Plant Cell Rep (2010) 29:495–502
DOI 10.1007/s00299-010-0838-4

ORIGINAL PAPER

An efficient method for the production of marker-free transgenic plants of peanut (*Arachis hypogaea* L.)

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Received: 9 November 2009 / Revised: 12 February 2010 / Accepted: 17 February 2010 / Published online: 9 March 2010

Abstract Recombinant genes conferring resistance to antibiotics or herbicides are widely used as selectable markers in plant transformation for selecting the primary transgenic events. However, these become redundant once the transgenic plants have been developed and identified. Although, there is no evidence that the selectable marker genes are unsafe for consumers and the environment, it would be desirable if the marker genes can be eliminated from the final transgenic events. The availability of efficient transformation methods can enable the possibility of developing transgenic events that are devoid of the marker gene/s upfront. Taking advantage of the high and consistent transformation potential of peanut, we report a technique for developing its transgenics without the use of any selectable marker gene. Marker-free binary vectors harboring either the *phytoene synthase* gene from maize (*Zmpsy*1) or the chitinase gene from rice (*Rchit*) were constructed and used for *Agrobacterium tumefaciens*-mediated transformation of peanut. The putative transgenic events growing in vitro were initially identified by PCR and further confirmed for gene integration and expression by dot blots assays, Southern blots, and RT-PCR where they showed a transformation frequency of over 75%. This system is simple, efficient, rapid, and does not require the complex segregation steps and analysis for selection of the transgenic events. This approach for generation of marker-free transgenic plants minimizes the risk of introducing unwanted genetic changes, allows stacking of multiple genes and can be applicable to other plant species that have high shoot regeneration efficiencies.

Keywords *Arachis hypogaea* · Groundnut · Marker-free transgenics · Peanut · Transgenic plants

Communicated by P. Kumar.

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Introduction

Genetic transformation of peanut or groundnut (*Arachis hypogaea* L.) for various biotic and abiotic constraints has been an important area of research (Bhatnagar-Mathur et al. 2008). In a typical plant transformation process, marker genes are used mainly for the initial screening of the putative transgenic shoots to identify the transformed plants from the untransformed ones. Selectable marker genes are conditionally dominant genes that confer an ability to grow in the presence of applied selective agents that are normally toxic to plant cells or inhibitory to plant growth, such as antibiotics and herbicides, e.g., *bar*, *pat*, *aroA* (or *epsps*), *csr1* (or *ahas*), *npt*II, *hem*L, *hppd* and *hpt* (Aragao and Brasileiro 2002). However, in view of the biosafety requirements, it is recommended to phase out the selectable marker genes since these are unnecessary once an intact transgenic plant has been identified and established (Ow 2001; Puchta 2003; Darbani et al. 2007). Besides, there are public concerns about the widespread

occurrence of selectable marker genes in novel ecosystems as these are integrated into the plant genome along with gene of interest (Daniell 2002). In addition to the risk of horizontal gene transfer, there is also a "vertical cross-species" transfer risk that could potentially create enhanced weediness problems in some cases, especially the outcrossing plant species (Dale et al. 2002). Moreover, both pleiotropic and position effects can lead to unpredictable changes in the transgenic plants (Miki et al. 2009). Interaction between the selectable marker gene or its regulatory element and the genetic element at the site of insertion may result in position effects, leading to improper expression or knock-out mutations, induction of gene-silencing and chromatin remodeling (Kim et al. 2007). Using selectable marker genes also poses the potential concern of metabolic drain, since the expression of a marker gene and its regulatory element in a transgenic plant often utilizes a significant amount of the host cell's resources, and placing a metabolic load on the host which may dramatically alter biochemistry and physiology of the transgenics (Glick 1995). Furthermore, these may cause regulatory difficulties for approving transgenic crop release and commercialization.

