30

Pigeonpea (*Cajanus cajan* L. Millsp.)

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Summary

Pigeonpea [*Cajanus cajan* (L.) Millsp.], also known as redgram, is one of the major grain legume (pulses) crops grown in the semiarid tropics (SAT) extending between 30°N and 30°S; it is the second most important food legume of India. It is cultivated in about 50 countries of Asia, Africa, and the Americas for a variety of uses (food, fodder, fuel wood, rearing lac insects, hedges, wind breaks, soil conservation, green manure, roofing, and so on). The constraints of enhancing its productivity include the damage caused by various fungi, bacteria, viruses, and insect pests. Conventional plant breeding methods have not been successful for the improvement of pigeonpea because of genetic variation and incompatibility among the wild varieties. Genetic engineering technology can therefore be used as an additional tool for the introduction of agronomically useful traits into established varieties. The development of plant transformation techniques has been a major breakthrough in overcoming constraints to achieve precision in genetic manipulation. The development of efficient plant regeneration protocols is a prerequisite for recombinant technology to carry out genetic transformation. This chapter describes an *Agrobacterium*-mediated transformation protocol for pigeonpea, a simple, efficient, and reproducible method that is applicable across diverse genotypes of pigeonpea.

Key Words: *Agrobacterium tumefaciens*; *Cajanus cajan*; legumes; pigeonpea; genetic transformation; redgram; regeneration; transgenic plants.

1. Introduction

Pulses have been indispensable constituents of the Indian diet, and pigeonpea, an important grain legume, stands second in terms of cultivation and yield in India. It is a member of the Fabaceace family, with chromosome number 2n = 22. It is an important grain legume crop of rain-fed agriculture in the semiarid tropics of the Indian subcontinent and is also widely grown in Southeastern Africa, Latin America, and the Caribbean. Its productivity is constrained

From: *Methods in Molecular Biology, vol. 343:* Agrobacterium *Protocols, 2/e, volume 1*
Edited by: Kan Wang © Humana Press Inc., Totowa, NJ

by both biotic ***(1)*** and abiotic factors. Conventional plant breeding methods have not been successful in improving pigeonpea because of genetic variation and incompatibility among the wild varieties. The major difference between conventional breeding and biotechnology lies in the speed, precision, reliability, and scope. The development of plant transformation techniques has been a major breakthrough in overcoming constraints to achieve precision in genetic manipulation. With the establishment of vector-mediated and direct gene transfer methods, genes from taxonomically distant and or unrelated donors have been incorporated into plants. In pigeonpea there are numerous reports on tissue culture and regeneration ***(2–4)*** and few reports on genetic transformation ***(5–9)***.

Reliable tissue culture and plant regeneration systems play an important role in routine production of transgenic plants ***(10,11)***. Direct regeneration is preferred to indirect regeneration as the length of the callus phase is negatively correlated with regeneration ability: the somaclonal variation can influence the phenotype of the regenerated shoots. Here we describe a simple and highly reproducible *Agrobacterium*-mediated genetic transformation of pigeonpea using leaves as explants ***(12)***. Five- to 6-d-old leaf explants from germinated seedlings are cocultivated with *Agrobacterium tumefaciens* strain C58 harboring the plasmid pCAMBIA1301 carrying the genes encoding for hygromycin phosphotransferase (*hpt)* and β-glucoronidase (*uid*A). The putatively transformed shoots are selected on selection medium with hygromycin as the selection agent. The shoots are rooted on rooting medium and transferred to pots containing a 1:1 sand and soil mixture. Stable expression and integration is confirmed by GUS assay, polymerase chain reaction (PCR), and Southern blot analysis. With this protocol we obtain an average of 75% transformation efficiency, i.e., 75 antibiotic-resistant PCR-positive independent lines from 100 infected plants.

2. Materials

2.1. Tissue Culture

1. Healthy pigeonpea seeds of variety ICPL 88039 obtained from the gene bank of the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT).
2. 70% v/v Ethanol.
3. 0.1% w/v Mercuric chloride, Tween-20.
4. MS medium ***(13)***: MS inorganics + MS organics + 3% sucrose + 0.8% Difco Bacto agar. Adjust the pH of the medium to 5.8 with 1 *N* NaOH prior to autoclaving (**Table 1**).
5. Kinetin (Sigma): 1 m*M* stock. Weigh 21.52 mg of kinetin and dissolve in few drops of 1 *N* HCl, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.

