Agrobacterium–mediated transformation of chickpea using shoot meristem

Rekha Singh, N P Singh*, Subhojit Datta, Indu Singh Yadav and A P Singh
Biotechnology Unit, Indian Institute of Pulses Research, Kanpur 208 024, India

Received 7 September 2006; revised 23 July 2008; accepted 24 September 2008

Agrobacterium–mediated gene transfer to pre-organized meristematic tissue combined with axillary regeneration was standardized for transformation and regeneration of chickpea, which otherwise was difficult to achieve from other explants. Different Agrobacterium strains harbouring binary vectors pCGP1258, containing the GUS as a reporter and bar [gene for resistance to phosphinothricin (PPT)—the active ingredient of the herbicide Basta] as the selectable marker, were used for the transformation experiments. After co-cultivation, the shoot apex explants were transferred onto a PPT-free regeneration medium and their tops (2 mm) were thoroughly wetted with PPT solution (2 mg/mL). The multiple axillary shoots developing from the shoot apices were excised and placed onto a medium containing 10 mg/L PPT. The surviving shoots were subcultured every 2nd wk onto fresh medium containing 20 mg/L PPT. After each subculture, the number of surviving shoots decreased until it stabilized. Some of the chimeric shoots surviving the PPT selection eventually developed new healthier axillary shoots, which could be rooted or grafted on in vitro grown seedling. This whole process took 6-9 months. Average transformation frequency was found between 1.29-3.33%. Transmission of the transgenes into progeny was also studied following the inheritance of uid A gene in T1 and T2 progenies. The overall segregation ratio among progenies of plants derived from T0 plants appeared to be close to 3:1 Mendelian ratio, indicating integration of the transgene at single locus.

Keywords: Agrobacterium tumefaciens, chickpea, Cicer arietinum, genetic transformation, β-glucuronidase

Introduction

Chickpea is the most important pulse crop of Indian subcontinent and is used as protein rich food for human consumption and animal feed. Besides, it improves soil health by fixing atmospheric nitrogen into soil. On an average chickpea is grown on about 6.92 m ha producing 5.39 m tones of grains, which represents 32 and 40% of national pulse acreage and production, respectively. The chickpea production has increased only marginally in the last three decades due to its high sensitivity to various biotic and abiotic stresses. Among these stresses, pod borer, Fusarium wilt and drought are of major concern in India. Conventional breeding is not very successful in developing disease and pest resistant varieties due to unavailability of desired donor parents in gene pool of chickpea. Moreover, traditional breeding is laborious and time-consuming process and the chances of success are quite unpredictable. Biotechnology is considered to be a potential alternative in these situations. However, availability of simple and reproducible regeneration and genetic transformation protocol is pre-requisite for any genetic manipulation work in chickpea, which is lacking at present.

Agrobacterium-mediated transformation has been used commonly in grain legumes for more than a decade2. Genetic transformation system for lupin2, peas3–5, soybean6, peanut7 and chickpea8–15 has been developed. However, in most of the cases, successful routine recovery of large number of transgenic plants, gene integration, expression and inheritance of transgene into progenies have not been demonstrated8–10. The present paper describes the development of a highly reproducible transformation protocol using A. tumefaciens. The system involves gene transfer to pre-organized meristematic tissue combined with axillary regeneration, which demonstrates stable integration and expression of marker gene uid A exhibiting β-glucuronidase activities through two generations in the progenies of transformed chickpea plants.

Materials and Methods

Plant Materials

Pure seeds of three widely grown chickpea varieties, viz., C235, Annegiri and K850 (desi types) were obtained from Genetic Resource Unit of Indian
Institute of Pulses Research, Kanpur, India. Preliminary transformation experiments were conducted with all three chickpea genotypes. However, detailed studies were performed on gene integration, expression and inheritance with putative transformants generated only from genotype C235.

