PROTOCOL

High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants

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This protocol describes a method for high-frequency recovery of transgenic soybean, bean and cotton plants, by combining resistance to the herbicide imazapyr as a selectable marker, multiple shoot induction from embryonic axes of mature seeds and biolistics techniques. This protocol involves the following stages: plasmid design, preparation of soybean, common bean and cotton apical meristems for bombardment, microparticle-coated DNA bombardment of apical meristems and *in vitro* culture and selection of transgenic plants. The average frequencies (the total number of fertile transgenic plants divided by the total number of bombarded embryonic axes) of producing germline transgenic soybean and bean and cotton plants using this protocol are 9, 2.7 and 0.55%, respectively. This protocol is suitable for studies of gene function as well as the production of transgenic cultivars carrying different traits for breeding programs. This protocol can be completed in 7–10 months.

INTRODUCTION

The development of high-frequency plant gene transfer systems is one of the key stages for the effective evaluation of different traits' functional efficacy under *in vitro*, greenhouse and field conditions[1]. Several studies have been conducted to produce a highly efficient transformation system that is simple to execute, variety independent, and capable of solving the problems associated with host and tissue specificity and/or somaclonal variation, frequently related to plant regeneration from tissue culture.

Currently, *Agrobacterium*- and biolistics-based systems are the two main ways of transferring exogenous DNA into several crops, including soybean[2–14], common bean[15–19] and cotton[20–29]. In soybean, the first two successful reports involved the use of the *Agrobacterium* system to obtain plants regenerated from cotyledonary nodes[2] and the biolistic system[3], which was based on acceleration of microparticle-coated DNA into the apical meristematic region of embryonic axes. Nowadays, the utilization of optimized *Agrobacterium* systems has also been successfully applied to achieve high-frequency soybean transformation[13,14]. In common bean, due to the difficulties in regenerating plants through tissue culture procedures, the biolistic system has been the main effective option to generate fertile transgenic plants[15–18]. Recently, *Agrobacterium* has also been used[19]. In cotton, most varieties transformed by *Agrobacterium* are Coker or Coker-derived[23–27]. However, most cultivars of commercial interest are difficult to regenerate into fertile transgenic plants. Although genes introduced into regenerative cultivars could be bred into different cotton lines, the introgression process is lengthy. Stable gene integration and expression have been effectively achieved in cotton with biolistics[28]. However, most of the putative transgenic plants have shown only the transformation of the epidermal cortex cell layer of the plant and most of the progeny did not inherit the transgene. The utilization of microparticle bombardment of embryogenic cells in suspension has also enabled transgenic cotton plants to be produced[29]. A cultivar-independent procedure was also developed to produce transgenic cotton[30]. However, efficiency (the total number of fertile transgenic plants divided by the total number of explants) was low, varying from 0.027 to 0.2%.

As an additional and viable option over these existing procedures, we have developed a novel system to select transgenic meristematic cells from embryonic axes of soybean[31], bean[32] and cotton[33] mature seeds after the physical introduction of a mutant *ahas* gene using a selection system based on the use of the herbicide, imazapyr. The utilization of this selectable marker system (described further in EXPERIMENTAL DESIGN), combined with multiple shoot induction and biolistics, resulted in a significant increase in the recovery of fertile transgenic plants. However, the application of this protocol is limited to the utilization of a selective molecule capable of translocating and concentrating in the apical meristematic region of the embryonic axes and the availability of a regeneration procedure to induce multiple shooting and regenerate fertile plants. In soybean, this strategy allowed the recovery of transgenic plants at an increased frequency of up to 200-fold[31] over the existent nonselective biolistic system[3,4]. The ability to transform common bean is still not trivial[15,34], and the previously reported efficiencies of transformation were 0.9% (refs. 16,17) and 0.2–0.9% depending upon cultivar[18]. The use of the *ahas* gene as selectable marker resulted in a significant threefold increase in the recovery of fertile transgenic bean plants[32]. In cotton, the average germline transformation frequency was 0.55%[33], which is similar to other transformation systems using different selective molecules such as bialaphos, kanamycin and imidazolinones derived[20–30,35].

