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ORIGINAL PAPER

Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration

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Abstract An improved method of *Agrobacterium*-mediated transformation of cowpea was developed employing both sonication and vacuum infiltration treatments. 4 day-old cotyledonary nodes were used as explants for co-cultivation with *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pSouv-cry1Ac. Among the different injury treatments, vacuum infiltration and their combination treatments tested, sonication for 20 s followed by vacuum infiltration for 5 min with *A. tumefaciens* resulted in highest transient GUS expression efficiency (93% explants expressing GUS at regenerating sites). After 3 days of co-cultivation, the explants were cultured in 150 mg/l kanamycin-containing selection medium and putative transformed plants were recovered. The presence, integration and expression of *nptII* and *cry1Ac* genes in T_0 transgenic plants were confirmed by polymerase chain reaction (PCR), genomic Southern and qualitative reverse transcription (RT)-PCR analysis. Western blot hybridization and enzyme-linked immunosorbent assay (ELISA) detected and demonstrated the accumulation of Cry1Ac protein in transgenic plants. The *cry1Ac* gene transmitted in a Mendelian fashion. The stable transformation efficiency increased by 88.4% using both sonication-assisted *Agrobacterium*-mediated transformation (SAAT) and vacuum infiltration than simple *Agrobacterium*-mediated transformation in cowpea.

Keywords *Agrobacterium tumefaciens* · *cry1Ac* · Cowpea · Sonication · Vacuum infiltration · Wounding

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Abbreviations

BAP	6-Benzylaminopurine
TDZ	Thidiazuron
GUS	β-Glucuronidase
nptII	Neomycin phosphotransferase II
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is widely cultivated in Africa, India, Middle East and, South America mostly for dry grain and fodder (Ehlers and Hall 1997; Timko et al. 2007), and is a major source of high-quality dietary protein for millions of local poor people (Singh 2002; Diouf and Hilu 2005; Xu et al. 2010). Cowpea production is seriously affected by a number of biotic and abiotic constraints, of which notably insect pests and viral diseases cause substantial yield loss worldwide (Solleti et al. 2008a). Despite its economic importance, progress in genetic improvement of cowpea for insect pest and disease resistance through conventional breeding is slow primarily due to narrow genetic base and barriers in crossing with distant wild species (Gomathinayagam et al. 1998; Fang et al. 2007). Consequently, the transfer of insect pest and virus resistance genes by genetic transformation could potentially aid plant breeders in overcoming these constraints and accelerate the development of resistant cultivars for breeding programs. Furthermore, efficient genetic transformation system would provide a valuable tool for functional genomics studies of cowpea. *Agrobacterium*-mediated transformation has been extensively applied to many crop plants including grain legumes, because this method offers several advantages such as the defined

integration of transgenes, potentially low copy number, and preferential integration into transcriptional active regions of the chromosome (Koncz et al. 1989; Hiei et al. 1994). *Agrobacterium*-mediated transformation of cotyledonary explants has led to the generation of stable transgenic plants in cowpea (Muthukumar et al. 1996; Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b). Cotyledonary explants are preferred for *Agrobacterium*-mediated transformation of cowpea as T-DNA delivery to axillary meristem followed by regeneration via adventitious bud formation minimizes the risks of chimeras and somaclonal variation (Tzfira et al. 1997). However, cowpea transformation still remains inefficient and consequently, production of transgenic cowpea is far from being a routine procedure due to poor transformation efficiency and low numbers of regenerated transgenic plants. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Joersbo and Brunstedt 1992; Trick and Finer 1998; Santare′m et al. 1998) and vacuum infiltration (Charity et al. 2002; Park et al. 2005; Paz et al. 2006) methods have been reported to enhance the efficiency of *Agrobacterium*-mediated transformation of recalcitrant plant species. Exposure of the explants to short periods of sonication in the presence of *Agrobacterium* carrying desired T-DNA vector is thought to produce large numbers of micro wounds across the tissue which permits the *Agrobacterium* to penetrate deeper and more completely throughout the tissue as compared to the natural infection obtained during co-cultivation (Trick and Finer 1997; Santare′m et al. 1998; Tang et al. 2001; Liu et al. 2005), thus enhancing the bacterial colonization and infection of the tissue. Performed scanning electron and light microscopy observations revealed that ultrasound treatment produces small and uniform fissures and channels throughout the plant tissue, which allows *Agrobacterium* access to internal plant tissue (Trick and Finer 1997). SAAT method has been successfully employed in improving transformation of a number of recalcitrant plants (Oliveira et al. 2009).

Agroinfiltration is an effective method in enabling the regenerating cells, often located a few cell layers beneath the surface of explants, rapid access to *Agrobacterium* and consequently increasing transient transgene expression in many recalcitrant plant species (Bechtold and Pelletier 1998; Tague and Mantis 2006). This method has been adapted for the successful transformation of number of recalcitrant plants (Subramanyam et al. 2011).

Although the benefits of sonication and vacuum infiltration during *A. tumefaciens*-mediated transformation methods are evident, no effort has been made to apply these methods to cowpea. In order to improve the *Agrobacterium*-mediated transformation in cowpea for routine generation of transgenic plants with candidate genes, we investigated the effect of sonication and vacuum infiltration on *Agrobacterium*-mediated transformation of cowpea cotyledonary node explants. Stable transgenic cowpea plants expressing *cry1Ac* were recovered using both SAAT and vacuum infiltration, which showed presence, integration, expression and inheritance of transgenes.