Table 1
Composition of Murashige and Skoog (1962) Basal Medium (MS)[a]

Salt	Concentration required (mg/L)	Stock (g/L)	Use (mL/L)
Major salts			
NH_4NO_3	1650	165	10
KNO_3	1900	95	20
KH_2PO_4	170	17	10
$CaCl_2$	440	44	10
$MgSO_4 \cdot 7H_2O$	370	37	10
Minor salts			
H_3BO_3	6.20	0.620	10
KI	0.83	0.083	10
$MnSO_4 \cdot H_2O$	22.3	2.230	10
$ZnSO_4 \cdot 7H_2O$	8.6	0.860	10
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.025	10
$CuSO_4 \cdot 5H_2O$	0.025	0.0025	10
$CoCl_2 \cdot 6H_2O$	0.025	0.0025	10
Iron			
$Na_2EDTA \cdot 2H_2O$	37.3	3.73	10
$FeSO_4 \cdot 7H_2O$	27.8	2.78	10
or			
$FeNa_2EDTA$	40	4.00	10
Organics			
Glycine	2.0	0.200	10
Nicotinic acid	0.5	0.050	10
Thiamine HCl	0.1	0.010	10
Pyridoxine HCl	0.5	0.050	10
m-Inositol	100	10.0	10

[a]*See* **ref. *13***.

6. N^6-benzyl adenine (BA; Sigma): 1 m*M* stock. Dissolve 22.5 mg of BA in few drops of 1 *N* NaOH, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.
7. Giberellic acid (GA_3; Sigma): 1 mM stock. Dissolve 34.63 mg of GA_3 in 1 mL of sterile water, filter-sterilize, and store at 4°C in aliquots.
8. Indole-3-acetic acid (IAA; Sigma): 1 m*M* stock. Dissolve 17.52 mg of IAA in a few drops of 1 *N* NaOH, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.
9. Cefotaxime: 125 mg/mL stock. Dissolve 125 mg of cefotaxime in 1 mL distilled water, and filter-sterilize the stock prior to use.

10. Kanamycin monosulfate (Sigma): 100 mg/mL stock. Weigh 100 mg of kanamycin, dissolve in 1 mL of distilled water, filter-sterilize, and store at –20°C for not more than 15 d.
11. Hygromycin (Sigma): 50 mg/mL stock. Weigh 50 mg of hygromycin, dissolve in 1 mL of distilled water, and store at –20°C.
12. Shoot induction medium (SIM): MS medium + 250 mg/L cefatoxime + 5 µ*M* BA + 5 µ*M* kinetin.
13. Shoot development medium (SDM): MS medium + 250 mg/L cefotaxime + 0.25 µ*M* BA + 0.25 µ*M* kinetin.
14. Shoot elongation medium (SEM): MS medium + 250 mg/L cefotaxime + 0.58 µ*M* GA_3.
15. Root induction medium (RIM): MS basal medium + 1% sucrose.

2.2. Agrobacterium tumefaciens *Strains and Selectable Markers*

We have used C58 as the *Agrobacterium* strain harboring the pCAMBIA1301 plasmid with the *hpt* and *uid*A genes as selectable and screenable markers.

2.3. Culture Media for Agrobacterium tumefaciens

1. Yeast extract medium (YEB): for 1 L. Weigh and dissolve 5 g of Bacto-peptone, 1 g of yeast extract, 5 g of beef extract, 5 g of sucrose, and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 800 mL of water. Adjust the pH to 7.0 with 1 *N* NaOH, make the volume to 1000 mL, and autoclave prior to use. Then cool to 50°C before adding the appropriate selective agent (depending on the vector).
2. LB agar: weigh and dissolve 10 g of Bacto-peptone, 5 g of yeast extract, and 10 g of sodium chloride in 800 mL of water, adjust the pH to 7.0 with 1 *N* NaOH, add 15 g of agar, make the volume to 1000 mL, and autoclave. Then cool to 50°C before adding the appropriate selection agent (depending on the vector).

