Agrobacterium tumefaciens - mediated transformation of blackgram: an assessment of factors influencing the efficiency of *uidA* gene transfer

R. SAINI* and P.K. JAIWAL

Department of Biosciences, M.D. University, Rohtak-124001, India

Abstract

Agrobacterium tumefaciens strain EHA105 carrying a binary vector pCAMBIA2301, which contains a neomycin phosphotransferase gene (*nptII*) and a β-glucuronidase (GUS) gene (*uidA*) interrupted with an intron, was used for transformation of *Vigna mungo* cotyledonary node explants. Various factors such as preculture and wounding of explants, manipulations in inoculation and co-cultivation conditions were found to play a significant role in influencing tissue competence, *Agrobacterium* virulence and compatibility of both, for achieving the maximum transformation frequencies. The stable transformation with 4.31 % efficiency was achieved using the optimized conditions. The transformed green shoots that were selected and rooted on medium containing kanamycin and tested positive for *nptII* gene by polymerase chain reaction were established in soil to collect seeds. GUS activity was detected in leaves, roots, pollen grains and T_1 seedlings. Southern analysis of T_0 plants showed the integration of *nptII* into the plant genome.

Additional key words: cotyledonary node, GUS activity, transgenic plants, *Vigna mungo.*

Introduction

Development of transformation procedure for any plant, especially for recalcitrant species involves a gene delivery method for targeting foreign DNA to regenerable cells. The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capability of *Agrobacterium tumefaciens*. In spite of the broad natural host range of *Agrobacterium tumefaciens*, till today relatively a few grain legumes have been stably transformed using disarmed vectors (Jaiwal and Singh 2003, Somers *et al*. 2003, Popelka *et al*. 2004). *Vigna mungo* – an important but highly recalcitrant grain legume has recently been transformed using cotyledonary node explants and *A. tumefaciens*. However, the transformation frequency was 1 % (Saini *et al*. 2003). Cotyledonary nodes are good explants for plant transformation because regeneration *via* direct shoot formation minimizes the risks of somaclonal variation. The optimization of some important aspects of transformation system components that affect the overall transformation efficiency is therefore, essential to enhance the virulence so as to increase the transformation frequency. There are no reports on systematic optimization of conditions for *V. mungo* transformation, in the present study, the effects of several parameters known to influence *Agrobacterium*-mediated DNA transfer were optimized using the *uidA* gene with intron marker system and effect of the optimized conditions on stable transformation was also evaluated.

Materials and methods

Seeds of *Vigna mungo* (L.) Hepper cv. PS-1 procured from Division of Genetics, IARI, New Delhi were surface sterilized and cotyledonary node explants were excised as described by Saini *et al*. (2003).

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Abbreviations: BAP - 6-benzylaminopurine; CTAB - cetyl trimethyl ammonium bromide; IBA - indole-3-butyric acid; RM - rooting medium; SR - shoot regeneration medium.

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* Corresponding author; fax: (+91) 1262 294817; e-mail: ramanksaini@rediffmail.com

The disarmed *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCAMBIA2301, which contains a β-glucuronidase (GUS) gene (*uidA*) and a neomycin phosphotransferase gene (*nptII*) both driven by CaMV 35S promoter, was used for transformation studies. The *uidA* gene contains an intron in the coding region to ensure that the observed GUS activity occurred in the plant cell and not due to residual *Agrobacterium* cells. The transformation events were quantified by directly observing the GUS blue staining in the explants tissues.

A. tumefaciens strain EHA105 (pCAMBIA2301) was cultured on 20 cm^3 of YEM medium (1 g dm^{-3} yeast extract, 10 g dm^{-3} mannitol, 0.1 g dm^{-3} NaCl, 0.2 g dm^{-3} $MgSO_4 \cdot 7 H_2O$, 0.5 g dm^{-3} K_2HPO_4, pH 7.2) containing 50 mg dm^{-3} kanamycin and 50 mg dm^{-3} rifampicin and grown for overnight at 28 ºC on a shaker at 200 rpm. Bacteria were pelleted at 4 000 rpm for 10 min and resuspended in liquid shoot regeneration medium (SR) containing Murashige and Skoog (MS) salts, B_5 vitamins, 3 % sucrose and 0.5 μM 6-benzylaminopurine (BAP). The density of bacterium suspension was checked at 600 nm and dilutions were made for different concentrations of bacterial cells. The cotyledonary node explants excised from 16 h water soaked seeds were inoculated for 10 - 60 min with different concentrations of bacterial cells (10^6 - 10^9 cells cm^{-3}) and co-cultured on SR medium for 1 - 4 d under 16-h photoperiod (cool-white fluorescent tubes, irradiance of 80 μmol m^{-2} s^{-1}) and temperature of 25 ± 2 °C. In an effort to increase transformation efficiency, acetosyringone (50 μM) was added to inoculation medium and the transformation of the freshly excised explants in the absence or presence of acetosyringone in the co-cultivation medium was compared. The effect of pre-culture of explants, on SR medium for 0 - 3 d and mechanical injury (gently stabbed 4 - 5 times using a sterile fine hypodermic needle), both the factors either alone or in combination prior to inoculation with bacterium, was also investigated. After co-cultivation, the explants were washed 3 - 4 times with liquid SR medium with vigorous stirring and blotted dry on sterile filter paper and analyzed for GUS histochemical assay (Jefferson *et al*. 1987). For each treatment, 40 explants were used and each experiment was repeated twice. The frequency of transient GUS expression was calculated for each experiment.

