Ferrous Sodium Stock (100x)[3]	EDTA-Na_2•$2H_2O$, 3.7 g/L $FeSO_4$•$7H_2O$, 2.79 g/L	dH_2O	-	No sterilization	4 ˚C, up to 3 months
Gentamicin	50 mg/mL	dH_2O	-	Filter sterilization	-20 ˚C, up to 2 years
Imazapyr	2 mg/mL	dH_2O	-	Filter sterilization	-80 ˚C, up to 1 year
N6 Macro Salts Stock (10x)	$CaCl_2$•$2H_2O$, 1.66 g/L $(NH_4)_2SO_4$, 4.62 g/L KH_2PO_4, 4 g/L $MgSO_4$•$7H_2O$, 1.85 g/L KNO_3, 28.3 g/L	dH_2O	-	Autoclave	Room temp, up to 1 year
Silver Nitrate[4]	1 mg/mL	dH_2O	-	Filter sterilization	4 ˚C, up to 1 year
Spectinomycin	100 mg/mL	dH_2O	-	Filter sterilization	-20 ˚C, up to 2 year
Thiamine HCl	2 mg/mL	dH_2O	-	Filter sterilization	4 ˚C, up to 6 months
Thidiazuron	1 mg/mL	1N KOH	dH_2O (1:50)	Filter sterilization	-20 ˚C, up to 2 year
Thymidine[5]	50 mg/mL	dH_2O	-	Filter sterilization	4 ˚C, up to 6 months
Timentin	100 mg/mL	dH_2O	-	Filter sterilization	-20 ˚C, up to 2 years
Zeatin	1 mg/mL	1N NaOH	dH_2O (1:50)	Filter sterilization	-20 ˚C, up to 2 years

