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3.1. Introduction

Soybean, besides being the world’s most important oil- and protein-producing crop, serves as a model plant in the functional genomics studies. The introduction of useful or novel gene(s) into soybean is important and it requires an efficient protocol for transgene integration and regeneration of transformed plants. Generally, soybean remains extremely recalcitrant to transformation (Trick and Finer, 1997). This problem may be acute due to non-availability of sufficient amount and nature of target tissues competent enough for integration of T-DNA from *Agrobacterium*. Enzymatic browning, tissue necrosis, death of target tissue due to wounding- and/ or plant-pathogen defense response, age of target tissue, explant nature, and genotype dependency are additional factors that decide the success of transformation in soybean.

In soybean, *Agrobacterium tumefaciens*-mediated transformation methods have been used only for few cultivars due to genotype specificity. The cells that are commonly targeted in soybean transformation include the meristematic cells located at the cotyledonary node (Hinchee et al., 1988; Townsend and Thomas, 1994; Zhang et al., 1999; Olhoft et al., 2003; Paz et al., 2006), and meristematic cells of freshly germinated soybean seeds (Chee et al., 1989; Martinell et al., 2002). Other reports of successful transformation protocols include the callus tissue induced from cotyledonary nodes and primary nodes (Hong et al., 2007), and cells on the surface of immature cotyledons (Parrott et al., 1989; Yan et al., 2000; Ko et al., 2003). Considerable efforts have also been made to enhance the efficiency of soybean transformation using various parameters like improvement in *vir* gene constructs (Hansen et al., 1994; Palanichelvam et al., 2000), identification and selection of susceptible soybean genotypes...
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responding (Byrne et al., 1987; Delzer et al., 1990; Meurer et al., 1998; Cho et al., 2000), addition of chemical inducers of the vir genes expression like acetosyringone (Bolton et al., 1986; Dye et al., 1997), improving selection and plant regeneration (Zhang et al., 1999; Olhoft et al., 2003; Paz et al., 2004; Zeng et al., 2004), application of thiol compounds (L-cysteine, dithiothreitol, and sodium thiosulfate) during co-cultivation (Olhoft et al., 2001), using alternate explants like embryonic tip and half-seed (Liu et al., 2004; Paz et al., 2006), using multi needle to improve efficiency of infection in cotyledonary nodal cells (Xue et al., 2006), and addition of surfactant like Silwet L-77 to the co-cultivation medium (Liu et al., 2008) etc. While these previous studies represented substantial progress towards improving the soybean transformation, none of them studied the combined effect of sonication and vacuum infiltration procedures in soybean transformation.

Sonication-assisted Agrobacterium-mediated transformation (SAAT) is an effective method to transfer foreign genes into especially recalcitrant target plants (Trick and Finer, 1997). This method involves subjecting the target explant to brief periods of sonication in presence of Agrobacterium carrying desired gene (Liu et al., 2006). The cavitation-induced microwounds created by SAAT facilitated the Agrobacterium to travel deeper and more completely throughout the tissue than normal co-cultivation would have permitted (Trick and Finer, 1997; Santarém et al., 1998; Tang et al., 2001; Liu et al., 2005), thus enhancing the bacterial colonization and infection of the tissue. SAAT has been successfully applied in loblolly pine, black locust, flax, and citrus (Santarém et al., 1998; Tang et al., 2001; Zaragozá et al., 2004; Beranová et al., 2008; De Oliveira et al., 2009).

Vacuum infiltration is another effective method for rapidly transforming and inducing transient transgene expression in many plant species. Bringing A. tumefaciens into contact with susceptible host plant cells is important for effective agroinfiltration. Vacuum infiltration, where plant tissue is submerged
in a liquid suspension of *Agrobacterium* and subjected to decreased pressure followed by rapid repressurization, is a common method for introducing bacteria to the interior of the plant tissue (Bechtold and Pelletier, 1998; Tague and Mantis, 2006). It has been proved that vacuum application substantially increases transient expression levels when compared to infiltrations at atmospheric pressure in lettuce (Joh *et al*., 2005). This method has been successfully applied to the transformation of *Brassica napus* (Wang *et al*., 2003), cabbage (Liu *et al*., 1998) and radish (Curtis and Nam, 2001).

Hence, the present study was conducted to (1) investigate the use of sonication, vacuum infiltration, and combination of these two treatments in *Agrobacterium*-mediated transformation on cv. PK 416 with EHA105 harboring pCAMBIA1301 using the cotyledonary node and half-seed as target tissue. In addition, to assess the conditions affecting *Agrobacterium*-mediated soybean transformation, the experiments were designed (2) to determine the optimal co-cultivation period and acetosyringone concentration in co-cultivation medium (3) to assess the effect of various concentrations and combinations of thiol compounds (L-cysteine, dithiothreitol and sodium thiosulfate) in co-cultivation medium (4) and to investigate the use of hygromycin B as the selection agent to determine the efficient selection concentration.

### 3.2. Materials and Methods

#### 3.2.1. Plant material and explant preparation

Cotyledonary node and half-seed explants were used for transformation studies and the explants were prepared as described in materials and methods sections 2.2.3 and 2.2.4 of chapter 2, respectively.
3.2.2. Sensitivity of cotyledonary node and half-seed explants to hygromycin B

Cotyledonary node and half-seed explants were cultured in SIPAM [MS salts and vitamins, sucrose (87.65 mM), BA (2.22 µM), and spermidine (137.69 µM)] along with different concentrations (2, 4, 6, 8 and 10 mg/l) of hygromycin B (Sigma, St. Louis, USA) to determine the sensitivity concentration (MIC) of hygromycin B that inhibits the shoot regeneration. After 10 days of initial culture, the explants of both types were subcultured 3 times (at 10 days interval) into fresh SIPAM medium containing respective concentrations of hygromycin B. Hygromycin B (50 mg/1ml) was filter sterilized (0.22 µm) and required aliquots were added to the warm autoclaved medium. A control without hygromycin B was also maintained for both the types of explants.

3.2.3. Sensitivity of elongated shoots on rooting to hygromycin B

Elongated shoots (>4 cm) were excised and transferred to RIPAM [MS salts and vitamins, sucrose (87.65 mM), IBA (4.93 µM), and putrescine (62.08 µM)] supplemented with different concentrations (1, 2, 3, 4 mg/l) of hygromycin B to determine the minimum inhibitory concentration (MIC) of hygromycin B that inhibits root formation. A control without hygromycin B was also maintained for both the types of explants.

3.2.4. Experimental design and statistical analysis for hygromycin B sensitivity tests

For hygromycin B sensitivity test on shoot regeneration, 50 explants of both the types were cultured per treatment and repeated thrice. Number of shoots/explant obtained after 40 days of culture were tabulated. For hygromycin B sensitivity test on rooting, 50 elongated shoots above 4 cm were cultured per treatment and repeated thrice. Percentage of root formation was tabulated after 30 days of culture. Data were statistically analyzed using analysis of variance (ANOVA). Data are presented as means±standard error. The mean separations
were carried out using Duncan’s multiple range test and significance was determined at 5% level (SPSS 11.5).

3.2.5. Agrobacterium strain and plasmid vector

*Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) harboring the binary vector pCAMBIA1301 (Cambia, 2006) was used in the transformation experiments (Fig. 3.1). The vector contains *hygromycin phosphotransferase* (*hpt*) gene which confers resistance to hygromycin B and an intron-containing *β-glucuronidase* gene (*uidA*) in its T-DNA. Both *hpt* and *uidA* genes are under the control of CaMV35S promoter and nos terminator. The *uidA* gene is expressed upon transfer into plant cells, but not in the *Agrobacterium* due to the presence of intron.

3.2.6. Optimization of parameters affecting Agrobacterium-mediated soybean transformation

3.2.6.1. *Agrobacterium* culture and pre-incubation with acetosyringone

Glycerol stock of *A. tumefaciens* EHA105/pCAMBIA1301 stored at -80°C was streaked on LB medium (Himedia, Mumbai, India) containing rifampicin (10 mg/l) and kanamycin (50 mg/l) [Sigma St. Louis, USA] solidified with agar (0.8%). Streaked *Agrobacterium* cultures were incubated under total darkness at 28°C until bacterial colony formation. A single colony was inoculated into 30 ml of liquid LB broth containing rifampicin (10 mg/l) and kanamycin (50 mg/l) and incubated in an orbital shaker (Orbitek-Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rpm for 16–18 hr at 28°C under total darkness. A single colony was inoculated into 30 ml of liquid LB broth containing rifampicin (10 mg/l) and kanamycin (50 mg/l) and incubated in an orbital shaker (Orbitek-Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rpm for 16–18 hr at 28°C under total darkness. A single colony was inoculated into 30 ml of liquid LB broth containing rifampicin (10 mg/l) and kanamycin (50 mg/l) and incubated in an orbital shaker (Orbitek-Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rpm for 16–18 hr at 28°C under total darkness. Subsequently, 200 μl of the *Agrobacterium* culture was transferred to a 250-ml Erlenmeyer flask containing 100 ml of LB broth containing the two antibiotics [rifampicin (10 mg/l) and kanamycin (50 mg/l)]. The cultures were incubated in an orbital shaker under total darkness at 200 rpm at 28°C until its OD$_{600}$ nm reached 1.0 ($\sim 5 \times 10^8$ cells/ml). About 30 ml of *Agrobacterium* culture was centrifuged at 5,000 rpm for 10 min and the bacterial cell pellet was
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re-suspended in 30 ml of liquid infection medium (LIM) comprising 1/2X MS salts and vitamins, sucrose (87.65 mM), and MES buffer (3 mM) [pH 5.4] (Sigma, St. Louis, USA). Acetosyringone (200 µM) [Fluka, Zurich, Switzerland] was added to the LIM containing Agrobacterium cell suspension, one hr prior to infection. Stock solutions of rifampicin, kanamycin and acetosyringone were filter sterilized (0.22 µm) before adding them to the respective medium.

3.2.6.2. Effect of co-cultivation period on GUS expression

The cotyledonal node explants were pricked gently (5 times), and randomly at the axillary and apical meristematic areas using a sterile hypodermic needle (27G1/1) [Dispovan, New Delhi, India]. The half-seed explants were wounded by making ten scratches at the surface of cotyledonal nodal regions with a number 11 Personna Plus surgeon’s blade (Himedia, Mumbai, India). Wounded-explants of each type were then transferred to 30 ml of Agrobacterium suspension and incubated at room temperature for 30 min with occasional gentle agitation. The explants of both the types were blot-dried using Whatman No.1 filter paper [Whatman (A part of GE healthcare), Mumbai, India]. Co-cultivation was performed for 1, 2, 3, 4, 5 and 6 days in co-cultivation medium (CCM) containing 1/2X MS salts and vitamins, sucrose (87.65 mM), MES buffer (20 mM), and 0.2% (w/v) phytagel (pH 5.4). The cotyledonal node explants were placed horizontally over a layer of sterile Whatman No.1 filter paper on CCM whereas half-seed explants were placed flat in such a way that adaxial side touching the sterile filter paper placed on CCM. During co-cultivation period, all the cultures were incubated at 25±2°C under total darkness.

3.2.6.3. Regeneration of shoots in selection medium

After co-cultivation, cotyledonal node and half-seed explants were washed initially with sterile distilled water thrice and then with liquid washing medium (LWM) comprising 1/2X MS salts and vitamins, sucrose (87.65 mM), MES buffer (3 mM) along with filter-sterilized cefotaxime (200 mg/l) [Duchefa,
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Haarlem, Netherlands, and vancomycin (50 mg/l) [Duchefa, Haarlem, Netherlands] twice to remove excess of *Agrobacterium*.

After washing, both the types of explants were blot-dried using sterile filter paper (Whatman No.1) and cultured in SIPAM with MES buffer (3 mM), cefotaxime (100 mg/l), and vancomycin (25 mg/l) without hygromycin B to stimulate shoot induction for the first 7 days. After this time, both the types of explants containing the newly developed shoot buds were subcultured 4 times at 10 days interval into fresh SIPAM containing the aforesaid antibiotics along with hygromycin B (10 mg/l) for selection of transformed shoots. The cultures were maintained at 25±2°C at 16/8 hr light/dark conditions with a light intensity of 50 µmol m−2 s−1.

3.2.6.4. Effect of acetosyringone on GUS expression

Effect of acetosyringone treatment during co-cultivation was tested by co-cultivating the infected cotyledonary node and half-seed explants on co-cultivation medium containing different concentrations (50–250 µM) of acetosyringone for 5 days. Both the types of explants were co-cultivated as mentioned in section 3.2.6.2. Washing of explants after co-cultivation, regeneration of transformed shoots and culture conditions were same as mentioned in section 3.2.6.3.