2.4. GUS Assay (14)

1. 100 mL of 1 *M* Sodium phosphate stock solution: dissolve 14.2 g of Na_2HPO_4 (dibasic sodium phosphate) and 13.8 g of $NaH_2PO_4 \cdot H_2O$ (monobasic sodium phosphate) in approximately 90 mL of sterile distilled water. Adjust the pH to 7.0 using 1 *N* NaOH. If necessary to reduce the pH, use phosphoric acid. Bring the volume to 100 mL with distilled water
2. Assay mixture: 100 mL of X-Gluc solution. Weigh out 0.052 g of X-Gluc. Dissolve the X-Gluc in about 50 mL of sterile distilled water. Add 5 mL of 1 *M* sodium phosphate stock solution and 0.1 mL of the detergent Triton X-100 and mix. Bring the volume up to 100 mL with distilled water. Store the solution in the dark in the refrigerator (*see* **Note 1**).

3. Methods

3.1. Explant Preparation and Culture Conditions

1. Sterilize pigeonpea seeds by rinsing in 70% ethanol for 2 min and 0.1% mericuric chloride containing 1 to 2 drops of Tween-20 for 8 min on a rotary shaker at room temperature (*see* **Note 2**).

2. Wash the seeds five to six times with sterile water under aseptic conditions (in a laminar flowhood) and soak in autoclaved water overnight.
3. Wash the presoaked seeds and remove the seed coat using a scalpel and bent-ended sharp forceps.
4. Place the dehusked seeds on Murashige and Skoog's basal medium supplemented with 3% sucrose and 0.8% agar in a Petri dish (90 × 15 mm) and incubate under standard culture conditions of a 16-h photoperiod at 25 ± 1°C and a light intensity of 60 μE/m²/s.
5. Excise primary leaves with their petiolar region intact from 4- to 5-d-old aseptically grown seedlings and cocultivate with *Agrobacterium* culture

3.2. Agrobacterium *Inoculum Preparation and Cocultivation*

1. Inoculate a single colony of *Agrobacterium tumefaciens* strain C58 harboring the binary plasmid pCAMBIA1301 in 25 mL of YEB media containing 50 mg/L kanamycin and incubate at 28°C on a shaker at 100 rpm for 16 to 18 h. Use the culture at late log phase (OD of 0.6 at A_{600}) for cocultivation.
2. Centrifuge the culture at 9000*g*, discard the supernatant, and dissolve the pellet in 20 mL of half-strength MS liquid medium.
3. Put the cell suspension in a sterile Petri dish (3 mL) by placing in a slanted position with support from the other sterile Petri dish, so the suspension settles at the base of the Petri dish.
4. Dip the petiolar end of the freshly cut leaf explants into the suspension culture for few seconds for infection, blot on sterile filter paper (*see* **Note 3**), and place them on shoot induction medium (SIM) with their abaxial side in contact with the medium (*see* **Note 4**) for 2 d at 26 ± 1°C under continuous cool white light provided by fluorescent lamps (60 μE/m²/s) (**Fig. 1A**).

3.3. Shoot Induction, Selection, and Elongation

1. After 2 d, transfer them to fresh SIM medium supplemented with filter-sterilized cefotaxine (250 mg/L) to arrest the growth of *Agrobacterium* (16-h photoperiod at 25 ± 1°C and a light intensity of 60 μE/m²/s). Cefotaxime at 250 mg/L is included in all the media during subsequent subcultures until all the *Agrobacterium* overgrowth is arrested.
2. Observe for shoot bud initiation from the cut end of the leaf (**Fig. 1B**) and transfer leaves to half-strength shoot development medium (SDM) after removing half the lamina of the leaf (**Fig. 1C**). Shoot bud initiation can be observed within 7 to 10 d.
3. At this stage supplement SDM medium with 2 mg/L hygromycin as an initial selection pressure to select the transformed shoot from infected culture. With a gap of 7 d (**Fig. 1D**), transfer the explants to the same media with increased concentrations of hygromycin such as 6, 8, and 10 mg/L.
4. Maintain the explants in SDM with frequent subculture for 10 to 21 d (**Fig. 1E** and **F**). Transfer the explants with differentiated and proliferated multiple shoot buds to culture tubes (25 × 150 mm) for elongation in shoot elongation medium (SEM) and maintain for 7 to 14 d (**Fig. 1G**).