1. Medium 605T may have light yellow color after autoclave.
2. 2,4-D and dicamba are prepared by dissolving in KOH at low heat while stirring.
3. Disodium EDTA Dihydrate cannot be substituted for other forms of EDTA.
4. Silver nitrate is light sensitive, and plates containing it should be kept in the dark. Discard stock if solution is clouded or black.
5. Heat to 55 ˚C and vortex to resuspend if crystallized.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2,4-D	Millipore Sigma	D7299	
6-Benzylaminopurine (BAP)	Millipore Sigma	B3408	
Acetosyringone	Millipore Sigma	D134406	
Agar	Millipore Sigma	A7921	
Aluminum foil			To cover the flask
Ammonium Sulfate	Millipore Sigma	A4418	
Analytical balance			To weigh small quantities of chemicals
Autocalve	Primus (Omaha, NE)	PSS5-K	To autoclave media and tools
Bacterial culture loop (10 µl)	Fisher scientific	22-363-597	Collects Agrobacterium from plate to transfer to liquid
Bactoagar	BD bioscience	214030	
Beakers (1 L, 2 L, 4 L)			To mix the chemicals for media
Benomyl	Millipore Sigma	#45339	
Bleach (8.25% Sodium Hypochlorite)	Clorox		For seed sterilization
Boric Acid	Millipore Sigma	B6768	
Calcium Chloride Dihydrate	Millipore Sigma	C7902	
Carbenicillin	Millipore Sigma	C3416	
Casein Hydrolysate	Phytotech	C184	
Cefotaxime	Phytotech	C380	
Conical tube (50 mL)	Fisher scientific	06-443-19	Contain liquid medium and Agro suspension
Cuvette (Semi-micro)	Fisher scientific	14955127	To hold liquid for measuring OD
Dicamba	Phytotech	D159	
Digital hygrometer			Checking temperature and humidity for heat treatment
EDTA, Disodium Salt, Dihydrate	Millipore Sigma	324503	
Eppendorf tube (2.0 mL)	ThermoFischer Scientific	AM12475	
Eriksson's Vitamins	Phytotech	E330	1000x in liquid
Ethanol (70%)			Sterilizing tools and surfaces
Ferrous Sulfate Heptahydrate	Millipore Sigma	F8263	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Fertilizer, Osmocote Plus 15-9-12	ICL Specialty Fertilizers (Dublin, OH)	A903206	Fertilizer
Flask (2 L)	Pyrex	10-090E	To autoclave media and tools
Flats (Standard 1020, open w/holes, 11"W x 21.37"L x 2.44"D)	Hummert International (Earth City, Mo)	11300000	Tray to hold soil and pot insert, fits Humidome
Forceps (fine-tipped and large)			Fine for handling embryos; larger for large plant materials and use as ear holders
Gentamicin	Gold Biotechnologies	G-400	
Glass bottle (1 L)	Pyrex	06-414-1D	To autoclave medium
Graduated cylinder			To adjust volume of media
Imazapyr	Millipore Sigma	37877	
Incubator, 20 °C	Percival Scientific	Model I-36NL	To grow mother plate and incubate embryos during Agro infection
Incubator, 27 °C	Percival Scientific	Model I-36NL	To grow co-cultivation plate and maize embryo culture
Incubator, 45 °C			Heat shock treatment
Insert TO Standard, pots	Hummert International (Earth City, Mo)	11030000	For transplanting plants from rooting to soil, fits flat and Humidome
Laminar flow hood			Maintains sterile conditions
L-proline	Phytotech	P698	
Magnesium Sulfate Heptahydrate	Millipore Sigma	M1880	
Maize inbred seed B73	U.S National Plant Germplasm	id=47638	
Maize inbred seed Mo17	U.S National Plant Germplasm	id=15785	
Maize inbred seed W22	U.S National Plant Germplasm	id=61755	
Manganese Sulfate Monohydrate	Millipore Sigma	M7899	
Milli-Q Water purification systems	Millipore sigma	MILLIQ	For tissue culture grade water
MS Basal Medium	Millipore Sigma	M5519	
MS Basal Salt Mixture	Millipore Sigma	M5524	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
N6 Basal Salt Mixture	Millipore Sigma	C1416	
Paperclips, non-skid			Holding on tassel bags
Peptone	BD bioscience	211677	
Petri dish (100x15 mm)	Fisher scientific	FB0875713	For bacteria culture medium
Petri dish (100x25 mm)	Fisher scientific	FB0875711	For the plant tissue culture medium
pH meter	Fisher scientific	AB150	To adjust pH of media
Pipette (1 mL)	ThermoFischer Scientific	4641100N	
Plastic Boxes	The Container Store	10048430	For tissue culture storage and incubation
Plastic humidy dome (Humi-Dome)	Hummert International (Earth City, Mo)	14385100	Platic cover for soil flat
Potassium Iodide	Millipore Sigma	793582	
Potassium Nitrate	Millipore Sigma	P8291	
Potassium Phosphate Monobasic	Millipore Sigma	P5655	
Scale			To weigh chemicals for media
Scalpel Blade (No. 11, 4 cm)	Thermo Scientific	3120030	remove the top of the kernel crowns for embryo dissection
Scalpel handle			Holding scalpel blades
Schenk & Hildebrandt Vitamin (S&H vitamin)	Phytotech	S826	100x powder
Scissors			Cutting ear shoots
Shoot bag (Canvasback-semi-transparent)	Seedburo (Des Plaines, IL)	S26	Semi-transparent bag to cover ear shoots
Silver Nitrate	Millipore Sigma	S7276	
Sodium Molybdate Dihydrate	Millipore Sigma	M1651	
Soiless substrate LC1	SunGro Horticulture (Agawam, Ma)	#521	For growing maize plants
Spatula (Double Ended Micro-Tapered)	Fischer Scientific	2140110	Dissecting embryos from kernels
Spatula (with spoon)	Fisher scientific	14-375-10	To measure chemicals for media
Spectinomycin	Millipore Sigma	S4014	
Spectrophotometer (Genesys 10S UV-Vis)	Thermo Scientific	840-300000	Measure OD of Agro suspension
Stirring bar	Fisher scientific	14-513-67	To mix media
Stirring hotplates			To mix media

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Syringe (without needle, 60 mL)	Fisher scientific	14-823-43	For filter sterilization
Syringe filter (0.22 µm)	Fisher scientific	09-720-004	For filter sterilization
Tassel bag (Canvasback-brown)	Seedburo (Des Plaines, IL)	T514	Bag to cover tassels of non-transgenic plants
Tassel bag (Canvasback-green stripe)	Seedburo (Des Plaines, IL)	T514G	Bag to cover tassels of transgenic plants
Thiamine HCl	Phytotech	T390	
Thidiazuron	Phytotech	T888	
Thymidine	Millipore Sigma	T1895	
Timentin	Phytotech	T869	
Tween 20	Fisher Scientific	Cas #9005-64-5	surfactant
Vortex Genie 2	Scientific Industries	SI0236	Homogenizes liquids (Agro suspension)
Water bath (large - Precision model 186)	Fisher scientific	any that can fit 4+ 2L flasks and reach 55 °C	Keeps autoclaved media at optimal temperature
Weigh dish	Fisher scientific	08-732-112	To measure chemicals for media
Weighing paper	Fisher scientific	09-898-12A	To measure chemicals for media
Yeast Extract	Fisher Scientific	BP14222	
Zeatin	Millipore Sigma	Z0164	

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Our response:
We have thoroughly reviewed and revised the manuscript.