3.2.6.5. Effect of thiol compounds on GUS expression

To study the effect of thiol compounds on efficiency of soybean transformation, compounds such as L-cysteine, dithiothreitol (DTT) and sodium thiosulfate (Sigma, St. Louis, USA) were tested. The CCM containing acetosyringone (200 µM) was supplemented with various concentrations of L-cysteine (0.83–4.13 mM) individually and in combination with different concentrations of DTT (0.50–1.5 mM), and sodium thiosulfate (0.50–1.5 mM). All tested thiol compounds were filter sterilized (0.22 µm) before adding to
warm autoclaved medium. Both the types of explants were co-cultivated as mentioned in section 3.2.6.2. Washing of explants after co-cultivation, regeneration of transformed shoots and culture conditions were same as mentioned in section 3.2.6.3.

3.2.6.6. Effect of sonication and vacuum infiltration on GUS expression

To evaluate the effect of sonication and vacuum infiltration time period on GUS expression, two different experiments were carried out. Cotyledonary node and half-seed explants (wounded manually as mentioned in section 3.2.6.2) were placed in 150 ml Erlenmeyer flasks and covered with the previously prepared \textit{Agrobacterium} suspension. Sonication was accomplished with a water-bath sonicator (Branson Ultrasonics, Danbury, USA). Flasks containing the explants were sonicated by placing them into the water-bath chamber and holding in place for 5, 10, 15, 20 and 25 sec. \textit{Agrobacterium} suspension was discarded after sonication and replenished with fresh culture and incubated at room temperature for 30 min with occasional gentle agitation. In the second set of experiment the explants of both the types (wounded manually as mentioned in section 3.2.6.2) were sonicated as mentioned above for 20 sec. After sonication, the \textit{Agrobacterium} suspension was discarded and replenished with fresh suspension and subjected to vacuum infiltration (75 in of Hg) for 5, 10, 15, 20 and 25 min. The explants were incubated in the same \textit{Agrobacterium} suspension at room temperature for 30 min with occasional gentle agitation. The infected-explants were co-cultivated for 5 days in the CCM containing acetosyringone (200 µM) along with optimized concentration of thiol compounds. Both the types of explants were co-cultivated as mentioned in section 3.2.6.2. Washing of explants after co-cultivation, regeneration of transformed shoots and culture conditions were same as mentioned in section 3.2.6.3.
3.2.6.7. Experimental design and statistical analysis for optimization of parameters affecting Agrobacterium-mediated soybean transformation

Infection of explants was performed in batches of 25 explants/30 ml of Agrobacterium suspension for all the parameters/treatments. For standardization of factors, parameters and various treatments influencing transient GUS expression in the explants, 50 cotyledonary nodes and half-seeds were infected for each parameters/treatments and the experiment were repeated five times. Out of 50, 30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for transient GUS activity after (i) 1, 2, 3, 4, 5 and 6 days of co-cultivation for optimization of co-cultivation time period and (ii) 5 days of co-cultivation (optimized time period) for other parameters/treatments. Percentage of explants showing GUS expression was determined as the number of cotyledonary nodes or half-seeds with at least one qualifying GUS focus divided by the total number of cotyledonary node or half-seed explants assayed for GUS expression × 100.

For standardization of stable GUS expression in shoots, 125 cotyledonary nodes and half-seeds were infected for each parameters/treatments and the experiments were repeated five times. 30 randomly selected explants (both types with shoots) that responded in each replicate were assayed for GUS activity after 40 days of culture on selection medium (SIPAM + 10 mg/l hygromycin B). The percentage of explants that responded in selection medium (SIPAM + 10 mg/l hygromycin B) [after 40 days of selection] and mean numbers of GUS positive shoots/30 explants of both types were tabulated.

Data were statistically analyzed using analysis of variance (ANOVA). Data are presented as means±standard error. The mean separations were carried out using Duncan’s multiple range test and significance was determined at 5% level (SPSS 11.5).
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3.2.7. Cotyledonary node versus half-seed explants

To produce transgenic plants and to compare the final transformation efficiency among the two explants, 10 independent experiments were done for cotyledonary node and half-seed.

3.2.7.1. Agrobacterium infection, co-cultivation and regeneration of shoots in selection medium

Cotyledonary node and half-seed explants were infected and co-cultivated using the optimized parameters/treatments. Washing of explants after co-cultivation, regeneration and selection of transformed shoots from both the types of explants were carried out as mentioned in section 3.2.6.3.

3.2.7.2. Elongation, rooting and hardening

Cotyledonary node and half-seed explants with regenerated shoots were transferred to SEPAM [MS salts and vitamins, sucrose (87.65 mM), GA3 (1.45 µM), and spermine (74.13 µM)] amended with MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l), and hygromycin B (10 mg/l) for elongation of shoots. After 15 days, the explants were subcultured once on fresh SEPAM containing hygromycin B (10 mg/l) for another 15 days. The elongated shoots (>4 cm) were then transferred to RIPAM amended with same concentration of aforesaid antibiotics except hygromycin B, in which the concentration was reduced to 4 mg/l to enable rooting. All the cultures were incubated in same culture condition as mentioned in section 3.2.6.3.

After 30 days of culture in the RIPAM, the rooted-plantlets were removed from the culture tubes and were washed gently in running tap water to remove the gelling agent from the root surface. Then, the rooted-plantlets were transferred to plastic cups containing sterile sand, soil, and vermiculate (1:1:1 v/v/v). The plantlets were covered with polythene bags with minimum puncture and grown in growth chamber at 25±2°C with 85% relative humidity (RH) for
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2–3 weeks. The plantlets were irrigated once in two days. Upon growth, the plantlets were transferred to earthen pots containing sterile sand, soil, and vermiculate (1:1:1 v/v/v) and grown in greenhouse under controlled conditions.

3.2.7.3. Experimental design to compare transformation efficiency of cotyledonary node and half-seed explants

Ten independent experiments (for both the types of explants) were carried out by co-cultivating 70–83 explants. Number of explants responded in selection medium, number of hygromycin B resistant shoots produced [after 70 days of culture in selection medium (SIPAM + 10 mg/l hygromycin B – 40 days and SEPAM + 10 mg/l hygromycin B – 30 days)], number of hygromycin B resistant shoots forming roots (after 30 days of culture in RIPAM + 4 mg/l hygromycin B), number of putative T₀ transformants (PCR positive for \( hpt \) gene) and number of transgenic plants (Southern positive for \( hpt \) gene) for both the types of explant were recorded. Transformation efficiency (%) is calculated as number of transgenic plants obtained divided by total number of explants infected × 100.

3.2.8. Transgene analyses

3.2.8.1. Assay for β-glucuronidase activity

Expression of the \( gus \) gene was assayed by the histochemical staining procedure of Jefferson (1987) as listed below.

(1) Performed in cotyledonary node and half-seed explants after (i) 1, 2, 3, 4, 5 and 6 days of co-cultivation for optimization of co-cultivation time period and (ii) 5 days of co-cultivation (optimized time period) for other parameters/treatments along with respective controls (non-infected explants of both the types) to check the transient GUS expression in explants.

(2) Performed in cotyledonary node and half-seed explants with regenerated shoots after 40 days of culture on selection medium (SIPAM + 10 mg/l
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hygromycin B) along with respective controls (non-infected explants of both the types with regenerated shoots) to check the stable GUS expression in shoots.

(3) Performed in stems, leaves, flowers and floral parts of putative T₀ transformants regenerated from cotyledonary node explants along with respective controls of non-transformed plants to check the transgenic status.

(4) Performed in hand-cut tissue sections of stems and leaves (midrib) tissues of putative T₀ transformants regenerated from cotyledonary node explants along with respective controls (tissue sections) of non-transformed plants for microscopic analysis of GUS expression in transformed tissues sections.

All the plant materials and tissue sections were washed with distilled water and incubated for 10 min in phosphate buffer [NaH₂PO₄ (0.5 mM) and Na₂HPO₄ (0.5 mM)] (Sisco Research Laboratories, Mumbai, India) with pH 7.0 containing potassium ferri- and ferrocyanide (0.5 mM) and Na₂EDTA (10 mM) [Sisco Research Laboratories, Mumbai, India]. The buffer was replaced with fresh phosphate buffer containing 1% (v/v) Triton X-100 (Sigma, St. Louis, USA) and incubated for 1 hr at 37°C. After draining the solution, again fresh phosphate buffer containing solution of 5-bromo-4-chloro-3-indolyl β-D-glucuronide (1.0 mM) [X-Glu; Sisco Research Laboratories, Mumbai, India] was added and further incubated for 8 hr at 37°C. After incubation, the plant materials and tissue sections were subjected to dechlorophyllation by washing in acetone:methanol mixture (1:3 v/v) [Sigma, St Louis, USA] and then visually observed for blue staining. For microscopic analysis of GUS expression, the sections of stems and leaves midribs of putative T₀ transformants along with respective controls were fixed with Formalin-Acetic-Alcohol (FAA) solution.
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[ethyl alcohol (50 ml), glacial acetic acid (5 ml), 37–40% formaldehyde (10 ml) (Sigma, St Louis, USA), and distilled water (35 ml)]. The photographs were taken in Nikon color digital camera system DS-Fil-U2 (100-240 V) consisting NIS elements software package (Nikon, Tokyo, Japan).

3.2.8.2. Molecular confirmation of transformants

3.2.8.2.1. Isolation of genomic DNA

Soybean genomic DNA was isolated according to the method described by Dellaporta et al. (1983) by homogenizing 100 mg leaf tissue of putative T₀ transformants and control (non-transformed plant) in a 1.5 ml microfuge tube containing 500 µl of extraction buffer [Tris-HCl (100 mM, pH 8.0), EDTA (20 mM), NaCl (1.4 M), 2% (w/v) CTAB, and 0.5% (v/v) β-mercaptoethanol] (Sigma, St. Louis, USA). The extract was treated with 500 µl of the phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) [Sigma, St. Louis, USA] and centrifuged at 12,000 rpm for 12 min. The aqueous phase was collected, mixed with 1 µl of 10 mg/ml RNase A (Sigma, St. Louis, USA) and incubated at room temperature for 20 min. The samples were re-extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated by adding 2.5 volumes of ice cold ethanol and spooled out. The spooled DNA was washed with 70% (v/v) ethanol, dried and resuspended in 100 µl sterile 1X TE [(Tris-HCL (10 mM) and EDTA (1.0 mM)] buffer (pH 8.0).

3.2.8.2.2. Estimation of DNA

Quantification of DNA was done by measuring absorbance at 260 and 280 nm using UV spectrophotometer (Jasco, Tokyo, Japan). The isolated DNA (2 µl) was added to 998 µl of 1X TE, mixed well and taken in a quartz cuvette for measuring absorbance. Based on the optical density of the sample at 260 nm, the DNA concentration was calculated according to standard notion that an optical density value of one corresponds to 50 µg/ml of DNA.
3.2.8.2.3. **Polymerase chain reaction (PCR)**

To confirm the integration of the *hpt* gene into the genomic DNA of the putative T₀ transformants regenerated from cotyledonal node and half-seed explants, standard PCR analysis was performed using primers specific to *hpt* gene (forward, 5´-GATGTTGCGACCTCGTATT-3´ and reverse, 5´-GTGTCACGTTGCAAGACCTG-3´). Non-transformed soybean plant genomic DNA and Plasmid pCAMBIA1301 (positive control) were also subjected to amplification. The PCR reaction contains 50 ng of genomic DNA or 50 ng of plasmid DNA, 0.2 mM dNTPs, 1.0 U of *Taq* DNA polymerase (Sigma Genosys, Texas, USA), 0.4 µM of each primer and 2.5 µl of 10X Taq buffer in a total of 25 µl reaction. PCR amplifications were carried out in a PTC-200™ thermal cycler (MJ research Inc, Waltham, Mass, USA) programmed with one cycle of initial denaturation of DNA at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min (for denaturation), 55°C for 1 min (for annealing) and 72°C for 1 min (for extension), followed by a final extension at 72°C for 7 min. The amplified fragments were analyzed by electrophoresis at 100 V for 45 min in a 1% (w/v) agarose (Sigma, St. Louis, USA) gel containing 0.5 µg/ml ethidium bromide (Sigma, St. Louis, USA), and visualized on UV transilluminator.

3.2.8.2.4. **Southern blot analysis**

For Southern blot hybridization, 10 µg of genomic DNA from putative T₀ transformants regenerated from cotyledonal node and half-seed explants along with non-transformed plant and positive control (5 µg of pCAMBIA1301 plasmid) were digested overnight with *Eco*RI (MBI Fermentas, St. Leon-Rot, Germany), which cuts at one site within the T-DNA region. The digested DNAs were separated on a 1% (w/v) agarose gel.

