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GENETIC TRANSFORMATION AND HYBRIDIZATION

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An efficient protocol for shoot regeneration and genetic transformation of pigeonpea [*Cajanus cajan* (L.) Millsp.] using leaf explants

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Abstract A protocol for efficient plant regeneration from leaf explants of pigeonpea [*Cajanus cajan* (L.) Millsp.] was developed for the production of transgenic plants. Leaf explants from 4- to 5-day-old in vitro raised seedlings were most efficient in producing multiple adventitious shoots in 90% of the explants on shoot induction medium [Murashige and Skoog (MS) medium +5.0 μM benzyladenine +5.0 μM kinetin]. Shoot buds originated from the petiolar cut end of the explants and elongated rapidly on medium containing 0.58 μM gibberellic acid. Over 80% of the elongated shoots rooted well on MS medium containing 11.42 μM indole-3-acetic acid and were transplanted with 100% success. The procedure reported here is very simple, efficient and reproducible, and is applicable across diverse genotypes of pigeonpea. The usefulness of this system for further studies on the genetic transformation of pigeonpea has been demonstrated in biolistics-mediated gene transfer by using *npt*II and *uid*A as marker genes, where 50% of the selected plants showed gene integration and expression.

Keywords Biolistics · *Cajanus cajan* (L.) · Organogenesis · Pigeonpea · Transformation

Abbreviations *BA:* N^6-Benzyladenine · *GA_3:* Gibberellic acid · *IAA:* Indole-3-acetic acid · *IBA:* Indole-3-butyric acid · *2-iP:* N^6-[2-Isopentenyl]adenine · *MS:* Murashige and Skoog medium · *RIM:* Root induction medium · *RT-PCR:* Reverse transcriptase polymerase chain reaction · *SEM:* Shoot elongation medium · *SIM:* Shoot induction medium

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Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.], one of the major grain legumes of the semi-arid tropics (SAT), is cultivated on over 3.4 million hectares world-wide with an annual production of 2.7 million tons and an average yield of 790 kg ha^{-1} (Nene and Sheila 1990). Because of its high protein content, pigeonpea forms a significant component of the diet of vegetarians in the SAT. However, its production and productivity are constrained by several diseases, including sterility mosaic, *Fusarium* wilt, *Phytophthora* blight, *Alternaria* blight and stem canker (Reddy et al. 1990), and by *Helicoverpa armigera* (the legume pod borer), which is a major insect pest of pigeonpea.

Biotechnological approaches such as gene transfer for enhanced disease and pest resistance offer opportunities for rapid improvement of pigeonpea. However, the availability of an in vitro regeneration system is a prerequisite for effective genetic transformation. The regeneration of shoot buds from different seedling explants of pigeonpea has been reported previously (George and Eapen 1994; Shiva Prakash et al. 1994; Naidu et al. 1995; Geetha et al. 1998; Mohan and Krishnamurthy 1998). In these reported regeneration systems, the time required for the formation of shoot buds and their complete differentiation into shoots was long, and the recovery of fully differentiated plants was low thus making such systems inefficient for genetic transformation work. Hence, in the present study, the major emphasis was on the establishment of a regeneration protocol that would provide transgenic plants in large numbers for routine work on the genetic enhancement of pigeonpea. The usefulness of this system for further studies on the genetic transformation of pigeonpea was demonstrated by biolistics-mediated gene transfer leading to recovery of a large number of transgenic plants.

Materials and methods

Plant material

Different genotypes of pigeonpea [*Cajanus cajan* (L.) Millsp.] were obtained from the gene bank of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). These belong to different maturity groups, including ICPL 87, ICPL 88039, ICPL 87119, ICPL 85063, ICPL 88009, ICPL 87091, ICPL 2376, ICPL 87051, ICPL 91011, ICPL 332, and ICPL 84031. Unless mentioned otherwise, all experiments were carried out with var. ICPL 88039.

Explant preparation and shoot regeneration

The seeds of pigeonpea var. ICPL 88039 were surface sterilized with 70% ethanol for 2 min and further washed with 0.1% (w/v) mercuric chloride containing 1–2 drops of Tween-20 for 8 min, followed by rinsing in sterile water 4–5 times prior to soaking for 4 h. The seed coat was removed from pre-soaked seeds and germinated on semi-solid Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962). Primary leaves from 4- to 5-day-old aseptically germinated seedlings were used as explants for initiating tissue cultures. While preparing the leaf explants, care was taken to excise the petiolar region sufficiently away from the axillary meristem so as to completely eliminate any preformed meristematic tissues. In each Petri dish, 10–12 explants were cultured with the petiolar cut end and the abaxial surface of the lamina in contact with the medium. To achieve efficient organogenesis, the effect of age of the explants was studied by taking leaves from 2- to 15-day-old aseptically germinated seedlings and culturing them on shoot induction medium (SIM).

