Agrobacterium tumefaciens mediated transformation and regeneration of ginger (*Zingiber officinale* Rosc.)

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Abstract

The absence of seed set in ginger makes conventional breeding methods inapplicable warranting genetic modification through biotechnological means. *Agrobacterium tumefaciens* strain *EHA105/p35SGUSInt*, effective in expressing β-glucuronidase activity, was used to standardize the pre-culture of explants, bacterial dilution, and co-cultivation period, besides evaluating the effect of acetosyringone and post cultivation in darkness, and to assess the optimum concentration of kanamycin as selection agent for transformation. Transformants were recovered on selection media containing 100 mg L^{-1} kanamycin and a combination of 2,4-D 1.0 mg L^{-1} and BA 0.5 mg L^{-1}, and regenerated in half strength MS media of BA 3.0 mg L^{-1} and 2,4-D 0.5 mg L^{-1}. Successful transformation was confirmed by histochemical GUS assay and polymerase chain reaction analysis.

Keywords: Genetic transformation, β-glucuronidase, GUS expression

Introduction

Ginger (*Zingiber officinale* Rosc.) is a major spice crop of the humid tropics. India is the second largest producer of ginger in the world with the states of Assam, Orissa, Kerala, Meghalaya and West Bengal accounting for 68% area and 61% production in this country (Spices Board, 2004-05). Soft rot (*Pythium aphanidermatum*) and bacterial wilt (*Ralstonia solanacearum*) are two major diseases causing substantial yield losses of ginger (Joshi and Sharma, 1980; Dake, 1995). Lack of resistance in the cultivated types and the absence of natural seed set, however, hamper the resistance breeding programmes (Valsala et al., 1996), necessitating the search for alternate approaches. Plant tissue culture techniques combined with genetic engineering have considerable potential in this respect. *Agrobacterium* based DNA transfer system has advantages in plant transformation due to its simplicity, precision, integration of DNA sequence with defined ends, linked transfer of genes of interest along with the transformation marker,

high frequency of stable transformation with many single copy insertions, and the ability to transfer long stretches of T-DNA (Veluthambi et al., 2003). One of the pre-requisites for successful plant transformation, however, is the availability of a regeneration protocol that is compatible with the gene transfer. As on date, no such protocols have been published for ginger. The objective of this study was to develop a reliable transformation and regeneration system that could be effectively used for genetic improvement of ginger. For optimizing the conditions for transformation, the effects of various factors such as co-cultivation period, pre-culture of explants, bacterial density, infection time, post cultivation in darkness, and the use of inducers such as aceto-syringone were evaluated.

Materials and Methods

Bud sprouts from the ginger cv. Rio-de-Janeiro were surface sterilized in 0.1% (w/v) HgCl$_2$ for 10 min. and rinsed in sterile water three times. The buds were

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inoculated on to half MS media containing BA 3.0 mg L^{-1}. The decapitated crown sections cultured in the same medium at $25 \pm 1°C$ under 16/8 h photoperiod gave more shoots and young buds. Leaf discs, pseudostem, and young buds from the 4-week-old aseptic cultures were examined for regeneration.

Agrobacterium tumefaciens *strain and co-cultivation*

A. tumefaciens EHA 105 (Hood et al., 1993) containing the binary plasmid *p35SGUS INT* (Vancanneyt et al., 1990) was the vector system used. Plasmid *p35SGUS INT* contains the *npt II* gene under regulatory control of the *nos* promoter and terminator and the *gus* A coding region, containing a plant *intron* linked to the cauliflower mosaic virus *35 S* (*CaMV35S*) promoter, located near the left border. Strain EHA 105 was maintained at 28°C on YEM (yeast extract manitol) medium supplemented with kanamycin 50 mg L^{-1} and rifampicin 20 mg L^{-1}. For co-cultivation, isolated colonies of the bacteria were picked from the selection plates and grown overnight (200 rpm) at 28°C in the same medium without agar pellets and resuspended to an OD_{550} of 0.3. The bacterial suspension was then diluted (1:20 v/v) to give a density of 1 x 10^9 cfu ml^{-1} in a pH 5.4 MS liquid medium. Bud explant sections were immersed in the bacterial culture, blotted dry on sterile filter paper, and transferred to the induction medium. To determine the effect of acetosyringone (AS) on transformation efficiency, 0, 100, 200, 300, 400, 500, and 1000 µM AS were added to the induction medium. *A. tumefaciens* strain EHA 105 was grown overnight at 28°C in AB minimal medium at pH 7.0 (Chilton et al., 1977) containing sucrose 5 g L^{-1}, kanamycin 50 mg L^{-1}, rifampicin 20 mg L^{-1}, and acetosyringone 200 µm to activate *Vir* genes. Before co-cultivation, the bacterial cells were co-cultivated for 8 h in 1:20 diluted bacterial suspension with YEM liquid medium. Callus induction medium containing 0, 100, 200, 300, 400, 500, and 1000 µM AS was prepared and the air dried explants after infection were inoculated and kept in the dark for 2 days. The number of GUS spots per 100 mg cell clumps were calculated.