The gel was depurinated with 250 ml of diluted HCl (250 mM) for 10–12 min (till the bromophenol blue dye turns turmeric yellow color). After brief rinsing of gel with sterile Milli-Q water, DNA was denatured by soaking the gel...
in 250 ml of denaturation solution [(NaCl (1.5 M) and NaOH (0.5 M))] for 25 min (till bromophenol dye retains its blue color). Finally, the gel was rinsed with sterile Milli-Q water and neutralized with 250 ml of neutralization solution [(NaCl (1.5 M) and Tris-Cl (0.5 M), pH 7.5)] for 30 min. The agarose gel was subjected to capillary blotting (Southern, 1975) using 20X SSC buffer [(NaCl (3 M) and tri-sodium citrate (0.3 M), pH 7.0)] for transfer of DNA onto Hybond N+ membrane (Amersham Biosciences, Buckinghamshire, England). After blotting, the membrane was removed and rinsed briefly for 1 min in 6X SSC buffer to remove the agarose pieces. The membrane was air dried and subjected to UV cross linking for 5 min to fix the DNA firmly to the membrane.

The PCR amplified product of hpt gene (407 bp) was purified using QIA quick PCR purification kit (Qiagen, GmbH, Germany) and used for the probe preparation. The DNA was labeled by non-radioactive labeling kit (AlkPhos Direct Labeling and Detection System with CDP-Star) [GE healthcare limited, Buckinghamshire, England]. DNA (100 ng) was denatured for 5 min in boiling water bath and immediately plunged into ice for 5 min. DNA labeling reagent and cross linker was added to denatured probe and incubated at 37°C for 30 min.

The blot was prehybridized at 55°C for 1 hr in hybridization buffer containing 5% (w/v) blocking agent and NaCl (0.5 M). The labeled probe (ALP-labeled 407 bp PCR amplified product of hpt gene) was added to the hybridization buffer and the blot was incubated in hybridization oven (Amersham Biosciences, Buckinghamshire, England) with gentle agitation for 8 hr at 55°C. Post hybridization washes were performed in high stringency conditions.

The blot was washed twice with an excess of primary wash buffer [urea (2 M), NaCl (150 mM), sodium phosphate buffer (50 mM, pH 7.0), MgCl₂ (1 mM), 0.1% (w/v) SDS, and 0.2% (w/v) blocking agent] for 10 min each at
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55°C. Further, the blot was washed two times with secondary wash buffer [Tris base (0.25 M) and NaCl (0.5 M), pH 10.0] for 5 min each at 37°C. The membrane was air dried and inundated with detection reagent (Alkphos CDP-star) for 5 min. The membrane was placed in saran wrap (SC Johnson, Racine–Wisconsin, USA) and exposed to X-ray film in an X-ray cassette (Kodak, Mumbai, India) under dark condition. After 10 min, the X-ray film was developed and the signal was detected in X-ray film using developer and fixer solutions (Kodak, Mumbai, India).

3.3. Results

3.3.1. Plant material

Cotyledonary node (Fig. 3.11a) and half-seed explants (Fig. 3.12a) were used for Agrobacterium-mediated gene transfer through direct regeneration system.

3.3.2. Sensitivity of cotyledonary node and half-seed explants to hygromycin B

Cotyledonary node and half-seed explants were cultured in different concentrations of hygromycin B to determine the inhibitory concentration to screen the transformed shoots. Both the types of explants when cultured on SIPAM without hygromycin B (control) produced 39.02 and 34.31 shoots/explant respectively after 40 days of culture. Fig. 3.2 shows a progressive decrease in shoot number as the hygromycin B concentration was increased from 0 to 10 mg/l. Complete inhibition of shoot production occurred at 10 mg/l concentration hygromycin B for both the types of explants in SIPAM. Therefore, 10 mg/l hygromycin B was assigned as sensitivity concentration for selection of transformed shoots.

3.3.3. Sensitivity of elongated shoots on rooting to hygromycin B

Elongated shoots (>4 cm), regenerated from both the types of explants were excised and cultured on RIPAM supplemented with different
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concentrations of hygromycin B (0–4 mg/l). After 30 days, the rooting response was scored. In the medium without hygromycin B (control), maximum rooting percentage of 94.04% was noticed (Fig. 3.3). Increase of hygromycin B concentration from 0 to 3 mg/l in RIPAM, resulted in a gradual decrease in the percentage of root induction response, and at a concentration of 4 mg/l, complete inhibition of root production was observed (Fig. 3.3). Therefore, 4 mg/l hygromycin B was selected as sensitive concentration for rooting of the transformed elongated shoots derived from both types of explants.

3.3.4. Agrobacterium strain and plasmid vector

*Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) harboring the binary vector pCAMBIA1301 with culture OD$_{600nm}$ of 1.0 was used in the transformation experiments.

3.3.5. Optimization of parameters affecting Agrobacterium-mediated soybean transformation

Factors affecting *Agrobacterium*-mediated soybean transformation were optimized by performing transient GUS assay in cotyledonary node and half-seed explants (i) after 1, 2, 3, 4, 5 and 6 days of co-cultivation for optimization of co-cultivation time period and (ii) 5 days of co-cultivation (optimized time period) for other parameters/treatments. Stable GUS expression was assessed in shoots regenerated from both the types of explants after 40 days of culture on selection medium (SIPAM + 10 mg/l hygromycin B).

3.3.5.1. Effect of co-cultivation period on GUS expression

Six different time periods (1, 2, 3, 4, 5 and 6 days) were used to test the optimal co-cultivation period for maximum GUS expression of infected cotyledonary node and half-seed explants with *Agrobacterium* strain EHA105.
3.3.5.1.1. Effect of co-cultivation on transient GUS expression in the explants

Among the different co-cultivation periods tested, 5 days co-cultivation was found to be optimal at which the maximum transient GUS expression was observed in both cotyledonary node (17.33%) and half-seed explants (12.00%) [Fig. 3.4]. Increase (>5 days) or decrease (<5 days) in co-cultivation time showed a decline in percentage of explants showing transient GUS expression in both the types of explants (Fig. 3.4).

3.3.5.1.2. Effect of co-cultivation on stable GUS expression in the regenerated shoots

Explants co-cultivated for 5 days produced maximum number of GUS positive shoots (2.60 shoots/30 cotyledonary node explants and 1.60 shoots/30 half-seed explants) [Table 3.1] when compared to other co-cultivation periods tested. The explants responded at the same co-cultivation period were found to be 37.60% for cotyledonary node explants and 31.04% for half-seed explants (Table 3.1). Increase (>5 days) or decrease (<5 days) in time period, reduced the number of GUS positive shoots as well as percentage of explant responding for both the types of explants compared to the optimal co-cultivation period of 5 days (Table 3.1).

3.3.5.2. Effect of acetosyringone on GUS expression

The effect of acetosyringone treatment during co-cultivation of cotyledonary node and half-seed explants infected with EHA105 strain has been studied. Addition of acetosyringone to the CCM increased GUS expression (transient as well as stable) in both the types of explants when compared to control (explants co-cultivated without acetosyringone supplementation in CCM) [Fig. 3.5; Table 3.1].
3.3.5.2.1. Effect of acetosyringone on transient GUS expression in the explants

Acetosyringone at a concentration of 200 µM resulted with higher level of transient GUS expression in both the types of explants (26.66% for cotyledonary node explants and 20.66% for half-seed explants) when compared to that of other concentrations tested (Fig. 3.5). Increase or decrease in concentration of acetosyringone (200 µM) showed a decrease in percentage of explants showing transient GUS expression for both the types of explants (Fig. 3.5).

3.3.5.2.2. Effect of acetosyringone on stable GUS expression in the regenerated shoots

Among the different concentrations of acetosyringone tested, a concentration of 200 µM was most effective for both the types of explants and produced maximum number of stable GUS positive shoots (4.60 shoots/30 cotyledonary node explants and 3.00 shoots/30 half-seed explants) [Table 3.1]. The percentage of explants responded at the same concentration in selection medium (SIPAM + 10 mg/l hygromycin B) were found to be 41.28% for cotyledonary node explants and 34.24% for half-seed explants (Table 3.1). Increase or decrease in acetosyringone concentration (200 µM) affected the production of GUS positive shoots as well as percentage of explant responding for both the types of explants (Table 3.1). Results confirmed that supplementation of acetosyringone to CCM generated some results in transformation for both the types of explants, but still the percentage of explants showing transient GUS expression, number of explants responding in the selection medium (SIPAM + 10 mg/l hygromycin B) as well as production of stable GUS positive shoots remained low even at the optimal concentration of acetosyringone (200 µM) [Fig. 3.5; Table 3.1]. The reason for such poor T-DNA delivery in to the regenerable cells in both the types of explants was assumed due to formation of enzymatic browning and tissue necrosis at the wounded-sites.
of the explants following co-cultivation (Fig. 3.13i – cotyledonary node; 3.13j – half-seed). Hence, standardization with supplementation of thiol compounds (L-cysteine, DTT, and sodium thiosulfate) in different concentrations and combinations to CCM containing acetosyringone (200 µM) has been carried out to determine their role on controlling the enzymatic browning and tissue necrosis at the wounded-sites of both the types of explants.

3.3.5.3. Effect of thiol compounds on GUS expression

Addition of thiol compounds to CCM containing acetosyringone (200 µM) greatly controlled enzymatic browning and tissue necrosis. On the other hand it increased transient GUS expression in both the types of explants (Fig. 3.6, 3.7 & 3.8) as well as stable GUS expression in shoots regenerated from both the types of explants and also increased the percentage of cotyledonary node and half-seed explants responding in selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.2] when compared to control (explants co-cultivated without thiol compounds supplementation in CCM).

3.3.5.3.1. Effect of thiol compounds on transient GUS expression in the explants

L-cysteine (0.83–4.13 mM) amendment to the CCM containing acetosyringone (200 µM) controlled the enzymatic browning and tissue necrosis and increased the percentage of cotyledonary node and half-seed explants showing transient GUS expression when compared to that of control (without L-cysteine) [Fig. 3.6]. Percentage of explants showing transient GUS expression increased from 28.66 to 42.66% for cotyledonary node explants and 22.00 to 30.66% for half-seed explant (Fig. 3.6) respectively with increasing the concentration of L-cysteine (0.83–3.31 mM) in CCM containing acetosyringone (200 µM). Further increase of L-cysteine concentration (>3.31 mM) showed a decline in percentage of explants showing transient GUS expression for both the
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types of explants (38.66% for cotyledonary node explants and 28.00% for half-seed explants) [Fig. 3.6].

Further, addition of DTT (0.50–1.50 mM) to CCM containing optimal concentration of acetosyringone (200 µM) and L-cysteine (3.31 mM) decreased enzymatic browning (compared to L-cysteine treatment) and increased the percentage of explants showing transient GUS expression in both the types of explants (Fig. 3.7). Among the different combinations tested, CCM containing acetosyringone, L-cysteine and DTT (1.00 mM), resulted with a maximum of 51.33% of cotyledonary node and 37.33% of half-seed explants showing transient GUS expression (Fig. 3.7).

In order to reduce tissue necrosis further, sodium thiosulfate at different concentrations (0.50–1.50 mM) was added to CCM containing optimized concentration of acetosyringone (200 µM), L-cysteine (3.31 mM), and DTT (1.00 mM). Addition of sodium thiosulfate decreased enzymatic browning to a greater extent, prevented tissue necrosis and further increased the percentage of explants showing transient GUS expression (Fig. 3.8) in both the types of explants (compared to 3.31 mM L-cysteine and 1.00 mM DTT combinations tested for both the types of explants). Among the several combinations tested, acetosyringone, L-cysteine, DTT, and sodium thiosulfate (1.00 mM) was found to be most effective and at this combination 60.00% of cotyledonary node explants and 44.66% of half-seed explants showed transient GUS expression (Fig. 3.8). At this combination, the morphological appearance of the wounded explants increased with very less enzymatic browning and tissue necrosis.

3.3.5.3.2. Effect of thiol compounds on stable GUS expression in the regenerated shoots

The percentage of explants (cotyledonary node and half-seed) responding in selection medium (SIPAM + 10 mg/l hygromycin B) and number of GUS
positive shoot production from both the types of explants increased positively with the increasing concentration of L-cysteine (0.83 to 3.31 mM) in CCM containing acetoxyringone (200 µM) and at a optimal concentration of 3.31 mM, 56.80% of cotyledonary node explants and 48.16% of half-seed explants responded in the selection medium (SIPAM + 10 mg/l hygromycin B) and produced maximum number of GUS positive shoots (8.40 shoots/30 cotyledonary node explants and 5.40 shoots/30 half-seed explants) [Table 3.2]. Further increase of concentration in L-cysteine (>3.31 mM) showed a reduction in percentage of explants responding in selection medium (SIPAM + 10 mg/l hygromycin B) [54.88% for cotyledonary node explants and 46.40% for half-seed explants] as well as production of GUS positive shoots (7.60 shoots/30 cotyledonary node explants and 4.80 shoots/30 half-seed explants) [Table 3.2].

Addition of DTT (0.50–1.50 mM) to CCM containing optimal concentration of acetoxyringone (200 µM) and L-cysteine (3.31 mM) further increased the percentage of explants responding in the selection medium (SIPAM + 10 mg/l hygromycin B) and also increased the number of GUS positive shoots in both the types of explants (Table 3.2). Among several combinations tested, CCM containing acetoxyringone, L-cysteine, and DTT (1.00 mM) showed better results and a maximum of 60.96% of cotyledonary node explants and 52.16% of half-seed explants responded in the selection medium (SIPAM + 10 mg/l hygromycin B) and produced maximum number of GUS positive shoots (10.40 shoots/30 cotyledonary node explants and 7.20 shoots/30 half-seed explants) [Table 3.2].