Materials and methods

Plant material and explant preparation

The mature seeds of cowpea cultivar Pusa Komal (IARI, New Delhi) were surface sterilized with 70% ethanol (v/v) for 30 s followed by 0.2% of mercuric chloride (w/v) for 5 min. The sterilized seeds were rinsed 5 times with sterile water and cultured on MSB5 medium [MS salts (Murashige and Skoog 1962) + B_5 vitamins (Gamborg et al. 1968)] supplemented with 3% sucrose (w/v), 0.8% agar agar (w/v) and 10 μM TDZ. The cultures were incubated at 26 ± 2°C under 16 h-photoperiod regime provided by cool white fluorescent lamps (36 μmol m^{-2} s^{-1}). Cotyledonary node explants (5–6 mm) were excised from 4-day-old seedlings by removing both the cotyledons, and decapitating epicotyls as close as possible and hypocotyls 3 mm below the nodal region, and used for transformation experiments.

Binary plasmid, bacterial strain and culture conditions

The binary plasmid pSouv:cry1Ac (Bt*cry1Ac* expression cassette cloned in binary vector pCAMBIA2301) (Fig. 1) was mobilized into the disarmed hypervirulent *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments. The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*nptII*) and *β*-glucuronidase gene (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter. The *A. tumefaciens* strain harboring pSouv:cry1Ac was maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l of rifampicin, and 50 mg/l of kanamycin. Single bacterial colony was inoculated into 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics and grown overnight at 28°C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid co-cultivation medium, LCM (MSB5 medium containing 1 μM BAP, pH adjusted to 5.5) supplemented with 100 μM acetosyringone and used for inoculation.

Inoculation of explants with *A. tumefaciens*

For each experiment, 30–40 explants were subjected to wounding treatment either by mechanical injury with

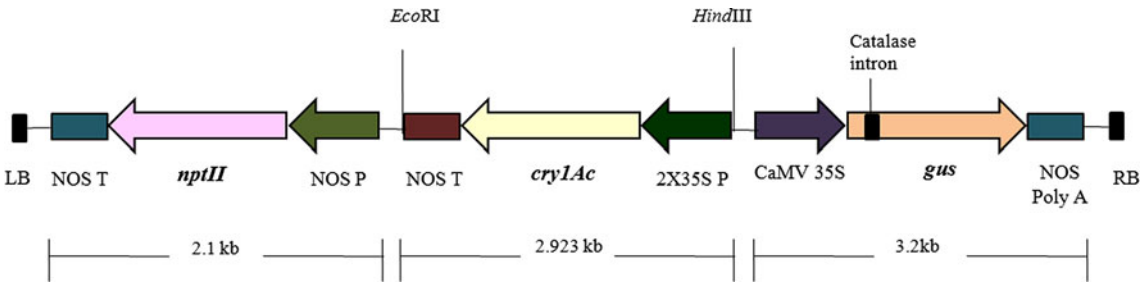

Fig. 1 Schematic construction of pSouv:cry1Ac (14.5 kb). The 2.9 kb (*Eco*RI–*Hin*dIII) fragment containing Bt*cry1Ac* under the control of CaMV 2X35S promoter and NOS terminator was cloned at the *Eco*RI–*Hin*dIII sites of T-DNA of pCAMBIA2301. *LB* and *RB* left border and right border of T-DNA region, *NOS T* nos terminator, *2X35SP* double 35S promoter, *npt*II neomycin phosphotransferase II

needle or by sonication, and inoculated in bacterial suspension by occasional shaking for 30 min or by vacuum infiltration. The explants inoculated in bacterial suspension without prior wounding treatment were considered as control. After inoculation in all cases, explants were blotted on a sterile filter paper to remove excess liquid and co-cultivated for 3 days under dark condition at 22°C, in petri dishes lined with filter paper moistened with LCM supplemented with 100 μM acetosyringone. Following co-cultivation, the explants were rinsed three to four times with LCM and blotted dry on sterile filter paper and placed onto initial multiple shoot induction and selection medium, SISM (MSB5 medium containing 5.0 μM BAP and 0.5 μM kinetin supplemented with 150 mg/l kanamycin and 500 mg/l cefotaxime) for 20 days with three rounds of subculture at an interval of 5, 7 and 8 days, respectively.

Wounding and SAAT treatments

The cotyledonary node explants were wounded at axils by puncturing approximately 1.5 mm in depth with a sterile hypodermic needle (0.56 mm in diameter.) prior to inoculation with *Agrobacterium* cell suspension.

For SAAT, the explants were immersed in 15 ml flat bottom glass culture tubes (Borosil, India) containing 6 ml of *Agrobacterium* cell suspension. The tubes were capped, placed in a float at the center of a bath-type sonicator (Telsonic ultrasonic TPC-40, Switzerland) and then subjected to ultrasound at a frequency of 30 kHz. The treatments differed as to sonication duration (5, 10, 15, 20, 25, and 30 s). Following sonication, explants were removed from the tubes, placed on sterile filter paper surface to blot off excess bacteria and then transferred to co-cultivation medium.