2. Please rewrite the title to avoid the use of a subtitle.

Our response:
We have revised the title.

Original title: Recalcitrant maize genotype transformation: *Agrobacterium*-mediated immature embryo transformation of maize inbred lines using morphogenic genes

Revised title: *Agrobacterium*-mediated immature embryo transformation of recalcitrant maize inbred lines using morphogenic genes

3. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Our response:
Done.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Please number references in the order they appear in the manuscript.

Our response:
Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Parafilm, Sigma, Humi-Dome, Osmocote, etc.

Our response:
Done.

Protocol:

1. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Our response:
We have highlighted steps to be demonstrated by the video.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Our response:
We have been following this guideline in revision.

Specific Protocol steps:

1. 3.1: Please avoid lengthy paragraphs in the protocol. Please move this paragraph to the introduction.

Our response:
This paragraph is moved to the Introduction.

Figures:

1. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, or .pdf file.
2. Please remove 'Figure 1/2/etc.', titles, and legends from the figures themselves.

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
2. Please do not abbreviate journal titles.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Our response:
We have been following these guidelines in revision.

Reviewer #1:

Manuscript Summary:

In this manuscript the use of the QuickCorn technology to improve maize transformation is explained in great detail. This manuscript will be welcomed by the maize research community. The QuickCorn technology is widely seen as a breakthrough technology in the field and many academic labs are eager to introduce it in their labs. It is much appreciated that researchers from Corteva share their knowledge and show with an academic partner how the technology works. The experiments are very well explained and contain all the details that are needed for replication.

Major Concerns:

Comment 1.

It would be nice to include also a time line/time schedule

Our response:

We have added a sentence to the end of the introduction as following:

"Using this method, rooted plants should be ready to transfer to soil in just 5-7 weeks after the day of the infection, rather than 3+ months required by traditional protocols (Frame et al., 2015)."

Comment 2.

Add paragraph in the protocol how one can determine if plants are transgenic:

- By PCR or can it be assumed by their resistance to imazapyr? Does one need to take care of escapes, especially looking at Figure 5a?
- How are the final percentages of transformation frequency calculated in this protocol: "transgenic shoots/100 IE" or "transgenic shoots with successful excision by cre/Lox /100IE"?
- If multiple non-grassy shoots appear from one zygotic embryo, can one assume these as independent in this protocol?
- Do you expect a developmental phenotype when morphogenic genes were not removed?

Our response:

We thank the reviewer for the comments. We added the following sentences in the Discussion to address this comments.

"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 the sampling one plant from each immature embryo that produced plants, and divide 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 would require sequencing border regions around transgenes; this would be prohibitively expensive and time consuming for most applications, though there could be cases where these data are useful."

Minor Concerns:

Abstract

L67 "regulate by *the* maize Pltp promoter"

Our response:

Corrected

L69 carrying the morphogenic genes *on the T-DNA* and extra copies…"

Our response:

Corrected

L71 "embryos can be selected". Do you mean *by the herbicide imazapyr*. It can also be interpreted as "picked".

Our response:

We added words to clarified the sentence. "… can be selected and germinated …" is changed to "… can be selected by herbicide resistance and germinated …".

Introduction

L97 I would describe this as *another* limitation (next to the long time lines)

Our response:

We have revised the sentence. The modification stresses that the genotype dependency is a much larger, more absolute limitation with the conventional protocols.

Original:

"…conventional maize transformation protocols have one major limitation, …"

Revised:

"…conventional maize transformation protocols have a much larger limitation, …"

L118 herbicide reistan*ce* Zm-Hra gene

Our response:

We did not make change because we think that either could be correct, as this is an allele of the naturally occurring Zm-ALS that produces an enzyme that is resistant to sulfonylurea and imidazolinone herbicides.

L120 I would add also a map of PHP71539 to the manuscript. The hypervirulence is another important part of QuickCorn, next to BBM/WUS

Our response:

We respectfully disagree. The map of PHP71539 was published in Anand et al (2018) and is copy-right protected. The readers can find this publication readily online because it is an open accessed article.

Protocol

L217 LBA4404(Thy-). You still are using antibiotics in the media (Cefo, etc.) to kill of the agro. Is the auxotrophy used for removal of the agro or for biocontainment? Also, what is the genotype in this strain for recA? As you are working with very complex plasmids, you would expect plasmid instability.

