Agrobacterium-mediated transformation of maize

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Maize may be transformed very efficiently using *Agrobacterium tumefaciens*-mediated methods. The most critical factor in the transformation protocol is the co-cultivation of healthy immature embryos of the correct developmental stage with *A. tumefaciens*; the embryos should be collected only from vigorous plants grown in well-conditioned glasshouses. With the protocol described here, approximately 50% of immature embryos from the inbred line A188 and 15% from inbred lines A634, H99 and W117 will produce transformants. About half of the transformed plants are expected to carry one or two copies of the transgenes, which are inherited by the progeny in a mendelian fashion. More than 90% of transformants are expected to be normal in morphology. The protocol takes about 3 months from the start of co-cultivation to the planting of transformants into pots.

INTRODUCTION

Transgenic maize plants were first obtained from protoplasts by an electroporation method¹, but fertile plants have never been produced by this method. Other direct gene transfer methods, which did not require the prior culture of protoplasts, were then tried^{2–5}, and microprojectile bombardment⁶ of cells in suspension cultures or immature embryos became quite popular in basic and applied studies. Efficiency of transformation by microprojectile bombardment has been higher than other direct methods, and quite a few fertile plants have been generated to date⁷. Microprojectile bombardment is also useful for the analysis of the transient expression of foreign genes in intact, fully developed tissues. However, high copy numbers and extensive rearrangement of the foreign DNA have frequently been found in plants transformed with direct gene transfer methods^{8,9}.

For the last two decades, dicotyledonous plants have been transformed using the soil phytopathogen *A. tumefaciens*. *A. tumefaciens* is first transformed with the DNA construct of interest (T-DNA); this modified bacterial strain is then used to introduce the T-DNA into plants. A major advantage of *Agrobacterium*-mediated transformation is that a small number of copies (often one or two) of relatively large segments (can be larger than 10 kb) of T-DNA with defined ends are integrated into the plant genome with minimal rearrangement, resulting in transgenic plants of high quality. Initially, it was not clear if this technology could be extended to monocotyledonous plants, as they are not natural hosts of *A. tumefaciens*. However, a highly efficient method of transformation of rice by *A. tumefaciens* was reported¹⁰, followed by successful reports of the transformation of important cereals such as maize¹¹, wheat¹², barley¹³ and sorghum¹⁴ by *A. tumefaciens*. Key factors in these achievements include the optimization of types of plant material for infection with *A. tumefaciens*, choice of vectors, choice of strains of *A. tumefaciens* and optimization of tissue culture techniques. Transformation mediated by *A. tumefaciens* is now highly recommended for maize varieties with good tissue culture responses.

For the successful production of transgenic plants in any species, foreign genes must be delivered to undifferentiated, dedifferentiated or dedifferentiating cells that are actively dividing or about to divide and that are capable of regenerating plants. In maize, the material of choice is immature embryos, and all protocols mediated by particle bombardment or *A. tumefaciens* for efficient production

of transgenic maize have solely employed immature embryos. Thus, the primary determinants of a successful transformation are the response of immature embryos in tissue culture, the types of cells that grow from immature embryos and subsequent characteristics in growth and regeneration. Unfortunately, many genotypes of maize, especially so-called elite varieties, are poor in these aspects, and thus only a limited number of genotypes have been efficiently transformed so far.

In the original protocol of Ishida *et al.*¹¹, transgenic plants were obtained from between 5% and 30% of *A. tumefaciens*-infected immature inbred A188 embryos. The protocol was successfully employed in a number of studies in molecular biology and biotechnology^{15–19}. Since then, the methods have been greatly improved, and a highly optimized protocol routinely used in our laboratory is presented in this article^{20,21}. An overview and typical timeline of this optimized protocol is given in **Figure 1**. The modifications made to the protocol include pretreatment by heat and centrifuging addition of silver and copper ions to the co-cultivation medium and extension of the co-cultivation period from 3 to 7 days. The effects of heat, centrifuging and ions are evident²¹, but the mechanisms are not understood. In protocols described by other authors, a resting culture, which is a non-selective incubation of embryos on a medium that contains an antibiotic to kill bacteria, is performed after co-cultivation²². In our experience, the growth of transformed cells was better in the selection culture if the resting culture was performed; however, the same effect was produced by an extended period of co-cultivation. Thus, if the co-cultivation is extended, no resting culture is necessary. In general, the *bar* gene²³, which confers resistance to the herbicide phosphinothricin, is a more effective selection agent than the *hpt* gene²⁴, which confers resistance to the antibiotic hygromycin. Further, use of vectors that carry additional *virB* and *virG* genes from pTiBo542 (see refs. 25,26) gives much higher transformation frequencies. The function of *virB* is related to formation of a transmembrane channel between the bacterium and the plant cell and the function of *virG* is related to the activation of the other *vir* genes²⁷.