The development of marker-free transgenic plants could thus solve the issues of biological and biosafety in the genetically engineered (GE) crops, besides supporting multiple transformation cycles for transgene pyramiding (Vaucheret et al. 1998). Several strategies that have been used for the elimination of selectable markers include co-transformation, multi-auto-transformation system (MAT), site-specific recombination system, transposon-based marker methods, intrachromosomal recombination system and transplastomics (Miki and McHugh 2004; Darbani et al. 2007). However, these methods involve multiple steps and are time consuming, besides seriously reducing the efficiencies of stable transformants. There have been earlier reports on using binary vectors devoid of selection marker gene for genetic transformation in crops such as potato (de Vetten et al. 2003), alfalfa (Popelka et al. 2003; Rosellini et al. 2007), apple (Malnoy et al. 2007), wheat (Doshi et al. 2007) and tobacco (Li et al. 2009) where the recovery of transformed events has been low in the range of 0.93–25%. Although, several reports on regeneration and transformation using selectable markers have been published in peanut, the highest regeneration and transformation efficiency was reported by Sharma and Anjaiah (2000) using cotyledon explants. In the present study, this protocol was further exploited to generate clean transgenic plants of peanut. The system has been tested with two gene constructs where the transgenic plants were recovered at high frequencies. The system provides a novel way of generating marker-free transgenics, especially in an edible crop like peanut.

Materials and methods

Plant material

For all the experimental procedures on peanut transformation, the cultivar JL 24, a medium duration Spanish type variety was used. The cotyledon explants from presoaked mature seeds were used for the development of transgenic plants. All the conditions used for tissue culture and co-cultivation with *Agrobacterium tumefaciens* were as described previously (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006).

Construction of binary vectors

Binary vector pCAMBIA2300 was used as the backbone for construction of plasmids for *A. tumefaciens*-mediated genetic transformation. To construct the plasmid suitable for marker-free transformation, the 800 bp of *npt*II gene fragment was removed from the plasmid pCAMBIA2300 by restricting with *Xho*I and was re-ligated to obtain the vector pCAMBIA2300*φnpt*II. Two different binary plasmids were then constructed, each with a different gene of interest, viz., rice chitinase (*Rchit*) and maize phytoene synthase (*Zmpsy*1).

The 1.57 kb DNA fragment carrying the 1.1 kb *Rchit* from rice genomic clone along with the CaMV 35S promoter was recovered from the *Hin*dIII digested plasmid pCAMBIA1302:*Rchit*. This 1.57 kb DNA fragment was subsequently cloned into the *Hin*dIII site of the linearized plasmid pCAMBIA2300*φnpt*II. Similarly, the 2.9 kb DNA fragment carrying a 1.2 kb *Zmpsy*1 gene driven by the *Arabidopsis thaliana* oleosin promoter was recovered from the *Bam*HI digested plasmid pCAMBIA2300:*oleopsy*1. This DNA fragment was cloned into the *Bam*HI site of the linearized plasmid pCAMBIA2300*φnpt*II. The ligated DNA products were then introduced into *Escherchia coli* strain DH5α. The orientation and alignment of the fused fragments in the new plasmid were confirmed by restriction analysis and polymerase chain reaction (PCR) with appropriate primers. The selected plasmids were designated as pCAMBIA2300*φnpt*II:*Rchit* (Fig. 1a) and pCAMBIA2300*φnpt*II:*oleopsy*1 (Fig. 1b), respectively. The modified binary plasmids were introduced into the disarmed *Agrobacterium tumefaciens* strain C58 through electroporation.

Genetic transformation

Agrobacterium-mediated genetic transformation of peanut as reported earlier (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006) was employed for the development of transgenic plants. During the whole process of

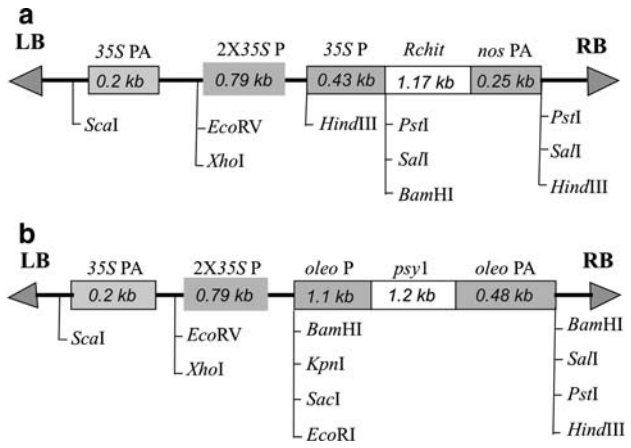

Fig. 1 Schematic representation of the T-DNAs used for peanut transformation without the marker gene. **a** The T-DNA region of pCAMBIA2300*φnpt*II:*Rchit* used to introduce the rice *Rchit*. **b** The T-DNA region of pCAMBIA2300*φnpt*II:*oleopsy*1 used to introduce a phytoene synthase (*Zmpsy*1) gene