Following the optimized conditions, the co-cultivated explants were cultured on semi-solid SR medium containing 75 mg dm^{-3} kanamycin and 500 mg dm^{-3} cefotaxime for shoot regeneration. The explants were transferred onto fresh medium containing the same concentrations of antibiotics every 2 weeks for a total of 4 - 6 weeks, until the shoots attained a height of 2 - 3 cm. Green shoots were transferred to rooting medium (RM) containing half-strength MS salts, full-strength MS vitamins, 2.5 μM indole-3-butyric acid (IBA), 3 % sucrose and 10 mg dm^{-3} kanamycin. The putative transformed plants were established in soil and grown to maturity to collect T_0 seeds.

Total genomic DNA was extracted from fresh leaves of non-transformed (control), putative transformants (T_0) plants and their progeny by the CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988).

Putative transformants were screened by polymerase chain reaction (PCR) for the presence of the *nptII* gene. The 540 bp coding region of *nptII* was amplified by using 20 bp oligonucleotide primers (I: 5′-CCACCATGATAT TCGGCAAC-3′ and II: 5′- GTGGAGAGGCTATTC GGCTA-3′), 100 ng of purified genomic DNA and *Taq* polymerase (www.invitrogen.com) in a thermal cycler (www.perkinelmer.com) under the conditions optimized by Saini *et al*. (2003).

The stable GUS activity was monitored in the leaves after 4 - 6 weeks culture of *Agrobacterium* infected explants on SR medium and in the germinated T_1 seedlings by a histochemical assay according to Jefferson *et al*. (1987). The leaves and germinated T_1 seedlings of putative transformed and non-transformed plants were immersed in freshly prepared 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) solution and incubated overnight at 37 ºC. The staining solution was removed the following day, and plant tissues were decolorized using ethanol and examined under a microscope.

Ten μg of DNA samples from transformed and non-transformed (control) plants were digested with *Kpn*I (www.fermentas.com) and separated on 0.9 % agarose gel, blotted on positively charged nylon membrane (www.roche-applied-science.com) and hybridized with $dCTP^{32}$ labeled probe (500 bp fragment of *nptII*) following supplier's instructions (www.roche-applied-science.com).

Results and discussion

The transformation frequency increased with increase in concentration of *Agrobacterium* cells up to 10^8 cells cm^{-3} and, thereafter, decreased with further increase in number of *Agrobacterium* cells (Table 1). Similar results were obtained in *Nicotiana tabacum* and *Arabidopsis thaliana* (Lin *et al*. 1994) and in most of the grain legumes (Bean *et al*. 1997). Much higher concentrations lead to cause hypersensitive response of explants with decrease in regeneration potential, aggregation of *Agrobacterium* cells or difficulty in killing them after co-cultivation.

The significant differences on transient GUS expression were observed when the explants inoculated with bacterial suspension for 10 to 60 min with gentle shaking at 90 rpm. Maximum transformation frequency

was observed in explants inoculated for 20 or 30 min with no significant difference between them (Table 1). Further increase in inoculation time did not increased transformation frequency and caused problems in eliminating the bacteria.

Table 1. Effect of different transformation factors on transient GUS expression frequency of cotyledonary node explants of blackgram cv. PS-1 inoculated with *Agrobacterium tumefaciens* strain EHA105 harboring vector pCAMBIA2301.