Transgenic plants may be routinely obtained from more than 50% of the immature embryos from the A188 genotype, and the range of transformable genotypes has been extended to inbreds A634, H99 and W117 and hybrids between pairs of these varieties

in our laboratory^{20,21}. Hybrid genotype Hi-II (ref. 28) and inbreds Mo17, B104, B114 and Ky21 have been transformed by similar protocols in other laboratories^{22,29–31}.

It should be noted that two types of embryogenic callus, type I and type II, may proliferate from the scutellum of immature embryos, depending on the genotypes of maize³². The type I callus is a relatively hard and compact embryogenic callus, whereas the type II callus is relatively soft and friable. A type I callus is usually obtained from inbreds A188, A634, H99 and W117 on the media described in this article. For genotypes such as Hi-II, from which the type II callus is frequently produced, modification of the media is recommended^{22,29,33}. Protocols recommended for Hi-II and some other genotypes have been reported by other groups^{22,29–31}. These protocols are similar to those presented in this article and differ essentially only in the composition of the media. While the macro components in all of the media in this article are based on the LS medium, those in the protocols of other groups are based mostly on the N6 medium. In addition, Frame *et al.*²⁹ used media that contained cysteine for co-cultivation. Thus, especially when other genotypes are to be transformed, media based on N6 and media with cysteine should also be considered.

Elimination of selection marker genes from transgenic plants for commercialization is an important task because the presence of selection marker genes, which are unnecessary once transgenic plants are established, is of high public concern. A simple approach to remove selection markers is to perform *A. tumefaciens*-mediated co-transformation of plants with two T-DNA segments, one with a selection marker and the other with genes of interest, followed by segregation of marker-free progeny³⁴. Because the frequency of co-transformants among initial transformants is never 100% and about a half of the co-transformants do not segregate marker-free progeny, this approach requires a high frequency of transformation, desirably more than 20% (independent transgenics/immature embryo). With the highly optimized protocol presented here, production of marker-free transgenics has become a realistic option in maize; selection-marker-free transformants may be obtained from about 50% of co-transformed plants when co-transformation vectors are employed.

It is clear that the protocol presented in this article still needs to be improved. Despite the high frequency of transformation in A188 and related genotypes, the majority of genotypes—including the so-called elite inbreds—are not expected to be transformed

Figure 1 | Timeline of maize transformation protocol. It is assumed that 100 immature embryos are collected in Step 3 and that materials derived from the 100 embryos are handled in the subsequent steps by a single, skilled technician. Similarly, seeds from ten transgenic plants are examined in Steps 32–35.

efficiently. In addition, there are many critical parameters in the protocol and the ‘windows’ of optimal conditions are very narrow; the assistance of an experienced tissue culture specialist may be necessary to establish and maintain efficient maize transformation programs.

MATERIALS

REAGENTS

- Maize seeds of inbreds A188, A634, H99 and W117 were supplied from the National Institute of Agrobiological Sciences of Japan (http://www.nias.affrc.go.jp/index_e.html)
- *A. tumefaciens* strain carrying appropriate T-DNA-containing vectors (see **Table 1**)
- 10× LS major salts (see REAGENT SETUP)
- 100× FeEDTA (see REAGENT SETUP)
- 100× LS minor salts (see REAGENT SETUP)
- 100× modified LS vitamins (see REAGENT SETUP)
- 100 mg liter⁻¹ 2,4-dichlorophenoxy-acetic acid (2,4-D) (see REAGENT SETUP)
- 100 mg liter⁻¹ Zeatin (see REAGENT SETUP)
- 100 mg liter⁻¹ indole-3-butyric acid (IBA) (see REAGENT SETUP)
- 100 mg liter⁻¹ 6-benzylamino-purine (6BA) (see REAGENT SETUP)
- 100 mM acetosyringone (see REAGENT SETUP)