To study the role of the lamina and petiolar region of the leaf explant, different portions of the lamina were surgically removed so as to have explants with full, one-half, one-quarter or no lamina along with the petiolar cut end (Table 1).

The effect of various cytokinins [N^6-benzyladenine (BA), kinetin, N^6-[2-isopentenyl]adenine (2-iP)], used either alone or in combination, on the regeneration of multiple shoot buds was studied. Aseptic leaf explants (4–5 days old) were placed on MS containing four different combinations of BA, kinetin and 2-iP concentrations (Table 2). In the first combination, the concentration of BA ranged from 0 to 10 μM, while the kinetin concentration was kept constant at 5 μM (C1–C6); in the second combination the concentration of BA and kinetin was vice versa (K1–K6). In the third combination, the concentration of kinetin ranged from 0 to 10 μM, while the 2-iP concentration was kept constant at 5.0 μM (P1–P6). In the fourth combination, the concentration of kinetin was kept constant (5.0 μM), while the concentration of 2-iP varied between 0 and 10 μM (I1–I6). Furthermore, various concentrations of BA and kinetin were tested to standardize the best combination of cytokinin for multiple shoot induction. Based on the morphogenic response, MS in combination with 5.0 μM BA and 5.0 μM kinetin (SIM) was optimal for shoot bud differentiation. The leaf explants with multiple shoot buds that were obtained from SIM were transferred to shoot elongation medium (SEM) consisting of MS supplemented with gibberellic acid (GA_3) ranging from 0.58 to 2.89 μM (data not shown).

Table 1 Effect of size of the lamina on shoot bud induction from the petiolar region of leaf explant of in vitro-germinated seedlings of pigeonpea

Explant	No. of explants cultured	No. of explants producing shoot buds	Frequency of shoot bud induction (%)
Full lamina	30	27	90.0
One-half lamina	30	26	86.6
Three-quarter lamina	30	22	73.3
Minus lamina	30	6	20.0

Table 2 The effect of N^6-benzyladenine (BA), kinetin and N^6-[2-isopentenyl]adenine (2-iP) on shoot bud induction from leaf explants of in vitro-germinated seedlings of pigeonpea

Medium	Growth regulators (μM)			No. of explants cultured	Explants producing shoots (mean±SE)	Explants forming shoots (%)
	Kinetin	BA	2-iP			
C1	5.0	10.0	–	30	15.0±1.4	50.0
C2	5.0	7.5	–	30	20.5±0.7	68.3
C3	5.0	5.0	–	30	28.5±0.7	95.0
C4	5.0	2.5	–	30	26.5±0.7	88.3
C5	5.0	1.0	–	30	22.0±0.7	73.3
C6	5.0	0.0	–	30	24.5±0.7	81.7
K1	10.0	5.0	–	30	15.0±1.4	50.0
K2	7.5	5.0	–	30	23.5±0.7	78.3
K3	5.0	5.0	–	30	27.0±1.4	90.0
K4	2.5	5.0	–	30	20.5±0.7	68.3
K5	1.0	5.0	–	30	22.0±0.0	73.3
K6	0.0	5.0	–	30	23.5±0.7	78.3
P1	10.0	–	5	30	25.5±0.7	85.0
P2	7.5	–	5	30	21.0±1.4	70.0
P3	5.0	–	5	30	18.5±0.7	61.7
P4	2.5	–	5	30	17.5±0.7	58.3
P5	1.0	–	5	30	21.5±0.7	71.7
P6	0.0	–	5	30	20.5±0.7	68.3
I1	5.0	–	10	30	11.0±1.4	36.7
I2	5.0	–	7.5	30	21.0±0.7	70.0
I3	5.0	–	5.0	30	23.5±0.7	78.3
I4	5.0	–	2.5	30	10.5±0.7	35.0
I5	5.0	–	1.0	30	13.5±0.7	45.0
I6	5.0	–	0.0	30	21.5±0.7	71.7

Table 3 Effect of explant donor genotype on shoot bud regeneration from leaf explants from in vitro-germinated seedlings of pigeonpea after culture on shoot induction medium

Genotype	Duration type	No. of explants cultured	No of explants producing shoots	Frequency of shoot bud induction (%)
ICPL 91011	Extra short	39	24	61.5
ICPL 88009	Short	39	16	41.0
ICPL 84031	Short	42	25	59.2
ICPL 87091	Short	50	31	62.0
ICPL 87	Short	73	52	71.2
ICPL 88039	Short	54	45	83.3
ICPL 2376	Medium	77	34	44.2
ICPL 87051	Medium	57	31	54.4
ICPL 332	Medium	35	19	54.3
ICPL 85063	Medium	69	44	63.8
ICPL 87119	Medium	35	23	65.7