Factors		GUS expression [%]
Bacterial concentration [cells cm^{-3}]	10^6	65
	10^7	78
	10^8	90
	10^9	86
Inoculation time [min]	10	60
	20	88
	30	90
	60	50
Co-cultivation period [d]	1	65
	2	85
	3	87
	4	70
Pre-culture [d]	0	88
	1	40
	2	15
	3	4

The length of co-cultivation period required for achieving maximum gene transfer was found to be 2 - 3 d with no significant difference between them for cotyledonary node explants of *Vigna mungo* (Table 1). Further extension in co-culture time decreased the transformation frequency resulting in bacterial overgrowth and had detrimental effect on regeneration potential of explants. A short co-culture period of 2 or 3 d has also been found to be optimum in other plant species such as *Antirrhinum majus* (Holford *et al*. 1992), *Vigna unguiculata* (Muthukumar *et al*. 1996), *Vigna radiata* (Jaiwal *et al*. 2001), *Cajanus cajan* (Mohan and Krishnamurthy 2003), *Glycine max* (Li *et al*. 2004) and *Nicotiana tabacum* (Uranbey *et al*. 2005).

The T-DNA transfer is mediated by products encoded by the *vir* (virulence) region of the Ti-plasmid, which is composed of at least six inducible operons that are activated by signal molecules, mainly small phenolics, certain class of monosaccharides and acidic pH acts synergistically with phenolic compounds (De la Riva *et al*. 1998). Induction of *vir* operons by inclusion of phenolics like acetosyringone in the co-cultivation medium could therefore enhance T-DNA transfer to plant cells. Addition of 50 μM acetosyringone to the bacterial re-suspension medium as well as co-cultivation medium resulted in non-significant increase in transformation frequency from 88 % in cultures without acetosyringone to 95 % with large GUS positive sector(s). Acetosyringone enhances *vir* functions during transformation (Stachel *et al*. 1986) and has been shown to increase transformation potential of *Agrobacterium* strain with moderately virulent *vir* region in several plant species (Atkinson and Gardner 1991, Janssen and Gardner 1993, Kaneyoshi *et al*. 1994).

Injuries implicated with the help of hypodermic needle, enhanced the frequency of transient GUS expression, at the regeneration and cotyledons detachment sites of the cotyledonary nodes up to 98 %. Wounding the plant material before co-cultivation allows better bacterial penetration into the tissue facilitating the accessibility of plant cells for *Agrobacterium* or possibly stimulated the production of potent *vir* gene inducers like phenolic substances such as acetosyringone and hydroxyacetosyringone (Stachel *et al*. 1985) and enhanced the plant cell competence for transformation (Binns and Thomashow 1988). Wounding the plant material before co-cultivation has also been shown to increase transformation frequency (Bidney *et al*. 1992). Mechanical injury of the meristematic region probably induces meristem reorganizations promoting formation of large transgenic sectors and enhanced recovery of transformants.

Pre-culture of explants on regeneration medium prior to inoculation and co-cultivation with *Agrobacterium* has been reported to enhance efficiency of transformation in some grain legumes, *e.g. Vigna unguiculata* (Muthukumar *et al*. 1996) and *Cajanus cajan* (Geetha *et al*. 1999). However, in present study, no such results were obtained. This may be due to the specificity of species to pre-culture. In contrast, pre-culture (0 - 3 d) of cotyledonary node explants prior to co-culture with bacteria reduced the frequency of transient GUS expression very much (Table 1). This may be due to the healing of the wounding site, which is a prerequisite for *Agrobacterium*-mediated transformation. The reduction may be attributed to the secretion of compounds that inhibit *vir* gene induction or dilution of the *vir* gene inducing signal molecules released as result of wounding. Wounding induced division and production of phenolic compounds such as acetosyringone and hydroxyacetosyringone. These signal molecules are recognized especially by *Agrobacterium* to induce *vir* gene expression and thereby activate T-DNA transfer (Zambryski 1992). Pre-culture was found to reduce transformation efficiency in other plant species also such as kiwifruit (Janssen and Gardner 1993) and apple (De Bondt *et al*. 1994) and had no effect in peanut transformation (Sharma and Anjaiah 2000). When preculture was combined with mechanical injury the results were reversed that leads to increase in transient GUS expression up to 100 % and specifically at the regeneration site of the cotyledonary node explants. This may be attributed to visually more clear regeneration site on the pre-cultured explants for mechanical injury as