- 100 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) (see REAGENT SETUP)
- 50 mM Na₂HPO₄ (see REAGENT SETUP)
- 50 mM NaH₂PO₄ (see REAGENT SETUP)
- YP plates (see REAGENT SETUP)
- LS-inf medium (see REAGENT SETUP)
- LS-inf-AS medium (see REAGENT SETUP)
- LS-AS medium (see REAGENT SETUP)
- LSD1.5A medium (see REAGENT SETUP)
- LSD1.5B medium (see REAGENT SETUP)
- LSZ medium (see REAGENT SETUP)
- LSF medium (see REAGENT SETUP)
- ELA medium (see REAGENT SETUP)
- Soil for pots (see REAGENT SETUP)
- Buffer P, for β-glucuronidase (GUS) staining (see REAGENT SETUP and **Box 1**)
- Buffer X, for GUS staining (see REAGENT SETUP and **Box 1**)

TABLE 1 | Typical frequency of transformation.

Bacterial strain (binary vector)	Gene				Transformation frequency ^c (%)	
	<i>virB</i> , <i>G</i> ^a	Selection marker	Reporter (<i>intron gus</i>) ^b	Inbred line	Original protocol	Optimized protocol
LBA4404(pSB131) ¹¹	+	<i>bar</i>	+	A188	15	50
LBA4404(pSB131)	+	<i>bar</i>	+	H99	0	10
LBA4404(pSB131)	+	<i>bar</i>	+	W117	0	20
LBA4404(pSB131)	+	<i>bar</i>	+	A634	0	20
LBA4404(pNB131) ²¹	—	<i>bar</i>	+	A188	0	3
LBA4404(pSB134) ³⁹	+	<i>hpt</i>	+	A188	5	20
LBA4404(pIG121Hm) ¹⁰	—	<i>hpt</i>	+	A188	0	3

^a+: additional *virB* and *virG* from pTiBo542 (see refs. 25,26). ^b*intron gus*: a β -glucuronidase (GUS) gene that carries an intron in the coding sequence (*intron-gus*⁴⁰). ^cIndependent transgenic plants/inoculated immature embryos $\times$ 100.

EQUIPMENT

- Scalpel blade (no. 19, Futaba Co., Ltd)
- Surgical tape made of unwoven fabric (no. 25, Nichiban Co., Ltd, <http://www.nichiban.co.jp/>)
- Parafilm (Pechiney Plastic Packaging)
- 270 mm pot (270 mm in diameter and 270 mm in height) for maize cultivation
- 230 mm pot (230 mm in diameter and 190 mm in height) for maize cultivation

REAGENT SETUP

10 $\times$ LS major salts Dissolve 19.0 g KNO₃, 16.5 g NH₄NO₃, 4.4 g CaCl₂ · 2H₂O, 3.7 g MgSO₄ · 7H₂O and 1.7 g KH₂PO₄ in 900 ml distilled water and make up the volume to 1,000 ml (see ref. 35). Store at 4 °C. The final concentrations of components in this solution are 188 mM KNO₃, 206 mM NH₄NO₃, 30 mM CaCl₂ · 2H₂O, 15 mM MgSO₄ · 7H₂O and 12.5 mM KH₂PO₄.

100 $\times$ FeEDTA Dissolve 2.78 g FeSO₄ · 7H₂O in 900 ml of hot distilled water and add 3.73 g ethylenediamine-N,N,N',N'-tetraacetic acid, disodium salt (Na₂EDTA). Cool and make up the volume to 1,000 ml. Store at 4 °C. The final concentrations of components in this solution are 10 mM FeSO₄ · 7H₂O and 10 mM Na₂EDTA.

100 $\times$ LS minor salts Dissolve 2.23 g MnSO₄ · 5H₂O, 1.06 g ZnSO₄ · 7H₂O, 620 mg H₃BO₃, 83 mg KI, 25.0 mg Na₂MoO₄ · 2H₂O, 2.5 mg CuSO₄ · 5H₂O and 2.5 mg CoCl₂ · 6H₂O in 900 ml of distilled water and make up the volume to 1,000 ml. Store at 4 °C. The final concentrations of components in this solution are 9.3 mM MnSO₄ · 5H₂O, 3.7 mM ZnSO₄ · 7H₂O, 10 mM H₃BO₃, 0.5 mM KI, 0.1 mM Na₂MoO₄ · 2H₂O, 0.01 mM CuSO₄ · 5H₂O and 0.01 mM CoCl₂ · 6H₂O.