**Agrobacterium Strains and Plasmids**

Three different disarmed strains of *Agrobacterium tumefaciens*, viz., AgL0, LBA4404 and EHA105, all harbouring the same binary vector pCGP1258 were used for preliminary transformation experiments. However, detailed studies were performed only with AgL0 (pCGP1258). The binary vector pCGP1258 contains a GUS gene, and a phosphinothricin (Basta) resistance gene bar, both controlled by a cauliflower mosaic virus (CaMV) 35S constitutive promoter. The T-DNA of pCGP1258(13.5 Kb) contains one EcoRI site located near the left border region. The uid A gene is not expressed in bacteria due to deletion of the bacterial ribosomal site.

**Explant Preparation**

Mature seeds were sterilized with sodium hypochlorite (10%) for 10 min, followed by washing with 70% alcohol for 5 min and three rinses with sterilized doubled distilled water. These seeds were germinated on moistened sterilized germination paper at 25°C and under 12 h photoperiod. After 2 d, germinating seeds with 2 to 10 mm long shoots were used for explant preparation. To excise the whole shoot axes from the germinating seed, the seed coat was first removed, the cotyledon excised and finally the two pairs of leaves present in the plumule were also removed. The apical dome and primordial of the third pair of leaves were injured 5-10 times with a 30 G needle under the microscope. The explants (10-15 mm) thus prepared were placed upright (apex 3-5 mm above the surface of the medium) on the co-cultivation medium containing MS salts + B5 vitamins + 2.0 mg/L BAP + 0.1 mg/L NAA + 40 gm/L sucrose and 0.8% agar-agar, and pH was maintained to 5.7 (Fig. 1).

**Co-cultivation of Explant with *A. tumefaciens***

*Agrobacterium* strains AGl0, LBA4404 and EHA105 containing the binary vector pCGP1258 were cultured overnight at 28°C in liquid YEM medium, containing 50 μg/mL tetracycline. This overnight grown culture were spun down and re-suspended in 1 mL of liquid MS solution. A drop of about 2 μL of the suspension was gently placed on the wounded shoot apex of each explant and was allowed to completely dry. To study the effect of co-cultivation period on transformation frequency, different co-cultivation periods, viz., 12, 24, 48, 72 and 96 h were checked.
Selection and Regeneration

After co-cultivation with Agrobacterium, the explants were decontaminated with 300 mg/L cefotaxime and blotted dry. The explants were then transferred to medium containing MS salt supplemented with B5 vitamins + 2.0 mg/L BAP + 0.1 mg/L NAA + 40 gm/L sucrose + 400 mg/L carbenicillin. Later (2 d), a drop of PPT solution (2 mg/mL) was applied to the tip of the explant and then withdrawn from the explant using a pipette so as to leave a wet film over the shoot apex. Cultures were grown at 25°C under fluorescent light with 12 h photoperiod. After 15 d, the shoot from shoot clumps were excised and transferred to a fresh selection regeneration medium (MS + B5 vitamins + 2.0 mg/L BAP + 0.1 mg/L NAA + 40 gm/L sucrose + 10 mg/L PPT). The non-transformed shoots (control) were also sub-cultured on the same medium at an interval of 15 d and after 2 months the surviving shoots were grafted onto 10 d-in vitro grown seedlings of their corresponding cultivar.

GUS Assay

Histochemical GUS assay was performed according to Jefferson et al. For visual identification of GUS activity in explants at the end of co-cultivation, or in regenerated plants and their progenies, entire explant or leaf discs were sterilized in 70% ethanol followed by incubation at 37°C temperature for 48 h in 0.5 mL 5-bromo-4 chloro-3 indolyl β-D-glucuronoside (X-Gluc) solution. After staining, the sections were rinsed in 70% ethanol for 5 min, mounted on slides and examined under stereomicroscope. For quantitative determination of GUS activity, assays using 4-methyl umbelliferyl β-D-glucuronoside (MUG) were performed on individual leaflets from the regenerated chickpea plants and their progeny. Aliquots of the sample were also removed for measurement of the protein concentration using a dye-binding assay. The concentration of 4-methyl umbelliferone (MU) was determined with spectrophotometer. GUS activity was expressed as pmoles MU per mg protein per min.