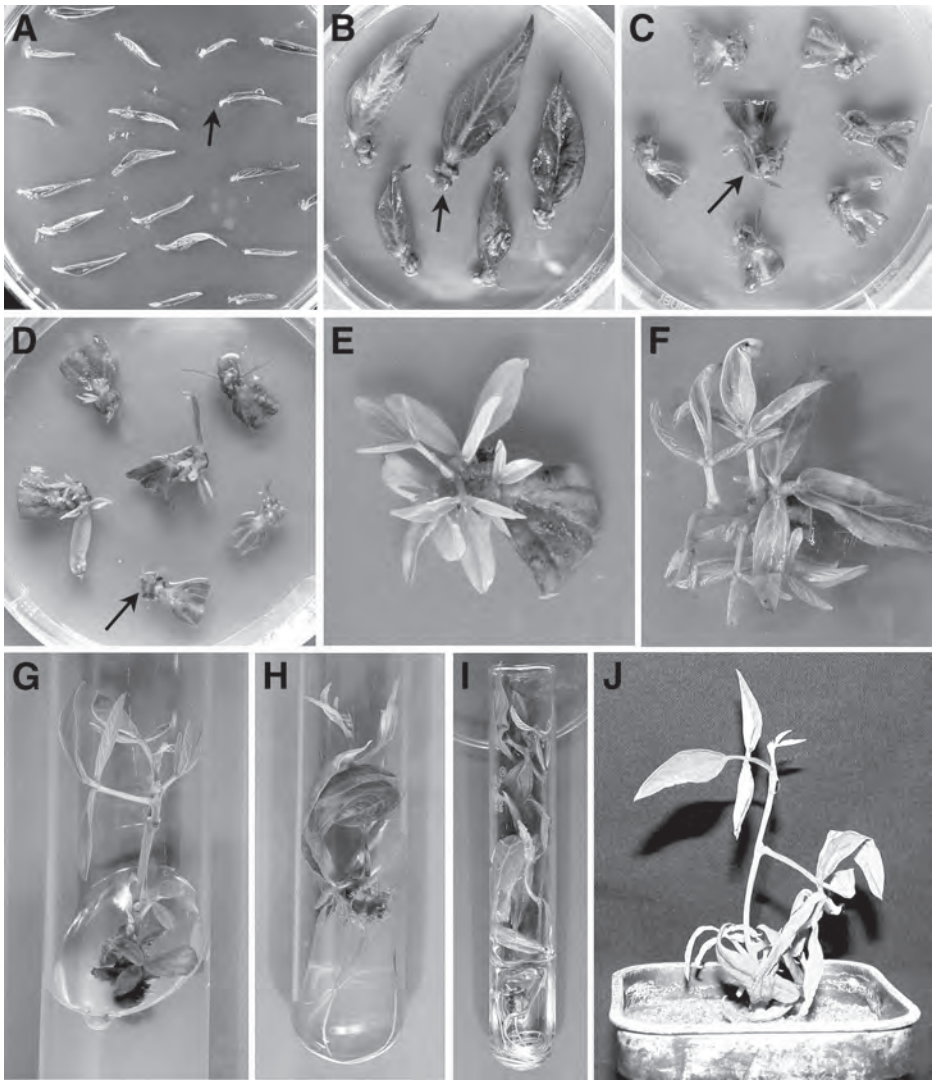

Fig. 1. Regeneration of adventitious shoots and development of plants from leaf explants of *Cajanus cajan* L. (Millsp.) Arrows indicate the petiolar cut end undergoing shoot bud differentiation. **(A)** Leaf explants at 0 d on MS medium supplemented with 5 μ*M* BA and 5 μ*M* kinetin. **(B)** Differentiation of shoot buds from petiolar cut end. **(C)** Leaf explants with proliferating multiple shoots on shoot development medium with half-cut lamina. **(D)** Development of shoot buds into shoots on reduced SIM. **(E)** Formation of multiple shoots from the petiolar region after 18 d. **(F)** Formation of multiple shoots from the piteolar region after 25 d. **(G)** Individual shoot on MS medium supplemented with 0.58 μ*M* GA_3 (elongation medium) after 7 d. **(H)** Elongated shoot in rooting media, MS basal with 1% sucrose, after pulse treatment after 7 d. **(I)** Putative transformants showing profuse rooting on MS basal with 1% sucrose after 14 d. **(J)** Well-rooted putative transformant in a 3-inch pot after hardening.