Supplementation of sodium thiosulfate (0.50–1.50 mM) to CCM containing acetoxyringone (200 µM), L-cysteine (3.3 mM), and DTT (1.00 mM) further increased the percentage of explants responding in the selection medium (SIPAM + 10 mg/l hygromycin B) and also favored the increase in GUS positive shoot production from both the types of explants (Table 3.2). A combination of
acetosyringone, L-cysteine, DTT and sodium thiosulfate (1.00 mM) was found to be most effective when compared to other combinations tested and at this combination, 65.60% of cotyledonary node explants and 57.28% of half-seed explants responded and produced 12.60 GUS positive shoots/30 cotyledonary node explants and 9.20 GUS positive shoots/30 half-seed explants (Table 3.2).

The combination of thiol compounds (L-cysteine (3.31 mM), DTT (1.00 mM), and sodium thiosulfate (1.00 mM) to CCM containing acetosyringone (200 µM) greatly reduced enzymatic browning and tissue necrosis at the wounded-sites of cotyledonary node and half-seed explants making regenerable cells competent for T-DNA delivery and increased the transient GUS expression in explants as well as stable GUS expression in shoots from both the types when compared to control (Fig. 3.6, 3.7 & 3.8; Table 3.2). With an interest to further improve the T-DNA delivery into cells of cotyledonary node and half-seed explants, in addition to wounding of explants done manually, methods like sonication and vacuum infiltration were adopted in the present study. Role of such treatments in increasing T-DNA delivery for both the types of explants were checked by performing transient and stable GUS assay.

3.3.5.4. Effect of sonication and vacuum infiltration on GUS expression

Sonication and combination of sonication and vacuum infiltration treatment adopted for cotyledonary node and half-seed explants greatly increased the percentage of explants showing transient GUS expression in both the types of explants as well as number of GUS positive shoots in both the types of explants when compared to control (explants co-cultivated without subjecting them to above treatments) [Fig. 3.9 & 3.10; Table 3.3]. There was a slight increase in enzymatic browning and tissue necrosis in both the types of explants subjected to sonication and vacuum infiltration treatment even though CCM contained optimized concentration of thiol compounds. However, the increase is very meager and not affected the T-DNA delivery.
3.3.5.4.1. **Effect of sonication and vacuum infiltration on transient GUS expression in the explants**

Out of different time periods tested (5–25 sec), a 20 sec sonication was found to be optimal for both the types of explants when compared to other time period tested. At the same time period, 72.66% of cotyledonary explants and 54.66% of half-seed explants showed transient GUS expression (Fig. 3.9). Increase (>20 sec) or decrease (<20 sec) in sonication time period showed decrease in percentage of transient GUS expression in both cotyledonary node and half-seed explants (Fig. 3.9). Further, the sonicated explants (20 sec) were subjected to different vacuum infiltration time period (1–20 min) which showed better results with increased percentage of transient GUS expression in both the types of explants. A 5 min vacuum infiltration showed greatest effect for both the types of explants. At this time period, 86.00% of cotyledonary explants and 65.33% of half-seed explants showed transient GUS expression (Fig. 3.10). Increase (>5 min) or decrease (<5 min) in vacuum infiltration time period showed decrease in percentage of transient GUS expression in both cotyledonary node and half-seed explants (Fig. 3.10).

3.3.5.4.2. **Effect of sonication and vacuum infiltration on stable GUS expression in the regenerated shoots**

The production of GUS positive shoots was found more when both the types of explants were sonicated for a time period of 20 sec (16.00 shoots/30 cotyledonary node explants and 12.20 shoots/30 half-seed explants) when compared to other time periods tested (Table 3.3). At this time period, 74.72% of cotyledonary node explants and 66.24% of half-seed explants responded in the selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.3]. Increase (>20 sec) or decrease (<20 sec) in sonication period led to the reduction of GUS positive shoots as well as percentage of explants responding in the selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.3].
Combining sonication (20 sec) with vacuum infiltration treatment further increased the production of GUS positive shoots. A vacuum period for 5 min combined with sonication (20 sec) was found to be optimal and resulted in maximum number of GUS positive shoots from both the types of explants (20.60 shoots/30 cotyledonary node explants and 15.60 shoots/30 half-seed explants) compared to other treatments tested (Table 3.3). At this optimal treatment, a maximum of 81.76% of cotyledonary node explants and 72.80% of half-seed explants responded in the selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.3]. Increase (>5 min) or decrease (<5 min) in vacuum infiltration time period led to the reduction of GUS positive shoots as well as percentage of explants responding in the selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.3].

From the overall standardization of various parameters influencing Agrobacterium-mediated transformation of cotyledonary node and half-seed explants of cv. PK 416, both types of wounded explants (manually) subjected to 20 sec sonication, 5 min vacuum infiltration and co-cultivated in CCM containing acetosyringone (200 µM), L-cysteine (3.31 mM), DTT (1.00 mM), and sodium thiosulfate (1.00 mM) generated maximum GUS activity. Transformation experiments were conducted further for both the types of explants with the above optimized treatments for production of transgenic plants.

3.3.6. Cotyledonary node versus half-seed

The sum total of 10 individual co-cultivation experiments with 748 cotyledonary node explants and 783 half-seed explants were infected with Agrobacterium strain EHA105 harboring pCAMBIA1301 to produce transgenic plants. Transformation efficiencies between the two explants were compared based on Southern blotting results. The transformation efficiency was found to range between 1.25 to 5.26% (average = 3.20%) for cotyledonary node explants and 0.0 to 2.66% (average = 1.15%) for half-seed explants (Table 3.4 & 3.5).
3.3.6.1. Shoot regeneration, elongation, rooting and hardening

After infection and 5 days co-cultivation, cotyledonary node and half-seed explants when cultured on SIPAM containing MES buffer (3 mM) cefotaxime (100 mg/l), and vancomycin (25 mg/l) without hygromycin B (10 mg/l) for 7 days initiated shoot buds [Fig. 3.11b – cotyledonary node; Fig. 3.12b – half-seed]. After 7 days of culture, explants of both types were transferred to SIPAM containing aforesaid antibiotics with 10 mg/l hygromycin B and subcultured at 10 days interval for further growth and selection of transformed shoots. The regenerated shoots from cotyledonary node and half-seed explants after 8, 30 and 45 days of selection in SIPAM containing 10 mg/l hygromycin B were photographed (Fig. 3.11 c, d &e – cotyledonary node; Fig. 3.12 c, d &e – half-seed). Out of 748 cotyledonary node explants co-cultivated, 613 explants responded in the selection medium and in the case of half-seed explants, out of 783 explants co-cultivated, 569 explants responded in the selection medium and produced shoots (Table 3.4 – cotyledonary node; Table 3.5 – half-seed). Regenerated shoots from both the types of explants were elongated in SEPAM in which hygromycin B concentration was maintained at 10 mg/l (Fig. 3.11f – cotyledonary node; Fig. 3.12f – half-seed). After 30 days of culture in such a medium, a total of 96 and 56 hygromycin B resistant elongated shoots were produced from the cotyledonary node and half-seed explants, respectively (Table 3.4 – cotyledonary node; Table 3.5 – half-seed). The elongated-shoots from cotyledonary node and half-seed explants were successfully rooted in RIPAM containing 4 mg/l hygromycin B (Fig. 3.11g – cotyledonary node; Fig. 3.12g – half-seed). Out of 96 and 56 elongated shoots of cotyledonary node and half-seed explants, 38 and 21 shoots responded respectively for rooting by developing roots (Table 3.4 – cotyledonary node; Table 3.5 – half-seed). The rooted-plantlets of cotyledonary node and half-seed explants were hardened in the plastic cups containing sterile sand, soil, and vermiculate (1:1:1 v/v/v). The plantlets were covered with polyethylene bags with minimum puncture and grown in the growth chamber at 25±2°C with 85% of relative humidity (RH) for
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2–3 weeks. The plantlets were irrigated once in two days. Upon growth, the plantlets were transferred to earthen pots containing sterile sand, soil, and vermiculate (1:1:1 v/v/v) and grown in the greenhouse under controlled condition. A final of 35 plantlets regenerated from cotyledonary node explants and 18 plantlets regenerated from half-seed explants survived successfully after hardening and acclimatization procedures (Fig. 3.11h &i – cotyledonary node; Fig. 3.12h &i – half-seed).

3.3.7. Transgene analyses

3.3.7.1. Assay for $\beta$-glucuronidase activity

Histochemical localization of GUS activity was employed to test the effect of various factors influencing the transformation efficiency i.e., co-cultivation time period, acetylsyringone concentration, thiol compounds concentration, sonication and vacuum infiltration time duration. The cotyledonary node and half-seed explants, shoots from transformed explants (both types) were subjected to GUS assay. Intense blue color was observed in both transformed cotyledonary node and half-seed explants (Fig. 3.13a – cotyledonary node; Fig. 3.13c – half-seed) and shoots regenerated on both the types of explants (Fig. 3.13e – cotyledonary node; Fig. 3.13g – half-seed). No GUS activity was observed in non-infected explants (Fig. 3.13b – cotyledonary node; Fig. 3.13d – half-seed) as well as shoots of both the types of explants (Fig. 3.13f – cotyledonary node; Fig. 3.13h – half-seed) [control]. Intense blue color was observed in stems (Fig. 3.14a), leaves (Fig. 3.15a), flowers (Fig. 3.16a) and floral parts (Fig. 3.16b–h: gynoecium; i–j: calyx; k–l: standard petal; m–n: keels; o: anther; p–q: pollen grains) of putative T0 transformants regenerated from cotyledonary node explants subjected to GUS assay and no GUS activity was observed with respective controls from non-transformed plants [stems (Fig. 3.14b), leaves (Fig. 3.15b), flowers (Fig. 3.16g) and floral parts (Fig. 3.16b–h: gynoecium; i–j: calyx; k–l: standard petal; m–n: keels; o: anther; p–q: pollen grains)]. Microscopic
observation revealed blue staining in hand-cut tissue sections of stems (Fig. 3.14c, e–k) and leaves midrib tissue (Fig. 3.15c, e–h) of putative T₀ transformants regenerated from cotyledonary node explants. The respective control sections from non-transformed plants showed no such staining when observed under microscope (Fig. 3.14d – stem section; Fig. 3.15d – leaf midrib section).

3.3.7.2. Molecular confirmation of transformants

3.3.7.2.1. Polymerase chain reaction (PCR)

Genomic DNA of 35 putative T₀ transformants regenerated from cotyledonary node explants and 18 regenerated from half-seed explants were subjected to PCR amplification of the hpt gene. Genomic DNA of non-transformed plant and positive control (plasmid of pCAMBIA1301) were also subjected to PCR. The primers used for PCR amplification and the amplification procedure are described in section 3.2.8.2.3. PCR amplification analysis of DNA from the putative T₀ transformants derived from both the types of explants yielded the expected amplicon (407 bp) of hpt gene which was visible by exposing the EtBr stained gel in UV light. The presence of hpt gene was confirmed in 29/35 genomic DNA samples of plants regenerated from cotyledonary node explants and 14/18 genomic DNA sample of plants regenerated from half-seed explants (Table 3.4 – cotyledonary node; Table 3.5 – half-seed). The amplification was not seen in non-transformed control plant. The amplification of the 407 bp was also observed with the plasmid pCAMBIA1301. PCR analysis results of 5 putative T₀ transformants regenerated from cotyledonary node (randomly chosen from 29 PCR positive plants) and half-seed explants (randomly chosen from 14 PCR positive plants) along with non-transformed plants and positive control has been grouped together in Fig. 3.17 (a) and Fig. 3.18 (a) respectively.
3.3.7.2.2. Southern blot analysis

To further ensure the transgene integration and copy number analysis, genomic DNA from putative T₀ transformants (PCR positive) regenerated from cotyledonary node explants and half-seed explants, genomic DNA from non-transformed plant, and positive control (plasmid of pCAMBIA1301) were subjected to Southern blot hybridization. These genomic DNAs and plasmid of pCAMBIA1301 were digested with restriction enzyme EcoRI and PCR amplified hpt gene fragment was used as probe. The detailed procedure for Southern hybridization is described in section 3.2.8.2.4. 24/29 genomic DNA samples of plants regenerated from cotyledonary node explants showed Southern positive (single copy T-DNA integration in 16 plants, two T-DNA copy in 5 plants and three T-DNA copy in 3 plants) by developing the signals when the blot was exposed to X-ray film in dark (Table 3.4). In the case of plants regenerated from half-seed explants, 9/14 genomic DNA samples showed Southern positive (single copy T-DNA integration in 5 plants, two T-DNA copy in 2 plants and three T-DNA copy in 2 plants) [Table 3.5]. The results of the Southern hybridization from 5 transgenic plants regenerated for cotyledonary node (randomly chosen from 24 Southern positive plants) and half-seed explants (randomly chosen from 9 Southern positive plants) along with non-transformed plant and a positive control have been grouped together in Fig. 3.17 (b) and Fig. 3.18 (b) respectively.