For vacuum infiltration experiment, the explants with or without wounding and 20 s sonication treatments were placed in vacuum system consisted of a vacuum pump at 600 mm Hg (Rocker 400, Tarson, India) to which a desiccator was attached. Glass petri dishes containing explants immersed in *Agrobacterium* cell suspension were placed in the desiccator and vacuum was applied for different durations (2.5, 5, 10, 15 and 20 min).

The best treatments achieved in SAAT and vacuum infiltration experiments were combined to evaluate the effect of sonication followed by vacuum infiltration in contrast to the use of these methods alone.

In all experiments, the frequency of transient GUS expression was analyzed after 3 days of co-cultivation. The optimal wounding, sonication and vacuum infiltration treatments were determined as the levels that led to a perceived increase in GUS positive foci in explants at the site of regeneration without any perceived decrease in explant viability. Control treatments consisted of explants either uninoculated or inoculated with *Agrobacterium* without wounding, sonication and vacuum infiltration treatments.

Histochemical GUS assays

GUS activity was visualized using the histochemical assay (Jefferson 1987). Transient expression was examined after 3 days of co-cultivation (Solleti et al. 2008a). The explants were bleached with 100% ethanol for 24 h prior to examination under a stereomicroscope. Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each cotyledonary node explant. The blue foci were the discrete areas of cells with GUS activity.

Shoot recovery

Following three rounds of kanamycin selection on SISM, the survived explants were transferred to SIEM [shoot induction and elongation medium (MSB medium containing 5.0 μM BAP, 0.5 μM kinetin and 500 mg/l cefotaxime)] and cultured for 10 days for optimal elongation and selective regeneration of transformants. Elongated putative transformed shoots (>1.5 cm) were transferred to rooting medium (MS + 2.5 μM IBA) devoid of any antibiotics for root induction. Rooted putative transformed plants were

transferred to pots containing sterile soil:compost (1:1) and were acclimatized in greenhouse containment for 3 weeks.

Evaluation of transgenic plants

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, GUS histochemical analysis of different plant tissues, RT-PCR, ELISA and Western blot hybridization analysis for confirmation of the presence, integration, expression and inheritance of the introduced genes.

Stable GUS assay

Stable *gus* expression was detected in various plant parts including flower, anthers, pollens and pistils following the histochemical procedure as described previously.

Screening of putative transformed plants using polymerase chain reaction (PCR)

Genomic DNA was isolated from the young leaves of T_0 putative transformants and T_1 transgenic plants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *npt*II and *Btcry1Ac* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pSouv:cry1Ac (positive control) as templates. The 540 bp region of *npt*II and 1 kb coding region of *Btcry1Ac* were amplified using respective 20 mers (*npt*II Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAG GCTATTCGGCTA) and 24 mers (*Btcry1Ac* Fw: CCCAG AAGTTGAAGTACTTGGTGG; Rv: CCGATATTGAAG GGTCTTCTGTAC) oligonucleotide primers. The amplification reaction was carried out under the following conditions: 94°C for 5 min (1 cycle), 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension) for 35 cycles followed by the final extension at 72°C for 7 min (1 cycle). PCR was performed using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

Southern hybridization

Randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the *cry1Ac*. 10 µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *Hin*dIII. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 1 kb PCR product, corresponding to the coding region of *cry1Ac* gene. The probe labeling and Southern hybridization were performed using the non-radioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. Pre-hybridization and hybridization were carried out using high hybridization buffer containing 5XSSC, 1% blocking solution, 0.1% (w/v) *N*-lauroyl sarcosine and 0.02% (w/v) sodium dodecyl sulfate. Washing and detection were performed according to the instruction of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany).

Qualitative reverse transcription (RT)-PCR analysis

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR of the coding sequences of *Btcry1Ac* gene in the cDNA was carried out using respective primers as described earlier.

Western blot hybridization

Proteins were extracted from 1 g of young leaves of T_0 transgenic plants using an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT). The protein concentration was determined by the method of Bradford (1976). 30 µg of protein was fractionated on 12% acrylamide gels with sodium dodecyl sulfate (SDS–PAGE) and blotted on to a PVDF membrane by electro transfer blotting unit. Blots were blocked for 2 h at room temperature in 5% blocking buffer (non-fat powdered milk in Tris-buffered saline with 0.1% Tween-20). Goat polyclonal antibodies (Amar Diagnostics, India) were used at 1/500 dilution in blocking buffer and incubated for overnight at 4°C. The samples were washed three times in TBST (tris-buffered saline tween-20) for 5 min each. A secondary rabbit anti-goat antibody alkaline phosphatase conjugate (Amar Diagnostics, India) was then used for final detection, at a dilution of 1/1,000. Blots were incubated for 40 min at 4°C, washed 5 times for 5 min each with TBST followed by development in nitro blue tetrazolium/bromo chloro indolyl phosphate (NBT/BCIP) substrate solution (Sigma, USA) for 15–20 min. The reaction was stopped by washing the membrane with distilled water.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the accumulated levels of Cry1Ac protein in T_0 transgenic plants using Desigen Quan T-ELISA-96 well plate kit (Desigen, Maharashtra, India) following manufacturer's protocol. Total protein was extracted from 5 mg of dry leaf powder using 500 µl of sample extraction buffer. The sample was chilled and spun at 8,000 rpm for 15 min and 100 µl of supernatant was used for loading to anti-Cry1Ac pre-coated plate. For the estimation of Cry1Ac, the 96-well titre plate was coated with 150 µl per well (1:1,000) of goat anti-Cry1Ac antibodies. The plate was then loaded with 100 µl samples and buffer was used in control wells. The plate was incubated at 37°C for 1.5 h, followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1:1,000 with 250 µl per well for 45 min at 37°C. The plate was then washed with wash buffer twice and 250 µl of freshly prepared substrate (*p*-nitro phenyl phosphate, 1 mg/ml) was added per well. The plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a micro plate reader (Tecan, Switzerland).

