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Bar as a Potential Selectable Marker Gene to Obtain Putative Transformants in Indian Chickpea (*Cicer arietinum* L.) Cultivars

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ABSTRACT

A reproducible and efficient transformation method was demonstrated for two Indian desi chickpea (*Cicer arietinum* L.) cultivars using *bar* as a selectable marker gene. In order to select the transformants in tissue culture medium, 2.5 mg/l of phosphinothricin was optimized to use as a selective agent in regeneration and selection medium. Cotyledonary explants containing half embryonic axes were infected and co-cultivated with *Agrobacterium* strain harboring binary plasmid (pBK16.2) containing a chimeric *cre* (Cyclization recombination) gene of bacteriophage P1 and a *bar* gene as selectable marker gene. The frequencies of putative transformed plants were 1.23% in cv. 'ICCV 89314' and 1.12% in cv. 'Vijay'. Explant to plant duration was 61-71 days. Putative transformed plants/shoots that were selected on regeneration and selection medium were confirmed by PCR analysis for the presence of transgenes. A "leaf painting assay" using 0.6 mg/ml of phosphinothricin confirmed expression of the *bar* gene in the putative transformed plants. This protocol generated a slightly higher frequency of putative transformed plants in our laboratory when compared to generation of transformed chickpea plants using *npt*II as the selection marker gene.

Keywords: *Agrobacterium*, *nptII*, phosphinothricin, genetic transformation

INTRODUCTION

India accounts for more than 67.3% of the world's chickpea production (FAOSTAT Report 2004, hptt://foastat.fao.org). Chickpea occupies highest position in production as well as in area under cultivation among the grain legumes in India and the third position in the world. In spite of its important contribution to the world agriculture, it yields only around 0.8 tons/hectare whereas its estimated yield potential is 5 tons/ha (Sharma and Lavanya 2002). Genetic improvement of chickpea by conventional breeding methods is limited mainly due to the non-availability of desired traits in chickpea germplasm or the linkage of desired traits with undesired characteristics (Popelka and Higgins 2007). But recent advances in gene transfer technology have made it possible to incorporate foreign genes for desired agronomic traits. One of the most widely used genetic engineering tools to develop transgenic plants is *Agrobacterium tumefeciens*-mediated genetic transformation (Karami 2008). Chickpea transformation has been extensively reviewed (McPhee *et al.* 2006; Uncuoglu *et al.* 2008) and the literature shows that even though there are only two reports of using phosphinothricin (ppt) as a selective agent (Tewari-Singh *et al.* 2004; Krishnamurthy *et al.* 2000), in both experiments the phosphinothricin-acetyltransferase (*pat*) gene was used, which is very similar to *bar* in action (Wehrmann *et al.* 1996) and there is only one report of chickpea transformation using the *bar* gene (Senthil *et al.* 2004).

Phosphinothricin is a commonly used herbicide that inhibits a key enzyme of nitrogen assimilation in both bacteria and plant cells. The *bar* gene encoding Phosphinothricin Acetyl Transferase enzyme, can detoxify its effect, (Thompson *et al.* 1987) is often used as a plant selectable marker gene. In this work we are reporting optimization of ppt concentration for four desi chickpea cultivars to use *bar* gene as selection marker gene and genetic transformation is demonstrated in two cultivars, namely 'ICCV-89314' and 'Vijay'. A robust and reliable protocol for transformation of chickpea using *nptII* as selection marker gene has already been reported (Sarmah *et al.* 2004). The purpose of developing a protocol using *bar* gene in our laboratory is to develop transgenic chickpeas with *cre* gene from bacteriophage P1. Such Cre plant will be then utilized for crossing with transgenic chickpeas harbouring *Bt-Cry1Ac* gene (developed under the Indo-Swiss collaborative projects at Assam Agricultural University {AAU}, Jorhat and at Bose Institute, Kolkata) and Floxed *nptII* (*nptII* gene flanked by Lox sites of bacteriophage P1) to facilitate site specific recombination at Lox sites in order to eliminate *nptII* gene in segregating progeny. *Cre* gene encodes the enzyme recombinase and facilitates recombination at Lox sites. Crossing Cre plant, harbouring *bar* gene to Cry1Ac plant that harbours Floxed *nptII* gene will facilitate easy identification of segregating progeny that are devoid of *nptII* gene through simple PCR analyses.