regeneration, recovery and rooting of the putative transgenic plants, no selection agent was used. To identify the transgenic plants, the genomic DNA from the in vitro growing shoots was subjected to PCR by using the respective gene primer pairs. The control plants were cultured simultaneously to regenerate untransformed control plants. The T_0 plants were grown to maturity and seeds harvested to obtain the T_1 generation. The transgenic plants in T_0 and T_1 generations were subjected to molecular analysis. Transformation frequency was calculated as [(number of PCR-positive plants/total number of plants produced) × 100].

Molecular analysis

Polymerase chain reaction (PCR)

Initially all the primary transformants in T_0 generation were screened by PCR to determine presence of the gene of interest and identify the putative transformants. Genomic DNA was extracted from the putative transgenics plants and untransformed control plants grown in vitro or in the greenhouse following the modified CTAB method (Doyle 1991). 150 ng of RNase-treated DNA was used for PCR with oligonucleotide primers: 5′ CGC TAA GGG CTT CTA CAC CTA C 3′ and 5′ AGC TTA TCG ATA CCG TCG ACC T 3′ for the *Rchit* gene and 5′ CGG CTT TAG AGA GAG AAT TGA GAG G 3′ and 5′ TCT TCG TCT TGA GCA GGG TGG AGC 3′ were used for *Zmpsy*1 gene. The PCR reaction was performed in a 25 µl reaction mixture containing 150 ng of genomic DNA, 1× PCR buffer (10× PCR buffer: 200 mM Tris HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 10 pM of primer I, 10 pM of primer II, and 1 U of Taq DNA polymerase (Invitrogen®). The total volume was made up to 25 µl with sterile distilled water. The amplification reactions were carried out using a gradient thermal cycler (Eppendorf®). The PCR conditions included an initial denaturation at 95°C for 5 min for 1 cycle followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C (*Rchit)* or 63.4°C (*Zmpsy*1) for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified PCR products were analyzed under UV light following their electrophoresis on 1.2% agarose gel for the detection of 814 bp *Rchit* and 663 bp *Zmpsy*1 amplification fragments.

RT-PCR

RT-PCR analysis of the putative transformants (T_0) was carried out using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, Calif.) on total RNA isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol using the above-mentioned oligonucleotide primers and PCR conditions.

Southern hybridization and dot blot analysis with the genomic DNA

Genomic DNA was isolated from the leaves of the putative transgenic peanut plants carrying the *Rchit* gene using the procedure described by Dellaporta et al. (1983). The genomic DNA (20–25 µg) was digested with *Hin*dIII to release the integrated T-DNA and was separated on a 0.8% (w/v) agarose gel followed by its transfer to a positively charged nylon membrane (Roche Molecular Biochemicals®). Similarly, dot blot assay was performed on *Zmpsy*1 carrying peanut transgenic samples prepared by boiling the genomic DNA for 10 min that was denatured with 1 M NaOH. The denatured DNA was loaded onto nitrocellulose membrane. A non-radioactive DIG-based system (Roche Molecular Biochemicals®) was used for conducting pre-hybridization, hybridization, washing, and detection of the membranes following the manufacturer's protocol. The 1.57 kb rice *Rchit* coding sequence was used as probe for Southern analysis, whereas, for dot blot analysis a 1.2 kb *Zmpsy*1 coding sequence was used as probe. For autoradiography, the blots were exposed to X-Omat film (Eastman Kodak Company, Rochester, NY, USA) for 15–30 min.