3.4. Discussion
3.4.1. Plant material

The cotyledonary node explants derived from 7-day-old in vitro seedlings and half-seed explants derived from 1-day-old imbibed seeds were used in the present study. Protocols for soybean transformation using cotyledonary node explants offered one of the better methods for regeneration of fertile soybean plants due to a short seed-to-seed generation time and no requirement for the maintenance of parental donor plants or long term cultures (Meurer et al., 1998).
Hinchee et al. (1988) reported that the cotyledonal node (cot-node) method is frequently used in soybean transformation system, which is based on *Agrobacterium*-mediated T-DNA delivery into regenerable cells in the axillary meristems of the cotyledonal node. In soybean, cotyledonal node was the choice of explant for many transformation protocols (Zhang et al., 1999; Clemente et al., 2000; Donaldson and Simmonds, 2000; Olhoft and Somers, 2001; Paz et al., 2004; Hoa T Thi Cuc 2008; Liu et al., 2008; Ye and Qin, 2008; Kim et al., 2011). Xue et al. (2006), Paz et al. (2006), and (Zia et al., 2010b) used half-seed explants in soybean transformation experiments. In *Cicer arriatrum*, Akbulut et al. (2008) preferred half-seed explants for production of transgenic plants.

### 3.4.2. Sensitivity of cotyledonal node and half-seed explants to hygromycin B

Generally, in any transformation protocol, only a minor fraction of the target tissue is transformed while a majority of the tissues remain non-transformed. Hence, an effective selection system is virtually necessary to selectively allow the transformed cells to grow. Usually, it is done using a compound that inhibits the growth of the non-transformed cells and allows only those cells that are transformed to grow.

Many soybean transformation reports described the use of kanamycin (Meurer et al., 1998; Donaldson and Simmonds, 2000), PPT (Zhang et al., 1999; Clemente et al., 2000; Xing et al., 2000; Olhoft and Somers, 2001), and hygromycin B (Olhoft et al., 2003; Liu et al., 2008) for selection of transformed plants. In soybean, Donaldson and Simmonds (2000) reported that use of kanamycin in the selection medium resulted in high number of escapes with low recovery of transgenic plants from the co-cultured cotyledonal node explants infected with EHA105 harboring pBI121 due to poor selection regime. Olhoft et al. (2003) reported that the selection regime based on PPT resulted in slow death of PPT-sensitive shoots and callus tissue on soybean explants, high
frequency of escapes, and slow growing, unhealthy, or chlorotic shoot development. In addition, they also reported that, elongation of the majority of transformed shoots on ‘Bert’ (soybean genotype) explants typically occurred between 6 to 12 months after co-cultivation under PPT selection, while only 2 to 6 months under hygromycin B selection. Hygromycin B is a potent antibiotic that inhibits polypeptide elongation in protein synthesis of bacteria (Gonzalez et al., 1978). D'Halluin et al. (1992) reported that transformed cells are not likely to cross-feed non-transformed cells with metabolites that override the effect of the selective agent when hygromycin B was used, but which can occur using PPT selection. Olhoft and Phillips (1999) reported that the rapid production of multiple, transgenic shoots in the presence of hygromycin B not only increased the probability of producing fertile plants from a single transformation event but also reduced the probability of inducing somaclonal variation in soybean. Liu et al. (2008) reported that hygromycin B selection method can be applied for wide range of soybean genotypes for efficient selection of transformed plants. Hence, in the present study, hygromycin B was used as selection agent to select transformants.

3.4.3. Sensitivity of elongated shoots on rooting to hygromycin B

In order to avoid the major problem of chimerism, which is most prevalent in organogenic protocols, the transformed shoots from both the types of explants initiated and elongated in the presence of 10 mg/l hygromycin B were isolated and rooted on RIPAM containing different concentrations of hygromycin B. The results confirmed that no shoots from both the types of explants were able to produce roots at 4 mg/l hygromycin B (Fig. 3.3). Hence, in the present study, the selection pressure is reduced from 10 mg/l to 4 mg/l hygromycin B in the medium at the time of rooting of transformed shoots from both the types of explants. In soybean, Clemente et al. (2000) decreased the selection pressure of PPT from 0.075–0.15 mM to 0.025–0.05 mM at the time of shoot elongation of cotyledonary node explants and maintained the same
selection pressure until rooting. In a similar study, Xue et al. (2006) decreased the selection pressure of PPT from 5 mg/l to 3 mg/l at the time of shoot elongation of regenerated shoots from half-seed explants of soybean. In soybean, Liu et al. (2004) selected shoots from embryonic tips in the medium containing 100 mg/l kanamycin and reduced the concentration to 25 mg/l at the time of rooting. In *Cucumis sativus*, Vengadesan et al. (2005) selected transgenic shoots in the presence of 6 mg/l PPT and reduced the PPT concentration to 2 mg/l during root induction.

### 3.4.4. *Agrobacterium strain*

Virulence of *Agrobacterium tumefaciens* strains varies widely among the plant hosts (Hobbs et al., 1989; Bush and Pueppke, 1991; Davis et al., 1991). The super virulent strains contain wide host range than normal strains and resulted in higher transformation frequency (Hooykaas and Beijersbergen, 1994). The selection of appropriate strain is a prerequisite for efficient transformation. In the present study, *Agrobacterium tumefaciens* strain EHA105 was the choice for infecting the cotyledonary node and half-seed explants. EHA105 was the preferred strain of choice for high percentage recovery of transformants that too in a wide number of legume genera (Schroeder et al., 1993; Samac, 1995; Cheng et al., 1996; Trieu and Harrison, 1996) including the present study. The reason being the derivation of EHA105 from a super virulent strain A281 and the opine background *i.e.*, the super virulent EHA105 strain has L,L-succinamopine background (Hood et al., 1986; Hobbs et al., 1989; Du et al., 1994). This hypervirulent strain EHA105 has been commonly used for soybean transformation (Trick and Finer, 1997; Trick and Finer, 1998; Zhang et al., 1999; Donaldson and Simmonds, 2000; Yan et al., 2000; Olhof et al., 2001; Liu et al., 2004; Xingping and Deyue, 2006; Liu et al., 2008; Wang and Xu, 2008; Chen et al., 2011). Meurer et al. (1998) used EHA105 strain and found effective in promoting cotyledonary node transformation in soybean. In contrary,
Ko et al. (2003) reported that EHA105 was ineffective in producing transgenic somatic embryos of soybean.

Bacterial-induced stress is said to affect/inhibit regeneration. A decrease in the transformation rate is accountable due to high bacterial concentration and longer than optimal co-cultivation time (Fillatti et al., 1987). In the present study, culture OD\textsubscript{600nm} of EHA105 was adjusted to 1.0 using LIM and used for transformation experiments. Yu et al. (2002) reported that a culture OD\textsubscript{600nm} of 1.0 prevented overgrowth of bacteria and at the same time favored maximum number of GUS positive shoots in sweet orange and citrange. While enumerating various factors that affect transformation in soybean using cotyledonary node explants, Meurer et al. (1998) tested various OD\textsubscript{600nm} of different Agrobacterium strains EHA105 (Hood et al., 1993), KYRT1 (Torisky et al., 1997), and LBA 4404 (Hoekema et al., 1983) and reported that OD\textsubscript{600nm} = 1.0 was optimum for high frequency transformation for all Agrobacterium strains. Ke et al. (2001), Liu et al. (2008) and Zia et al. (2010b) reported same optical density for effective transformation of soybean.

3.4.5. Optimization of parameters affecting Agrobacterium-mediated soybean transformation

3.4.5.1. Effect of co-cultivation period on GUS expression

Co-cultivation of explants with Agrobacterium is an important phenomenon in the plant genetic transformation and the duration of co-cultivation greatly influences the transformation efficiency. In soybean transformation, co-cultivation period of 2 days (Trick and Finer, 1997; Trick and Finer, 1998), 3 days (Meurer et al., 1998; Zhang et al., 1999; Clemente et al., 2000; Donaldson and Simmonds, 2000; Ye and Qin, 2008) and 5 days (Olhoft et al., 2001; Olhoft et al., 2003; Liu et al., 2004; Zeng et al., 2004; Paz et al., 2006; Xinping and Deyue, 2006; Xue et al., 2006; Hoa T Thi Cuc 2008; Liu et al., 2008; Wang and Xu, 2008; Zia et al., 2010b; Chen et al., 2011) have been
employed to increase the transformation frequency. In the present study, 5 days co-culture of cotyledonary node and half-seed explants with the *Agrobacterium* strain EHA105 resulted in the highest frequency of transient GUS expression (Fig. 3.4) and produced maximum number of GUS positive shoots in both the types of explants (Table 3.1). In some legumes such as pea (Grant *et al.*, 1995) and *Medicago* species (Samac, 1995), an extended duration of 6 days was prescribed for high frequency of transformation.

Wounding is an integral step in *Agrobacterium*-mediated transformation as it allows the bacterium to infect the target tissue. The exudates of wounded tissue often produce acetosyringone and α-hydroxyacetosyringone, which induce the entire vir regulon in *Agrobacterium* as well as the formation of T-DNA intermediate molecules (Stachel *et al.*, 1985). The examples include wounding during explant preparation (Horsch *et al.*, 1985; Charity *et al.*, 2002), delivery of the bacterium to the target tissue via syringe (Chee *et al.*, 1989), gentle stabbing of explants a few times with a sterile multi needle (7 cm-long piece of electric copper wire consisting of 30 fibers) [Xue *et al.*, 2006], the use of particle gun-mediated micro-wounding prior to *Agrobacterium*-mediated transformation (Bidney *et al.*, 1992). Wounding of excised embryonic axes from immature seeds using a multi-needle wounding prong (Ko *et al.*, 2003) and cotyledons using forceps (Yan *et al.*, 2000) have also been reported. In the present study, cotyledonary node explants were wounded by pricking gently and randomly in the axillary and apical meristematic areas using a sterile hypodermic needle and half-seed explants were wounded by making ten scratches at the surface of cotyledonary node regions with a number 11 Personna Plus surgeon’s blade. Similar type of wounding procedures were adopted in soybean (Olhoft *et al.*, 2001; Olhoft and Somers, 2001; Paz *et al.*, 2004; Xinping and Deyue, 2006; Olhoft *et al.*, 2007; Hoa T Thi Cuc 2008; Liu *et al.*, 2008) and black gram (Muruganantham *et al.*, 2007).
3.4.5.2. Effect of acetosyringone on GUS expression

Acetosyringone, an inducer of T-DNA transfer (Stachel et al., 1985), enhances the transformation process and is now routinely used in transformation experiments. Acetosyringone has been shown to enhance the transient expression of GUS in different species due to activation of vir genes (Atkinson and Gardner, 1991). Our results conclusively showed that the addition of acetosyringone to the co-cultivation medium increased the transient GUS expression in explants of both types (Fig. 3.5) and stable GUS expression in shoots regenerated from explants of both the types when compared to control (Table 3.1). Among different concentrations of acetosyringone tested, a concentration of 200 µM showed the maximum GUS expression (transient as well as stable) for both the types of explants (Fig. 3.5; Table 3.1). Similar to the results obtained in the present study, an acetosyringone concentration of 200 µM was employed to the co-cultivation medium to increase the transformation efficiency in soybean (Zhang et al., 1999; Clemente et al., 2000; Olhoft and Somers, 2001; Paz et al., 2004; Olhoft et al., 2007; Liu et al., 2008).

3.4.5.3. Effect of thiol compounds on GUS expression

In the present study, we observed that cotyledonary node and half-seed explants exhibited enzymatic browning and tissue necrosis at the wounded-sites following co-cultivation, which were likely to affect the efficient transformation. The tissue browning and necrosis observed on soybean cotyledonary node and half-seed explants following Agrobacterium infection are likely due to defense responses to wounding and/or pathogen infection common to many plants (Olhoft and Somers, 2001). Upon wounding or pathogen infection, one of the earliest defense mechanisms activated is the production of reactive oxygen species, referred to as an ‘oxidative burst’ (Wojtaszek, 1997). The reactive-oxygen intermediates produced during the oxidative burst are thought to activate the programmed cell death, or the hypersensitive response, to generate a barrier of dead cells around the site of infection. Also activated by wounding are the
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deposition of tannins and the release of phytoalexins by phenolic oxidation via the coordinated action of polyphenol oxidases (PPOs) and peroxidases (PODs) in plants (Vámos-Vigyázó and Haard, 1981), presumably to provide further protection against pathogen infection. The enzymatic production of tannins results in the browning of wounded-tissues.