We have also demonstrated that chickpeas were transformed using *bar* as a selection marker gene with similar or even slightly higher efficiency than the use of *nptII* as the selection marker gene.

MATERIALS AND METHODS

Plasmid and *Agrobacterium* strain

The plasmid pBK16.2 containing the T-DNA harbouring *cre* gene (of bacteriophage P1) and *bar* as the selectable marker gene (**Fig. 1A**) was reconstructed in the laboratory of B. Hohn at Fredrich Meischer Institute (FMI), Basel under the Indo-Swiss Collaboration in Biotechnology Programme, which is being operated at Department of Agricultural Biotechnology, AAU, Jorhat. The *cre* gene is the part of the "*cre-lox*" system used for marker gene elimination. Both *cre* and *bar* genes are driven by 35S promoter from the cauliflower mosaic virus (CaMV) and terminated by 35S terminator (original vector was pCAMBIA 33001).The binary

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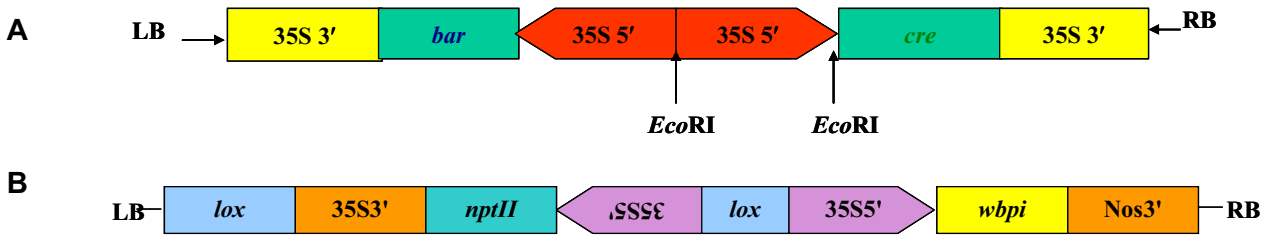

Fig. 1 Plasmid constructs used in this study. (**A**) T-DNA region of plasmid pBK 16.2 used in transformation experiment. LB: Left border, RB: Right border, 35S 3′: Terminator from CaMV, 35S 5′: Constitutive promoter from CaMV, *cre*: Cyclization recombination gene of bacteriophage P1, *bar*: Bialaphos resistance gene. (**B**) T-DNA region of plasmid pBK11/35S/747 used in transformation experiment. LB: Left Border; RB: Right Border; *lox*: Locus of crossing over from bacteriophage PI; Nos 3′: Terminator from *Agrobacterium tumefaciens*; *nptII*: neomycin phosphotransferase II from transposon Tn5 (selectable marker gene); 35S 5′: Constitutive promoter from CaMV; *wbpi*: wing bean proteinase inhibitor gene.

plasmid pBK11/35S/747 (**Fig. 1B**) carries chimeric wing bean proteinase inhibitor (*wbpi*) gene and neomycin phosphotransferase II (*nptII*) as selection marker gene. This vector was used to transform chickpeas using *nptII* as selection marker gene. The *Agrobacterium* strain GV-3101 (obtained from B Hohn of FMI, Basel) was used for the present investigation.

Transformation of plasmid into *Agrobacterium*

The plasmid pBK16.2 and pBK11/35S/747 were transformed into GV-3101 strain in separate experiments using the Bio-Rad Micro Pulser System wherein a high voltage electrical pulse was applied to the sample suspended in a small volume of high resistance medium. Electrocompetent cells of the *Agrobacterium* strain GV-3101 was prepared following the method described by Lin (1995). Electroporation of plasmid into the electrocompetent cells was done following the protocol provided by the manufacturer of Bio-Red Micro Pulser System.

Planting materials

In the present investigation, seeds of four desi chickpea cultivars namely 'Vijay', 'ICCV-89314', 'C-235' and a variety collected from the local market were used as sources of plant material. Seeds of 'Vijay' were obtained from Mahatma Phule Krishi Vidyalaya, Rahuri, Maharastra and 'ICCV 89314' and 'C-235' seeds were obtained from the International Crop Research Institute for the Semi Arid Tropics (ICRISAT), Patencheru.