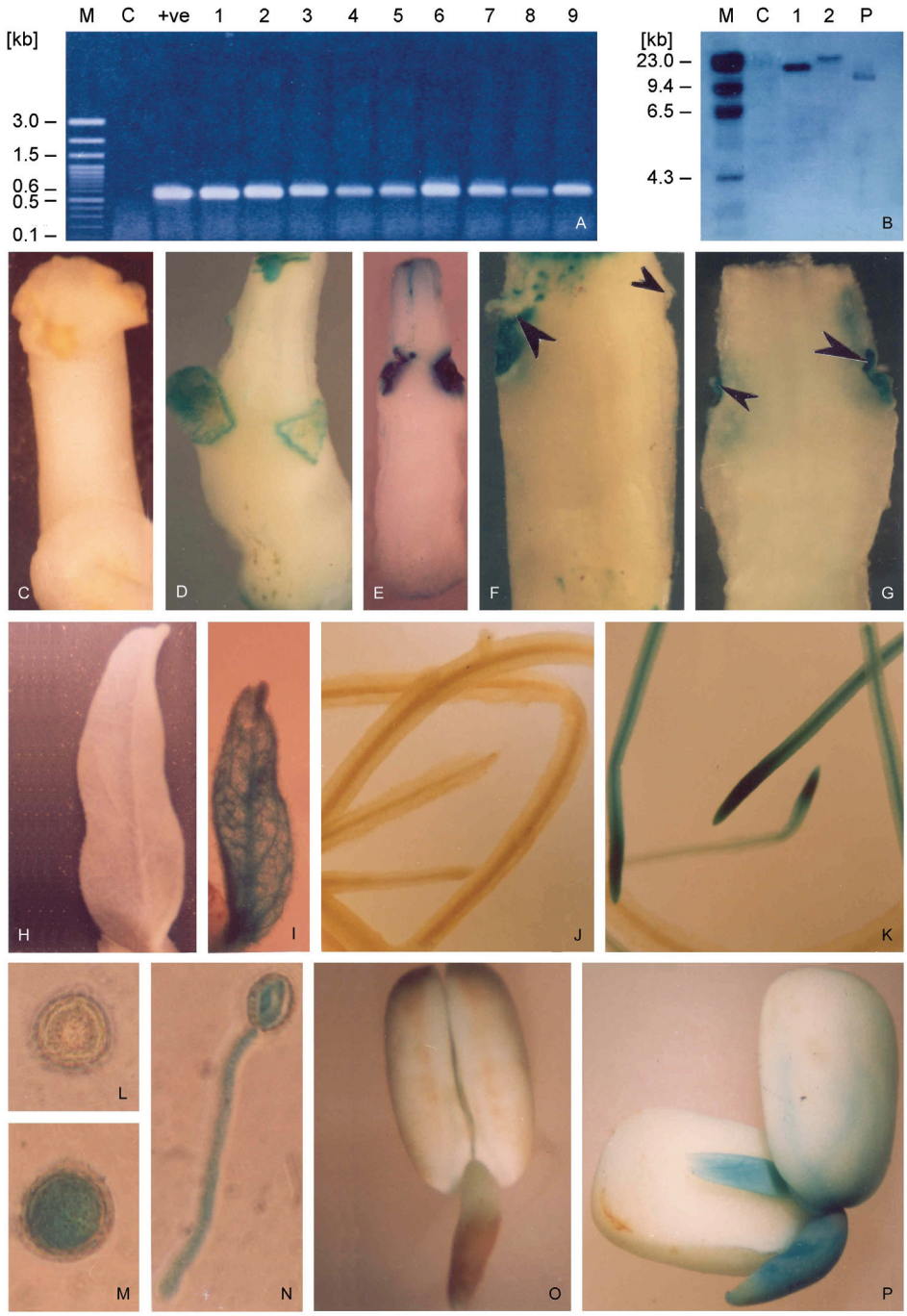

Fig. 1. Transient GUS activity of cotyledonary nodes, molecular analysis and stable GUS activity of putative transformed and non-transformed (control) plants *V. mungo* cv. PS-1 co-cultured with *A. tumefaciens* strain EHA105 (pCAMBIA2301). *A*: PCR analysis of primary transformants using *nptII* primers. *Lane M* - marker DNA, *lane C* - DNA from untransformed control, *lanes 1 to 9* - T_0 transformed plants and *lane +ve* - plasmid DNA. *B*: Southern blot analysis of genomic DNA of transformed and non-transformed (control) plants. *Lane M* - marker DNA, *lane C* - DNA from untransformed control, *lanes 1,2* - transformed plants, *lane P* - plasmid DNA (pCAMBIA2301) of size 11.6 kb. *C* - *G*: Transient GUS activity of cotyledonary nodes, *C* - control explants showing no GUS activity, *D* - explants without pre-culture and mechanical injuries showing less intensive GUS staining only at the cotyledons detachment sites, *E* - explants with mechanical injuries and without pre-culture showing a high level of GUS expression at the injured region, *F* - precultured explants without mechanical injuries showing GUS activity only at the cotyledons detachment sites while no GUS activity at the site of regeneration (*arrowheads*), *G* - a high level of GUS activity observed specifically at the site of regeneration (*arrowheads*) in pre cultured explants with mechanical injuries. *H* - *P*: Stable GUS activity in the leaves (*H* - control and *I* - transformed), roots (*J* - control and *K* - transformed), pollen grains (*L* - control and *M*,*N* - transformed), T_1 seedlings (*O* - control and *P* - transformed).