Rooting of shoots and transplantation

Shoots over 3 cm in length were transferred to root induction medium (RIM) consisting of auxins such as indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). Various concentrations of IBA (0.98–9.8 μM) and IAA (1.14–11.42 μM), and MS medium with reduced sucrose concentration (1% w/v) were tested individually (data not shown). The elongated shoots were cut and directly dipped in 11.4 μM IAA solution for pulse treatment and transferred to culture tubes containing MS containing 1% sucrose (w/v) and devoid of any growth regulators, where they formed adventitious roots within 6 days. Regenerated plantlets with well-developed roots were transferred to small pots, filled with autoclaved sand and thiram (fungicide), for hardening. The plantlets in the pots were kept covered with polythene bags for 5 days and later transferred to the greenhouse. The plants from smaller pots were transferred to bigger pots (30.5 cm diameter) that contained autoclaved sand and soil (1:1) supplemented with farm manure and di-ammonium phosphate.

Culture medium and conditions

MS basal medium containing 3% sucrose was used for all in vitro cultures. The pH of the medium was adjusted to 5.8 prior to adding 0.8% agar; media were autoclaved at 121°C for 15 min. Cultures were maintained at 26±1°C under continuous light provided by white cool fluorescent tubes of 60 $\mu E\ m^{-2}\ s^{-1}$ light intensity. The growth regulators BA, kinetin, GA_3 and IAA were filter-sterilized prior to addition to culture media. The explants were cultured on sterile Petri dishes (90×15 mm) containing SIM; explants bearing adventitious shoot buds were subsequently transferred to culture tubes (25×150 mm) for shoot elongation and rooting of shoots. Data on the frequency of shoot bud regeneration from each explant was recorded. All experiments were repeated three times and the data were analyzed by calculating mean and standard error.

Effect of genotype

To study the effect of genotype of the explant donor seedlings, 11 genotypes of pigeonpea belonging to different maturity groups were selected (see Table 3). Leaf explants from 4- to 5-day-old in vitro germinated seedlings were cultured on SIM and their shoot bud regeneration responses were compared.

Genetic transformation via biolistics

DNA preparation, microprojectile DNA delivery and analysis of transgenics

Plasmid pRT99GUS (Fig. 1) containing the *uid*A and *npt*II genes, both under the control of the 35S promoter of cauliflower mosaic virus (Topfer et al. 1988) was used to optimize genetic transformation of leaf explants. The plasmid was isolated by the alkaline lysis method (Sambrook et al. 1989) and DNA delivery was carried out using the particle delivery system PDS-1000He (Bio-Rad, Hercules, Calif.) following the manufacturer's recommendations. Gold particles (1 μm diameter) were coated with 5 μg plasmid DNA per 50 μl particle preparation by using the $CaCl_2$ precipitation method. About 50 leaf explants were placed in each Petri dish containing SIM and explants were placed in such a way that the petiolar cut end of all the leaves faced towards the center. In each experiment, 400 explants were taken and each experiment was repeated three times. The plates containing the explants were placed at a distance of 6 cm from the stopping plate under a vacuum of 22 inches of Hg (74.5kPa) and rupture disk rated for a pressure of 1,300 psi (8.96 MPa). After each bombardment, the explants were incubated on the same plate overnight and transferred to fresh plates containing SIM at a plating density of 10–12 explants per plate. The regeneration protocol as described above was followed. Once shoot differentiation was observed from the petiolar ends, the explants were subjected to 25 mg l^{-1} kanamycin in a shoot development medium consisting of half-strength SIM. After 2 weeks of culture, the explants were transferred to elongation medium consisting of 0.58 μM GA_3 along with 50 mg l^{-1} kanamycin. In the following 2–3 subcultures of 2 weeks each on SEM, the concentration of kanamycin was increased to 100 mg l^{-1} for stringent selection of transformed shoots. This was followed by rooting of the selected and elongated shoots, and transplantation of the rooted shoots to the glasshouse.