Our response:
The primary utility of the auxotroph is for biocontainment but it has the additional benefit of reducing Agro over-growth. The auxotrophic Agro strain does not grow without supplemental thymidine but THY can, presumably, be supplied by dying plant tissue. So there is still a need to provide an antibiotic in the medium to completely control the auxotrophic Agro. But it will be easier to control due to compromised growth in the absence of THY. This explanation is now added to Step 3.1 as a note).

The LBA4404(Thy-) is a Ach5-based disarmed octopine-type Agrobacterium strain. We did not observe or experience much plasmid instability.

L252 I think this is a description of the "streak plate method" for obtaining single colonies, but without sterilizing the loop in between quadrants.
Our response:
The reviewer's comment is essentially correct, this is a "streak plate method" and there is no sterilization of the loop between "quadrants" because we use disposable plastic loops.

L261 Not exactly sure how the "thin film with denser interspersed pockets" looks like. Maybe include as a picture.
Our response:
This description has been revised.

Original:
"…use a loop or similar tool to collect Agrobacterium from a region of the "working" plate where bacterial growth is visible as a thin film with some denser interspersed pockets (but avoid areas of the plate with a dense lawn)."

Revised:
"…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 (avoid areas of the plate with a dense lawn of bacterial growth)."

L254 YEP = YP?
Our response:
Corrected

L300 Vortex on a "low setting" could be more precise. Why is the vortexing included? Is this to wound the embryos?
Our response:
Vortexing has a two-fold purpose. First, it allows for better mixing and distribution of the agro. Secondarily, it may also have the additional benefit of slightly wounding the embryos to increase transformation. We have revised the sentence to clarify the vortex setting.

Original: Add 1 mL of the Agrobacterium suspension and vortex on a low setting (3/10) for 30 seconds.

Revised: Add 1 mL of the Agrobacterium suspension and vortex on a low setting (3/10) for 30 seconds or invert tube 12-15 times to mix.

L329 "Transfer the heat-treated *zygotic* embryos".

Our response:

Corrected and clarified step 4.8 to say "immature zygotic" embryo because we had failed to use that term, and we wanted to avoid confusion by novices who might believe that the zygotic embryos were the new somatic embryos that had formed on the IZE.

Representative Results

L402 Independent shoots. This is confusing as the word independent is used to also indicate that they are derived from independent zygotic embryos. Maybe remove here.

Our response:

Corrected. We removed the word "independent". We also made a correction in the Fig 5 legend.

Table media

- Glucose is not very visible in 605T

Our response:

We are reformatting the table and this should be addressed.

- The same media are named differently in Jones et al.

Our response:

- Resting media is named 605T in this manuscript but 605J in Jones et al (2019). This is because the different antibiotics are used in the two media.
- Media 13329A is called "Maturation medium" in this manuscript but was called "Shoot formation" in Jones et al. The only difference was the concentration of imazapyr, which was 50% of what used in Jones et al. We have revised the "Maturation" to "Shoot formation" to be consistent with Jone et al.
- 131582 should be 13158. It was a typo with an extra 2 added at the end of 13158 that had formerly been a subscript. This is corrected.

Finally, it can be mentioned that the materials are available from Corteva through an MTA.

Our response:

We have added a sentence in step 3.1 in the revision. "These materials can be obtained from Corteva Agriscience through a Material Transfer Agreement."

Reviewer #2:

Manuscript Summary:

The MS is easy reading and following. All essential information was given. The steps listed in the procedure are clearly explained. There are just a few things that are minor concerns.

Minor Concerns:

Line 229: Bbm is a transcription factor associated … Should Bbm be written in a regular format?

Our response:

The authors are unsure of what Reviewer #2 means by "regular format"

Line 236: Wus2 is another transcription factor… Should Wus2 be written in a regular format?

Our response:

The authors are unsure of what Reviewer #2 means by "regular format"

Line 254: The authors mentioned a new YEP plate… but there is no YEP in the list of table 1. Is it YP?

Our response:

It should be YP plate, we have made the correction.

Line 335: …rooting medium (13158)… but in the Table 1 is 131582. Which one is correct?

Our response:

131582 should be 13158. It was a typo with an extra 2 added at the end of 13158 that had formerly been a subscript. This is corrected.

Lines 405-408: Was the transformation frequency calculated from all transgenic shoots or only ones with the cre-mediated excision. It should be indicated.

Our response:

The transformation frequency was calculated including all the transgenic shoots, we have added a sentence stating this at the end of Representative Results.

Line 421: repABC does not correspond to the vector graphic.

Our response:

This has changed to Rep A, B, C to match the graphic.