We have used this protocol effectively to generate a soybean line presenting reduction of up to 94.5% of phytate (InsP6) content[36] and a transgenic elite plant[31] tolerant to imidazolinone herbicides that was integrated in a breeding program, making a viable option available to soybean producers worldwide. In common bean, a noncommodity crop but an important staple food in numerous developing countries, the use of this protocol has allowed the production of transgenic elite plants resistant to *Bean golden mosaic virus*[32] which are at regulatory approvals stage[1] and planned to reach the market within the next few years. In cotton, we are currently evaluating insect tolerance genes (M.F. Grossi de Sá, unpublished data) and a silk spider gene expression under

control of the *actin2* promoter (N.B. Cunha, unpublished data). In addition, this protocol has been used to generate transgenic soybean plants expressing pharmaceutical molecules codifying the human growth hormone, human factor IX, single-chain variable antibodies, human insulin (N.B. Cunha, unpublished data) and microbicides against HIV (L. Madeira, unpublished data) coding genes under control of seed-specific promoters. There is nothing intrinsic to this technology that would limit its wide use in any transformation system employing the selection of embryonic axes' apical meristematic cells from mature seeds. This protocol is compatible with several cultivars and may utilize mature seeds. In addition, it may be applied to generate transgenic plants in other crops that are known to be recalcitrant, allowing them to be transformed.

Experimental design

Selection system. This protocol exploits the morphogenic potential of embryonic axes derived from mature seeds of soybean, common bean and cotton to regenerate into fertile plants utilizing a simple tissue culture step. The physical process of introducing foreign genes using microparticle bombardment has a wide application to almost any living cell[37]. However, the application of this protocol is limited to the use of a selective molecule capable of translocating and concentrating in the apical meristematic region of the embryonic axes and the availability of similar regeneration procedures as described. Despite the wide and appropriate use of other molecules such as hygromycin, kanamycin, phosphinothricin and glufosinate ammonium as selective markers for cells from cotyledons, embryogenic calli and cell suspension, these molecules are not appropriate to select transgenic cells from embryonic axes effectively after microparticle bombardment. This is due to the fact that the putative transgenic apical meristematic cells are not in contact with the selective marker in the culture medium.

In this protocol, the exposed apical meristematic region of the embryonic axes derived from the mature seeds is positioned (**Fig. 1**) in the appropriate bombardment culture medium and microparticle bombardment is then carried out with the plasmid vector carrying the selectable marker *ahas* gene and the gene of interest. Under *in vitro* culture, the herbicidal molecule imazapyr utilized for selection is capable of systemically translocating through the embryonic axes radicle in a nonlethal manner and concentrating in the apical meristematic region. Imazapyr's action mechanism is the inhibition of acetohydroxyacid synthase enzy-

Figure 1 | Positioning of embryonic axes. Soybean embryonic axes are disposed in circle, equidistant between 6 and 12 mm from the center of the dish (5 cm diameter) and with the apical meristematic region directed upward. Common bean and cotton embryonic axes are disposed in a similar manner.

matic activity, which catalyzes in the apical meristematic region the initial step in the biosynthesis of three essential amino acids: isoleucine, leucine and valine[38]. The *ahas* selectable marker gene, isolated from *Arabidopsis thaliana*, contains a mutation that confers imidazolinone-specific resistance[39]. As a consequence, after the microparticle bombardment, the putative transgenic cells in the apical region of the embryonic axes can be effectively selected among the nontransgenic cells.

Plasmid design. The 5,718-bp fragment containing the mutated *Atahas* gene (GenBank accession number AL133315, position 35,863–41,580) is cloned in *Xba*I site of the vector pBS SK+ (Stratagene) to generate the vector pAC321. The *ahas* gene is under control of the *Arabidopsis* AHAS promoter and terminator (**Fig. 2**). The *Atahas* gene contains a mutation at position 653 bp of the coding sequence[39] (GenBank accession number X51514) resulting in a Ser-to-Asp substitution that confers imidazolinone-specific resistance. The unique sites can be used for cloning a cassette containing the gene of interest. The sites for *Bss*HII, *Pvu*II, *Bgl*I and *Fsp*I can be used to remove totally or partially the ampicilin resistance gene (*bla*) before particle bombardment.

MATERIALS
REAGENTS
- Absolute ethanol (Aldrich-Sigma Chemical Co. Ltd., cat. no. 459844)
- Agar (Acros Organics, cat. no. 400395000)
- 6-Benzylaminopurine (BAP; Aldrich-Sigma Chemical Co. Ltd., cat. no. 852430)
- Calcium chloride (CaCl2; Aldrich-Sigma Chemical Co. Ltd., cat. no. 10043524)
- Charcoal, activated (Merck, cat. no. 1021860250)
- Glycerol (J.T. Baker, cat. no. 213601)
- Isopropanol (2-propanol; Aldrich-Sigma Chemical Co. Ltd., cat. no. 67630)
- Imazapyr technical grade (2-(4,5-dihydro-4-methylethyl)-5-oxo-1H-imidazol-2-yl)-3-pyridinecarboxylic acid); BASF)
- MS medium (Aldrich-Sigma Chemical Co. Ltd., cat. no. M5524-50L)
- Plasmid DNA isolation Kit (QIAGEN Plasmids Kits, cat. no. 12162)
- Plasmid vector for selection (BASF) (see REAGENT SETUP)

Figure 2 | Basal map for plasmid vector design (unique sites in bold).