Molecular Analysis

The leaflets of putative transformants (T0) and their subsequent progenies (T1 & T2) were used to isolate genomic DNA by CTAB method. DNA was subjected to Southern hybridization analysis by digestion with EcoRI and probed with a PCR-amplified GUS fragment (1.4 kb). The probe was labeled with [³²P]–d CTP using random primer-labeling (Gibco BRL) and hybridized with the QuikHyb system (Stratagene). Hybridization was carried out at 65°C for 16-18 h. The blots were then washed twice with 2× SSC, 0.1% sodium dodecyl sulphate (SDS) for 10 min each time (first wash at 37°C and the second one at 65°C), followed by a third wash with 1× SSC, 0.1% SDS for 10 min at 65°C and a final wash with 0.5× SSC, 0.1% SDS for 1 min at 65°C. The blots were exposed to X-ray film with a High-Speed-X intensifying screen for 48-72 h at −70°C.

Segregation Analysis

In order to evaluate the inheritance pattern of the GUS gene and the stability of transformants, T0 and T2 progenies, derived from original transformants (T0 plants) of chickpea genotype C235, were planted under the same conditions (containment condition) as the T0 plants. The leaflets were collected for analysis by both the X-Gluc and MUG assays. In addition, transformed plants and their progenies were subjected to Southern hybridization blot analysis.

Plant Establishment

Putative transformed shoots (T0 plants) were established through rooting as well as micrografting. PPT resistant shoots were rooted (¼ MS + 2.0 mg/L NAA + 20 g/L sucrose) in PPT free rooting medium and were grafted in some cases on to the root stock of 10 days old seedlings of the corresponding cultivar with the help of small pieces of straw pipe and nourished with Hogland solutions. After grafting these plants were shifted to greenhouse with high relative humidity (95%).

Results and Discussion

Genetic Transformation and Regeneration

To study the effect of co-cultivation period on transformation frequency, different co-cultivation periods, viz., 12, 24, 48, 72 and 96 h were compared. The 48 h co-cultivation period was found best as it resulted swelling of the explants indicating infection (Fig. 2a) as well as produced highest number of PPT resistant shoots. In a preliminary study, the interaction of all the Agrobacterium strains with the three chickpea cultivars was examined for identifying the most efficient combination of host and bacteria for transformation (data not shown here). Among different Agrobacterium strains tested (AgL0, LBA 4404, EHA105), AgL0 gave a higher level of histochemical GUS-staining for the three genotypes (C235, K850 and Annegeri) than any other strains.
All the cultivars tested had a comparably low transformation frequency (2.43%). Among genotypes, C235 showed highest transformation frequency (3.33%) followed by Annegiri (2.70%) and K850 (1.29%) (Table 1).

Although, strong GUS activities were noticed in apical shoot meristem, leaves, petiole and stems of most of the putative transformants, the highest activity was mainly localized in the meristematic regions of shoot apices. Besides, leaf sections and