Results and discussion

Plant transformation

The optimized plant regeneration and transformation system using cotyledon explants of peanut was reliable,

reproducible, efficient and capable of producing independently transformed plants directly through organogenesis via the development of multiple shoots without any callus phase. The regeneration frequencies ranged from 32 to 48% which is lower than what we observed in our previous studies with peanut where the regeneration frequency was over 75% (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006). It is assumed that this could be due to the nature of the transgenes and the promoters used in these studies. However, the average transformation frequencies in both the previous and the present studies ranged between 55 (Sharma and Anjaiah 2000) to 77% (Table 1). Survival rate of the in vitro regenerated plants was over 90% and about 30 and 60 putative transgenic plants transformed with *Zmpsy*1 and *Rchit* genes, respectively, were transferred to the greenhouse (Table 1). Molecular analysis of T_0 and T_1 plants through PCR, RT-PCR, Southern blot and dot blot analyses proved the existence of transgenes and absence of selectable marker in the transformants. It is interesting to note that in our ongoing studies on the genetic transformation of other legumes including pigeonpea and chickpea, the transformation efficiency in the former using the marker-free system was as good as peanut (Sharma et al., unpublished results). However, the transformation efficiency was much lower (~35%) in the latter species (Sharma et al., unpublished results). These observations indicate that the success of this marker-free system in different crops lies in the robustness of the regeneration protocol besides the amenability of the tissues to *Agrobacterium*.

Polymerase chain reaction (PCR) and RT-PCR

Out of 30 *oleo:psy1* transformants (T_0), 23 were found to be positive for the amplification of the 663 bp fragment of the *Zmpsy*1 gene (Fig. 2a), while of the 60 transformants carrying *35S:Rchit* (T_0), 45 were found to be positive for the amplification of 814 bp *Rchit* fragment by PCR (Fig. 2b). No amplification was observed in DNA from the untransformed control plants. The transformation efficiency using *Zmpsy*1 and *Rchit* in the T_0 transformants was over 75%. The expression of *Rchit* and *Zmpsy*1 gene in the T_0 plants was analyzed by RT-PCR where out of the 45 PCR-positive T_0 plants, only 20 plants tested positive for *Rchit* gene and out of the 6 PCR-positive T_0 plants tested, 4 plants were positive for *Zmpsy*1 gene (Fig. 3a, b). Nine events carrying *Zmpsy*1 gene and five events carrying the *Rchit* gene from T_0 generation when advanced to T_1 generation showed inheritance and segregation of the introduced genes in a 3:1 Mendelian ratio (Table 2) based on amplification of *Zmpsy*1 gene and *Rchit* gene in T_1 generation plants (Fig. 4a, b).

Southern hybridization and dot blot analysis

PCR-positive events were analyzed by Southern blot hybridization and dot blot assay using 1.57 kb *Rchit* and 1.2 kb *Zmpsy*1 fragment, respectively, as probes to determine the T-DNA integration. Southern blot analysis of nine selected events indicated the integration of *Rchit* gene in three events, viz., 18, 32 and 70. No hybridization signal was detected in the untransformed control plants (Fig. 5a). Dot blot assay was performed with nine selected events using denatured genomic DNA (10 μg) where five events, viz., C, E, F, G, T showed integration of the transgene (Fig. 5b).

The antibiotic resistance genes have gained importance in selecting the transformants from the non-transformants in the process of producing transgenic plants. Since these are used only as a tool of selection and do not code for any desirable traits, their presence in the transgenic plants is not only unnecessary but may disturb the genetic constituency of the plant and its wild varieties in some cases. Therefore, gene products need to be assessed for safety and environmental impact (Bryant and Leather 1992; Gressel 1992). In view of this, it is necessary to look for alternatives for safer

Table 1 Frequency of recovery of marker-free transgenic plants of peanut by using cotyledonary explants from three separate experiments with pCAMBIA2300*φnpt*II:*Rchit* and pCAMBIA2300*φnpt*II:*oleopsy*1 gene constructs

Expl no.	Gene	Number of explants	Independent events recovered post selection	Regeneration frequency of transformants post selection (%)	PCR/RT-PCR positives	Transformation frequency (%)
1	*Rchit*	50	20	40	12	60
2	*Rchit*	50	22	44	17	77
3	*Rchit*	50	18	36	16	88
4	*Zmpsy*1	25	8	32	8	100
5	*Zmpsy*1	25	10	40	7	70
6	*Zmpsy*1	25	12	48	8	66
Mean primary transformants obtained						77

Transformation frequency was determined on the basis of PCR analysis of the independent transformants