- Phytagel (Aldrich-Sigma Chemical Co. Ltd., cat. no. P8169)
- Plant material: mature seeds from the following commercial cultivars are used in the protocol: soybean seeds from the cultivars BR-16, BR-91, Celeste, Conquista, Doko RC, Nina, Indiana and Itaipu were supplied by Embrapa Soybean, Rodovia Carlos João Strass, Distrito de Warta, C.P. 231, CEP 86001-970, Londrina, PR, Brazil; common bean seeds from the cultivars Olathe Pinto, Pontal (Carioca) were supplied by Embrapa Rice & Beans, Rodovia GO-462, km 12 Zona Rural C.P. 179, 75375-000, Santo Antônio de Goiás, GO, Brazil; cotton seeds from the cultivars 7MH, Antares, CD-401 and ITA 94 were supplied by Embrapa Algodão, Rua Osvaldo Cruz, 1143, Centenário, C.P. 174, 58107-720, Campina Grande, PB, Brazil.
- Silica gel (Fisher Scientific Ltd., cat. no. 241640010)
- Spermidine (Aldrich-Sigma Chemical Co. Ltd., cat. no. S0266)
- Sucrose (Aldrich-Sigma Chemical Co. Ltd., cat. no. S5016)
- Sterile distilled deionized water
- Tris, ultra pure (Invitrogen, cat. no. 15504-020)
- Tungsten microparticles (Atlantic Equipment Engineers, Micron Metals, cat. no. WP-100-M10 equivalent)

EQUIPMENT
- The high pressure helium-driven microparticle acceleration system utilized was built in our laboratory essentially as described previously[37] (Fig. 3). ▲ CRITICAL The PDS-1000/He system (Bio-Rad, cat. no. 165-2257) has also been used by other laboratories showing similar results.
- Kapton 200 HN (a polyimide film, DuPont; rupture disks (13.2 mm diameter) and microparticle-coated DNA carrier membrane (24 mm diameter))
- Stopping screen (stainless steel AISI 304; 16 mesh; 18 mm diameter)
- Stereomicroscope (standard)
- Ultrasonic (Branson, cat. no. B1210E-MTH) ▲ CRITICAL Other ultrasonic models/brands have shown similar results.
- 1.5-ml Polypropylene microcentrifuge tubes (Fisher Scientific Ltd., cat. no. 05-669-32). Tubes are sterilized by autoclaving
- Wide-mouth bottles, 250 ml (Fisher Scientific Ltd., cat. no. 02911916)
- Filters, 0.22 µm (Fisher Scientific Ltd., cat. no. MPGL02GH2)
- Vessels for plant tissue culture (baby food jars; Aldrich-Sigma Chemical Co. Ltd., cat. no. V8630)
- Forceps (tissue forceps, 20 cm; Fisher Scientific Ltd., cat. no. 08953G)
- Forceps (microforceps, 10.8 cm; Fisher Scientific Ltd., cat. no. 08953E) and a dissecting knife handle (Fisher Scientific Ltd., cat. no. 089135) with a stainless-steel sterile blade (number 11; Fisher Scientific Ltd., cat. no. 089165B)
- Whatman paper (12.5 cm; Fisher Scientific Ltd., cat. no. 0979012E)
- Standard plant tissue culture room and greenhouse with photoperiod, humidity and temperature control ▲ CRITICAL Experiments utilizing genetically modified organisms should be performed in accordance with relevant guidelines and regulations.

REAGENT SETUP
BAP stock solution (10 mg ml^{-1})   Dissolve 50 mg of 6-BAP in 1 N NaOH, complete the volume to 10 ml with distilled water, sterilize by autoclaving along with the culture medium. Store at -20 °C up to 6 months.