100 $\times$ modified LS vitamins Dissolve 10 g myoinositol, 100 mg thiamine hydrochloride, 50 mg pyridoxine hydrochloride and 50 mg nicotinic acid in 900 ml of distilled water and make up the volume to 1,000 ml. Store at 4 °C. The final concentrations of components in this solution are 55.5 mM myoinositol, 0.30 mM thiamine hydrochloride, 0.24 mM pyridoxine hydrochloride and 0.41 mM nicotinic acid.

100 mg liter⁻¹ 2,4-D Add 1 N NaOH dropwise to 100 mg 2,4-D until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 °C. The final concentration of 2,4-D in this solution is 0.45 mM.

100 mg liter⁻¹ zeatin Add 1 N NaOH dropwise to 100 mg zeatin until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 °C. The final concentration of zeatin in this solution is 0.46 mM.

100 mg liter⁻¹ IBA Add 1 N NaOH dropwise to 100 mg IBA until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 °C. The final concentration of IBA in this solution is 0.49 mM.

100 mg liter⁻¹ 6BA Add 1 N NaOH dropwise to 100 mg 6BA until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 °C. The final concentration of 6BA in this solution is 0.44 mM.

100 mM acetosyringone Dissolve 392.4 mg acetosyringone in 10 ml of dimethyl sulfoxide and dilute with 10 ml distilled water. Filter-sterilize and store in the dark at 4 °C.

100 mM X-gluc Dissolve 52 mg X-gluc (Sigma B6650) in 1 ml of ethylene glycol monomethyl ether (Sigma E5378). Store in the dark at -20 °C.

! CAUTION Ethylene glycol monomethyl ether is toxic. Wear suitable protective clothing, gloves and eye/face protection.

50 mM Na₂HPO₄ Dissolve 17.91 g Na₂HPO₄ · 12H₂O in 900 ml of distilled water and make up to 1,000 ml.

50 mM NaH₂PO₄ Dissolve 7.8 g NaH₂PO₄ · 2H₂O in 900 ml of distilled water and make up to 1,000 ml.

YP plate (for *A. tumefaciens*) Dissolve 5 g yeast extract, 10 g peptone and 5 g sodium chloride in 900 ml of distilled water and adjust pH to 6.8 with NaOH. Make up to 1,000 ml and add 15 g agar (Difco). Autoclave at 121 °C for 15 min. Cool the medium to 50 °C, add appropriate antibiotics, which depend on the type of plasmid(s) in the strain, and pour 20 ml aliquots into Petri dishes (90 $\times$ 15 mm).

LS-inf medium (for preparation of immature embryos) Add 100 ml of 10 $\times$ LS major salts, 10 ml of 100 $\times$ FeEDTA, 10 ml of 100 $\times$ LS minor salts, 10 ml of 100 $\times$ modified LS vitamins and 15 ml of 100 mg liter⁻¹ 2,4-D (final concentration is 1.5 mg liter⁻¹) to 700 ml of distilled water. Dissolve 68.46 g sucrose, 36.04 g glucose and 1.0 g Casamino acids in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.2 and sterilize with a 0.22 μ m cellulose-acetate filter.

LS-inf-AS medium (for infection) Add 1 μ l of 100 mM acetosyringone to 1 ml of LS-inf medium.

BOX 1 | EXAMINATION OF TRANSIENT EXPRESSION OF GUS AFTER CO-CULTIVATION (OPTIONAL FOR STRAINS THAT CARRY AN INTRON-GUS GENE)

Monitoring of the level of expression of a transgene in the immature embryos gives very useful information for optimization of the protocol. The *intron-gus* gene⁴⁰, in which there is an intron in the coding sequence of the β -glucuronidase (GUS⁴¹), has been especially convenient because the presence of this gene does not result in the expression of GUS activity in *Agrobacterium*. As this is a destructive assay, the embryos examined in this method cannot be returned to the culture.

1. After Step 18, incubate the embryos in the dark at 25 °C for 3 days.
2. After 3 days of inoculation, immerse the embryos in 0.5 ml of buffer P in a microcentrifuge tube and incubate at 37 °C for 1 h.
3. Remove the buffer and add 0.3 ml of buffer X.
4. Place under mild vacuum (half an atmosphere) for 5 min.
5. Incubate for 18 h at 37 °C.
6. Examine embryos for change in color under stereoscopic microscope. **Figure 6** shows typical results of a GUS-staining experiment.