Reviewer #3:

Manuscript Summary:
Plant transformation has become a keystone for plant genomics and improvement research. Yet the methods used for transformation have not been improved in decades, and today, are not up to the task. Transformation remains very labor intensive, and is the purview of just a handful of highly specialized laboratories.

The technology presented here is the first major breakthrough in the field, and thus, very worthy of dissemination. At the same time, plant tissue culture and transformation is notoriously fickle, and even the tiniest of details stand between success and failure of a protocol. Therefore, the detail provided in this manuscript is welcome and easy to justify.

Overall, the manuscript is well written and clear. I just have a few points:

Major Concerns:

Comment 1.
Line 464 - the discussion on frequency is not interpretable without knowing more about the experimental design and the number of replicates used to get the data for this manuscript. Are the lower frequencies due to lack of optimization as claimed, or could they be random, due to the lack of replication? Hence, the word 'likely' on line 476 is not warranted. Replace with 'possible'

Our response:
Thank you for this comment. We have changed to word “likely” to “possible” to better reflect the claims made in this paper.

Comment 2.
Is the way the AS, benomyl, imazapyr, silver nitrate, thidiazuron and thymidine stocks correct? Normally one dissolves in less than the final volume, and then brings up to volume, rather than as described here, which is starting with a full volume, then adding the compound. This latter way is the way you have the instructions for BAP and Dicamba.

Our response:
We have revised Table 1 to better inform readers about non autoclavable reagents and stock solution preparations.

Minor Concerns:

Comment 3.
Line 89 - what is the current criterion for a 'high throughput' system in maize? It helps to have a base line
Our response:
In our paper high throughput refers to an easily replicable/repeatable protocol that makes it possible to scale to any desired experiment size large or small. We have revised the sentence to more clearly reflect this thought.

Original:
The discovery that immature embryos in maize could be used for the production of regenerable callus dates back to 1975 (Green and Phillips, 1975). Since then, in a high throughput system, maize has required callus formation and selection prior to regeneration (Ji et al., 2013).

Revised:
The discovery that immature embryos from maize could be used for the production of regenerable callus dates back to 1975 (Green and Phillips, 1975). Since this revelation, most scalable maize transformation protocols have required callus formation and selection prior to regeneration (Ji et al., 2013).

Comment 4.
Through out the manuscript—the use of 'media' and 'medium' is very inconsistent. In some parts, media is used as plural and medium as singular, particularly in the step-step section. In other parts, media is used as both the plural and the singular. The preference should be one medium, two media.
Our response:
We have reviewed and revised according to the context in the revised manuscript.

Comment 5.
Line 129--- need supporting data or citation - how much faster? How much more efficient?
Our response:
We have added a sentence to address this comment.

Original:
Compared to previously published maize transformation methods (Que et al., 2014), the QuickCorn method is faster, more efficient, and less genotype dependent.

Revised:
Compared to previously published maize transformation methods (Que et al., 2014), the QuickCorn method is faster, more efficient, and less genotype dependent. Using this method, rooted plants should be ready to transfer to soil in just 5-7 weeks after the day of the infection, rather than 3+ months required by traditional protocols (Frame et al., 2015).

Comment 6.
Missing from the protocol—where does one get the necessary Agrobacterium strain and the vector with the morphogenesis genes?
Our response:
We have added a sentence in Step 3.1:
"These materials can be obtained from Corteva Agriscience through a Material Transfer Agreement.

Comment 7.
Line 148 - what type of agar is used for the plants? Can gellan gum be used?
Our response:

All or the reagents used in the paper are listed in detail in Table 1. Agar is used to harden the growth medium. While other hardening reagents may be used, they may not be as effective.

Comment 8.

Lines 405-408. How reliable are these results, given they have no standard error? Are they the representative results, or are they just from 1 rep?

Our response:

We appreciate this comment. This manuscript is a protocol paper in which representative results are described. These results were from two repetitions involving four researchers and over 3700 immature embryo explants. Details of these experiments are in preparation for another manuscript.

Comment 9.

Line 442—in conventional maize regeneration, do the embryos form from callus, or is it a repetitive embryo system, where globular stage embryos give rise to more globular stage embryos?

Our response:

In conventional transformation immature zygotic embryos (IZEs) are used as target explants for either *Agrobacterium*-mediated or biolistic-mediated transformation. When cultured on appropriate media with growth regulators, these IZEs can develop embryogenic callus with globular appearance. This callus is then further developed into shoots and roots with appropriate growth regulators. Using the morphogenic genes in the Quickcorn protocol described in this manuscript, Agro-infected IZEs can develop somatic embryos from the scutella tissue directly. Each of these somatic embryos is an independent transformation event which can be regenerated into a transgenic plant. We have made changes in the revision to better convey this concept.