Analysis of transgenic plants

Genomic DNA from the putative transformants growing in the glasshouse was analyzed for the presence of the introduced genes by PCR amplification of *uid*A and *npt*II, and Southern hybridization for the *npt*II gene according to Sharma and Anjaiah (2000). For Southern blot hybridization of the *npt*II gene, the DNA was digested with *Xho*I, which is a unique site within the pRT99GUS plasmid DNA. The blot was probed with a non-radioactively labelled (Alkphos Direct Labelling and Detection System; Amersham Biosciences, Uppsala, Sweden) 700 bp PCR-amplified *npt*II gene fragment. For the positive control, the plasmid pRT99GUS was restricted with *Pst*I to release the 700 bp *npt*II gene fragment. RT-PCR analysis of the putative transformants growing in the glasshouse was carried out using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, Calif.) on total RNA isolated with the TRIzol

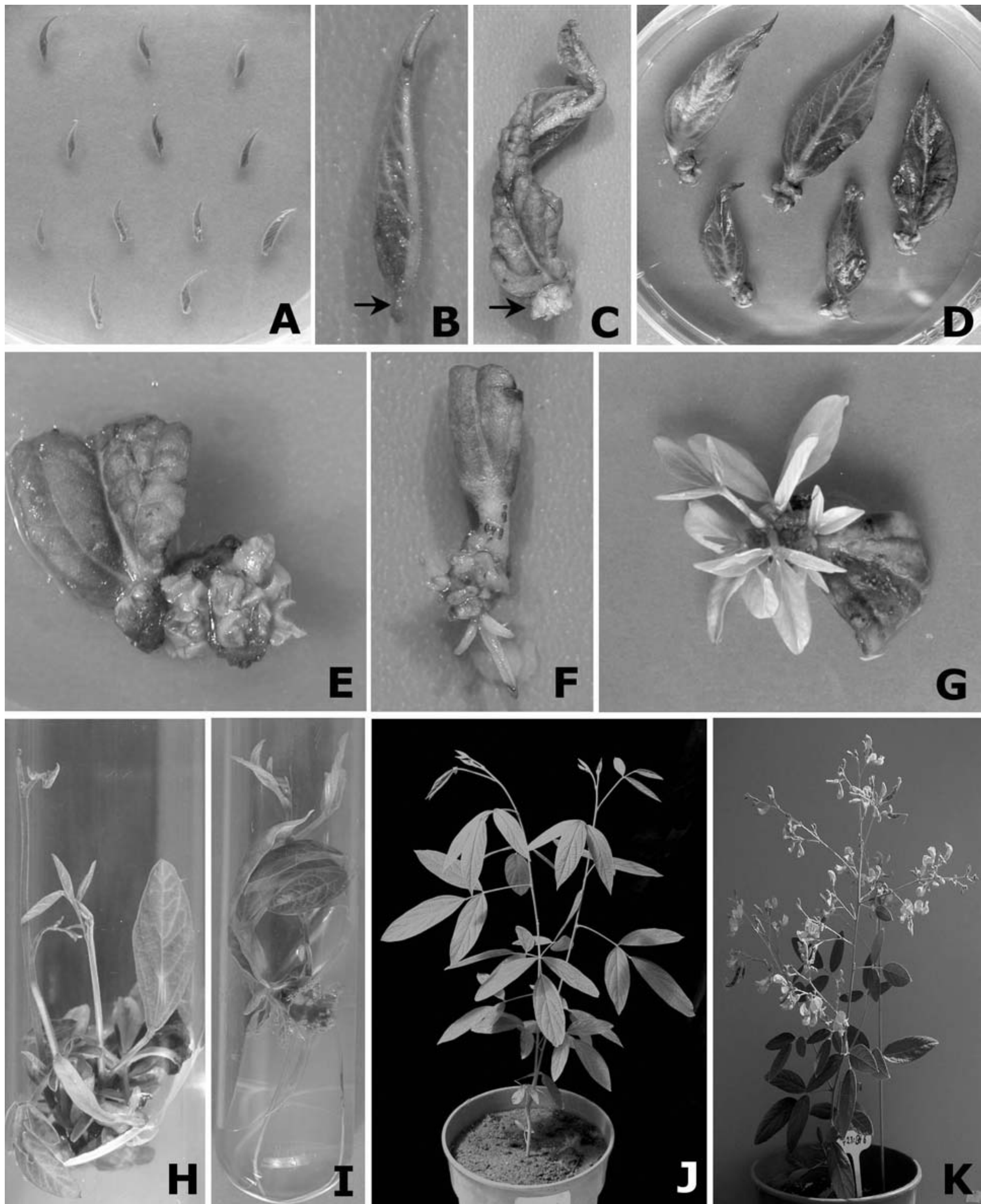

Fig. 1A–K Regeneration of multiple shoots from leaf explants derived from in vitro-germinated seedlings of pigeonpea, *Cajanus cajan* L. (*arrows* indicate the petiolar cut end undergoing shoot bud differentiation). **A** Leaf explants at day 0 cultured on Murashige and Skoog (MS) medium supplemented with 5.0 μM N^6-benzyladenine (BA) and 5.0 μM kinetin [shoot induction medium (SIM)]. **B** Enlargement and swelling of the petiolar cut end within 5 days of culture on SIM. **C** Initiation of adventitious shoot buds from leaf explants observed after 7 days on SIM from the swollen tissue of the petiolar cut end. **D** Differentiation of shoot buds from the petiolar cut end after 8 days on SIM. **E** Proliferation of multiple shoot buds after 10 days on SIM. At this stage, the explants with reduced lamina are ready for transfer to reduced SIM