LS-AS medium (for co-cultivation) Add 100 ml of 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× modified LS vitamins, 15 ml of 100 mg liter⁻¹ 2,4-D (final concentration is 1.5 mg liter⁻¹) and 0.05 ml of 100 mM CuSO₄ to 700 ml of distilled water. Dissolve 20 g sucrose, 10 g glucose, 0.7 g proline and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agarose. Autoclave and cool to 50 °C, and add 1 ml of 100 mM acetosyringone and 0.05 ml of 100 mM AgNO₃ and pour 30 ml aliquots into Petri dishes (90 × 20 mm). Store in the dark at room temperature (20–25 °C).

LSD1.5A medium (for first selection of transformed cells) Add 100 ml of 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× modified LS vitamins and 15 ml of 100 mg liter⁻¹ 2,4-D (final concentration is 1.5 mg liter⁻¹) to 700 ml of distilled water. Dissolve 20 g sucrose, 0.7 g proline and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at 121 °C for 15 min. Cool to 50 °C and add 1 ml of 250 g liter⁻¹ carbenicillin (final concentration is 250 mg liter⁻¹), 0.4 ml of 250 g liter⁻¹ cefotaxime (final concentration is 100 mg liter⁻¹), 0.1 ml of 100 mM AgNO₃ and either 0.25 ml of 20 g liter⁻¹ phosphinothricin (Riedel-de Haën AG) (final concentration is 5 mg liter⁻¹) for *bar* selection or 0.3 ml of 50 g liter⁻¹ hygromycin (Calbiochem) (final concentration is 15 mg liter⁻¹) for *hpt* selection. Pour 30 ml aliquots into Petri dishes (90 × 20 mm) and store in the dark at room temperature.

LSD1.5B medium (for second and third selection of transformed cells) This is identical to LSD1.5A medium except for the amount of selective agent. Add 0.5 ml of 20 g liter⁻¹ phosphinothricin (final concentration is 10 mg liter⁻¹) instead of 0.25 ml or 0.6 ml of 50 g liter⁻¹ hygromycin (final concentration is 30 mg liter⁻¹) instead of 0.3 ml in this case.

LSZ medium (for regeneration of transformed plants) Add 100 ml of 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× modified LS vitamins, 50 ml of 100 mg liter⁻¹ zeatin (final concentration is 5 mg liter⁻¹) and 0.1 ml of 100 mM CuSO₄ to 700 ml of distilled water. Dissolve 20 g sucrose and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at

121 °C for 15 min. Cool to 50 °C, and add 1 ml of 250 g liter⁻¹ carbenicillin (final concentration is 250 mg liter⁻¹), 0.4 ml of 250 g liter⁻¹ cefotaxime (final concentration is 100 mg liter⁻¹) and either 0.25 ml of 20 g liter⁻¹ phosphinothricin (final concentration is 5 mg liter⁻¹) for *bar* selection or 0.6 ml of 50 g liter⁻¹ hygromycin (final concentration is 30 mg liter⁻¹) for *hpt* selection. Pour 30 ml aliquots into Petri dishes (90 × 20 mm) and store in the dark at room temperature.

LSF medium (for rooting of transformed plants) Add 100 ml of 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× modified LS vitamins and 2 ml of 100 mg liter⁻¹ IBA (final concentration in 0.2 mg liter⁻¹ to 700 ml of distilled water. Dissolve 15 g sucrose and 0.5 g MES in the mixture and make up the volume to 1,000 ml and adjust pH to 5.8. Add 3 g gellan gum and warm to 90 °C to dissolve. Pour 10 ml aliquots to glass test tubes (25 mm in diameter × 100 mm in height). Cover the tubes with polypropylene caps and autoclave at 121 °C for 15 min. Store at room temperature.

ELA medium (for detached leaf analysis) Add 100 ml of 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts and 5 ml of 100 mg liter⁻¹ 6BA (final concentration is 0.5 mg liter⁻¹) to 700 ml of distilled water. Dissolve 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at 121 °C for 15 min. Cool to 50 °C and add either 0.1 ml of Basta (Bayer Crop Science) for analysis of expression of *bar* gene or 2 ml of 50 mg ml⁻¹ (final concentration is 100 mg liter⁻¹) hygromycin for analysis of expression of *hpt* gene. Pour 30 ml aliquots into Petri dishes (90 × 20 mm) and store in the dark at room temperature.

Soil for pots A soil mixture for horticultural use that is commercially available and well drained is usually good. Adjust the major nutrients to 0.4 g N per liter, 0.4 g P per liter and 0.4 g K per liter by adding a commercial fertilizer.