---

**Table 1**—Effect of genotypes on transformation frequency of chickpea using *A. tumefaciens* strain AgL0 (pCGP1258)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of explants</th>
<th>No. of regenerated shoots (after cocultivation)</th>
<th>No. of independent of PPT resistant shoots</th>
<th>GUS +ve shoots</th>
<th>Av. transformation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 235</td>
<td>480</td>
<td>760</td>
<td>23</td>
<td>16</td>
<td>3.33</td>
</tr>
<tr>
<td>K 850</td>
<td>480</td>
<td>647</td>
<td>14</td>
<td>6</td>
<td>1.29</td>
</tr>
<tr>
<td>Annegiri</td>
<td>480</td>
<td>815</td>
<td>18</td>
<td>13</td>
<td>2.70</td>
</tr>
<tr>
<td>Over all</td>
<td>1440</td>
<td>2222</td>
<td>65</td>
<td>35</td>
<td>2.43</td>
</tr>
</tbody>
</table>
embryonic axes (Fig. 2f) also showed GUS positive activity. The application of 2 mg/mL PPT to the shoot apex of control explants co-cultivated with the A. tumefaciens strain AgL0 (without plasmid) alone prevented the swelling of the explants and eventually killed it (Table 2). The explants co-cultivated with other Agrobacterium strains (AgL0, LBA 4404, EHA105), containing the binary vector (pCGP1258), application of 2 mg/mL PPT to the shoot apex did not kill all the explants (Fig. 2b). After 3 wk of co-cultivation, the axillary buds developed into large (5-10 mm) green shoots (Fig. 2c). Axillary shoots were excised from the shoot apices 3 wk after co-cultivation and placed on the regeneration medium (MS + B5 vitamins + 2.0 mg/L BAP + 0.1 mg/L NAA + 40 gm/L sucrose) with 10 mg/L PPT. The PPT concentration present in this selection medium was strong enough to remove the non-transformed shoots. After 2 wk on this medium, shoots from untransformed explants (control) turned brown. Medium with 10 mg/L PPT is enough to kill them. By contrast, among the shoots from transformed explants, many still contained some green shoots. These surviving shoots were transferred onto fresh medium with 10 mg/L PPT. This procedure was repeated at 2 wk intervals and at each transfer the number of surviving shoots decreased. Besides, the chimeric shoots which may contain both transformed and untransformed sectors developed several new, green-healthier axillary shoots (Fig. 2d). These shoots were rooted (Fig. 2e) at separate medium (¼ MS + 2.0 mg/L NAA + 20 g/L sucrose) or grafted onto rootstalk of in vitro grown seedlings of their corresponding cultivars (Figs 2g & 2h).

In total, 16 transformed plants (T₀) were resulted from 9 independent transformation events (shoots arising from the same explant) of genotype C235. Of these, 11 putative transformants (T₀) derived from 7 independent events (P1-P7) could be rooted/micrografted and successfully established in soil and produced flowering and fruiting (Fig. 2i). These putative transformants were tested GUS positive and further confirmed by Southern hybridization analysis. Besides, such GUS positive clones were advanced to T₁ and T₂ and subjected to segregation analysis (Table 3). An approximate copy number could be revealed by the number of bands (Fig 3a).

Molecular Aanalysis, Transgene Expression and Inheritance

Of 15 putative transformants (T₀) tested for the presence of the transgenes (Table 3) by Southern hybridization analysis, 14 were confirmed as transgenic and only one was escape (P9) not having a GUS gene (lane 3; Fig 3a). This indicated that the concentration (10 ppm) of selection chemical PPT (phosphothricin) was good enough to minimize the frequency of escapes. The results of Southern blot analysis confirmed that all the putative transformed plants arising from the single explant (same transformation event) show very similar banding pattern, hence may be considered as clones. Genomic DNA derived from independent transformation events

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of explants used</th>
<th>Total no. of transformed shoots</th>
<th>No. of independent transformation events</th>
<th>GUS +ve shoots</th>
<th>Av. transformation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404</td>
<td>480</td>
<td>498</td>
<td>12</td>
<td>7</td>
<td>1.45</td>
</tr>
<tr>
<td>EHA105</td>
<td>480</td>
<td>754</td>
<td>20</td>
<td>11</td>
<td>2.30</td>
</tr>
<tr>
<td>AGLO</td>
<td>480</td>
<td>870</td>
<td>3</td>
<td>16</td>
<td>3.33</td>
</tr>
<tr>
<td>Over all</td>
<td>1440</td>
<td>2122</td>
<td>5</td>
<td>34</td>
<td>2.36</td>
</tr>
</tbody>
</table>
should yield unique patterns when digested with EcoRI. Two hybridization signals were obtained in event P8 (8a, 8b & 8c), indicating that this event carries two inserts at different locations. Further, sister lines with event P3 (3a, 3b & 3c), P5 (5a & 5b), P4, P2 and P1 carry similar banding pattern as well as equal number of inserts (one), indicating that these lines are clones (arising from single independent explant). However, single hybridization signal with different molecular weight (7.1 Kb) was obtained in event P7, indicating that this event also carry single insert (Fig. 3a).