for shoot development. **F** Development of shoot buds into shoots after 12 days on reduced SIM. **G** Formation of multiple shoots after 2 weeks on reduced SIM. **H** Explant bearing multiple shoots placed on shoot elongation medium (SEM) containing MS supplemented with 0.58 μM gibberellic acid (GA_3) for shoot elongation after 7 days. **I** A rooted pigeonpea plant on MS containing 1.14 μM indole acetic acid (IAA) [root induction medium (RIM)] after 3 weeks ready for transplantation. **J** A well-established and hardened plant successfully transplanted to the glasshouse at 4 weeks. **K** In vitro produced plants after 2 months in the glasshouse showing normal growth, flower production, and pods that contain viable seeds

Table 4 Inheritance of *uid*A gene in the T1 generation of transgenic pigeonpea plants

Plant	No. of T1 plants tested	PCR analysis of *uid*A gene		3:1 Segregation[a]	
		No. of plants		χ^2	*P*
		Positive	Negative		
PP1	5	3	2	0.6	0.4386
PP2	5	3	2	0.6	0.4386
PP3	5	5	0	1.67	0.1963
PP5	5	2	3	1.65	0.1990
PP6	5	4	1	0.03	0.8625
PP7	5	3	2	0.60	0.4386
PP8	5	4	1	0.03	0.8625
PP13	5	1	4	4.03	0.0447

[a] *df*=1; *P*=0.05; χ^2=3.841

reagent (Invitrogen) according to the manufacturer's procedures. To study the inheritance of the introduced genes in the T1 generation, five seeds from eight selected primary transformants were germinated and PCR analysis to detect the *uid*A gene was carried out (Table 4).

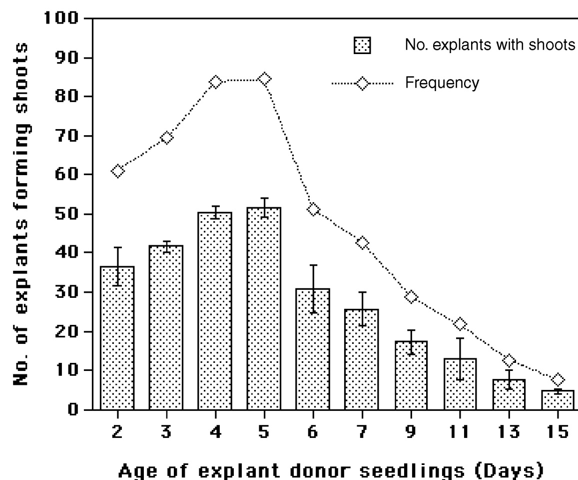

Fig. 2 Restriction map of plasmid pRT99GUS used for biolistic-mediated transformation of leaf explants from in vitro germinated seedlings of pigeonpea

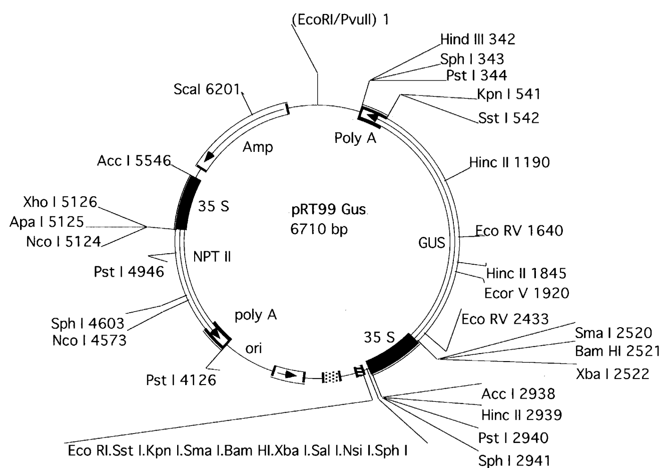

Fig. 3 Effect of age of leaf explant donor seedlings on the regeneration of multiple adventitious shoots in pigeonpea; 60 explants were cultured on SIM