Optimization of ppt concentration for transformation study

For the use of *bar* as selectable marker gene it was necessary to optimize a minimum concentration of ppt in regeneration and selection medium for elimination of untransformed cells. The cotyledonary explants with half embryonic axes were prepared in liquid MS0 (MS basal) medium from the four desi cultivars viz. 'Vijay', 'ICCV-89314', 'C-235' and the local market variety and kept for about an hour and then inoculated in B5 medium (Gamborg e*t al.* 1968) supplemented with 1 mg/l BAP + 1 mg/l NAA for three days. Explants were then washed thoroughly 3-4 times with sterilized distilled water and blotted dry on sterile filter paper. Then 40 numbers of explants from each cultivar were inoculated in first regeneration and selection (RSI) (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin + 0.05 mg/l NAA + 10 mM MES) medium with five different concentrations of ppt (Sigma Chemicals Co., US) i.e. 0, 0.5, 1.5, 2.5, 3.5 and 4.5 mg/l along with 250 mg/l cefotaxime (antibiotic used to kill *Agrobacterium*) to nullify any effect of it in presence of ppt. After 10-14 days the explants producing green healthy shoots were sub-cultured to second regeneration and selection (RSII) (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin + 10 mM MES + 250 mg/l cefotaxime) medium. After 10-14 days, on the number of dead shoots under selection at different concentrations of ppt and percentage of explants containing dead shoots was calculated in order to determine the optimum concentration of ppt that causes more than 90% death of the un-transformed explants and shall be considered as optimum concentration in transformation protocol.

Transformation protocol

Agrobacterium culture was prepared by growing single colony of *Agrobacterium* strain GV-3101 harbouring the binary plasmid pBK16.2 in liquid MGL medium (An 1987) supplemented with kanamycin (for bacterial selection) and rifampicin at 50 mg/l and 100 mg/l respectively. The culture was incubated at 28°C for 24 h in an environmental shaker at 150-200 rpm. The suspension having O.D. value of 0.6-1.0 at 600 nm was used to infect the explants. The cotyledonary explants with half embryonic axes (**Fig. 2A**) were infected by dipping them in *Agrobacterium* suspension culture in a Petri dish (90 mm diameter) for 45 min to 1 h at room temperature (22-25°C) under the Laminar hood and then the explants were inoculated in co-cultivation medium (B_5 + 10 mM MES + 1 mg/l NAA + 1 mg/l BAP + 10 mM acetosyringone) at 25