Segregation analysis

The leaves of T_1 transgenic plants generated from eight independent transformation events were analyzed for the presence of *npt*II and *Btcry1Ac* genes using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test (χ^2) as described by Solleti et al. (2008b).

Data analysis

Data were subjected to analysis of variance (ANOVA) and mean separation by Duncan's multiple-range test (DMRT) using single-factor completely randomized block design in order to study the effect of different treatments on frequencies of transient expression. All experiments were performed at least three times with a minimum of 30–40 explants per treatment.

Results and discussion

Effect of wounding

Efficient *Agrobacterium*-mediated transformation requires optimal delivery of the T-DNA to regenerable cells of the explants. Wounding of explants allows *Agrobacterium* to better access plant cells as it stimulates the production of potent *vir* gene inducers, like phenolic substances and enhances the plant cell competence for transformation (Stachel et al. 1985; Shimoda et al. 1990; Bidney et al. 1992). Only plants with an appropriate wound response develop larger populations of wound adjacent competent cells for regeneration and transformation (Potrykus 1991). Although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer via *Agrobacterium*-mediated transformation in recalcitrant species can be significantly enhanced by inducing wounds in the target tissue (Bidney et al. 1992). In cowpea, infection of cotyledonary node explants with most effective supervirulent *A. tumefaciens* strain EHA105, in absence of injury treatment resulted in 85% transient GUS expression frequency (Solleti et al. 2008a). However, the accounted GUS foci were located mostly at the cotyledons detachment site of cotyledonary node explants, and not at the regenerating site. The low stable transformation efficiency, 1.64% in cowpea (Solleti et al. 2008a) could be attributed to poor conversion of transient transformation to stable transformation. Wounding of regenerating sites of the cotyledonary node explants of cowpea by a hypodermic needle and co-cultivation with *A. tumefaciens* resulted in more efficient transient expression especially on needle-wounded explants, mainly in terms of the percentage of explants showing GUS foci at the regenerating sites as compared to unwounded explants infected with *A. tumefaciens* (Fig. 2). This clearly indicated that higher transient transformation of regenerating cells of meristematic tissue-based explants such as cotyledonary nodes, required an efficient wounding treatment. Wounding at the regenerating sites before co-cultivation allowed better bacterial penetration into the regenerating cells of cotyledonary node explants, facilitating the accessibility of plant cells for *Agrobacterium* infection. Such mechanical wounding treatments greatly enhanced transformation efficiency in a

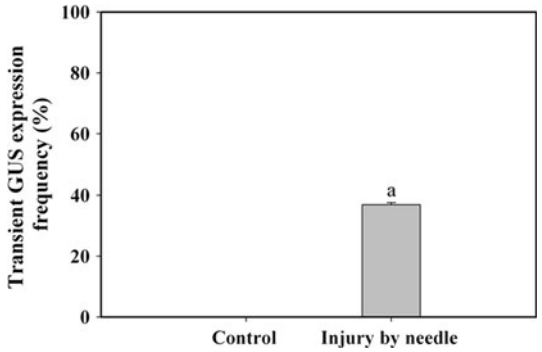

Fig. 2 Effect of mechanical injury by hypodermic needle on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. Control–injury is omitted in explants. The *bars* indicate ± standard errors. Means followed by the same letter are not statistically significant at $P < 0.05$

number of plant species including recalcitrant grain legumes (Roome 1992; Rohini et al. 2005; Supartana et al. 2006; Saini and Jaiwal 2007).

Effect of sonication and vacuum infiltration

To identify more efficient methods to improve access of *Agrobacterium* and also to create an area of wounding to induce cotyledonary node cells and to produce phenolic compounds for *vir* gene induction in cowpea, we evaluated the effect of sonication and vacuum infiltration on *A. tumefaciens*-mediated transformation of cotyledonary node explants. These treatments have the potential to increase transformation efficiency by improving penetration of *Agrobacterium* cells into the cell layers beneath the epidermis of cotyledonary node region. This is an important criterion as regenerating cells of cotyledonary node explants are positioned a few cells layers beneath the surface at the axils in *Vigna* species including cowpea, mungbean and blackgram (Sahoo and Jaiwal 2009). A control experiment with explants without inoculation with *Agrobacterium* was designed to determine whether these treatments could be used without a negative effect on shoot regeneration from cotyledonary node explant. Sonication was very effective in increasing transient GUS expression frequency (Figs. 3, 4a). With the increase in sonication treatment time, the number of transiently transformed explants increased significantly with a maximum of 79% of the explants showing GUS foci at the regenerating sites when sonication treatment was prolonged to 20 s (Figs. 3, 4a). The number of GUS foci appeared to be quite variable among cotyledonary node explants (data not shown). At lower sonication treatment time (10–20 s), the GUS foci were well defined, corresponding to probably one or a collection of small individual spots (Fig. 3a–f). With the increase in sonication treatment time beyond 20 s, a diffuse GUS expression was presented all over the surface of the cotyledonary node explants, making the quantification of the number of foci difficult (Fig. 3g–h). Moreover, with increase in sonication treatment time to 30 s, the untransformed explants showed a decrease in their bud-forming capacity indicating that longer sonication treatment compromised viability of regenerating cells (data not shown). SAAT has been used to enhance stable transformation of many recalcitrant plant species including soybean (Trick and Finer 1998), loblolly pine (Tang 2003), black locust (Zaragoza' et al. 2004), sweetpotato (Wang et al. 2006), rice (Yookongkaew et al. 2007), *Chenopodium rubrum* (Flores Solı́s et al. 2007), chickpea (Pathak and Hamzah 2008), flax (Beranova′ et al. 2008) and *Theobroma cacao* (Silva et al. 2009).