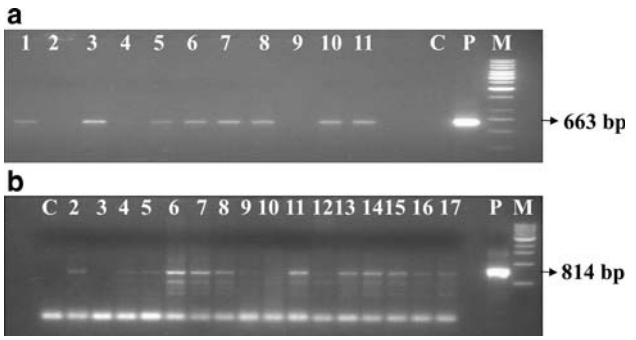

Fig. 2 PCR analysis for *Zmpsy*1 and *Rchit* gene in T_0 transformants of peanut. **a** Lanes *1–11* carry samples from putative transformants with *Zmpsy*1 gene, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*oleopsy*1 plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 663 bp. **b** Lanes *2–17* carry samples from putative transformants with *Rchit* gene, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*Rchit* plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 814 bp

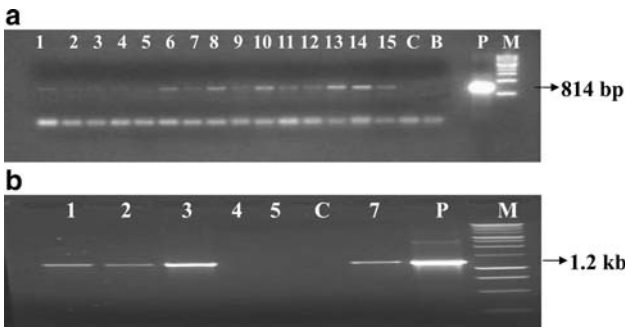

Fig. 3 RT-PCR analysis for *Rchit* and *Zmpsy*1 gene in T_0 transformants of peanut. **a** Lanes *1–15* carry samples from putative transformants, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*Rchit* plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 814 bp. **b** Lanes *1–7* carry samples from putative transformants, *B* blank, *P* pCAMBIA2300*φnpt*II:*oleopsy*1 plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 1.2 kb

marker genes or elimination of the marker genes from transgenic plants to produce environmentally safe transgenic plants and pyramid a number of transgenes by repeated transformation (Yoder and Goldsbrough 1994). Most alternatives are still in their development phase and are not widely available.

Here we report for the first time, the production of marker-free transgenic plants of peanut with such high transformation efficiency (over 75%). This study avoids the introduction of antibiotic resistance marker genes in plant cells, thus eliminating the risk of horizontal gene transfer, if any, and also mitigating vertical gene transfer. Our method is free from negative effects of selective agents that can limit the ability of transgenic cells to proliferate and differentiate into transgenic plants. In the present study,

Table 2 Inheritance of the *Zmpsy*1 or *Rchit* gene in the respective T_1 generation progeny of transgenic peanut carrying marker-free construct pCAMBIA2300*φnpt*II having either the *Zmpsy*1 or *Rchit* gene

Event#	Gene	Total no. of T_1 plants tested per event	PCR analysis of the transgene		$\chi^2_{3:1}$
			+ve	−ve	
A	*Zmpsy*1	9	7	2	0.037
B	*Zmpsy*1	7	4	3	1.190
C	*Zmpsy*1	14	12	2	0.857
D	*Zmpsy*1	6	6	0	2.000
E	*Zmpsy*1	5	3	2	0.600
F	*Zmpsy*1	4	4	0	1.330
G	*Zmpsy*1	14	11	3	0.095
H	*Zmpsy*1	7	4	3	1.190
J	*Zmpsy*1	3	3	0	1.000
18	*Rchit*	14	12	2	0.860
32	*Rchit*	16	14	2	1.330
70	*Rchit*	15	13	2	1.090
75	*Rchit*	13	11	2	0.640
77	*Rchit*	15	13	2	1.090