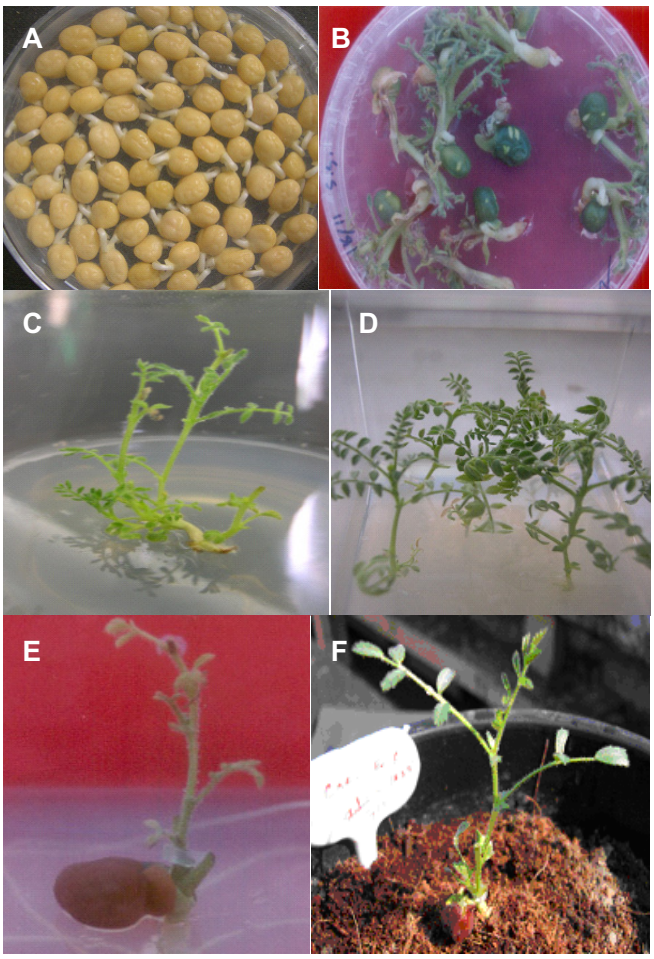

Fig. 2 *In vitro* regeneration and transformation of Chickpea using *bar* as selection marker gene. (**A**) Cotyledonary explants with half embryonic axes co-cultivated with *Agrobacterium.* (**B**) Initiation of shoots in RSI. (**C**) Elongated shoots in RSIII. (**D**) Individual shoots in RSIV and RSVI. (**E**) Grafted shoot. (**F**) Grafted plant transfer to soil.

± 2°C under light intensity of 3000-4000 lux with 16 h photoperiod. In co-cultivation medium 60-70 explants were plated per Petri dish (90 mm × 15 mm). After 3 days of co-cultivation, the explants were washed thoroughly 3-4 times with sterile distilled water, blotted on sterile filter paper and 10 numbers of explants were plated on RSI (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin + 0.05 mg/l NAA + 10 mM MES + 2.5 mg/l ppt + 250 mg/l cefotaxime) in 90 mm × 15 mm Petri dish (**Fig. 2B**). After 10 days, the cotyledons and roots if any were removed from the explants and sub-cultured into RSII medium (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin + 10 mM MES + 2.5 mg/l ppt + 10 mM MES + 250 mg/l cefotaxime) in a 250 ml conical flask. After 10 days, the green shoots were sub-cultured to RSIII medium (MS + 0.1 mg/l BAP + 0.1 mg/l Kinetin + 10 mM MES + 2.5 mg/l ppt + 250 mg/l cefotaxime) (**Fig. 2C**). In RSII and the subsequent cultures 5-6 explants were cultured per conical flask (250 ml). Only green, healthy graftable shoots were transferred to subsequent regeneration and selection medium. Individual shoots surviving selection were sub-cultured for another two or three cycles in the RSIII (**Fig. 2D**). All these cultures were kept at 25 ± 2°C temperature and under the light intensity of 3000-4000 lux with 16 h photoperiod. The green and healthy shoots were grafted onto the non-transgenic rootstocks following the procedure as described by Sarmah *et al*. (2004) (**Fig. 2E**). Grafted plants were kept in similar culture condition as described above for about 7 days. After 7 days, the grafted plants were transferred to earthen pots containing Cocopit mixture (Rajdeep Agency, New Delhi, India) and soil in ratio of 1:3 (**Fig. 2F**). The plants are hardened in polyhouse by gradual exposure to sunlight.

The transformation protocol using *nptII* as a selection marker gene followed that as described by Sarmah *et al*. (2004).

Molecular analyses of transgenic chickpea

Genomic DNA was isolated using Watman Gene Spin Plant DNA purification kit Cat. No. 4344004 and subjected to PCR analyses to confirm presence of transgenes. The *bar*, *cre* and *nptII* specific primers were designed from sequences available in the NCBI database and the *wbpi* specific primers were designed and kindly provided by Dr VS Gupta, NCL, India (**Table 1**). The annealing temperature for the PCR reactions was kept at 54°C and the elongation temperature was 72°C. The PCR amplification products were then subjected to electrophoresis in 1% agarose gel.

Table 1 PCR primers.

Primers	Sequence (5′–3′)
bar forward	GCACCATATCGTCAACCACTACATCG
bar reverse	AGCTGCCAGAAACCACGTCATG
crer forward	GCGCGCGAATTGTTCATCGAACAATGGCTACCG
cre reverse	GCGCGCAAGCTTGAATTAATTCCTAATCGCCATC
nptII forward	GGAGCGATACCGTAAAGC
nptII reverse	GAGGCTATTCGGCTATGACTG
wbpi forward	ATGAAGAGTACTACATTTCTTGC
wbpi reverse	GCATTCATTTAGTCATAAGC

Leaf painting assay for confirmation of *bar* gene expression

To optimize the ppt concentration for the assay, various concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of water) of ppt were used. With a cotton brass, around 250 µl of each solution was painted on both the sides of 5 tender leaves of untransformed chickpea plant in five replications. The lowest concentration of ppt, which killed the leaves in all replication after one week, was considered to be optimum for the leaf-painting assay.