Buffer P Add 50 mM Na₂HPO₄ (about 500 ml) to 1,000 ml of 50 mM NaH₂PO₄ until the pH reaches 6.8. Sterilize using a 0.22 μm cellulose-acetate filter and store at room temperature. Mix 9.9 ml of this buffer and 0.1 ml of Triton X-100 before use.

Buffer X Mix 8 ml of buffer P, 0.1 ml of 100 mM X-gluc and 2 ml methanol just before use.

PROCEDURE

Preparation of immature embryos ● **TIMING** 35 min for handling, 90 days for growing plants

1| Grow maize plants in individual 270 mm pots in a greenhouse. Maintain daytime temperature between 30 and 35 °C and night time temperature between 20 and 25 °C. Ideally, the light intensity should be stronger than 60,000 lx and the photoperiod should be more than 12 h.

▲ **CRITICAL STEP** The quality of the immature embryos is one of the most important factors for achieving highly efficient maize transformation. Good embryos are obtained only from vigorous plants grown in a well-conditioned greenhouse. Air-conditioning and supplemental lights are needed to ensure a supply of good embryos year-round. Usually, more than 150 kernels can be collected from a single ear of A188. Production of a much lower number of kernels on a single cob implies that the growth conditions are not optimal. If transformation efficiency is poor, the greenhouse conditions should be optimized before investigating other aspects of the protocol, such as types of vectors and strains, and media compositions.

2| Between 8 and 15 days after pollination (DAP), harvest an ear that contains immature embryos at the right developmental stage (see **Fig. 2**).

▲ **CRITICAL STEP** The use of immature embryos at the right developmental stage is a critical factor, and the size of the embryos is a very good indicator of the stage. Immature embryos that are between 1.0 and 1.2 mm in length along the axis are optimal for transformation. Time (DAP) required for embryos to reach the best stage differs depending on the genotypes and the season. So carefully examine the sizes of embryos and determine the time of collection. A typical pattern of the growth of embryos is shown in **Figure 2**. For example, the time of collection for A188 was 8 DAP in August, 10 DAP in June and 15 DAP in January at our facility. As long as the sizes of embryos are in the above range, the frequency of transformation is reproducible year-round.

3| Husk the ear and detach kernels from the cob by cutting the base of the kernel with a scalpel (**Fig. 3a,b**). Insert a scalpel into the detached kernels (**Fig. 3c**) and remove the immature embryos (**Fig. 3d**). If plants are grown in a greenhouse free from disease and pest, cobs in husks are clean and immature embryos can be removed without surface sterilization of the cobs.

4| Immerse the embryos in 2.0 ml of LS-inf medium in a 2.0 ml microcentrifuge tube at room temperature until the remaining embryos have been collected. Finish the collection of immature embryos within 1 h. More than 200 immature embryos can be collected in 1 h by a single, skilled technician.

5| Vortex the microcentrifuge tube at 2,700 r.p.m. at room temperature for 5 s and remove the medium.

Figure 2 | Size of immature embryo. Immature embryos of 9–12 DAP were collected from maize inbred line A188 on June 2004 in Iwata, Shizuoka, Japan. Immature embryo of 10 DAP is 1.2 mm in length and suitable for transformation in this case. Scale bar, 1 mm.

8| Cool the microcentrifuge tube on ice for 1 min.

9| Remove the medium and add 2.0 ml of LS-inf medium.

10| Centrifuge the microcentrifuge tube with a fixed-angle rotor with a maximum radius of 83 mm at 20,000g at 4 °C for 10 min.

▲ **CRITICAL STEP** The optimal centrifugation conditions will differ depending on genotype. The conditions described here work well for genotypes A188, A634, W117 and H99. Optimization will be necessary to find the best conditions for other strains and can be assessed by co-culturing with a strain of *A. tumefaciens* carrying an *intron-GUS* gene (Steps 11–13) and GUS staining at Step 18 (see **Box 1**).

Preparation of inoculum ● TIMING 5 min for handling, 2 days for cultivation

11| Culture *A. tumefaciens* strain on a YP plate that contains appropriate antibiotics in the dark at 28 °C for 2 days.

12| Collect the bacteria with a loop and suspend in 1.0 ml of LS-inf-AS medium at a density of 1×10^9 colony-forming units per ml (OD = 1.0 at 660 nm). Inoculum should be prepared fresh. Growth of *Agrobacterium* in liquid culture before transformation is not necessary.