χ^2 for $P = 5\%$ is 3.84

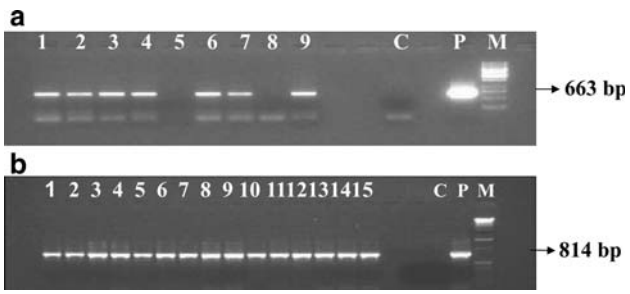

Fig. 4 PCR analysis for *Zmpsy*1 and *Rchit* gene in T_1 progenies of independent transgenic events of peanut. **a** Lanes *1–9* carry samples from putative transformants with *Zmpsy*1 gene, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*oleopsy*1 plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 663 bp. **b** Lanes *1–15* carry samples from putative transformants with *Rchit* gene, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*Rchit* plasmid as positive control, *M* 1 kb molecular weight marker, and *arrow* indicates a fragment of approximately 814 bp

plants were regenerated through direct organogenesis pathway using *Agrobacterium*-mediated gene transfer from cotyledon explants that involves a very short regeneration phase of 2–3 weeks only as reported earlier by Sharma and Anjaiah (2000). Direct regeneration systems have advantages due to the rapidity of morphogenesis and lack of frequent subcultures, besides an extremely rapid and synchronous de novo production of shoots (Bhatnagar-Mathur et al. 2008). Recently, Li et al. (2009) reported a non-selection approach for tobacco transformation where the

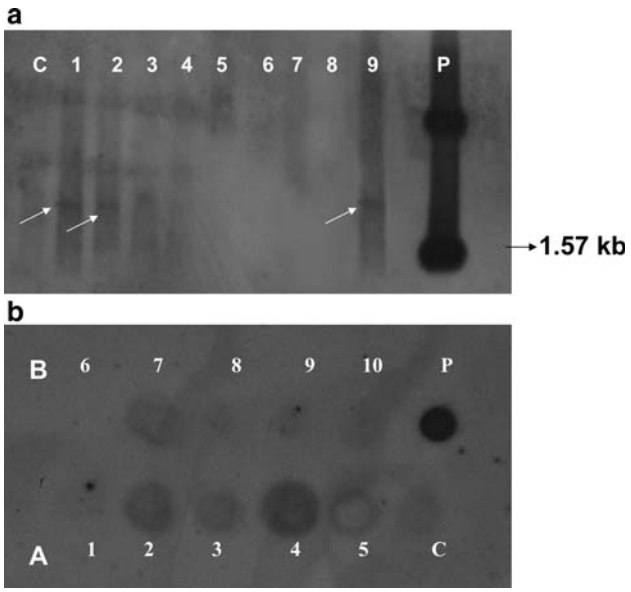

Fig. 5 Southern and dot blot analysis of primary transformants of peanut. **a** Southern blot analysis using the genomic DNA digested with *Hin*dIII enzyme to release the integrated T-DNA region and probed with 1.57 kb *Rchit*. Lanes *1*, *2*, and *9* carry sample from events 18, 32 and 70 and lanes *3–8* carry sample from events 15, 16, 56, 77, 75, and 72, *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*Rchit* plasmid restricted with *Hin*dIII to release the 1.57 kb *Rchit* gene fragment. **b** Dot blot assay of transformants carrying *Zmpsy*1 gene, probed with 1.2 kb *Zmpsy*1 coding sequence fragment. Lane *A*: *1–5* carry samples from events K, G, F, E, and C, and lane *B*: *6–10* carry samples from events S, T, L, M, and N. *C* untransformed control, *P* pCAMBIA2300*φnpt*II:*oleopsy*1 plasmid as positive control

transformation efficiency was quite low. However, in our study with peanut, the transformation efficiency obtained is comparable to that reported earlier using the selectable marker system (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006). In these studies, all the shoots arising from one explant were considered as a single event; however, upon molecular characterization, these differed in the gene integration pattern, and hence the efficiency could be much higher than what was reported (55%).

In the previously reported studies using non-antibiotic methods for producing transgenic plants, the efficiency obtained from these studies has been very low where the frequency of marker-free transgenic plants was 2.22% in rye (Popelka et al. 2003), 4.5–5% in potato (de Vetten et al. 2003), 6.25% in potato (Ahmad et al. 2008), 22–25% in apple (Malnoy et al. 2007), 1.55% in wheat and 0.93% in triticale (Doshi et al. 2007), 15% in tobacco (Jia et al. 2007), 7% in alfalfa (Weeks et al. 2008) and 2.8% in tobacco (Li et al. 2009). In contrast, the transformation efficiency reported here is the highest (over 75%) so far and is also comparable to frequencies obtained using the selectable marker system (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006). This could be attributed to the highly efficient regeneration and transformation protocol used in this study. Besides, the earlier reports show no data on stable transmission of transgene into progenies using non-antibiotic approach for the development of marker-free transgenic (De vetten et al. 2003; Jia et al. 2007), whereas our study demonstrated expression and integration of transgene into progenies and Mendelian inheritance of the transgenes similar to results obtained by Li et al. (2009).