compared to non-pre-cultured and freshly release of phenolics as a result of mechanical injury. High vigour of pre-cultured explants was also found to increases the regenerability of mechanically injured explants.

These optimized transformation factors were used for the stable genetic transformation of *Vigna mungo*. 255 cotyledonary node explants co-cultured with *Agrobacterium* produced a total of 113 shoots on kanamycin selection medium. The green shoots (2 - 3 cm) were subjected to a second round of selection at the rooting stage, where 38 shoots formed roots in the presence of kanamycin. These plantlets were subsequently transferred to soil, where 27 plants survived, grew to maturity and produced seeds. PCR analyses showed amplification of a 0.54 kb band corresponding to the coding region of *nptII* gene, indicating the presence of transgene in 11 out of 27 putatively transformed plants established in soil, with an overall transformation frequency of 4.31 % (11 PCR +ve shoots per 255 explants inoculated with *Agrobacterium*) (Fig. 1*A*). Southern analysis of putative transgenic plants revealed different patterns of junction fragments between the T-DNA and the plant genome, depending on the integration site (Fig. 1*B*). This indicates that these plants were derived from independent transformation events. The T-DNA of pCAMBIA2301 (5.3 kb) contains a single *Kpn*I site at the multiple cloning site located in the *lac*Z alpha region. The sizes of the bands detected were greater than that of the size of DNA fragment (*nptII* gene) from *Kpn*I site to the left border (2.1 kb) confirming the integration of T-DNA into the plant genome. The number of hybridization signals indicated the single copy insertion of T-DNA into the genome of the transgenic plants. DNA isolated from non-transformed plants did not hybridize with the *nptII* probe.

The cotyledonary nodes, which were not inoculated with *Agrobacterium*, showed no GUS activity (Fig. 1*C*). The cotyledonary nodes, without preculture and mechanical injuries, at the site of regeneration, upon co-cultivation with *Agrobacterium* showed less intensive GUS staining only at the cotyledons detachment sites (Fig. 1*D*), whereas extra wounding without preculture resulted in intense transient GUS activity at the wounded regions of the treated cotyledonary nodes (Fig. 1*E*). Pre-cultured explants without mechanical injuries showed no GUS activity at the site of regeneration except at the cotyledons detachment sites (Fig. 1*F*). Mechanical injuries of 3 d pre-cultured explants results a high level of GUS activity at the site of regeneration, possibly pre-culture of explants reduces the competence of the non-meristematic cells, wounded at the time of cotyledons detachment while mechanical injuries increased the competence of the meristematic cells, and thereby reduces the competitions between meristematic and non-meristematic cells to *Agrobacterium*-mediated transformation at the cotyledonary nodes (Fig. 1*G*). Stable GUS activity was detected in the leaves, roots and pollen grains of primary transformants and in the germinated T_1 seedlings indicating the inheritance and expression of the transgene in the progeny of transformed plants (Fig. 1*I*,*K*,*M*,*N*,*P*). There was no blue staining in the leaves, roots and pollen grains of control plants and in the germinated seedlings (Fig. 1*H*,*J*,*L*,*O*).

It is concluded that inoculation of pre-cultured and mechanically injured cotyledonary node explants of *V. mungo* for 30 min with *A. tumefaciens* at a density of 10^8 cells cm^{-3} followed by co-culture on SR medium for 3 d has been found more beneficial and resulted in the production of significant number of transgenic plants to efficiency of 4.31 %. In the present study, transgenic plants carrying *nptII* and *uidA* genes have been produced using *A. tumefaciens* strain EHA105 containing vector pCAMBIA2301. Transgenes inherited and expressed in the progenies of the transformants. This work will not only allow generating blackgram transgenics in near future with an array of genes for qualitative or quantitative improvements, with precision and simultaneously will be applicable to closely related *Vigna* species, *i.e. Vigna radiata* and *Vigna unguiculata*.

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