CaCl$_2$ 2.5 M   Autoclave and distribute aliquots in 200-µl microcentrifuge tubes. Store at -20 °C up to 12 months.
50% Glycerol (vol/vol)   Mix equal parts of glycerol and distilled water and autoclave. Store at room temperature (22 ± 2 °C) up to 12 months.
Imazapyr stock solution   The selective agent imazapyr is photolabile and thermolabile. Make sure that the temperature of the culture medium is < 55 °C before adding it. To prepare a 1 mM imazapyr stock solution (M_r: 261.3; purity: 99.2%): dissolve 13.17 mg of imazapyr in 50 ml of sterile purified water. Sterilize by filtration using a 0.22-µm filter and stock at -20 °C in 1–2 ml aliquots up to 2 months.
Plasmid DNA (1 µg µl^{-1} in TE: 100 mM Tris–HCl pH 7.0; 10 nM EDTA, pH 8.0)   The DNA must be of high purity. Pure preparation of plasmid DNA has OD$_{260}$:OD$_{280}$ value of 1.8 (see ref. 40). Store at -20 °C up to 24 months.
Plant culture media
Soybean   Bombardment medium: MS basal salts medium, 3% sucrose and 0.8% Phytagel, pH 5.7; induction medium: MS basal salts, supplemented with 22.2 µM BAP, 3% sucrose, 0.6% agar, pH 5.7; culture/selection medium: MS basal salts medium, 3% sucrose, 500 nM imazapyr and 0.6% agar, pH 5.7.
Common bean   Bombardment medium: MS basal salts medium, 3% sucrose and 0.8% Phytagel, pH 5.7; culture/selection medium: MS medium containing 44.3 µM BAP, 80 nM imazapyr, 0.6% agar, pH 5.7.
Cotton   Bombardment medium: MS basal salts medium, 3% glucose and 0.8% Phytagel, pH 5.7; culture/selection medium: MS basal salts medium, 3% glucose, 13.3 µM BAP, 300 nM imazapyr, 0.1% activated charcoal and 0.6% agar, pH 5.7; culture medium MS basal salts medium, 3% glucose, 0.1% activated charcoal, 0.6% agar, pH 5.7.
Sodium hypochlorite   1 and 2.5% (vol/vol) in sterile H$_2$O.
Spermidine 0.1 M   Dissolve the entire contents of a bottle (1 g) in 68.8 ml of distilled water. Sterilize by filtration (in 0.22-µm filter). Distribute aliquots in 200-µl microcentrifuge tubes. Store at -20 °C up to 12 months. ▲ CRITICAL Spermidine oxidizes easily in the presence of oxygen. The 200-µl microcentrifuge tubes should be filled. After defrosting, spermidine could be used two times at the most and discarded.
Plasmid vector for selection   A plasmid vector carrying the synthetic 5.7-kb fragment of the *Arabidopsis ahas* promoter, coding sequence and terminator cassette should be used for selection (see EXPERIMENTAL DESIGN for further details).

EQUIPMENT SETUP
The microparticle bombardment should be carried out according to the standard manufacturer's instructions (see EQUIPMENT). Basic setup conditions: (i) the distance between the high-pressure gas chamber (shockwave generator) and the carrier membrane containing the microparticle-coated DNA should be 8 mm; (ii) the distance between the carrier membrane and the retention screen is 13 mm; (iii) the distance between the retention screen and the material to be bombarded is 80 mm; (iv) the vacuum pressure should be 27 inches of Hg and (v) the helium gas pressure is 1,200 p.s.i. (Fig. 3).
Helium gas regulator: some gas regulators present the pressure gauge measurement in kgf cm^{-2}. Perform the conversion according to the following relation: 1 kgf cm^{-2} = 14.22 p.s.i.

Figure 3 | Diagram of the high-pressure helium microparticle acceleration system. The Petri dish carrying the embryonic axes to be bombarded is positioned (a) 80 mm below the stopping screen (stainless steel AISI 304; 16 mesh; 18 mm diameter) (b); the carrier membrane (Kapton, 2 mil = 50 µm thickness; 24 mm diameter) carrying the microparticle-coated DNA is positioned 13.2 mm from the stopping screen (c). The rupture disks (d) (Kapton, 2 mil = 50 µm thickness; 13.2 mm diameter) are positioned 8 mm away, as in (c,d). The required pressure of 1,200 p.s.i. is adjusted and the high-pressure chamber is pressurized with the helium gas (e); to deliver the helium shock wave, an electrical solenoid valve is activated (f), directing the energized needle (g) toward the rupture disks. Basically, the delivered helium shock wave hits and directs the carrier membrane carrying the microparticle-coated DNA toward the stopping screen (b). The screen retains the carrier membrane allowing the microparticle-coated DNA to penetrate the meristematic region of the embryonic axes (a). The cytoplasm liquid dissociates the DNA from the microparticles, which randomly integrate into the host genome. To reduce the aerodynamic forces and minimize the deceleration of the microparticle-coated DNA, the system should function under a partial vacuum of 27–28 mm Hg.
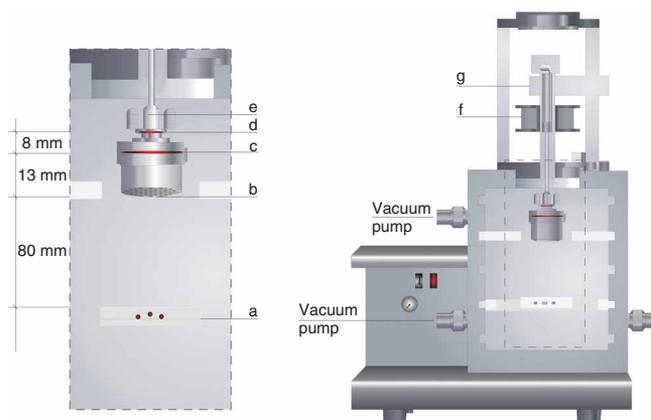
PROCEDURE