Hence, in the present study, an attempt has been made to reduce tissue browning and necrosis that happened during Agrobacterium-mediated transformation by trying several concentrations and combinations of thiol compounds. Addition of thiol compounds to CCM reduced the tissue browning and necrosis in the wounded-sites of both the types of explants and thereby increased transient GUS expression in explants, percentage of explants responding in selection medium (SIPAM + 10 mg/l hygromycin B) as well as production of GUS positive shoots to a reliable level when compared to control (without thiol treatment).

3.4.5.3.1. Effect of L-cysteine on GUS expression

Cysteine is a known inhibitor of PPOs and PODs and enzymatic browning, either directly or indirectly, through the action of its thiol group (Mayer and Harel, 1979; Richard-Forget et al., 1992; Negishi and Ozawa, 2000). By reducing wound- and pathogen-defense responses in plants, inhibitors like L-cysteine have the potential to increase the capacity of Agrobacterium to infect plant tissues and stably transfer its T-DNA and to increase the frequency of infected cells that remain viable and become transformed (Olhoft and Somers, 2001). In the present study, addition of L-cysteine (0.83–4.13 mM) to the CCM containing acetosyringone (200 µM) controlled enzymatic browning and tissue necrosis when compared to that of control (CCM without L-cysteine). Among the different concentrations of L-cysteine tested, our observation revealed that 3.31 mM was found optimal and at this concentration, both types of explants showed maximum transient GUS expression (Fig. 3.6) and produced maximum
number of GUS positive shoots (Table 3.2). The results agrees with the results of Olhoft and Somers (2001) in soybean using cotyledonary node explants in which, explants treated with 3.3 mM cysteine exhibited greater frequency of explants with a GUS\(^+\) sector in differentiating tissue and GUS\(^+\) shoot primordia than those explants not co-cultured with L-cysteine. In a similar study in soybean, Zeng et al. (2004) co-cultivated cotyledonary node explants in co-cultivation medium supplemented with 3.3 mM L-cysteine to control tissue browning. Frame et al. (2002) reported increase in the production of transgenic plants using Agrobacterium-mediated T-DNA delivery in Zea mays immature zygotic embryos by adding L-cysteine to the solid co-cultivation medium.

In the present study, transient GUS expression in explants as well as stable GUS expression in shoots increased positively with the increasing concentration of L-cysteine up to 3.31 mM for both the types of explants (Fig. 3.6; Table 3.2). Further increase in concentration of L-cysteine (>3.31 mM) in both the types of explants showed negative effect on GUS expression (Fig. 3.6; Table 3.2). On the other hand, Xue et al. (2006) reported requirement of an higher concentration of L-cysteine (8.2 mM) supplementation in CCM to achieve high transformation frequency in soybean cv. ‘Jungery’. In another study, Liu et al. (2008) investigated the effect of different concentrations of cysteine (1.6, 3.3, 4.9, 6.6 or 8.2 mM) on soybean gene transformation using cotyledonary node explants and reported that 6.6 mM L-cysteine gave rise to a higher rate of transgenic shoots (7.6%) than 8.2 mM (4.1%) in soybean cv. ‘Hefeng25’. Liu et al. (2008) also reported that high levels of cysteine in the CCM might also have some negative impacts on in vitro plant morphogenesis of soybean. Paz et al. (2006) reported that there was no significant difference on stable GUS expression in shoots regenerated from half-seeds explants of soybean co-cultivated in the presence or absence of cysteine i.e., GUS\(^+\) shoots were obtained with or without cysteine. In maize Agrobacterium-mediated transformation Frame et al. (2002) reported inhibitory effect of L-cysteine at
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3.3 mM concentration, which exhibited significant decrease in giving rise to embryogenic callus. A similar negative effect of 0.6 mM L-cysteine on embryogenesis in Japonica rice explants was also noticed by Enriquez-Obregon et al. (1999). The results from the present investigation and results of Enriquez-Obregon et al. (1999), Frame et al. (2002), Xue et al. (2006), Paz et al. (2006), and Liu et al. (2008) indicate that cysteine enhancement of Agrobacterium-mediated transformation might be a balanced consequence of its positive and negative roles in different plant species. Further it is suggested that L-cysteine concentration should be optimized for each explant, genotype, and plant species which were affected by enzymatic browning and tissue necrosis for efficient Agrobacterium-mediated transformation.

3.4.5.3.2. Effect of L-cysteine and DTT combination on GUS expression

In the present study, addition of DTT (0.50–1.50 mM) to CCM containing optimum concentration of acetosyringone (200 µM) and L-cysteine (3.31 mM) resulted with a notable decrease in enzymatic browning and further increased the percentage of explants showing transient GUS expression in both the types of explants as well as stable GUS expression in shoots regenerated from both the types of explants (compared to L-cysteine treatment). In the present observation, treatment of CCM containing acetosyringone, L-cysteine, and DTT (1.00 mM) resulted in maximum transient GUS expression in explants (cotyledonary node and half-seed) [Fig. 3.7] and produced maximum number of GUS positive shoots after selection in explants of both the types (Table 3.2) which was in agreement with the results of Olhoft et al. (2003) in soybean. They reported that the highest average transformation efficiency resulted from the combination of L-cysteine and DTT, which was significantly greater than either L-cysteine or DTT alone. The present results also corroborate with observations made by Paz et al. (2004) in soybean. The authors reported that the addition of cysteine (3.3 mM) and DTT (1.0 mM) in co-cultivation media decreased tissue necrosis and enzymatic browning and dramatically increased the transient GUS activity of
cotyledonary node explants (95% GUS activity was obtained in L-cysteine and DTT combination whereas only 10% GUS expression was obtained without the above combination) as evidenced in the present study. Similarly in soybean cotyledonary node transformation system, Zia et al. (2010b) supplemented 3.3 mM L-cysteine and 1.0 mM DTT combination to increase transformation efficiency by controlling tissue browning.

3.4.5.3.3. Effect of L-cysteine, DTT and sodium thiosulfate combinations on GUS expression

In the present study, supplementation of sodium thiosulfate (0.50–1.50 mM) to CCM containing optimum concentration of acetosyringone, L-cysteine, and DTT improved T-DNA delivery by inhibiting the activity of plant pathogen- and wound-response enzymes, such as peroxidases (PODs) and polyphenol oxidases (PPOs), which in turn increased GUS expression (transient and stable) in both the types of explants as reported by Olhoft et al. (2003) in soybean. Morphological observation of explants showed a significant improvement in reduction of enzymatic browning and tissue necrosis in both the types of explants (compared to L-cysteine and DTT combinations tested for both the types of explants). In our observation, addition of sodium thiosulfate (1.00 mM) to CCM containing optimal concentration of acetosyringone, L-cysteine, and DTT resulted in maximum transient GUS expression in both the types of explants (Fig. 3.8) and also produced maximum number of GUS positive shoots (Table 3.2). Similar results was recorded by Olhoft et al. (2003). They reported that exposing cotyledonary node explants to mixtures of thiol compounds [L-cysteine (3.3 mM), DTT (1.0 mM) and sodium thiosulfate (1.0 mM) during cocultivation with A. tumefaciens resulted in significant increase in transformation frequency as compared to single thiol compound. In a similar study conducted by Xingping and Deyue (2006) using cotyledonary node explants of soybean, higher transformation efficiency was achieved when combination of L-cysteine
(3.3 mM), DTT (1.0 mM) and sodium thiosulfate (1.0 mM) was supplemented in the CCM.

The results of present study confirmed the improved recovery of infected tissues by reducing the browning of wounded soybean tissue, when optimal concentration of L-cysteine (3.31 mM), DTT (1.0 mM), and sodium thiosulfate (1.00 mM) were added to the co-cultivation medium. In other tissues and plant species, the wounding and pathogen responses are likely different from those found in the soybean cotyledonary node explant, which could result in distinctive responses to either thiol compounds or phenol-absorbing agents according to the plant species and tissue used for transformation (Olhoft et al., 2003). For example, it was found in Vitis vinifera that the combination of DTT and the phenolic-absorbing agent, polyvinylpolypyrrolidone (PVPP), was the optimal treatment for increasing the transformation of grape callus over other treatments tested, including L-cysteine (Perl et al., 1996). Therefore, for optimization of T-DNA delivery into plant cells, experimentation with several thiol reagents in various combinations is prudent as described in the present study.

3.4.5.4. Effect of sonication and vacuum infiltration on GUS expression

In the present study, addition of thiol compounds [L-cysteine (3.31 mM), DTT (1.00 mM), and sodium thiosulfate (1.00 mM)] to CCM containing acetosyringone (200 µM) greatly reduced the enzymatic browning and tissue necrosis at the wounded sites of cotyledonary node and half-seed explants and thereby increased GUS expression in explants of both the types. Further adopting sonication and vacuum infiltration procedures with cotyledonary node and half-seed explants greatly increased transient GUS expression in both the types of explants as well as stable GUS expression in regenerated shoots from cotyledonary node and half-seed explants.
3.4.5.4.1. Effect of sonication on GUS expression

Sonication Assisted-Agrobacterium-mediated transformation (SAAT) has been shown to provide an efficient delivery of T-DNA into cells in a number of plants (Santarém et al., 1998; Tang et al., 2001; Zaragozá et al., 2004; Beranová et al., 2008), especially those that are typically more recalcitrant to Agrobacterium-mediated transformation (Trick and Finer, 1997). The basis for the increase in transient GUS expression from SAAT is believed to be caused by the infection by Agrobacterium of the target plant tissue (Trick and Finer, 1997).

In the present investigation, sonication treatment increased the GUS expression in both the types of explants. At an optimal time period of 20 sec, both cotyledonary node and half-seed explants showed a drastic increase in percentage of explants showing transient GUS expression (Fig. 3.9) as well as number of GUS positive shoots when compared to control (Table 3.3). Similar to the present study, SAAT increased both transient expression and stable transformation of soybean (Trick and Finer, 1997; Meurer et al., 1998; Santarém et al., 1998), black locust (Zaragozá et al., 2004), Chenopodium rubrum (Flores Solís JI et al., 2007), squash (Ananthakrishnan et al., 2007), chick pea (Pathak and Hamzah, 2008) and flax (Beranová et al., 2008). Gaba et al. (2006), Ananthakrishnan et al. (2007), and Beranová et al. (2008) reported that sonication treatment can also stimulate shoot regeneration in squash. The application of sonication treatment to various explants should ease efficient Agrobacterium T-DNA delivery to plant cells and at the same time it should not interfere in explants regenerating potential. In the present study, 20 sec sonication was found to be the optimal treatment over other time period tested in which the regeneration of explants was not affected for both the types. Extending sonication time period beyond 20 sec showed a reduction in regeneration of both cotyledonary node and half-seed explants in the selection medium (SIPAM + 10 mg/l hygromycin B) [Table 3.3]. In a similar study in soybean, Santarém et al. (1998) reported that sonication treatments of more than
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30 sec resulted in severe tissue disruption and death of all cotyledons after 5 days of sonication.

However, SAAT did not always produce positive results. For instance, SAAT was used in attempts to transform pre-cultured wheat inflorescence tissue, and although the number of explants showing transient GUS expression doubled with a brief sonication treatment, the number of expressing areas per explant was reduced, leaving no great benefit (Amoah et al., 2001). Transient gene expression in *Pinus pinea* was greatly increased by SAAT, but the cotyledonal explants were able to survive in SAAT and generated transgenic buds only at very low *Agrobacterium* concentrations (Humara et al., 1999). On the other hand, brief sonication enhanced the *Agrobacterium*-mediated transient and stable transformation of *Pinus taeda* (Tang, 2003). Hence, it is suggested that the duration of sonication should be optimized for a highly efficient *Agrobacterium*-mediated genetic transformation as evidenced in the present study.

### 3.4.5.4.2. Combined effect of sonication and vacuum infiltration on GUS expression

Another methodology widely adopted to enhance *Agrobacterium* infection is vacuum-infiltration. This process increases gene transfer efficiency by improving the penetration of *Agrobacterium* cells into the plant tissue layers. Combination of this method with sonication or other methods of wounding has improved T-DNA delivery into the target tissue (Bechtold et al., 1993; Charity et al., 2002; Liu et al., 2005). The use of vacuum infiltration after sonication may provide an additional entry sites for bacteria, allowing the transformation of cells deeper into the plant tissue layer compared to the cells from surface that are accessible by co-cultivation (De Oliveira et al., 2009). Franklin et al. (2004) subjected mature wounded soybean cotyledons under a mild vacuum for one hr with *Agrobacterium tumefaciens* strain LBA4404. In soybean, Paz et al. (2006)
could not get positive results by applying vacuum infiltration treatment using half-seed explants.