Our method for producing marker-free plants possesses several advantages as compared to other approaches such as co-transformation (Dutt et al. 2008), recombinase systems (Gleave et al. 1999; Zuo et al. 2001; Arumugam et al. 2007), transposase driven system (Goldsbrough et al. 1993), intrachromosomal recombination system, multi-auto transformation (MAT) vector system (Saelim et al. 2009) in terms of having a single step process without involving the genetic segregation and having less chances of producing chimeras. The phenomenon of chimerism due to incomplete DNA excision has also been reported (Gleave et al. 1999; Sugita et al. 1999; Zuo et al. 2001; Schaart et al. 2004). Moreover, transposon excision, recombinase-based methods and intrachromosomal recombination methods are more prone to somaclonal variations and lead to genomic instability in transgenic plants (Scutt et al. 2002; Darbani et al. 2007). Also, the efficiency of transposon-based system is low due to tendency of transposable elements to insert elsewhere in genome. Besides, the expression of microbial recombinases for prolonged periods in plant cells may result in unwanted changes to the genome at sites removed from transgene insertions (Scutt et al. 2002). Although, the isopentenyl transferase (*ipt*) gene has been used as a selectable marker for transformation of many plant species (Ebinuma and Komamine 2001), the system was not very efficient, and the use of the *ipt* selectable marker may require the optimization of transformation protocols due to changes in tissue culture conditions. The advantages of the present method over the previously reported ones are as follows:

1. No selection pressure is required during the transformation process and PCR analysis is done to distinguish transformed from the untransformed shoots/plants. Taking into account the labor involved in this approach, Popelka et al. (2003) applied sample pooling strategy to facilitate the identification of marker-free transgenic rye plants by PCR.
2. So far, most of the studies on developing marker-free transgenic plants had used the *uid*A (GUS) gene for establishing the proof of concept and also in the construct used for final event development (Jia et al. 2007; Weeks et al. 2008). Hence, such reporter gene has a potential to remain in the final selected event.

However, since our objective was to develop clean transgenic events for practical applications on crop improvement, no reporter gene was included in the transformation vector.

3. This method of producing a high number of independently transformed plants within a short period is applicable to all vegetatively and sexually propagated crops. In contrast, the co-transformation method cannot be used for vegetatively propagated crops as genetic segregation is needed to select the marker-free plants (Scutt et al. 2002). Besides, all recombinase and intrachromosomal recombination method-based systems require sexual crosses for removal of recombinase gene and hence, cannot be used with vegetatively propagated plants (Scutt et al. 2002).
4. A limited number of constitutive promoters are commonly used to express marker genes, and their repeated introduction could activate gene-silencing mechanisms with negative effects on the expression of one or more transgenes of interest (Puchta 2003). Therefore, a marker-free transformation system proves to be better option for multiple gene pyramiding.

In conclusion, marker free-based transformation system reported in this paper is highly reproducible and did not require further optimization of regeneration protocol, including the use of hormones or antibiotic selection, and could also very well be applied to other economically important crops for which efficient regeneration protocols are available. This is the first report on the development of marker-free (both selectable and reportable) transgenic peanuts using an one-step procedure. This technique has a potential for generating clean transgenic peanut plants with economically important traits. Besides, absence of selectable marker genes would circumvent the need to confirm the biosafety of these genes; thereby, facilitating the development of a regulatory approval package and also lower the costs for commercial release of new genetically modified products (Kuiper et al. 2001; Daniell 2002; Smyth et al. 2002).

Acknowledgments We acknowledge the financial support from HarvestPlus, the global Challenge Program on Biofortification for part of this work. MB and KP would like to thank the Jawaharlal Nehru Technological University (JNTU), Hyderabad, India for providing an opportunity to register for Ph.D. program. The excellent assistance by the technical staff of the Genetic Transformation laboratory at ICRISAT is duly acknowledged.

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