Microparticle preparation ● TIMING **1 h 40 min**

1| Weigh 60 mg tungsten microparticles, transfer into a 1.5-ml microcentrifuge tube and add 1.0 ml of 70% ethanol. Mix vigorously in a vortexer for 15 min.

2| Centrifuge at 3,000g for 5 min.

3| Discard the supernatant with the aid of a 1,000-µl micropipette. Take care not to disturb the microparticle pellet.

4| Add 1 ml sterile distilled water, mix vigorously in a vortexer for 15 min and centrifuge as in Step 2. Discard the supernatant and repeat this step two more times.

5| After the last washing, discard the supernatant and resuspend the microparticles in 1 ml 50% autoclaved glycerol (vol/vol).
■ **PAUSE POINT** The microparticles can be stored at room temperature for up to 1 month.

Preparation of microparticle-coated DNA ● TIMING **35 min**

6| Sonicate the microparticles for 5 min before removing aliquots, to assure a complete disaggregation of the microparticles and adequate suspension homogeneity.

7| Mix the microparticle suspension vigorously in a vortexer for 30 s and pipette an aliquot of 50 µl into a microcentrifuge tube.

8| Add 8 µl of the plasmid vector (1 µg µl^{-1}). Homogenize quickly (in a vortexer for 2 s).

9| Add 50 µl of 2.5 M CaCl$_2$ immediately and homogenize it quickly.

10| Add 20 µl of 0.1 M spermidine and homogenize it quickly.

11| Incubate the tubes at room temperature under slow stirring in a vortexer for 10 min.
▲ **CRITICAL STEP** The incubation time must not be <10 min, but at this stage you can wait up to 1 h.

12| Centrifuge for 10 s at 3,000g and discard the supernatant with the aid of a 100-µl micropipette pipette, without disturbing the pellet.

13| Add 150 µl absolute ethanol. Homogenize quickly. Centrifuge at 3,000g for 10 s and discard the supernatant with the aid of a 100-µl micropipette pipette, without disturbing the pellet. Repeat.

14| Add 24 µl absolute ethanol. Homogenize vigorously.

15| Sonicate for 2–3 s. Distribute aliquots of 3.2 µl on the central region of each carrier membrane previously positioned on the membrane support.

16| Transfer carrier membrane disks containing the DNA-coated microparticles to Petri dishes containing silica gel immediately and place inside a desiccator. Wait for at least 5 min for total drying of the ethanol.
■ **PAUSE POINT** At this point, you can wait up to 3 h at room temperature before using the microparticle coated–DNA in the bombardment procedure.

Preparation of cotton, soybean and common bean apical meristems for bombardment ● TIMING **21 h 55 min**

17| For cotton only, first remove the lint of ∼400 seeds through treatment with concentrated sulfuric acid (3 ml g^{-1} of seeds), mix well using a glass rod for ∼1 min. Immediately transfer the seeds into 5 l of distilled water, rinse three times in water and dry completely in paper towel.
! **CAUTION** Seeds must be dropped into water: never pour water onto the acid-treated seeds.

18| Using a 250-ml wide-mouth bottle, surface decontaminate the seeds using the following options for soybean and common bean seeds (option A) or cotton seeds (option B).
(A) Surface decontamination of soybean and common bean seeds
    (i) Place ∼300 seeds in 300 ml of 70% ethanol for 1 min and then in 300 ml 1% sodium hypochlorite for 20–30 min.
    (ii) Wash the seeds five times in sterile water.
(B) Surface decontamination of cotton seeds
    (i) Place the seeds from Step 17 in 300 ml of 70% ethanol for 10 min and then in 300 ml 2.5% sodium hypochlorite
        for 30 min.
    (ii) Wash the seeds five times in sterile water.