Inoculation and co-cultivation ● TIMING 30 min for handling, 7 days for cultivation

13| Remove the medium from the microcentrifuge tube in Step 10 and add 1.0 ml of bacterial suspension from Step 12.

14| Vortex the microcentrifuge tube at 2,700 r.p.m. for 30 s.

15| Incubate for 5 min at room temperature.

16| Transfer the suspension of the embryos and bacteria to an empty Petri dish (60 × 15 mm).

17| Remove and discard 0.7 ml of the liquid from the suspension.

18| Transfer the embryos onto fresh LS-AS solid medium with the scutellum face up and seal the Petri dishes with Parafilm. Up to 200 embryos may be placed on a single plate. If the procedure is being optimized using strains that contain the *intron-gus* gene, transient expression of GUS can be analyzed at this point, as detailed in **Box 1**.

19| Incubate in the dark at 25 °C for 7 days; this is the co-cultivation step.

6| Add 2.0 ml of LS-inf medium and vortex as in Step 5.

Pre-treatment with heat and centrifuging ● TIMING 30 min

7| Incubate the embryos in the microcentrifuge tube in a water bath at 46 °C for 3 min.

▲ **CRITICAL STEP** The optimal conditions for this heat treatment will differ depending on genotype. The conditions described here work well for genotypes A188, H99 and A634. Since immature embryos of W117 were more sensitive to heat than these genotypes, lower temperature and/or shorter treatment may be suitable for successful transformation in W117. Optimization will be necessary to find the best conditions for other strains and can be assessed by co-culturing with a strain of *A. tumefaciens* carrying an *intron-GUS* gene (Steps 11–13) and GUS staining at Step 18 (see **Box 1**). In addition, it is also important to assess callus induction from heat-treated immature embryos.

Figure 3 | Isolation of immature embryo. (a) Cut a kernel; (b) take out the kernel; (c) insert a scalpel into the kernel; and (d) lift out an immature embryo.

Selection of transformed calli ● **TIMING** 140 min for handling, 52 days for cultivation

20| Transfer the embryos to LSD1.5A medium and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate.

▲ **CRITICAL STEP** Do not rinse the embryos. Rinsing with an antibiotic solution tends to result in poor growth of cells. The surgical tape, which allows aeration, is much better for the growth of plant cells than air-tight tapes.

21| Incubate in the dark at 25 °C for 10 days; this is the first selection.

22| Transfer the embryos to LSD1.5B medium, and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate.

23| Incubate in the dark at 25 °C for 21 days; this is the second selection.

24| Cut type I calli proliferated from the scutellum into pieces of between 3 and 5 mm in diameter under a stereoscopic microscope (**Fig. 4**), transfer to LSD1.5B medium and seal the Petri dishes with the surgical tape. Up to 25 pieces may be placed on a single plate.

25| Incubate in the dark at 25 °C for 21 days; this is the third selection. Proliferated type I calli are transgenic.

Regeneration of transformed plants ● **TIMING** 240 min for handling, 28 days for cultivation, 100 days for growing plants

26| Cut the further proliferated type I calli from Step 25 into pieces of between 2 and 3 mm in diameter under a stereoscopic microscope, transfer to LSZ medium and seal the Petri dishes with Parafilm. Up to 25 pieces may be placed on a single plate.

27| Incubate under continuous illumination (5,000 lx) at 25 °C for 14 days.

28| Transfer a regenerated shoot to a tube of LSF medium, and cover with a polypropylene cap.

29| Incubate under continuous illumination (5,000 lx) at 25 °C for 14 days.

30| Transfer each plant to a 230 mm pot containing appropriately supplemented soil (see REAGENT SETUP).

31| Grow transgenic plants in a greenhouse as detailed in Step 1 for 3–4 months and harvest progeny seeds. Plants in the following generations may be grown as detailed in Step 1.

Figure 4 | Guide to cutting type I callus for third selection and regeneration step. Proliferated type I callus is cut with a scalpel as indicated by yellow dotted lines. The size of the cut fragment is 3–5 mm at Step 24 or 2–3 mm at Step 26. Scale bar, 5 mm.

Figure 5 | Assay of segregation of transgene in detached leaves. Leaves are excised from T1 plants of inbred line A188 transformed with LBA4404 (pSB131). Detached leaves are inserted in the medium containing 0.1% (w/v) Basta. The plate is incubated under continuous light at 25 °C for 3 days. Green leaves are resistant to Basta (R) and therefore transgenic, whereas yellow leaves are sensitive to Basta (S) and non-transgenic.