Original:
Traditional protocols for maize transformation require the development of a callus stage.

Revised:
Traditional protocols for maize transformation follow the paradigm of isolating immature zygotic embryos to produce transgenic callus tissue which is regenerated into fertile plants (Frame et al., 2015, Que et al. 2014).

Comment 10.

Line 486 - Shortened—how much shorter is this protocol?

Our response:

QuickCorn process skips callus induction step, it induces somatic embryos from the scutellum directly. The QuickCorn process generally requires approximately 45 days to obtain transgenic plantlets after infection. Practical Hi-II and B104 *Agrobacterium* mediated genetic transformation generally requires approximately 104 days and 130 days to harvest transgenic plantlets. We have added a sentence at the end of Introduction to address this comment.

"Using this method, rooted plants should be ready to transfer to soil in just 5-7 weeks after the day of the infection, rather than 3+ months required by traditional protocols (Frame et al., 2015)."

Comment 11.

Table 1: Not minor salts, but micro salts. Plant nutritionists stopped using minor b/c it sounds like they are of minor importance. They are not of minor importance, they are just used in small quantities. Thus they went from major/minor to macro/micro. This protocol is using macro/minor.

Our response:

We appreciate this comment that the macro/micro is the appropriate term for plant nutrients. "B5 minor salt stock" is a solution described in U.S. Patent No. 6025188A. It is not the same as the Gamborg B5 micro elements, although it is a subset of Gamborg B5 micro elements. To remain consistent with the literature, we will keep the name "B5 minor salt stock".

Comment 12.

Thidiazuron, not Thidazuron

Our response:

Corrected.

Comment 13.

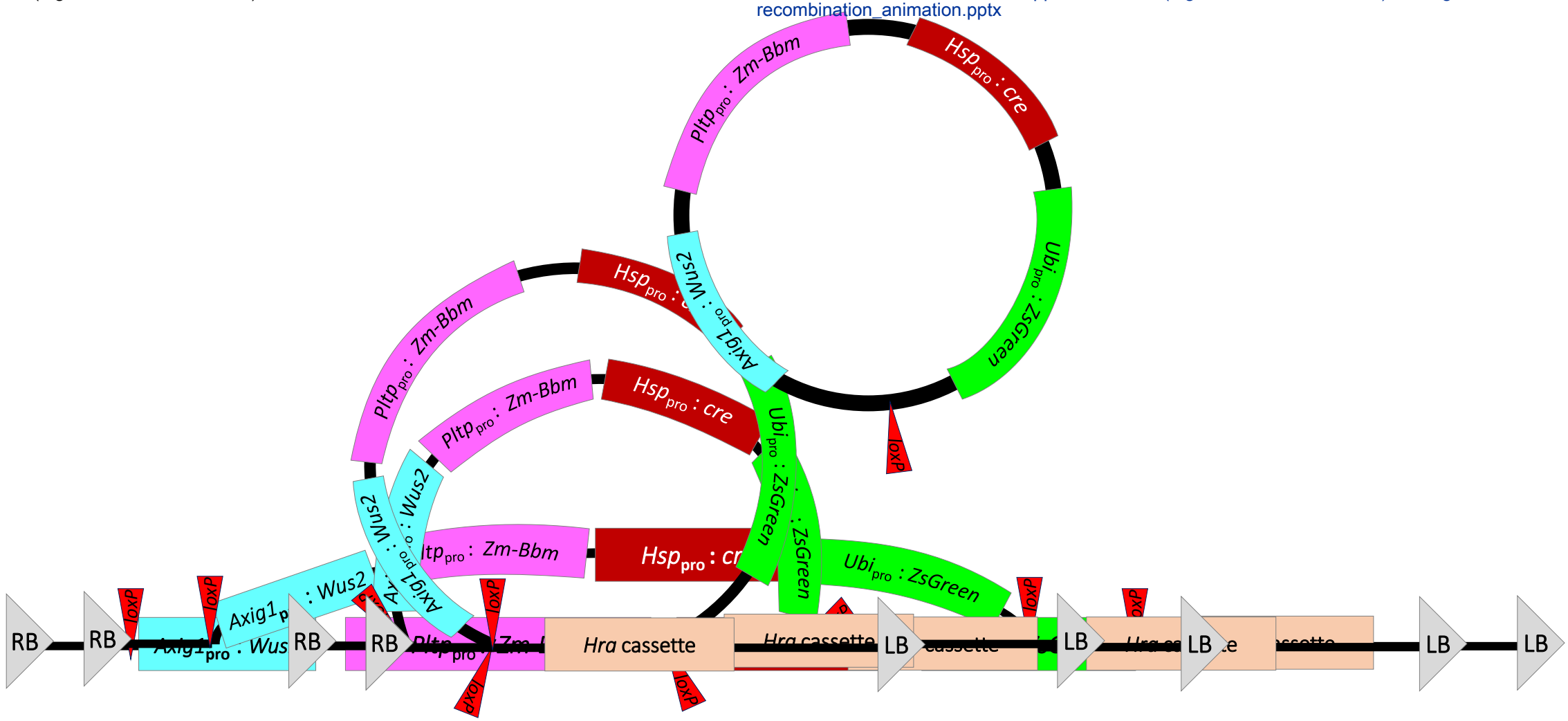
Carbenicillin and gentamycin need stocks as well—but rather than putting in column B, they are in parenthesis. This inconsistency is confusing. No instructions are given for proline, timentin, thiamin, or zeatin.