Figure 4 | Scanning electron micrographs showing the morphology of the vegetative apical meristematic region (m) after the removal of the embryonic axes' primary leaves (p) of common bean, soybean and cotton. In some common bean cultivars (e.g., Horizonte cv.), the apical meristematic region is partially covered by the primordial leaves (pl) (see panel **a**). Common bean cultivars in which the meristematic region is 100% exposed (**b,c**) (e.g., Olathe Pinto and Jalo 308 cvs.) are utilized for bombardment. In soybean (**d**) (e.g., BR-16 cv.) and cotton (**e**) (e.g., Araçá cv.) cultivars, the meristematic region is 100% exposed and utilized for bombardment. However, depending on the seed quality, a long germination may allow an unsuitable overgrowth of the primordium leaf (**f**). Bars represent 200 μm.
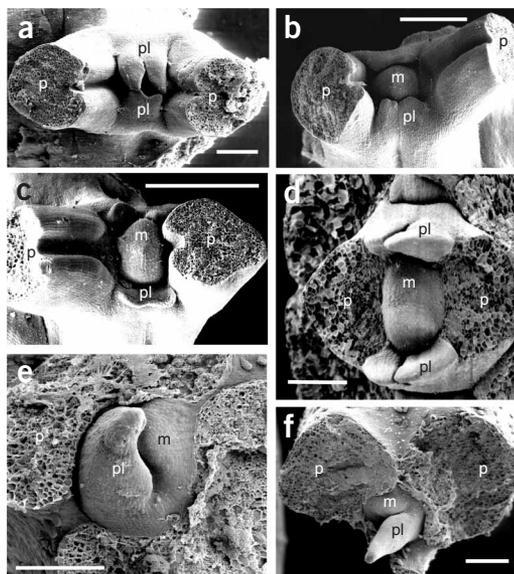
19| Incubate the decontaminated seeds for 16–18 h in sterile water at room temperature. Place at least an 8-cm layer of water on top of the seeds.

20| Embryonic axes are excised and apical meristems are exposed using the following options for soybean and common bean seeds (option A) or cotton seeds (option B).

(A) Excision of embryonic axes and exposure of apical meristems from soybean and common bean seeds

(i) Pick up approximately six to eight decontaminated seeds under sterile water with the help of forceps and transfer them to a sterile Petri dish (150 × 15 mm^2) containing sterile Whatman paper.

(ii) Using microforceps, individually hold each seed and open the cotyledons and excise the embryonic axes utilizing a dissecting knife handle with a sterile stainless-steel blade.

(iii) Under a stereomicroscope, cut the primary leaves to expose the apical meristematic region that will be bombarded with the microparticle-coated DNA (**Figs. 4** and **5**), and immediately transfer it to a sterile Petri dish containing sterile water.

(B) Excision of embryonic axes and exposure of apical meristems from cotton seeds

(i) For cotton seeds, remove the water from Step 19, close the bottle and leave them in the dark at 22–24 °C until the radicle is visible before excising the embryonic axes.

▲ CRITICAL STEP This process should take from 24 to 72 h, depending on the cultivar and/or seed quality.

(ii) Open cotton seeds and excise the embryonic axes as described in Steps 20A(i)–(iii) (**Figs. 4** and **5**).

Microparticle-coated DNA bombardment of apical meristems
● TIMING 90 min

21| Dry the exposed meristems partially from the embryonic axes on the surface of a sterile Whatman paper immediately before Step 22.

▲ CRITICAL STEP This procedure should be conducted under laminar flow.

22| Position the embryonic axes (10–15 axes per dish), on the 5 cm diameter culture dishes containing 12 ml of the appropriate bombardment medium (see REAGENT SETUP). The embryonic axes are disposed in a circle, equidistant between 6 and 12 mm from the center of the dish and with the apical meristem region directed upward (**Fig. 1**).