Examination of progeny for expression of a selection marker gene ● **TIMING** 10 days for growing plants, 30 min for handling, 2–4 days for incubation, 10 min for examination

32| This examination is performed by the modified method of Wang and Waterhouse³⁶. Sow individual progeny seeds of transgenic plants in soil in 40 mm × 40 mm plastic pots and grow as detailed in Step 1. It is preferable that more than 30 seeds are sown and examined.

33| Excise a leaf segment of 10 mm in length from a 10-day-old seedling, insert 3 mm of the tip of the segment in ELA medium, and seal the Petri dish with Parafilm.

34| Incubate the plates under constant illumination (5,000 lx) at 25 °C.

35| Examine the segment for changes in the color 2, 3 and 4 days after the start of incubation. A resistant segment, which

PROTOCOL

expresses the transgene, stays green whereas a sensitive segment (non-transgenic) turns yellow (**Fig. 5**).

? TROUBLESHOOTING

● TIMING

A typical timeline for the optimized protocol is given in **Figure 1**. It takes 87 days from the start of co-cultivation (Step 19) to the planting of transformants into soil in pots (Step 30).

? TROUBLESHOOTING

If transformation is unsuccessful and no transformants are obtained, ensure that the composition of all solutions and media is as indicated and perform all of the tissue culture steps without *A. tumefaciens*. Calli must grow vigorously from the immature embryos, and plants must be readily regenerated from the callus. Otherwise, ask an experienced maize breeder to examine the appearance of plants, temperature and light conditions of the greenhouse, soil conditions, watering frequency, etc. Take measures recommended by the expert. Check again if the sizes of the immature embryos are in the right range. If callus growth and plant regeneration without infection is fine, use an *A. tumefaciens* strain that carries the *intron-gus* gene to perform transient expression experiments (see **Box 1**). Typical results of this experiment are shown in **Figure 6**. If GUS expression is too low or too high, no transformants will be obtained. Adjust the concentration of *A. tumefaciens*, temperature and duration of heat treatment and centrifugal force and duration of centrifuging so that the expression is at the right level.

Figure 6 | Transient expression of GUS gene in immature embryos. Upper row: expression is too weak and not suitable for high-frequency transformation. Middle row: good expression and suitable for high-frequency transformation. Lower row: expression is too strong and not suitable for high-frequency transformation. Immature embryos of inbred line A188 were treated with X-gluc 3 days after inoculation. Scale bar, 1 mm.

ANTICIPATED RESULTS

Typical average transformation frequencies for a number of maize genotypes are shown in **Table 1** and raw data of transformation frequencies from a series of actual experiments are shown in **Table 2**. The optimized protocol has improved the frequency of transformation several fold over the original protocol for both selective markers, the *bar* gene and the *hpt* gene. In addition, some of the genotypes that could not be transformed using the original protocol have been successfully transformed using the optimized protocol (see **Table 1**).

TABLE 2 | Frequency of transformation (raw data).

Inbred line	Construct	46 °C (min)	Inoculated immature embryos (A)	Antibiotic-resistant plants produced (B)	Frequency of regeneration (B/A, %)	Antibiotic-resistant, GUS-positive plants produced (C)	Frequency of transformation (C/A, %)
A188	pSB131	3	29	20	69.0	15	51.7
A188	pSB134	3	28	13	46.4	8	28.6
H99	pSB131	3	38	3	7.9	3	7.9
W117	pSB131	1.5	79	18	22.8	15	19.0
W117	pSB131	2.5	74	30	40.5	20	27.0
W117	pSB131	3.0	75	12	16.0	9	12.0
A634	pSB131	3	29	7	24.1	7	24.1

All immature embryos were pretreated with centrifuging at 20,000g for 10 min.

To date, more than 90% of the maize plants transformed by *A. tumefaciens* have been normal in morphology and fertile^{11,20,21}. About half of them had one or two copies of the transgenes. The other half had more than three copies of the transgene, but a majority of them had less than six copies¹¹. Clear mendelian inheritance of the transgenes was observed in most of the cases¹¹.

Co-transformation vectors³⁴ have been tested in maize^{37,38}. About half of the transformants carried the nonselective T-DNA, and selection marker-free transformants with solely the nonselective T-DNA were obtained from about half of these co-transformants. Thus, marker-free transformants may be segregated from a quarter of the transformants. With the highly optimized protocol, co-transformation processes may be performed very efficiently.

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