The leaves of transformed plants were painted using optimum concentration of ppt as described above and observations were compared with control (untransformed plant) after 7 days.

RESULTS AND DISCUSSION

Optimization of ppt concentration

In the present investigation six different concentrations of ppt were used to determine the lowest level of ppt, which could inhibit the shoot proliferation and cause maximum shoot death (more than 90%) of wild type chickpea in RSII. The number of explants died due to the effect of ppt was recorded (**Table 2**). In RSI, it was observed that shoot regeneration ability of the explants reduced with the increased concentration of ppt (**Fig. 3**). A concentration of 2.5 mg/l of ppt was found to be optimum for inhibiting regeneration of 90.0% to 97.5% of the wild type (untransformed) explants in RSII in case of all four cultivars. In the subsequent cycles 100% mortality occurs. When ppt was used at a concentration of 3.5 or 4.5 mg/l, 100% explants died, mostly after the RSI. Such high concentration of selection pressure does not allow the weak expressers (for *bar* gene) to grow as ppt leads to accumulation of toxic levels of ammonia in untransformed plant cells. This high level of ammonia can even cause harm to the transformed cells. Therefore, it is not desirable to apply a very high concentration of ppt in the initial selection cycle. Sarmah *et al*. (unpublished) used 5 mg/l ppt to transform Australian desi chickpea cultivars 'Semsen' and 'Amethyst' while introducing the bean α-*amylase inhibitor* gene and *Nicotiana alata protease inhibitor* gene. The genotype-specific variation may be the reason of such different levels of resistance to the ppt in different chickpea cultivars of Indian and Australian origin. Similar findings were also reported by Senthil *et al.* (2004)

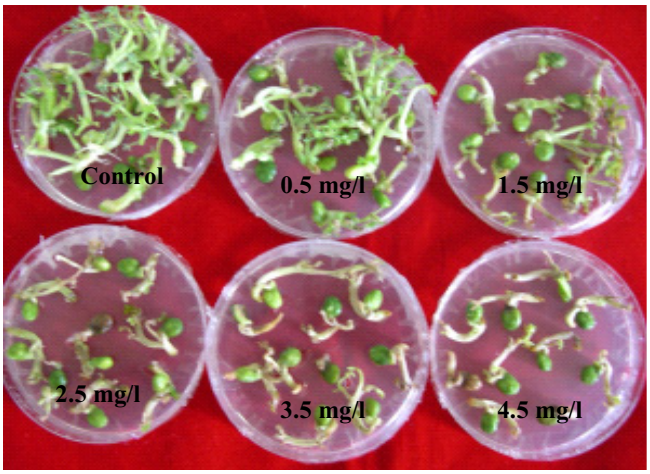

Fig. 3 Effect of different concentrations of ppt in wild type chickpea shoot regeneration during RSI (cv. 'ICCV-89314').

Table 2 Effect of ppt after second regeneration and selection cycle on wild type explants of four desi cultivars.

ppt conc.[n] (mg/l)	№ of shoots/explant								% Mortality			
	Survived in RSI				Survived in RSII							
	Vijay	ICCV	C235	Local	Vijay	ICCV	C235	Local	Vijay	ICCV	C235	Local
0	40	40	40	40	40	40	40	40	0	0	0	0
0.5	39	36	39	40	31	28	32	36	22.5	30	20	10
1.5	26	18	14	18	9	6	8	10	77.5	85	80	75
2.5	17	8	8	6	2	1	2	4	95	97.5	95	90
3.5	6	2	6	2	0	0	0	0	100	100	100	100
4.5	0	0	0	0	0	0	0	0	100	100	100	100

Table 3 Frequency of putative transformants in chickpea using binary plasmid pBK16.2 harbouring *bar* as selectable marker gene.

Cultivars	No explants co-cultivated	No. of explants survived after RSV/RSVI	No. of graftable shoots	Grafted plants transferred to poly house	No. of PCR-positive plants from individual transformation events	Frequency (%) of putative transformed plant
ICCV-89314	567	8	11	11	7	1.23
Vijay	247	3	3	3	3	1.21

Table 4 Frequency of putative transformants in chickpea using binary plasmid pBK11/35S/747 harbouring *nptII* as selctable marker gene.