Our response:

Thank you for the comment, all antibiotics, plant nutrients and hormones stock preparation are in the revised Table 1 with detailed description. In our protocol, the L-proline is added in the medium as powder. We have revised Table 1 to address this comment.

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);MJ_Figure 1_Cre recombination_animation.pptx

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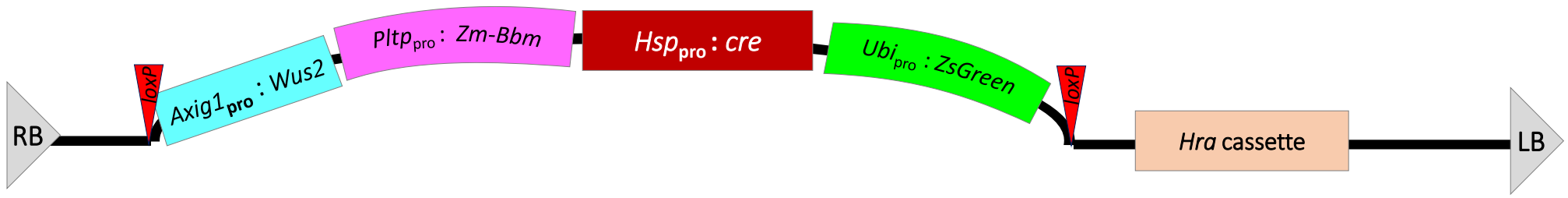