Results

The genotype ICPL 88039 of pigeonpea exhibited over 95% seed germination on MS basal medium. After the culture of leaf explants (Fig. 2A) on SIM, their lamina underwent considerable enlargement and swelling of the petiolar cut end within 5 days (Fig. 2B). Initiation of shoot buds from the swollen tissues of the petiolar cut end had occurred by 7 days (Fig. 2C). Differentiation of shoot buds from the proximal cut end of the petiole occurred at 8 days (Fig. 2D), and proliferation of these into a large mass of shoot buds by 10 days (Fig. 2E). Thereafter, the shoot buds continued to proliferate until transfer to SEM. Prior to subculture on reduced SIM for shoot development, half of the lamina of the explants was removed (Fig. 2F) and well-developed shoots developed by 2 weeks (Fig. 2G). After 21 days, explants bearing multiple shoots were separated and cultured on SEM, on which they underwent elongation (Fig. 2H). The semi-elongated shoots rooted easily on RIM within 3–4 weeks (Fig. 2I). The rooted shoots could be readily transplanted with a success rate of 100% and showed normal growth in the glasshouse (Fig. 2J). Upon maturity, the plants produced fertile flowers and pods that contained viable seeds (Fig. 2K). Following this protocol, more than 100 plants with normal morphology and seed fertility have been produced so far.

The effect of the age of the explant donor seedlings on shoot regeneration was determined. In general, younger seedlings (<5 days) provided explants that were highly regenerative while the regeneration potential declined with age thereafter (Fig. 3); 4- to 5-day-old leaf explants exhibited the highest frequency of multiple shoot regeneration where 90% of the explants responded. In studies on the role of the lamina tissue in shoot bud regeneration from the petiolar cut end, it was found that leaf explants containing intact lamina (Fig. 2A) were essential for the regeneration response, with shoot bud induction declining with reduced lamina tissue (Table 1). A very low frequency of shoot regeneration occurred if the entire lamina was removed from the petiolar explants. Hence, whole leaf explants from 4- to 5-day-old seedlings were used in the optimized protocol.

In preliminary experiments, it was found that a combination of two cytokinins was required for the regeneration of shoot buds from leaf explants. Hence, various combinations of cytokinins, such as BA with kinetin and 2-iP with kinetin, were tested to induce shoot bud differentiation. In general, BA in combination with kinetin was found to be more efficient in inducing multiple shoot buds as compared to combinations of kinetin with 2-iP, where only a single shoot was formed in a few explants (Table 2). Therefore, equimolar concen-

trations of BA and kinetin (5.0 μM) were used as standard SIM for all experiments. Once shoot bud differentiation was observed at the proximal cut end of the petiole, three-quarters of the lamina was removed and the remaining explants bearing adventitious shoot buds were cultured on medium containing 2.5 μM BA and 2.5 μM kinetin.

The elongation of multiple shoots was rapid when placed on SEM supplemented with GA_3 (data not shown). GA_3 at 0.58 μM was found to be optimal, resulting in elongation of shoots within 3–5 days (Fig. 2F). The amount of phenols produced by the explant was high and, if neglected, hindered further growth of the shoots. Shoot cultures were therefore sub-cultured onto fresh medium every 10–12 days. The cytokinins used for shoot induction were highly responsive at minimal concentration thus exerting less phytohormonal stress during plant development.

The orientation of the leaf explant played a vital role in the appearance of shoot formation. Hence, leaf explants were placed on SIM with their abaxial surface in contact with the medium. During shoot formation, the lamina of the leaf explant expanded towards the adaxial side of the explant, lifting the cut end of the petiole away from the medium. As a result, the petiole lost its contact with the medium resulting in drying of the explant as well as hindering further growth of the shoot buds. Physical contact of the petiole with the culture medium was essential to elicit a complete response. Therefore, cultures were kept under constant observation to combat this latter problem.

Once the shoots elongated to a reasonable length (3 cm), they were transferred to RIM, where IAA proved to be critical for the formation of adventitious roots on the in vitro formed shoots (Fig. 2I). In each passage of 2 weeks on RIM, 25–30% of shoots formed roots and all the shoots could be rooted within 3–4 passages. The rooted plants acclimatized upon transfer to pots, resulting in the formation of well-developed plants with healthy leaves at a survival rate of over 90% (Fig. 2J). The plants were phenotypically comparable with seedling-derived pigeonpea plants that produced normal flowers and pods with viable seeds (Fig. 2K). Overall, the time taken for complete regeneration of the plants under in vitro conditions was 45–50 days.

To study the effect of genotype on shoot regeneration potential using the optimized regeneration protocol, 11 genotypes of pigeonpea belonging to different maturity groups (extra short, short, and medium duration types) were also tested. In general, there was no significant variation amongst the genotypes belonging to different maturity groups with regards to the frequency of explants producing shoot buds, and the variation in regeneration frequencies between the maturity groups was as great as that within the maturity groups (Table 3).