Histochemical analysis using MUG assay revealed very high level of β-glucuronidase activities in all the 15 putative transformants (T0). However, different pattern of GUS expression was noticed in transformants arising from different transformation events. Besides, pattern of expression also differed from plant to plant. The strong GUS activities were noticed in apical meristem, embryonic axes, leaves, petiole and stems of all seven plants. The inheritance pattern of GUS gene was studied by X-Gluc and MUG assays as well as by Southern hybridization blot analysis using T1 progenies derived from a P1 event (T0). Of 9 progenies (T1) tested, 7 had similar banding pattern as the (T0) plant (Fig. 3b). This is very close to 3.84, expected 3:1 Mendelian ratio normally observed with a single gene insertion ($\chi^2 = 0.27$) (Table 3). Similar banding pattern was also observed with T1 progenies derived from other T0 plants. Besides, T1 progenies tested GUS positive were advanced to T2 generation by selfing and GUS activities (based on MUG assay values) were followed up to T2 generation. The GUS gene was inherited in all the T1 lines (derived from 7 T0 plants) and its activities were further retained in T2 generation, which indicated continued inheritance and expression of the GUS gene (Table 3).

Although successful transformation of chickpea was reported as early as a decade back8, stable integration, expression and inheritance were not demonstrated in most of the earlier studies8-11. Recently, however, some of the studies have shown successful gene integration, expression and inheritance of bar and GUS gene in Kabuli varieties of chickpea13-15. The present study, on the other hand, was based on desi chickpea varieties.

**Table 3**—Inheritance pattern of β-glucuronidase activities in the progenies of transgenic chickpea

<table>
<thead>
<tr>
<th>Parent plant</th>
<th>MUG value (pmol MU/mg protein/min)</th>
<th>T1 progenies</th>
<th>Mean MUG of progenies (pmol MU/mg protein/min)</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>26350</td>
<td>7 2</td>
<td>72867</td>
<td>128753</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>32874</td>
<td>8 3</td>
<td>87570</td>
<td>131674</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>44386</td>
<td>3 1</td>
<td>92865</td>
<td>108830</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>60378</td>
<td>8 2</td>
<td>112354</td>
<td>145694</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>42865</td>
<td>4 1</td>
<td>986759</td>
<td>156980</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>85642</td>
<td>9 4</td>
<td>121578</td>
<td>98678</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>68549</td>
<td>6 2</td>
<td>107329</td>
<td>127564</td>
<td></td>
</tr>
</tbody>
</table>

*a P5 was tested as escape
** P8(a,b,c) showed morphological abnormalities and did not produce flower and fruit

**Conclusion**

Though the system of chickpea transformation reported here shows low transformation frequency and is laborious exercise, the benefit of this system lies in its reproducibility. Hence, it can be used to genetically engineer chickpea using genes of economic importance on routine basis. The results obtained under the present study clearly demonstrate stable integration, expression and inheritance of marker gene (uid A) coding β-glucuronidase activities through two generations in progenies of transformed chickpea plants of variety C235. Histochemical analysis revealed high level of GUS activities in all seven T0 plants. However, pattern of expression differed from plant to plant. The strong GUS activities were noticed in apical meristem, leaves, petiole and stems of all seven plants. Transmission of the transgenes into progeny was also studied following the inheritance of uid A gene in T1 and T2 progenies. The overall segregation ratio among progenies of plants derived from all seven T0 plants appears to be close to 3:1 Mendelian ratio, indicating integration of...
the transgene at single locus. The χ² test indicated non-significant difference between expected 3:1 ratio and observed values. Besides, a varied pattern of GUS activities was also observed in the progenies (T₂). This protocol demonstrates the feasibility of its use in commercially viable constructs.

**Acknowledgement**
The authors acknowledge Dr Steve Wylie, West Australian State Agricultural Biotechnology Centre, Murdoch University, Perth, Australia for providing the gene constructs used in the study.

**References**