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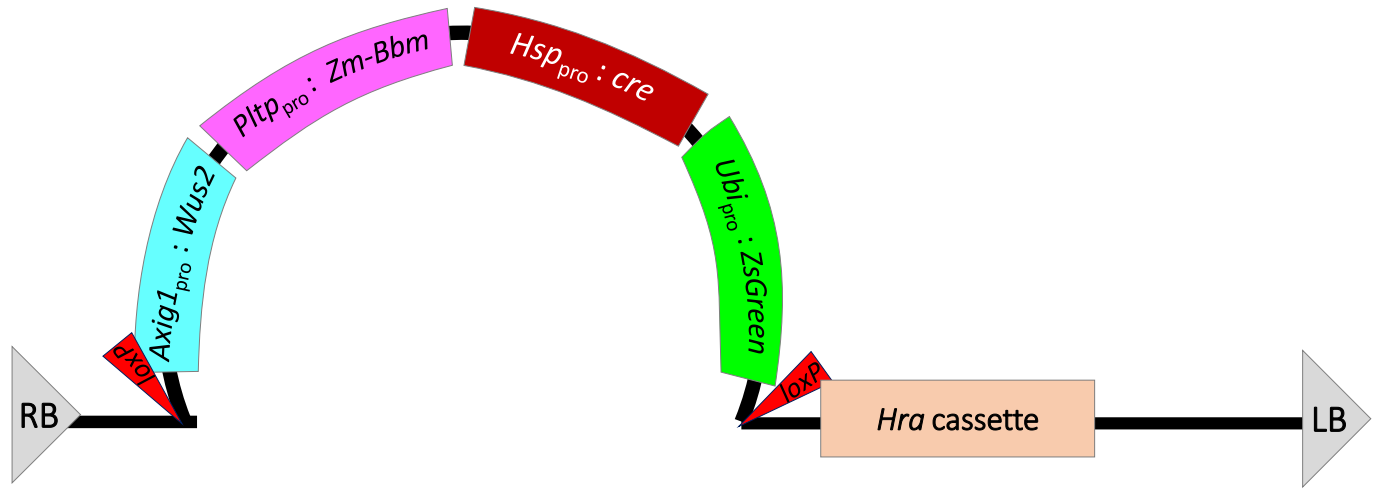
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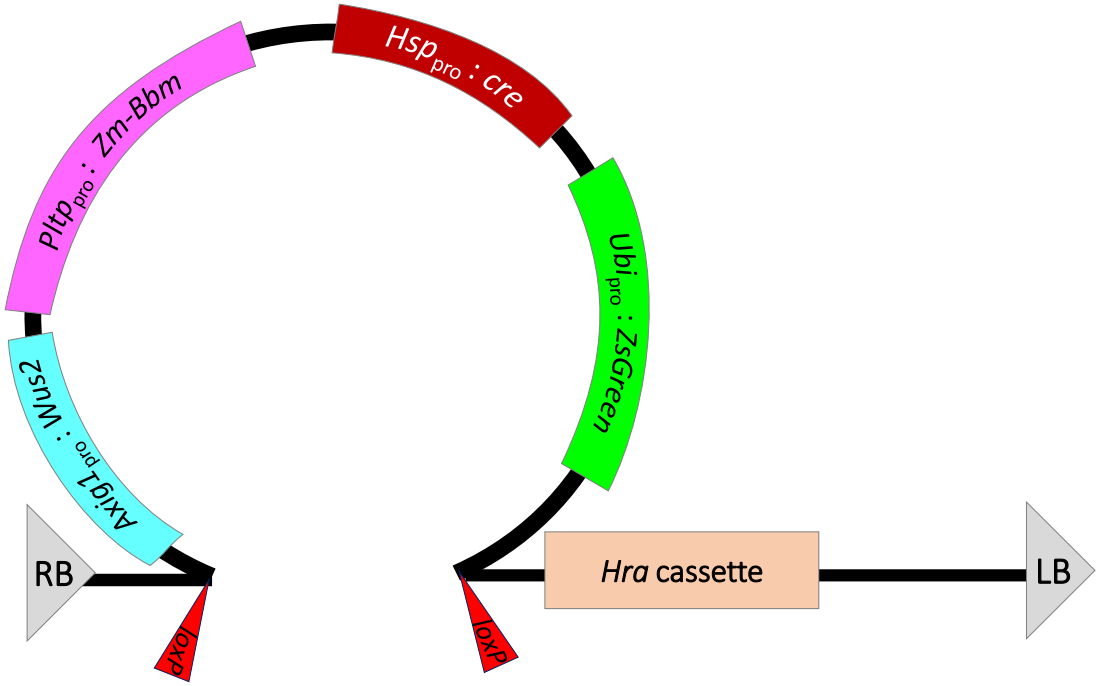
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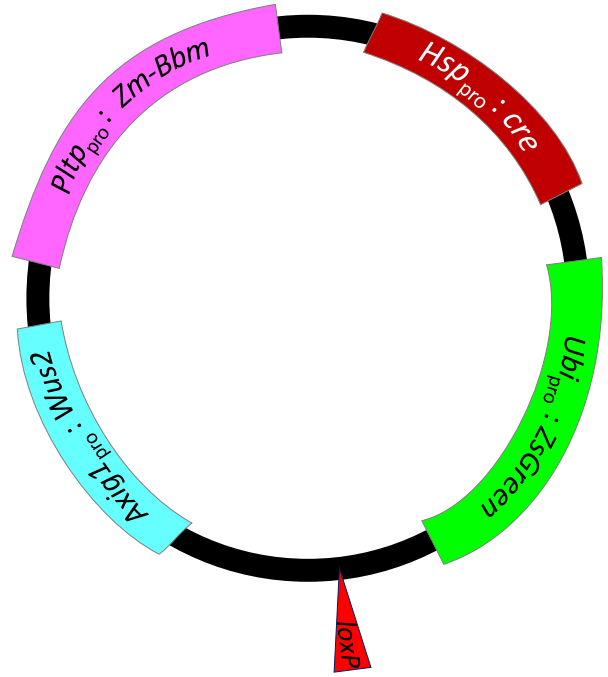
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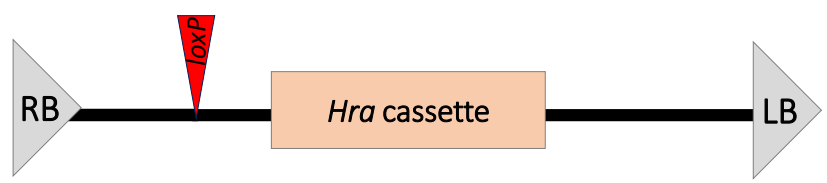
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