▲ CRITICAL STEP The positioning of the embryonic axes in the bombardment dishes is an important factor in making the tissues viable to obtain high frequency of transformation. They must be positioned with the apical meristematic region directed upward, inside the microparticle distribution radius, and outside the 'dead zone' (central region of the plate where the microparticle coated–DNA shockwave is more intense, making the tissues unviable). Immediately before the bombardment, using a stereomicroscope, check the correct positioning of the embryonic axes (it might change after a few hours in the bombardment culture medium).
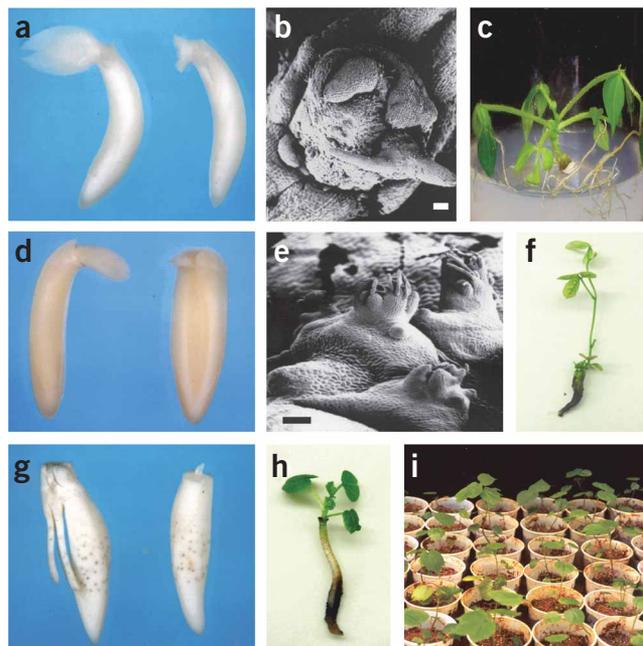
Figure 5 | Stages in the biolistic-mediated transformation of common bean, soybean and cotton embryonic axes. (**a–c**), common bean; (**d–f**), soybean; and (**g–i**), cotton. Embryonic axes are excised from mature common bean soybean and cotton seeds (**a,d** and **g**, left) and their primary leaves are removed (**a,d** and **g**, right); Common bean and soybean multiple shoot induction (**b** and **e**, respectively), and shoot elongation (**c** and **f**, respectively); Cotton shoot elongation (**h**) and acclimatized plantlets (**i**). Bars represent 100 μm.

23| The microparticle bombardment should be carried out according to the manufacturer's instructions (see EQUIPMENT SETUP for basic conditions).

Culture and selection of transgenic plants ● TIMING 3–8 weeks

24| Culture embryonic axes using the following options for soybean (option A), common bean (option B) and cotton (option C).

(A) Culture of soybean embryonic axes
   (i) Immediately after bombardment, transfer soybean embryonic axes into 10 cm diameter dishes and induce multiple shooting by the complete immersion in 15 ml of the induction medium (see REAGENT SETUP) for 16 h in the dark at 26 °C.
   (ii) After this period, transfer the explants into baby food jars containing 20 ml of the selection/elongation medium (see REAGENT SETUP) and culture at 28 °C with 16-h photoperiod 50 μmol m^{-2} s^{-1}.
   ▲ CRITICAL STEP After ~4 weeks in culture, the bombarded apical meristems produce elongated shoots and roots, which should be allowed to reach 3–4 cm in length (**Fig. 5**).

(B) Culture of common bean embryonic axes
   (i) Immediately after bombardment, transfer common bean embryonic axes to baby food jars containing 20 ml of selection/elongation medium (see REAGENT SETUP) and culture at 28 °C with 16-h photoperiod 50 μmol m^{-2} s^{-1}.
   ▲ CRITICAL STEP After ~3 weeks in culture, the bombarded apical meristems produce elongated shoots and roots, which should be allowed to reach 3 cm in length (**Fig. 5**).

(C) Culture of cotton embryonic axes
   (i) Immediately after bombardment, transfer cotton embryonic axes to baby food jars containing 20 ml of the culture/selection medium (see REAGENT SETUP) and culture at 28 °C with 16-h photoperiod 50 μmol m^{-2} s^{-1}.
   (ii) After 30 d, remove the necrotized radicles and transfer the explants into baby food jars containing 20 ml of the culture medium (see REAGENT SETUP) for elongation and rooting. Culture at 28 °C with 16-h photoperiod 50 μmol m^{-2} s^{-1}.
   ▲ CRITICAL STEP After ~4 weeks in culture, the bombarded apical meristems produce elongated shoots and roots, which should be allowed to reach 3–4 cm in length (**Fig. 5**).

Further growth and analysis of plants ● TIMING 24–32 weeks

25| Transfer the rooted plantlets individually to a plastic pot containing 0.2 dm^3 of autoclaved fertilized soil:vermiculite (1:1).

26| Cover with a plastic bag and seal with a rubber band.

27| Maintain in a greenhouse at 25 °C with 14-h photoperiod, relative humidity >80% and light intensity 350 μmol m^{-2} s^{-1}.

28| After 1 week, remove the rubber band and after an additional week remove the plastic bag also.

29| As soon as the acclimatized plantlets reach ~10 cm in length transfer them to a pot containing 5 dm^3 of fertilized soil and allow them to set seeds.
▲ CRITICAL STEP Under greenhouse conditions and a 10–14-h photoperiod, each soybean plant produces ~100 seeds in the progeny. Soybean is a short-day plant, and an increase in the photoperiod to 18–20 h induces a strong vegetative growth of the plant and flowering delay. After a period (up to 2 months) of vegetative growth, the photoperiod should be adjusted to 10–14 h to induce flowering. This procedure makes it possible to produce up to 900 seeds per plant.