The regeneration potential of leaf explants was not affected after biolistics treatment. While no bleaching of untransformed shoots was observed during the initial selection on 25 mg l^{-1} kanamycin, 25% of the explants showed bleached shoots when subjected to 100 mg l^{-1}

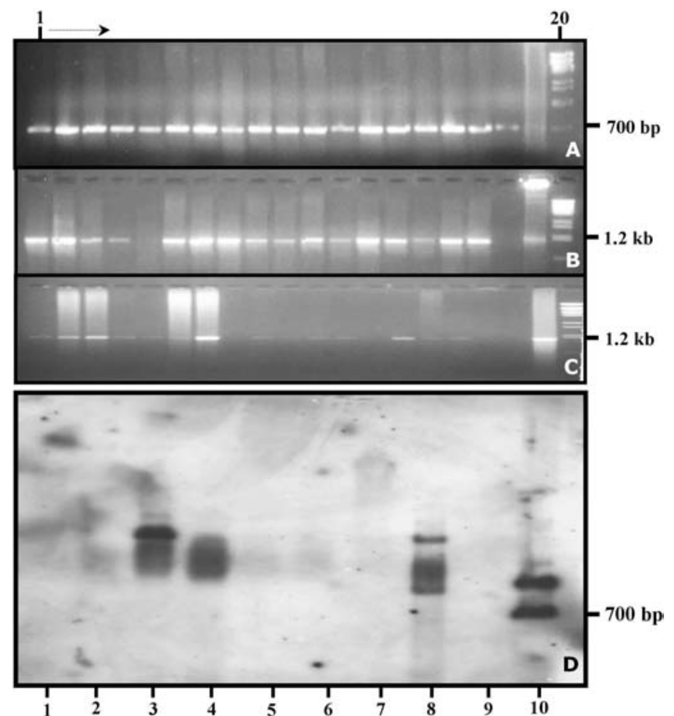

Fig. 4A–D Molecular analysis of putative transformants (T0) of pigeonpea obtained after biolistics delivery of the plasmid pRT99GUS. **A–C** Lanes: *1–17* Genomic DNA from putative transformants, *20* λ DNA restricted with *Hin*dIII. **A** PCR amplification of genomic DNA showing amplification of a 700 bp fragment of the *npt*II gene. Lanes: *18* pRT99GUS DNA, *19* DNA from untransformed control. **B** PCR amplification of the genomic DNA showing amplification of a 1,200 bp fragment of the *uid*A gene. Lanes: *18* Untransformed control, *19* pRT99GUS DNA. **C** RT-PCR of the cDNA showing amplification of a 1,200 bp fragment of the *uid*A gene. Lanes: *18* cDNA from untransformed control, *19* pRT99GUS DNA. **D** Southern blot hybridization of the *npt*II gene in genomic DNA from putative transformants. The DNA was digested with *Xho*I (a unique site within pRT99GUS plasmid DNA). The blot was probed with a 700 bp PCR-amplified *npt*II gene fragment. Lanes: *1–8* Genomic DNA from putative transformants, *9* untransformed control, *10* pRT99GUS DNA restricted with *Pst*I to release a 700 bp *npt*II gene fragment

kanamycin in shoot development medium. All the putatively transformed shoots thus obtained remained green on subsequent selection and, when these shoots were subjected to PCR analysis for the presence of the *uid*A and *npt*II genes, over 90% of shoots showed positive amplification of the respective gene fragments (Fig. 4A, B). Fidelity of the amplified gene fragments was verified by subjecting the PCR gels to Southern blot hybridization (data not shown). RT-PCR amplification of cDNA from the putative transgenic plants showed positive amplification of the 1,200 bp *uid*A gene fragment in all the selected transgenic lines (Fig. 4C). Southern blot analysis of these plants confirmed the transgenic nature of the selected shoots, where 50% of the transgenic lines showed single gene inserts and the rest contained two inserts (Fig. 4D). Thirty transgenic plants from the T0 generation have been analyzed so far, and all show a similar pattern. PCR amplification of the *uid*A gene in the T1 generation of

selected transformants showed that inheritance of the introduced genes segregated with a 3:1 Mendelian ratio for single copy integrations (Table 4).