We attempted various time intervals of vacuum infiltration of explants at 600 mmHg in an *Agrobacterium* suspension, and of the different time intervals tested, a

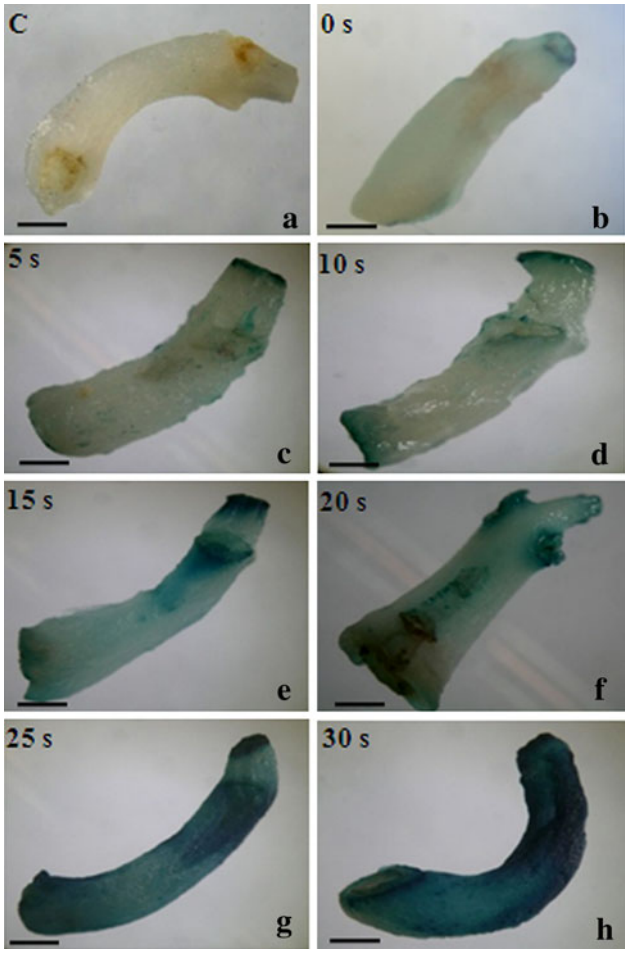

Fig. 3 Transient expression of GUS at the regenerating sites of sonication-treated cotyledonary node explants after 3 days of co-culture. **a** Control (untransformed). **b** *Agrobacterium*-treated explants (without sonication treatment). **c–h** sonication-treated cotyledonary nodes (**c** 5 s**, d** 10 s**, e** 15 s**, f** 20 s**, g** 25 s and **h** 30 s). *Bar* (in all figures) 1 mm

5 min vacuum infiltration resulted in a maximum of 93% transient transformation efficiency as accounted on the basis of number of explants showing GUS foci at the regenerating sites (Fig. 4b). Vacuum infiltration of cotyledon explants of *Pinus radiata* in an *Agrobacterium* suspension has allowed *Agrobacterium* to penetrate several layers deep through the sub-epidermal layer to mesophyll cells and vascular tissues (Charity et al. 2002), although the cells buried several layers deep, were not necessarily those that would induce shoots (Yeung et al. 1981). The vacuum infiltration of *Agrobacterium* has been successfully used to produce transgenic plants of model plant *Arabidopsis* (Clough and Bent 1998), and recalcitrant crop species including wheat (Cheng et al. 1997), mungbean (Jaiwal et al. 2001), pinus (Charity et al. 2002), cotton (Leelavathi et al. 2004), kidney bean (Liu et al. 2005), coffee (Canche-Moo et al. 2006), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