30| To detect the presence of foreign genes, plants are screened by PCR, Southern blot[3,31–33,36,40] and/or western blot[40,41] techniques, among other methods.
? TROUBLESHOOTING

● TIMING

This protocol takes ~7–10 months to complete from the embryonic axes bombardment until the plants set seeds and are ready for harvesting.
Steps 1–5: 1 h 40 min; Steps 6–14: 20 min; Steps 15 and 16: 15 min; Step 17: 15 min; Step 18: 40 min; Step 19: 18 h; Step 20: 3 h; Steps 21 and 22: 30 min; Step 23: 60 min; Step 24 option A (soybean): 4 weeks, option B (common bean): 3 weeks, option C (cotton): 8 weeks; Steps 25 and 26: 2 h; Steps 27 and 28: 2 weeks; Step 29: 28 weeks for soybean, 24 weeks for common bean and 32 weeks for cotton.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Low transformation frequency	Quality of the seed/culture contamination; if the batch of seeds has not been generated under adequate conditions for use in *in vitro* culture systems, the frequency of fungal or bacterial contamination could be high	Seeds should be produced in the greenhouse and manually collected to reduce and/or eliminate the appearance of contamination in the culture medium
	Inappropriate scale of microparticle-coated DNA preparation	Each precipitation (Steps 6–16) is sufficient to prepare six shots; a typical experiment includes 24 shots. In this case, prepare four precipitation tubes
	High humidity in the laboratory is detrimental to the transformation efficiency. The relative humidity of the air should not be >40% at the time of preparation of microparticle-coated DNA and microparticle-coated DNA bombardment of apical meristems. High humidity will cause particle aggregation, which damages the vegetal tissue, reducing the generation of transgenic plants	Utilize a dehumidifier to maintain adequate relative humidity in the laboratory
	Positioning of embryonic axes in the bombardment dishes	The positioning of the embryonic axes in the bombardment dishes is an important factor in making the tissues viable to obtain high frequency of transformation. Immediately before the bombardment, using a stereomicroscope check the correct positioning of the embryonic axes (it might change after a few hours in the bombardment culture medium)
	A liquid film in the surface of the meristematic region reduces microparticle penetration	Visually check whether the meristematic region to be bombarded is covered with a liquid film. If so, open the lid of the dish (under laminar flow) for 1–2 min to dry the meristematic region of the embryonic axes partially. Another option is to dry meristematic region of the embryonic axes manually with an autoclaved sheet of Whatman paper
Low transformation in common bean	For common bean cultivars where the apical meristematic region is only partially exposed, the number of meristematic cells that can be reached by the microparticle-coated DNA is drastically reduced and the frequency of transformation will be reduced (**Fig. 4**)	The choice of the cultivar is essential for high-frequency transformation during bombardment of the apical meristem. Both Pontal (Carioca) and Jalo cultivars have 100% of the embryonic axes with the apical meristematic region totally exposed[16]. However, owing to its intrinsic germplasm variability regarding the exposure of the apical meristematic region of the embryonic axes, a prior evaluation under stereomicroscope of other desirable cultivars should be conducted to determine the percentage of the apical meristematic region exposure

ANTICIPATED RESULTS

This protocol will yield an average frequency of transformation (the total number of fertile transgenic plants divided by the total number of bombarded embryonic axes) of transgenic soybean, common bean and cotton plants of 9, 2.7 and 0.5%, respectively. In soybean ∼88% of the putative transgenic shoots (R0 generation) that generate germline fertile transgenic plants were expressing the foreign genes in their progenies[31]. In common bean and cotton, the percentages were 60 and 50%, respectively[32,33].

In our study, the frequency of co-transformation of linked genes (genes in the same plasmid) is 100%. The frequency of co-transformation of unlinked genes should be drastically reduced and has an average of 40% for soybean, common bean and

cotton. However, most of the transgenic plants have presented the foreign gene integrated in one single locus and a Mendelian segregation. When comparing our results with other protocols using *Agrobacterium*-based systems[5,6,8–11,19,42,43], it can also be noted that the use of the biolistic system associated with the bombardment of the apical meristematic region of embryonic axes, followed by a short multiple shoot induction, has drastically reduced the problems of somaclonal variation frequently associated with several plant regeneration protocols from tissue culture. In addition, it can be seen that the transgenic plants generated using the biolistic and *Agrobacterium*-based systems are comparable in several molecular and biochemical aspects[9–12,43] such as copy number, rearrangements, silencing and preferential integration sites responsible for functional activity within the genome, among other regulation factors[44–46].

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