Till now, to our knowledge, there are no reports on combined effect of sonication and vacuum infiltration to enhance *Agrobacterium* infection in soybean. In the present study, coupling sonication with vacuum infiltration treatment enhanced the percentage of explants showing transient GUS expression as well as production of GUS positive shoots from both the types of explants (Fig. 3.10; Table 3.3). Among the different time periods of vacuum infiltration tested, a vacuum period for 5 min coupled with sonication (20 sec) was found optimal which resulted in maximum number of explants showing transient GUS expression (cotyledonary node and half-seed) [Fig. 3.10] and GUS positive shoots (Table 3.3). The results generated from the above treatment showed maximum GUS expression in both the types of explants when compared to any other parameter/treatment used in the present investigation indicating the advantage of coupled effect of sonication and vacuum infiltration in present soybean transformation protocol. Similar results were obtained by Liu *et al.* (2005), who described an efficient method for the transformation of kidney bean with *lea* gene using a combination of sonication and vacuum infiltration-assisted *Agrobacterium*-mediated transformation. Among 18 combinations of transformation methods, they reported that 5 min sonication combined with 5 min vacuum infiltration showed the highest transformation efficiency. In a similar study, 2 sec of SAAT followed by 10 min of vacuum infiltration increased frequency of transient GUS expression and stable transformation in *Agrobacterium*-mediated transformation of 'pineapple' sweet orange (De Oliveira *et al.*, 2009). Park *et al.* (2005) used 5 min sonication coupled with 5 min vacuum infiltration to transform radish plants. Subramanyam *et al.* (2011) reported 6 min sonication combined with the 6 min vacuum infiltration produced highest percentage of GUS positive shoots in banana sucker explants. The association of sonication and vacuum infiltration treatment has also been

### 3.4.6. Cotyledonary node versus half-seed

To compare transformation efficiencies of cotyledonary node and half-seed protocols, 10 independent experiments using both the types of explants were carried out. The results of Southern blot analysis confirmed that the transformation efficiency of 1.25 to 5.26% (Average = 3.20%) was obtained from cotyledonary node explants (Table 3.4) while half-seed method generated 0.0 to 2.66% (Average = 1.15%) [Table 3.5]. Overall, more consistent transgenic plant recovery was obtained using the cotyledonary node protocol when compared to half-seed protocol. The difference in transformation efficiencies might be due to the wounding procedures followed and age at which explants were prepared. It was assumed that the young half-seed explants (1-day-old) were not able to sustain such wounding procedures whereas the cotyledonary node explants (7-day-old) did. However, in the present study, deliberate wounding procedures were unavoidable since initial experiments carried out without adopting wounding procedures generated very poor transformation efficiency (data not shown). Precise wounding at the cotyledonary nodal area was believed to facilitate *Agrobacterium* infection by providing entry site for *Agrobacterium* (Townsend and Thomas, 1994; Meurer *et al.*, 1998).

#### 3.4.6.1. Regeneration of shoots, elongation, rooting and hardening

An effective selection strategy is very important for developing an efficient transformation procedure. In preliminary experiments, explants washed of excess *A. tumefaciens* after co-cultivation and transferred to solidified SIPAM containing hygromycin B (10 mg/l) exhibited severe tissue necrosis and no shoot formation was observed (data not shown). Delaying selection for 7 days greatly
stimulated the regeneration of transformed shoots from both the types of explants. This type of resting step was usually used to alleviate the suffering from the combined stress of Agrobacterium and selection agents (Liu et al., 2008). Zhao et al. (2001) reported a 4-day resting period increased the callus transformation frequency by 2.7 times higher than the control without resting. Olhoft et al. (2003) also obtained consistent results by eliminating the selection pressure during the first 14-day shoot initiation stage. Liu et al. (2008) reported that 7-day resting period was found adequate to sustain the combined stress of Agrobacterium and selection agents. In the present study, repeated transfer of cotyledonal node and half-seed explants to medium containing hygromycin B facilitated the production of uniformly GUS expressing shoots. The non-transformed hygromycin B-sensitive shoots of both the types of explants were found to be completely necrotic. Based on the observation, it is suggested that amendment of hygromycin B resulted in rapid cell death of hygromycin B-sensitive cells throughout the entire shoot regeneration period, enhanced the growth and development of healthy shoots that were resistant to hygromycin B and greatly avoided the problem of chimerism. In accordance to the present investigation, similar selection regime in the presence of hygromycin B was reported in soybean by Olhoft et al. (2003) and Liu et al. (2008).

3.4.7. Transgene analyses

3.4.7.1. Assay for β-glucuronidase activity

Gene transfer protocols developed for plant species require the use of visual or selectable markers due to the relatively small number of cells in which integration of the foreign DNA actually occurs (Xing et al., 2000). GUS histochemical assay allows a very rapid and sensitive screening of the transformed cells and tissues using the indigogenic substrate X-Gluc. While evaluating various parameters during standardization of the transformation protocols, the choice of GUS as histochemical marker was realized since the effect of different treatments could be assayed in the target tissues even before
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the plantlets grow and mature (Hinchee et al., 1988). The level of GUS activity was visually assessed based on the intensity of blue-staining areas. In the present study, the use of a binary construct carrying an intron-interrupted GUS gene (Narasimhulu et al., 1996) ensured that observed staining was due only to plant cell expression and to not contaminating *A. tumefaciens*. In the present study, transient GUS expression was very obvious in regions where cell division proceeds. This property is regulated by the promoter that drives the expression of the *gus* gene. Liu et al. (2004) reported a similar intense localization of GUS activity under the CaMV35S promoter in the meristematic cells of embryonic tip explants in soybean. CaMV35S promoter although being a constitutive promoter expressing in all plant parts, its expression is reported to be organ specific as it was more pronounced in immature leaves than mature leaves (Jefferson et al., 1987; Terada and Shimamoto, 1990). Further, its expression is dependent upon the phase of the cell cycle and cell types (Nagata et al., 1987). In the present study, intense GUS activity was observed in hand-cut stems and leaves (midrib) sections of putative T₀ transformants derived from cotyledonary node explants. Similar type of results was reported by Christou and McCabe (1992) in soybean. They reported intense GUS activity in sections of stems and leaves midrib as observed in the present study.

3.4.7.2. Molecular confirmation of transformants

PCR analysis confirmed the presence of the *hpt* gene in the putative T₀ transformants regenerated from cotyledonary node and half-seed explants and Southern hybridization using the PCR amplified *hpt* gene as a probe showed the integration of transgene into the soybean genome of cv. PK 416.

3.5. Conclusion

In the present investigation, we record the first report of a system utilizing cotyledonary node and half-seed explants for high efficiency *Agrobacterium*-mediated transformation with Indian soybean cultivar PK 416. Various factors
affecting transformation like co-cultivation duration, acetosyringone concentration, and thiol compounds supplementation were optimized for efficient gene delivery. In addition, it was observed that efficient T-DNA delivery to cells was greatly increased by combining sonication and vacuum infiltration treatments. This is the first report emphasizing the advantages of combined action of sonication and vacuum infiltration in increasing transformation efficiency of soybean at International level. From the overall results, it was concluded that cotyledonary node protocol showed better transformation efficiency when compared to half-seed protocol, and hence, used in further experiments to overexpress γ-tocopherol methyltransferase (γ-TMT) gene into cv. PK 416 to improve vitamin E content in seeds.
Table 3.1. Effect of co-cultivation duration and acetosyringone concentrations on stable GUS expression in shoots derived from cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

For each treatment, 125 explants were infected and repeated five times.

30 randomly selected explants of cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 40 days of culture on selection medium (SIPAM + 10 mg/l hygromycin B).

Percentage of explants responding (%) = Number of explants responded by producing shoots in selection medium/ total number of explants infected × 100.

Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan’s multiple range test at 5% level.

<table>
<thead>
<tr>
<th>Co-cultivation duration (days)</th>
<th>Acetosyringone (µM)</th>
<th>Percentage of explants responding (%)</th>
<th>Mean number of GUS positive shoots/30 explants</th>
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<tr>
<td></td>
<td></td>
<td>cotyledonary node</td>
<td>half-seed</td>
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<td>1</td>
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<td>33.76±0.46&lt;sup&gt;b&lt;/sup&gt;a</td>
<td>27.20±0.35&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>28.32±0.32&lt;sup&gt;g&lt;/sup&gt;</td>
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</tr>
<tr>
<td>4</td>
<td>-</td>
<td>36.48±0.54&lt;sup&gt;e&lt;/sup&gt;af</td>
<td>30.08±0.40&lt;sup&gt;e&lt;/sup&gt;af</td>
</tr>
<tr>
<td>5</td>
<td>- 50</td>
<td>37.60±0.25&lt;sup&gt;d&lt;/sup&gt;dae</td>
<td>31.04±0.46&lt;sup&gt;d&lt;/sup&gt;dae</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>36.16±0.46&lt;sup&gt;f&lt;/sup&gt;a</td>
<td>29.76±0.53&lt;sup&gt;f&lt;/sup&gt;a</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>38.24±0.29&lt;sup&gt;c&lt;/sup&gt;ad</td>
<td>31.68±0.40&lt;sup&gt;c&lt;/sup&gt;ad</td>
</tr>
<tr>
<td>5</td>
<td>250</td>
<td>40.16±0.46&lt;sup&gt;a&lt;/sup&gt;bab</td>
<td>33.60±0.25&lt;sup&gt;a&lt;/sup&gt;ab</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>41.28±0.19&lt;sup&gt;a&lt;/sup&gt;b</td>
<td>34.24±0.29&lt;sup&gt;a&lt;/sup&gt;b</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>39.52±0.40&lt;sup&gt;b&lt;/sup&gt;a</td>
<td>32.80±0.35&lt;sup&gt;b&lt;/sup&gt;a</td>
</tr>
</tbody>
</table>
Table 3.2. Effect of L-cysteine, DTT and sodium thiosulfate on stable GUS expression in shoots derived from cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM) without thiol compounds.

For each treatment, 125 explants were infected and repeated five times.

30 randomly selected explants of cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 40 days of culture on selection medium (SIPAM + 10 mg/l hygromycin B).

Percentage of explants responding (%) = Number of explants responded by producing shoots in selection medium/ total number of explants infected × 100.

Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan’s multiple range test at 5% level

<table>
<thead>
<tr>
<th>Thiol compounds (mM)</th>
<th>Percentage of explants responding (%)</th>
<th>Mean number of GUS positive shoots/30 explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cotyledonary node</td>
<td>half-seed</td>
</tr>
<tr>
<td>Control</td>
<td>41.28±0.19&lt;sup&gt;i&lt;/sup&gt;</td>
<td>34.24±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.83</td>
<td>46.72±0.40&lt;sup&gt;j&lt;/sup&gt;</td>
<td>38.24±0.29&lt;sup&gt;ia&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.66</td>
<td>50.08±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.44±0.29&lt;sup&gt;ij&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.48</td>
<td>52.96±0.46&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>44.48±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.31</td>
<td>56.80±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.16±0.46&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.13</td>
<td>54.88±0.19&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>46.40±0.25&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-cysteine+DTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.31 + 0.50</td>
<td>58.08±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.28±0.40&lt;sup&gt;jb&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.75</td>
<td>59.84±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.88±0.40&lt;sup&gt;fg&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.00</td>
<td>60.96±0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.16±0.29&lt;sup&gt;eg&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.25</td>
<td>60.16±0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.36±0.29&lt;sup&gt;efa&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.50</td>
<td>59.20±0.43&lt;sup&gt;fb&lt;/sup&gt;</td>
<td>50.24±0.46&lt;sup&gt;gah&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-cysteine+DTT+STS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.31 + 1.00 + 0.50</td>
<td>62.08±0.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>53.28±0.40&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.75</td>
<td>63.52±0.19&lt;sup&gt;bac&lt;/sup&gt;</td>
<td>55.04±0.39&lt;sup&gt;ca&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.00</td>
<td>65.60±0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.28±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.25</td>
<td>64.48±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.32±0.32&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.50</td>
<td>63.20±0.35&lt;sup&gt;ca&lt;/sup&gt;</td>
<td>55.36±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 3.3. Effect of sonication and vacuum infiltration on stable GUS expression in shoots derived from cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

<table>
<thead>
<tr>
<th>Sonication (sec)</th>
<th>Vacuum Infiltration (min)</th>
<th>Percentage of explants responding (%)</th>
<th>Mean number of GUS positive shoots/30 explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cotyledonary node</td>
<td>half-seed</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>65.60±0.43h</td>
<td>57.28±0.19h</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>67.52±0.40g</td>
<td>59.36±0.29g</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>69.12±0.54f</td>
<td>60.80±0.35f</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>71.84±0.29ea</td>
<td>63.52±0.48ea</td>
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<tr>
<td>20</td>
<td>-</td>
<td><strong>74.72±0.40da</strong></td>
<td><strong>66.24±0.29da</strong></td>
</tr>
<tr>
<td>25</td>
<td>-</td>
<td>72.16±0.29e</td>
<td>63.68±0.19e</td>
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<tr>
<td>20</td>
<td>1</td>
<td>77.76±0.46c</td>
<td>68.64±0.29ca</td>
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<td>20</td>
<td>5</td>
<td><strong>81.76±0.29a</strong></td>
<td><strong>72.80±0.43a</strong></td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>80.00±0.35b</td>
<td>71.36±0.29b</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>77.44±0.46ca</td>
<td>69.28±0.54c</td>
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<tr>
<td>20</td>
<td>20</td>
<td>75.36±0.53d</td>
<td>66.88±0.59d</td>
</tr>
</tbody>
</table>

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM), L-cysteine (3.31 mM), DTT (1.00 mM) and STS (1.00 mM) without sonication.