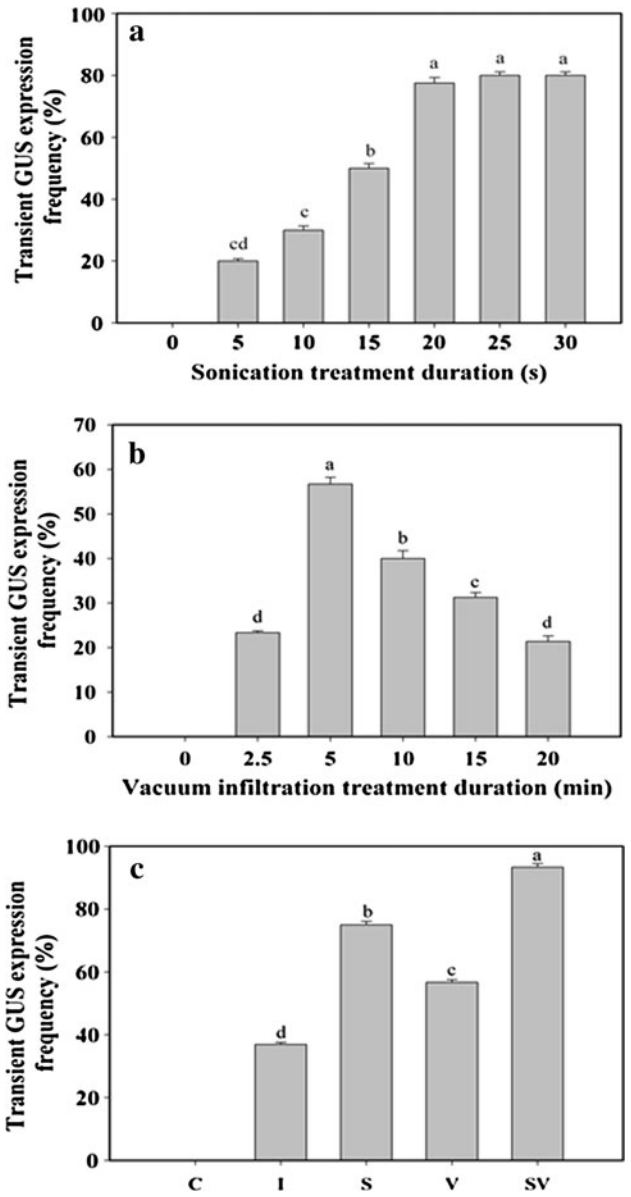

Fig. 4 **a** Effect of SAAT treatment duration and **b** vacuum infiltration treatment duration on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. **c** Effect of different wounding methods on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. *C* Without wounding. *I* Injury treatment by hypodermic needle. *S* 20 s sonication treatment. *V* Vacuum infiltration treatment for 5 min. *SV* 20 s sonication followed by vacuum infiltration treatment for 5 min. The *bars* indicate ± standard errors. Means followed by the same letter are not statistically significant at $P < 0.05$

Combined treatment of sonication and vacuum infiltration

In order to evaluate the combined action of sonication and vacuum infiltration on transient transformation, the effect of 20 s sonication and 5 min vacuum infiltration was tested as compared to the two treatments separately. The combination of 20 s sonication followed by 5 min vacuum infiltration resulted in maximum frequency of cotyledonary node explants expressing GUS at the regenerating sites (Fig. 4c). Sonication coupled with vacuum infiltration has increased transient and stable transformation of radish (Park et al. 2005), kidney bean (Liu et al. 2005), citrus (Oliveira et al. 2009), *Fraxinus pennsylvanica* (Du and Pijut 2009), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

Production of transgenic cowpea plants carrying *cry1Ac* gene

Putative transformed plants were regenerated from cotyledonary node explants, which were subjected to a combination of 20 s sonication followed by 5 min vacuum infiltration prior to *Agrobacterium* co-cultivation, on kanamycin selection medium and established in greenhouse containment (Fig. 5a–g). A strong, uniform and stable *gus* expression was detected in flower, anthers, pollens and pistils of PCR-positive T_0 plants and no endogenous *gus* expression was detected in the tissues of control plants (Fig. 5h–o).

Analysis of transgenic cowpea plants

The detection of the expected 540 bp and 1 kb amplified products corresponding to *nptII* and *cry1Ac* in PCR analysis confirmed the presence of the transgenes in T_0 transformed plants (Fig. 6a, b). No amplification was detected in the control untransformed plants.

Four randomly selected PCR-positive T_0 transgenic cowpea plants were further screened by Southern analysis to confirm the integration of *cry1Ac* gene. Southern blot analyses of four T_0 transgenic plants are shown in Fig. 6c. Hybridizations of DIG-labeled cry1Ac probe to total genomic DNA digested with *Hind*III were expected to identify DNA fragments unique to individual integration events greater than 5.0 kb (Fig. 1). All the four randomly selected T_0 transgenic plants were found positive for *cry1Ac* gene and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 6c, *lanes 1, 2, 3* and *4*). The T_0 transgenic plants exhibited simple hybridization patterns that ranged from single integration event to three loci and, in general, most fragments were greater than 5.0 kb (Fig. 6c). A signal of size less than 5.0 kb was detected in *lane 3* (Fig. 6c), suggesting the possibility of rearrangement of the T-DNA near the left border upon integration into the plant genome. No hybridization signal was detected in the untransformed plant (Fig. 6c, *lane C*).