Cultivars	No explants co-cultivated	No. of explants survived after RSV/RSVI	No. of graftable shoots	Grafted plants transferred to poly house	No. of PCR-positive plants from individual transformation events	Frequency (%) of putative transformed plant
ICCV-89314	1000	8	10	10	8	0.8
Vijay	830	5	9	9	5	0.6

but they started the selection process at a later stage with 2.5 mg/l of ppt in the selection media, which means a large number of explants had to be maintained throughout the selection cycles and the chance of escapes would have been higher. So by exposing the explants to the selection medium from first regeneration cycle itself, we reduced the handling time and cost. Moreover, the chance of escapes was also reduced.

Agrobacterium-mediated genetic transformation

As all four cultivars had similar response to various ppt concentrations, two cultivars viz. 'ICCV-89314' and 'Vijay' were selected for transformation experiments to confirm if the *bar* gene could be used to transform Indian desi chickpea cultivars. In cultivar 'ICCV-89314' out of 567 explants, 7 putative transformed plants were positive for the *cre* and *bar* genes while in 'Vijay' out of 247 explants, 3 putative plants survived a final cycle of selection, giving a putative transformation frequency of 1.23 and 1.21% for cultivar 'ICCV-89314' and 'Vijay', respectively (**Table 3**). In a similar report on chickpea transformation using the *bar* gene, the overall frequency of transformation was 5.1% (Senthil *et al.* 2004). The overall frequency of transformation was high compared to our protocol. However, many escapes were observed during early selection cylces and it took 22 to 28 weeks from explant to the plant establishment. In the present investigation, shoots were subcultured after 10 days of each regeneration and selection cycle. Under normal circumstances as described by earlier workers (Das *et al*. 2002; Das and Sarmah 2003; Sarmah *et al*. 2004), subculturing was done after 14 days. So, the present procedure saves around 21 days in an experiment starting from seed cutting to plant establishment, thus producing putative transformed plants within 61-71 days saving time, cost and labor, which is the most crucial point for the commercialization of the transformation protocol. Furthermore, as the ammonia produced in the untransformed parts could diffuse into the transformed shoots and cause death of the transformed shoots, frequent subculturing is also beneficial from this point of view. The time requirement for plant establishment from starting explant is also much shorter in present protocol compared to the protocols of Polowick *et al.* (2004) and Sanyal *et al.* (2005) where the *nptII* gene was used as the selectable marker gene.

As transformation frequency may vary from lab to lab depending on the skill of the researcher and laboratory conditions, a comparative study of *bar* and *nptII* as selectable marker genes was carried out in order to confirm if '*bar*' could be a better selection marker gene for chickpea transformation. The putataive transformation frequencies in 'Vijay' and 'ICCV-89314' using *nptII* as selectable marker gene were 0.6% and 0.8, respectively (**Table 4**). Therefore from the present study it has been clear that *bar* can be used with similar or even slightly higher efficiency as a selectable marker gene for the genetic transformation of chickpea compared to *nptII*.

DNA isolation and transgene detection through PCR

DNA was isolated from the leaves of eight putative plants of cultivar 'ICCV-89314'. Binary plasmid pBK16.2 containing *bar* and *cre* gene, was used as positive control in PCR reaction. All the samples were then amplified using *bar* and *cre* gene specific primers and the PCR product were run on 1% agarose gel (**Fig. 4A, 4B**). In both the gels Lane No. 1 contained 1 kb ladder as marker and in Lane No. 2, plasmid DNA (pBK16.2) was run as a positive control while in Lanes No. 3 and 4 water and untransformed chickpea leaves were run respectively as negative controls. DNA samples from the putative transformed plants were run in Lanes No. 5 to 12. In *bar* gene amplification an expected 0.45 kb amplified band was observed in the positive control and in 7 out of 8 samples tested (**Fig. 4A**). In *cre* gene amplification an expected 1.2 kb band was observed in the positive control and in all the test samples that showed amplification for *bar* gene (**Fig. 4B**).