For each treatment, 125 explants were infected and repeated five times.

30 randomly selected explants of cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 40 days of culture on selection medium (SIPAM + 10mg/l hygromycin B).

Percentage of explants responding (%) = Number of explants responded by producing shoots in selection medium/ total number of explants infected × 100.

Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan’s multiple range test at 5% level.
Table 3.4. Summary of 10 independent experiments of transformation in cotyledonary node explants derived from 7-day-old *in vitro* seedlings of soybean cv. PK 416.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of explants infected</th>
<th>No. of explants responded</th>
<th>No. of hygromycin B resistant shoots (After shoot elongation)</th>
<th>No. of hygromycin B resistant shoots forming roots</th>
<th>No. of putative T₀ transformants (After PCR)</th>
<th>No. of T₀ transgenic plants (After Southern)</th>
<th>Transformation efficiency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76</td>
<td>64</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>5.26</td>
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<tr>
<td>2</td>
<td>70</td>
<td>57</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2.85</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>67</td>
<td>9</td>
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<td>1</td>
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<td>1</td>
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<td>1.33</td>
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<tr>
<td>7</td>
<td>77</td>
<td>63</td>
<td>11</td>
<td>4</td>
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<td>8</td>
<td>4</td>
<td>3</td>
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<td>2.66</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>58</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2.85</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td><strong>748</strong></td>
<td><strong>613</strong></td>
<td><strong>96</strong></td>
<td><strong>38</strong></td>
<td><strong>29</strong></td>
<td><strong>24</strong></td>
<td><strong>32.08</strong></td>
</tr>
</tbody>
</table>

*Transformation efficiency (%) = Number of transgenic plants/Number of explants infected × 100

**Average transformation efficiency = 32.08/10 = 3.20%
Table 3.5. Summary of 10 independent experiments of transformation in half-seed explants derived from 1-day-old imbibed seeds of soybean cv. PK 416.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of explants infected</th>
<th>No. of explants responded</th>
<th>No. of hygromycin B resistant shoots (After shoot elongation)</th>
<th>No. of hygromycin B resistant shoots forming roots</th>
<th>No. of putative T₀ transformants (After PCR)</th>
<th>No. of T₀ transgenic plants (After Southern)</th>
<th>Transformation efficiency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83</td>
<td>60</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>55</td>
<td>9</td>
<td>3</td>
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<td>1.29</td>
</tr>
<tr>
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<td>60</td>
<td>5</td>
<td>1</td>
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<td>1</td>
<td>1.23</td>
</tr>
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<td>4</td>
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<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>59</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2.50</td>
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<td>75</td>
<td>55</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1.33</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>56</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>59</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>54</td>
<td>7</td>
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<td>3</td>
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<td>2.66</td>
</tr>
<tr>
<td>10</td>
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<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.26</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td><strong>783</strong></td>
<td><strong>569</strong></td>
<td><strong>56</strong></td>
<td><strong>21</strong></td>
<td><strong>14</strong></td>
<td><strong>9</strong></td>
<td><strong>11.52</strong></td>
</tr>
</tbody>
</table>

*Transformation efficiency (%) = Number of transgenic plants/Number of explants infected × 100

**Average transformation efficiency = 11.52/10 = 1.15%
Figure 3.1. Linear map of the plasmid vector pCAMBIA1301 present within the *Agrobacterium tumefaciens* strain EHA105 that was used for the transformation experiments.
Figure 3.2. Sensitivity of cotyledonary node and half-seed explants of soybean cv. PK 416 to various concentrations of hygromycin B on SIPAM containing BA (2.22 µM) and spermidine (137.69 µM) after 40 days of culture.

Control: Treatment without hygromycin B.

For each treatment, 50 explants were used and repeated three times. The bars represent mean±standard error.
Figure 3.3. Sensitivity of rooting of elongated shoots of soybean cv. PK 416 to various concentrations of hygromycin B on RIPAM containing IBA (4.93 µM) and putrescine (62.08 µM) after 30 days of culture.

Control: Treatment without hygromycin B.

For each treatment, 50 elongated shoots (>4 cm) were used and repeated three times. The bars represent mean±standard error.
Figure 3.4. Effect of co-cultivation time period on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

For each co-cultivation time period, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 1, 2, 3, 4, 5 and 6 days of co-cultivation with *A. tumefaciens* strain EHA105.

Percentage of explants showing GUS expression (%): Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.5. Effect of acetosyringone on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with Agrobacterium strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the absence of acetosyringone.

For each concentration, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with A. tumefaciens strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.6. Effect of L-cysteine on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with Agrobacterium strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM) without L-cysteine.

For each concentration, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with A. tumefaciens strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.7. Effect of DTT on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM) and L-cysteine (3.31 mM) without DTT.

For each concentration, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with *A. tumefaciens* strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.8. Effect of sodium thiosulfate on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with Agrobacterium strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM), L-cysteine (3.31 mM) and DTT (1.00 mM) without STS.

For each concentration, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with A. tumefaciens strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.9. Effect of sonication on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with Agrobacterium strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were co-cultivated for 5 days in the presence of acetosyringone (200 µM), L-cysteine (3.31 mM), DTT (1.00 mM) and STS (1.00 mM) without sonication treatment.

For each sonication time period, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with A. tumefaciens strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.10. Effect of vacuum infiltration on transient GUS expression in cotyledonary node (7-day-old) and half-seed (1-day-old) explants of soybean cv. PK 416 co-cultivated with *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid.

Control: The explants were sonicated (20 sec) and co-cultivated for 5 days in the presence of acetosyringone (200 µM), L-cysteine (3.31 mM), DTT (1.00 mM) and STS (1.00 mM) without vacuum infiltration treatment.

For each vacuum infiltration time period, 50 explants were infected and repeated five times. The bars represent mean±standard error.

30 randomly selected cotyledonary node and half-seed explants from each replicate were assayed for GUS activity after 5 days of co-cultivation with *A. tumefaciens* strain EHA105.

Percentage of explants showing GUS expression (%) = Number of explants with at least 1 qualifying GUS focus/ total number of explants assayed for GUS expression × 100.
Figure 3.11. *Agrobacterium*-mediated transformation of cotyledonary node explants using EHA105 harboring pCAMBIA1301: a) Explants prepared from 7-day-old *in vitro* seedlings; b) Shoot buds induction from explant in SIPAM supplemented with MES buffer (3 mM), cefotaxime (100 mg/l) and vancomycin (25 mg/l) without hygromycin B (after 7 days of culture); c–e) Selection of regenerated shoots in SIPAM supplemented with MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (10 mg/l) [c:– after 8 days, d:– after 30 days and e:– after 45 days of selection]; f) Elongated shoots in SEPAM amended with MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (10 mg/l) after 30 days of culture; g) Rooted shoot on RIPAM containing MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (4 mg/l) after 30 days of culture; h) Hardened putative *T₀* transformants in growth chamber; i) Transgenic plant in greenhouse.
Figure 3.12. Agrobacterium-mediated transformation of half-seed explants using EHA105 harboring pCAMBIA1301: a) Explants prepared from 1-day-old imbibed seeds; b) Shoot buds induction from explant in SIPAM supplemented with MES buffer (3 mM), cefotaxime (100 mg/l) and vancomycin (25 mg/l) without hygromycin B (after 7 days of culture); c-e) Selection of regenerated shoots in SIPAM supplemented with MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (10 mg/l) [c: after 8 days, d: after 30 days and e: after 45 days of selection]; f) Elongated shoots in SEPAM amended with MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (10 mg/l) after 30 days of culture; g) Rooted shoot on RIPAM containing MES buffer (3 mM), cefotaxime (100 mg/l), vancomycin (25 mg/l) and hygromycin B (4 mg/l) after 30 days of culture; h) Hardened putative T₀ transformants in growth chamber; i) Transgenic plant in greenhouse.
Figure 3.13. Transient (in explants) and stable (in shoots) GUS expression of cotyledonary node and half-seed explants infected with A. tumefaciens EHA105 harboring pCAMBIA1301: a) Transient expression of the gus gene in cotyledonary node explant after 5 days of co-cultivation; b) Non-infected cotyledonary node explant (control); c) Transient expression of the gus gene in half-seed explant after 5 days of co-cultivation; d) Non-infected half-seed explant (control); e) Stable expression of the gus gene in regenerated shoots after culturing cotyledonary node explants for 40 days in selection medium (SIPAM + 10 mg/l hygromycin B); f) Regenerated shoots of non-infected cotyledonary node explant (control); g) Stable expression of the gus gene in regenerated shoots after culturing half-seed explants for 40 days in selection medium (SIPAM + 10 mg/l hygromycin B); h) Regenerated shoots of non-infected half-seed explant (control); i & j) Co-cultivated cotyledonary node and half-seed explant on CCM in the absence of thiol compounds after GUS assay (arrows showing enzymatic browning).
Figure 3.14. GUS expression in stem and hand-cut stem sections of putative T₀ transformants regenerated from cotyledonary node explants infected with A. tumefaciens EHA105 harboring pCAMBIA1301: a) Stem showing GUS expression; b) Stem of non-transformed plant (control); c) Transformed stem section showing intense GUS expression under stereomicroscope (4X); d) Stem section of non-transformed plant (control) [4X]; e–k) Histochemical staining of GUS expression in different tissues (e–g, k = 10X; h & j = 40X, i = 100X).
Figure 3.15. GUS expression in leaves and hand-cut leaf midrib sections of putative T<sub>0</sub> transformants regenerated from cotyledonary node explants infected with A. tumefaciens EHA105 harboring pCambia1301: a) GUS expression in leaves; b) Leaves of non-transformed plant (control); c) Transformed leaf midrib section showing intense GUS expression under stereomicroscope (4X); d) Leaf midrib section of non-transformed plant (control) (4X); e–h) Histochemical staining of GUS expression in different tissues of leaf midrib section (e: -100X; f–h: -40X).
Figure 3.16. GUS expression in flower and floral parts of putative T₀ transformants regenerated from cotyledonary node explants infected with *A. tumefaciens* EHA105 harboring pCAMBIA1301: a) Expression of gus gene in flower; b–f) Expression of gus gene in floral parts (b:– gynoecium; c:– calyx; d:– standard petal; e & f:– keels); g) Flower of non-transformed plant (control); h–k) Respective controls of floral parts from non-transformed plant (h:– gynoecium; i:– calyx; j:– standard petal; k:– keels); l) GUS expression in androecium (4X) [9 + 1 arrangement of stamens (black arrow:– 9 stamens fused in bundles; blue arrow:– single stamen)]; m & n) Stamens showing GUS expression (m:– 4X; n:– 10X); o) Single anther showing GUS expression (40X); p & q) Pollen grains showing GUS expression (100X); r–t) Respective controls from non-transformed plant [r:– androecium (4X); s:– stamen (4X); t:– pollen grains (100X)].
Figure 3.17. a) PCR confirmation of putative T₀ transformants regenerated from cotyledonary node explants to detect the presence of hpt coding region: Lane 1- Marker (100 bp); Lane 2- Plasmid pCAMBIA1301; Lane 3-7- DNA sample from putative T₀ transformants (arrow indicate the amplification of hpt gene at 407 bp); Lane 8- DNA sample from non-transformed plant; b) Southern hybridization of genomic DNA isolated from PCR positive putative T₀ transformants: Lane 1- Linear plasmid of pCAMBIA1301; Lane 2-6- DNA sample from putative T₀ transformants (PCR positive) [Lane 3, 4, and 6 shows the integration of T-DNA at single site of transgenic line no 3, 17, and 21 respectively; Lane 2 shows the integration of T-DNA at two site of transgenic line no 9; Lane 5 shows the integration of T-DNA at three site of transgenic line no 19]; Lane 7- DNA from non-transformed plant (control).
Figure 3.18. a) PCR confirmation of putative T₀ transformants regenerated from half-seed explants to detect the presence of hpt coding region: Lane 1- DNA sample from non-transformed plant; Lane 2- Plasmid pCAMBIA1301; Lane 3- Marker (100 bp); Lane 4–8- DNA sample from putative T₀ transformants (arrow indicate the amplification of hpt gene at 407 bp); b) Southern hybridization of genomic DNA isolated from PCR positive putative T₀ transformants: Lane 1- Linear plasmid of pCAMBIA1301; Lane 2–6- DNA sample from putative T₀ transformants (PCR positive) [Lane 3, 4, and 6 shows the integration of T-DNA at single site of transgenic line no 2, 8, and 9 respectively; Lane 5 shows the integration of T-DNA at two site of transgenic line no 4; Lane 2 shows the integration of T-DNA at three site of transgenic line no 6]; Lane 7- DNA from non-transformed plant (control).