Fig. 5 Transient and stable *gus* expression and regeneration of transgenic plants. **a** cotyledonary node explants. *Bar* 2 mm. (**b** and **c**) Transient GUS expression, non-transformed (control) explants not showing GUS activity (**b**), cotyledonary node explants showing transient GUS activity after 3 days of co-cultivation (**c**). *Bar* 4 mm. **d** Shoot induction from axils of explant after 5-day culture on SISM. *Bar* 2 mm. **e** Proliferation of multiple shoots within 4 weeks of culture. *Bar* 10 mm. **f** In vitro rooting of elongated transformed shoot. *Bar* 12 mm. **g** Acclimatized plant maintained in transgenic green house. *Bar* 10 cm. **h** Non-transformed control flower. *Bar* 7 mm. **i** Transformed flower. *Bar* 7 mm. **j** Control anthers. *Bar* 8 mm. **k** Transformed anthers. *Bar* 8 mm. **l** Control pistil. *Bar* 8 mm. **m** Transformed pistil. *Bar* 8 mm. **n** Control pollens. *Bar* 3 mm. **o** Transformed pollens. *Bar* 3 mm

The expression of the *cry1Ac* genes in leaves of T_0 transgenic plants was determined by RT-PCR analysis. RT-PCR showed the presence of expected transcripts of transgenes in different T_0 transgenic plants. The amplification of a 1 kb fragment of *cry1Ac* confirmed the accumulation of transcripts of *cry1Ac* in T_0 transgenic plants (Fig. 6d, e) indicating the absence of gene silencing events. Furthermore, the amplification of the *cry1Ac* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination.

The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for Bt*cry1Ac* and *npt*II divided by the total number of explants co-cultivated. An average stable transformation efficiency of 3.09 was recorded (Table 1), which was significantly higher than the previously published report on *Agrobacterium*-mediated transformation of cowpea using extra copies of *vir* genes (Solleti et al. 2008a).

Cry1Ac expression analysis

The randomly chosen PCR-positive T_0 transgenic lines were subjected to Cry1Ac protein expression analysis by Western hybridization and ELISA. The expression of the Cry1Ac protein was analyzed in T_0 transgenic lines generated from four independent transformation events by Western blot hybridization. A single band of 68 kDa corresponding to Cry1Ac toxin protein was detected

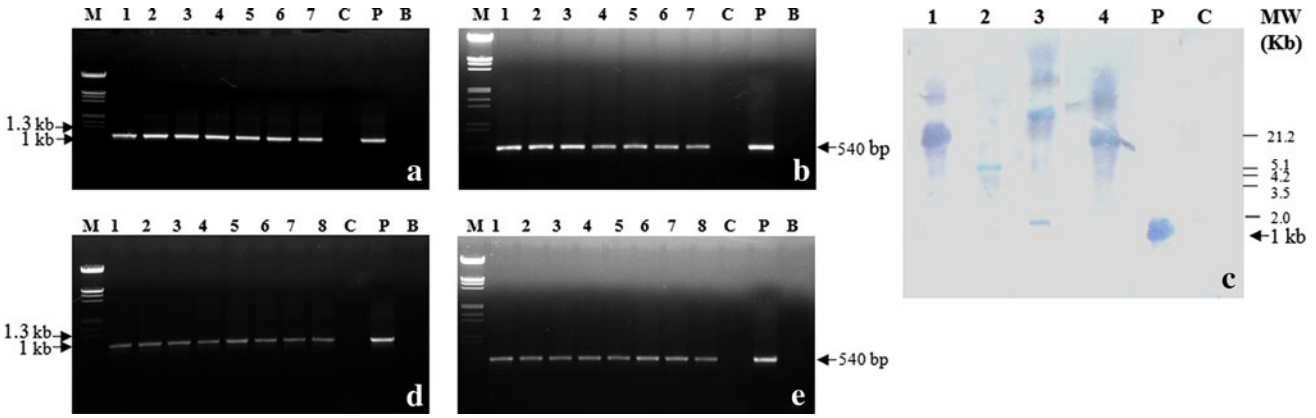

Fig. 6 Molecular analysis of T_0 transgenic plants. **a** PCR amplification of the 1 kb fragment of the *cry1Ac* gene, **b** PCR amplification of the 540 bp fragment of the *nptII* gene. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker, *lane P* pSouv:cry1Ac plasmid DNA (positive control), *lane C* DNA from untransformed plant (negative control), *lane B* blank, *lanes 1–7* DNA from independently transformed plants. **c** Southern blot hybridization analysis of junction fragments of four randomly selected PCR-positive T_0 lines. The plasmid and genomic DNA were digested with *Hin*dIII, and hybridized with cry1Ac probe. *Lanes 1–4* genomic DNA from four T_0 lines, *lane C* genomic DNA from untransformed plant, *lane P* cry1Ac PCR amplicon. **d** RT-PCR analysis of *cry1Ac* gene, **e** RT-PCR analysis of *nptII* gene. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker; *lane C* untransformed plant (negative control); *lane B* blank; *lanes 1–8* T_0 transgenic plants

Table 1 Summary of the *Agrobacterium*–mediated transformation of cowpea cotyledonary node explants subjected to 20 s sonication followed by vacuum infiltration for 5 min with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	Transient transformation efficiency[a] (%)	No. of shoots recovered on selection medium	No. of plants positive for *cry1Ac* and *npt*II genes by PCR	Transformation efficiency[b] (%)
1	247	91.10	15	8	3.20
2	239	95.00	12	7	2.93
3	204	92.12	11	6	2.94
4	243	95.10	12	8	3.30
Total[c]/average[d]	933[c]	93.33[d]	50[c]	29[c]	3.09[d]

[a] Number of explants showing GUS foci at the regenerating sites per number of explants co-cultivated with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

[b] Number of plants PCR-positive for *cry1Ac* and *npt*II per number of explants co-cultivated

[c] Total

[d] Average response

immunologically in T_0 transgenic plants confirming stability of *cry1Ac* expression. Protein extracts of control non-transformed plants did not show the 68 kDa protein band (Fig. 7a).