Discussion

The regeneration of shoot buds from various explants of pigeonpea, such as leaves (Eapen and George 1993; Eapen et al. 1998; Geetha et al. 1998), distal cotyledonary segments (Mohan and Krishnamurthy 1998), cotyledonary node (Shiva Prakash et al. 1994; Geetha et al. 1998) and shoot tips (Geetha et al. 1999) has been reported. Only 36% of callus cultures obtained from primary leaves regenerated shoot buds (Eapen and George 1993). Eapen et al. (1998) reported high frequency shoot regeneration from primary leaf segments, but the shoot buds appeared only after 45–50 days of induction. In cotyledonary segments, although shoot bud formation was observed in 83% of cultured segments, only 56% of these shoot buds developed further resulting in only 18% fully developed shoots (Mohan and Krishnamurthy 1998). Cotyledonary nodes have been used to produce multiple shoot buds that may not only have been contaminated with axillary shoots but also failed to elongate further (Shiva Prakash et al. 1994). We report an efficient system for the regeneration of multiple shoots in pigeonpea where 90% of cultures underwent differentiation of adventitious shoot buds from the petiolar cut ends of leaf explants that are devoid of any pre-existing meristems. The shoot regeneration method reported here is applicable across a wide range of pigeonpea genotypes belonging to different maturity groups.

Regeneration potential is affected by explant origin, culture maintenance conditions, and age of the explants (Sharma et al. 1990). The use of in vitro-raised seedlings provides juvenile explants that often have a better regenerability than explants derived from mature tissue. In the present study, age of the explant donor seedlings greatly influenced the differentiation of shoot buds, with younger leaves from 4- to 5-day-old seedlings exhibiting a greater regeneration potential, which then declined with increasing age of the explant donor seedlings.

One of the important features of regeneration in leaf explants of pigeonpea is a polarized regeneration response, where some of the tissues of an explant have a greater regeneration potential. In the present study, regeneration was restricted to petiolar tissues; earlier reports utilizing cotyledonary petioles of *Brassica juncea* (Sharma et al. 1990, 1991) also observed a similar phenomenon. In such cases, although regeneration occurs from the petiolar cut end, both tissues, viz., petiole and lamina, play an important role in the organogenic response and a direct correlation exists between the amounts of laminar tissue present and shoot regeneration response, thus suggesting the involvement of hormones and/or metabolites in expression of cellular totipotency of the petiolar tissue.

Cytokinins in general are required to induce shoot buds from cultured tissues. Thidiazuron has been used effectively for shoot bud formation in leaf explants (leaf discs) of pigeonpea (Eapen et al. 1998). Although in most previous reports on pigeonpea, BA alone was shown to be potent for shoot bud differentiation (Eapen and George 1993; George and Eapen 1994; Mohan and Krishnamurthy 1998; Geetha et al. 1998; Shiva Prakash et al. 1994), in the present study a combination of BA and kinetin produced optimum and reproducible shoot bud differentiation. However, longer exposure to this cytokinin combination was detrimental for further elongation of shoot buds and they had to be exposed to GA_3 for their further development.

The shoot-forming petiolar region of the leaf explant was used to test the efficiency of gene transfer by using a biolistic particle device. The leaf explants were found to be efficient targets for gene transfer by microprojectile bombardment since they resulted in the production of a large number of putative transformants of pigeonpea. Although 90% of the bombarded explants exhibited transient expression of the *uid*A gene, 50% of the selected plants that were transferred to the glasshouse showed positive gene integration.

In pigeonpea, previous reports on *Agrobacterium*-mediated transformation have utilized explants where direct regeneration from shoot apices and cotyledonary nodes (Geetha et al. 1999) as well as indirect regeneration from callus derived from embryonic axes (Lawrence and Koundal 2001) have been used. However, in the former report only three plants from the T0 generation were reported, while in the latter , of 898 explants, 213 (23.8%) produced callus on selection medium, of which only 11 (1.2%) produced 9 shoots and the effective frequency of transformed shoots (T0) was less than 1%. The present report is a significant improvement, with at least 50% of the selected transgenic plants showing positive gene integration and function as indicated in RT-PCR studies. Moreover, 50% of the transgenic plants showed single gene integration events. All the putative transgenic plants showed a Mendelian inheritance of the introduced genes in the T1 generation, thus confirming the success of this regeneration and transformation protocol.

In conclusion, the protocol reported here for pigeonpea is very efficient for the production of a high frequency of adventitious shoot regeneration across a wide range of pigeonpea genotypes where the site of shoot differentiation is predictable and occurs in a short span of 2–3 weeks. Whole plants can be obtained in less than 90 days. The present protocol describes an efficient method for high frequency regeneration of multiple adventitious shoots in over 90% of leaf explants that are devoid of pre-existing meristems. The adventitious nature of the regenerating shoot buds has been determined histologically and the system has also been successfully tested with *Agrobacterium*-mediated transformations (results to be published elsewhere). Such a system should be generally applicable to biotechnological improvement of pigeonpea, an important legume crop of the SAT.

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