3.4. Rooting

1. Transfer the shoots longer than 3 cm to root induction medium (RIM) with reduced concentration of sucrose (1%) after giving a pulse treatment, dipping the elongated shoots directly into a 11.42 µ*M* IAA solution for 30 to 60 s. Root initiation can be observed in 7 d (**Fig. 1H**), and upon subculture profuse rooting can be observed within 14 d (**Fig. 1I**).
2. The shoots that are not yet rooted can be given a second pulse treatment with IAA.

3.5. Hardening and Acclimatization of the Plants

1. Gently remove well-rooted plants and wash under tap water to remove media attached to the roots (*see* **Note 5**).
2. Transfer the regenerated plantlets with well-developed roots to 3-inch (7.62-cm) pots containing autoclaved sand and red soil in a 1:1 ratio for hardening. Every independent plant transferred should be numbered separately and should keep the same numbering in subsequent generations (T_1, T_2, and so on).
3. Cover the plantlets with polyethylene bags. This helps the plantlets to minimize loss of water through transpiration and maintains high humidity conditions.
4. Place the plants at 26°C and relative humidity of 40% for 1 d in a growth cabinet, and later transfer them to a greenhouse.
5. After transfer to the greenhouse, punch small holes in the sides of the polyethylene bag and after 3 d remove the top portion of the polythene bag by cutting; eventually remove the bag after 7 to 8 d. This helps the plant to withstand the sudden change in the atmosphere and get acclimatized (**Fig. 1J**).

3.6. Transplantation

1. Transfer the plants from smaller to larger pots (9.5 inches; 24.13 cm) containing autoclaved sand and red soil in a 1:1 ratio supplemented with a small amount of manure and di-ammonium phosphate (DAP).
2. Cover the plants with the same polythene bags with the top portion cut and provide support by placing small rods on either side.
3. After 3 d, uncover the plants totally, expose to withstand various climatic conditions, and maintain the plants in the same conditions till harvest. Depending on the variety used, it takes about 70 to 80 d to flower and 90 to 110 d to maturity; the yield is about 200 to 300 seeds per plant.

3.7. Screening of Transgenics

Independent transgenic plants transferred to the greenhouse are subjected to molecular analysis to check integration and expression of the transferred gene.

3.7.1. Histochemical GUS Analysis

Perform the GUS assay for all plants in all generations to check expression of the *uid*A gene.

1. Take 300 to 500 µL of assay mixture in a 1.5-mL microfuge tube.
2. Cut the leaves from regenerated and hygromycin-resistant plants and incubate in the assay mixture at 37°C overnight in the dark by wrapping in aluminum foil (*see* **Note 6**).
3. After staining, soak the tissue in 70 and 100% ethanol to clear the chlorophyll content, and finally fix in 70% ethanol until a blue color develops.
4. Observe under the light microscope and photograph.

3.7.2. Characterization of Transgenic Plants

1. To check for integration, isolate the genomic DNA separately using the cetyltrimethylammonium bromide (CTAB) protocol ***(15)***, digest with the restriction enzyme that has a single recognition site in the plasmid, resolve on 0.8% agarose gel, transfer onto nylon membrane (Hybond N+, Amersham) using a standard protocol, and probe the blot with labeled (radioactive or nonradioactive) PCR-amplified fragments of the *hpt* gene.
2. Carry out genetic analysis to ascertain the inheritance pattern of the introduced genes in the T_1 generation (progenies of primary transformants). This is done by growing all the progenies of the selected events (primary positively confirmed transformant) and then carrying out PCR analysis on the genomic DNA for the introduced genes. The segregation of a single-copy insert usually follows a Mendelian inheritance pattern of 3:1 (i.e., three positives to one negative progeny). In the case of pigeonpea, selection of the seedlings on hygromycin- or kanamycin-containing medium is not very reliable.

4. Notes

1. The assay mixture can be stored for several months at 0°C in a dark bottle.
2. The longer the time in ethanol and mercuric chloride solution lesser the germination efficiency.
3. Care should be taken that only the petiolar region is dipped into the culture; otherwise it becomes difficult to control bacterial growth and infection in the other cut parts that occur during explant preparation.
4. Orientation of the explant on the culture medium is one of the critical factors for obtaining higher regeneration.
5. Washing reduces the chance of bacterial and fungal contamination that may kill the plantlet in soil.
6. The enzyme glucoronidase cleaves the substrate X-Gluc into glucornic acid and an indoxyl derivative, which dimerizes and oxidizes to form insoluble, highly colored indigo dye.

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