The level of expression of Cry1Ac protein in transgenic lines ranged from 0.001 to 0.089% of the total leaf soluble protein (Fig. 7b). The results described above demonstrated that expression of the *cry1Ac* regulated by the double 35S-promoter led to the accumulation of Cry1Ac protein in transgenic plants.

Segregation analysis

The seeds from T_0 generation were advanced to T_1 generation and the T_1 transgenic lines generated from eight independent transformation events were analyzed for the segregation pattern of *cry1Ac* by PCR analysis. Presence of the expected 1 kb amplified product corresponding to *cry1Ac* in T_1 transgenic lines confirmed the inheritance of *cry1Ac* gene (Fig. 8). The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 2).

In conclusion, an improved *Agrobacterium*-mediated transformation system was developed for cowpea by employing sonication and vacuum infiltration was enhanced by 88.4% using SAAT in combination with vacuum infiltration as compared to simple *Agrobacterium*-mediated transformation. This is the first report on cowpea transformation using SAAT and vacuum infiltration.

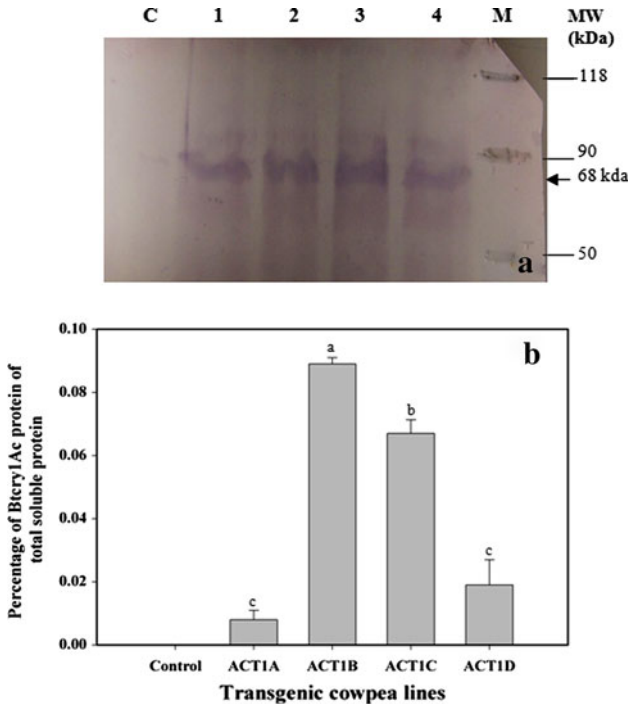

Fig. 7 **a** Detection of Cry1Ac protein by Western blotting analysis in transgenic cowpea leaves. *M* Protein molecular weight marker, *lanes 1–4 cry1Ac* transgenic lines (CT1A, CT1B, CT1C and CT1D), respectively, *lane 5* non-transformed plant. **b** Expression level of BtCry1Ac protein in transgenic cowpea lines (CT1A, CT1B, CT1C and CT1D) from enzyme-linked immunesorbent assay (ELISA). *Error bars* represent ± standard error of the means. Means followed by the same letter are not statistically significant at $P < 0.05$

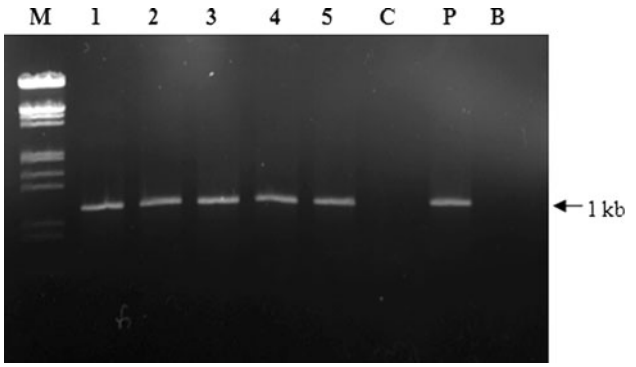

Fig. 8 PCR amplification of the 1 kb fragment of the *cry1Ac* gene of T_1 plants. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker, *lane P* pSouv:cry1Ac plasmid DNA (positive control), *lane C* DNA from untransformed plant (negative control), *lane B* blank, *lanes 1–5* DNA from T_1 transgenic plants

Furthermore, cowpea transgenics expressing *cry1Ac* is reported for the first time.

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Table 2 Segregation of *cry1Ac* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *cry1Ac*[a]			χ^2 value	Expected ratio
	Total	*cry1Ac* +ve	*cry1Ac* −ve		
C1	52	39	14	0.10	3:1
C2	35	24	11	0.77	3:1
C3	39	27	12	0.69	3:1
C4	55	40	15	0.15	3:1
C5	28	21	7	0.18	3:1
C6	43	30	13	0.56	3:1
C7	31	22	9	0.27	3:1
C8	44	40	4	0.61	15:1

[a] Presence of *cry1Ac* was analyzed by PCR

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