PCR amplification of *nptII* and *wbpi* genes were carried out with five putative transformed plants of 'Vijay' and eight putative transformed plants of 'ICCV-89314'. Binary plasmid pBK 11/35S/747 containing *nptII* and *wbpi* genes was used as a positive control in PCR reaction. All 13 putative plants ware found to be positive for both *nptII* and *wbpi* genes.

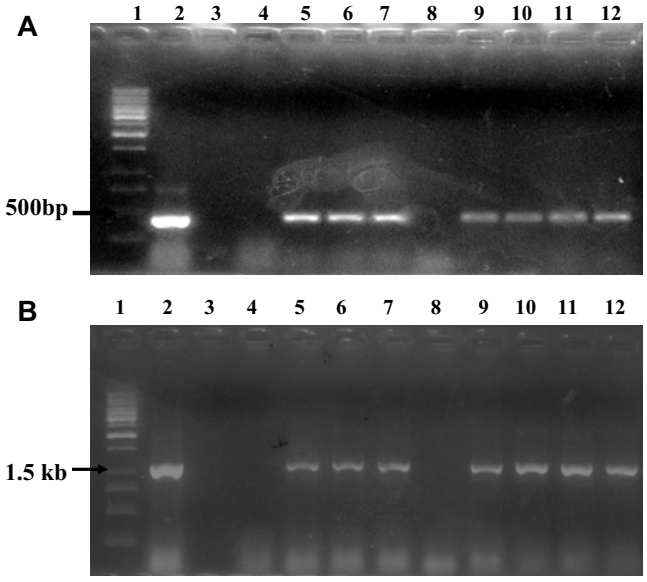

Fig. 4 PCR amplification. (**A**) *bar* gene. Lane 1: 1 kb ladder; Lane 2: Positive control; Lane 3: Negative control (water), Lane 4: Negative control (wild type chickpea leaves); Lanes 5-12: Putative transformed plant. (**B**) *cre* gene. Lane 1: 1 kb ladder; Lane 2: Positive control; Lane 3: Negative control (water), Lane 4: Negative control (wild type chickpea leaves); Lanes 5-9: Putative transformed shoots; Lanes 10-12: Putative transformed plants.

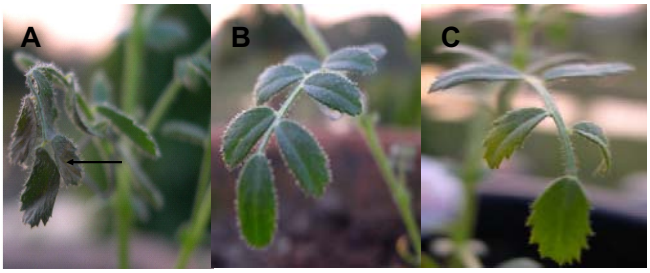

Fig. 5 Leaf painting assay using 0.6mg/l ppt to confirm expression of *bar* gene in two primary transgenics (Sh1 and Sh2). (**A**) Leaves of wild type chickpea painted with ppt. (**B**) Leaves of primary transgenic line Sh1 painted with ppt. (**C**) Leaves of primary transgenic line Sh2 painted with ppt.

Leaf painting assay

An attempt was made to detect the expression of *bar* gene in the established plantlets through leaf painting assay. The assay was performed with the plantlets that produced sufficient leaves after establishment in soil. A ppt concentration of 0.6 mg/ml was standardized for the assay. Two putative transformed plants 'sh1' and 'sh2' were subjected to the assay. Both the plants had shown mild yellowing of painted leaves (**Fig. 5B, 5C**). While the leaves of wild type plant were completely dried (**Fig. 5A**). Similar leaf painting assay was also performed on an Australian transgenic chickpea lines harbouring *bar* gene (Sarmah *et al*., unpublished). They used Basta, a commercial formulation containing ppt as an active ingredient for the painting assay. Senthil *et al.* (2004) used 1 g/l ppt paste prepared in carboxymethylcellulose for leaf painting assay in chickpea. In the present investigation a ppt solution was used for leaf painting assay without an additive.

In the future large numbers of transgenic plants will be generated and detailed molecular analyses carried out for obtaining stable transformation lines. However the present investigation demonstrated a protocol for transformation of Indian chickpea cultivar using *bar* as a selectable marker gene.

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