Plant tissue culture and transformation

Young buds produced aseptically from 4-week-old micropropagated shoot cultures were used as explants. Prior to co-cultivation, the buds were precultured on induction medium for a period of 0–5 days. The explants were infected with *Agrobacterium* at different densities of inoculum over varying infection periods. Co-cultivation was carried out for 15 min, 1, 2, 3 and 4 days in darkness on MS medium supplemented with 2,4-D (1.0 mg L^{-1}) + BA (0.5 mg L^{-1}). After co-cultivation, the explants were transferred to the same medium supplemented with kanamycin 100 mg L^{-1} + cefotaxime 300 mg L^{-1} and maintained under darkness for 2 and 4 weeks. The cultures surviving after four weeks were subcultured every three weeks on to the same medium at $25 \pm 1°C$ under 16/8 h photoperiod. Regenerating calli were further subcultured onto MS media containing BA 3.0 mg L^{-1} + 2,4-D 0.5 mg L^{-1}. For selection of transformants, *npt* II genes providing resistance to kanamycin was used. Sensitivity of uninoculated buds to kanamycin was tested by adding kanamycin (0, 20, 40, 60, 80, 100, 120, 140 mg L^{-1}) to the induction medium. Regeneration potential of non-transformed explants were also tested by adding cefotaxime and carbenicillin (0–300 mg L^{-1}) to the induction medium.

GUS assays

Explants were assayed for expression of the *gus A int* gene following the histochemical procedure described by Jefferson et al. (1987) with some modifications. Explants were incubated overnight at 37°C in 100 mM sodium phosphate buffer (pH 7) containing 0.5 mM potassium ferricyamide, 0.5 mM potassium ferro-cyamide, 10 mM Na$_2$ EDTA, 0.5% (v/v) Triton x-100, and 5-bromo-4 chloro-3 indolyl *β-D*-glucuronide (*X-Gluc*) at 0.5 mg L^{-1}. After incubation, the explants were cleared and fixed in 95% (v/v) ethanol +1 % (v/v) acetic acid. Transient *gus A int* expression was measured immediately after co-cultivation by counting the number of GUS-expressing zones appearing as blue spots. The number of GUS expressing calli that developed on

kanamycin selection medium after 4 to 5 weeks was determined by counting the number of calli with blue zones after *X-Gluc* incubation. GUS assays were replicated with a minimum of 5 to 10 explants. Putatively transgenic shoots were also assayed for GUS expression using the same procedure. Control explants were also treated as described. Histochemical *GUS* assay was performed on the transformed callus and leaf tissue of the regenerated shoot. For plant transformation, the *UidA (β*-glucuronidase*, GUS) intron* containing gene was used to prevent bacterial GUS expression.

DNA extraction and PCR analysis

Genomic DNAs were isolated from fresh leaf tissues of kanamycin resistant plants and a control plant following the method of Doyle and Doyle (1987). PCR (polymerase chain reaction) was performed to identify the presence of marker gene (*npt II*) in the transgenic plants. The primers used for amplification of a 700 bp fragment of the *npt* II gene were 5'CAATCG GCT GCT CTG ATG CCG3' and 5'AGG CUA TAG AAG GCA ATG CGC3'. For each PCR, the amount of DNA used was 15–20 mg of the appropriate plasmid as positive control and 75–100 mg of plant DNA. The reaction mixture contained 10 mM d NTP mix, *Taq* polymerase buffer, 0.6 units of *Taq* polymerase 2.5 mM $MgCl_2$, and 0.6 μM of each primer. After heating the samples to 94°C for 2 min, *Taq* polymerase was added and the reaction was proceeded with 30 cycles of 94°C for 2 min. A final extension step was carried out at 72°C for 5 min. PCR products were separated by electrophoresis and visualized in 0.8 % ethidium bromide stained agarose gel.

Results and Discussion

Although leaf discs, pseudostem, and young buds from the 4-week-old aseptic cultures were examined for regeneration, young buds were better than other explants for inducing embryogenic calli and plant regeneration. Culture of young bud explants on MS medium containing 2,4-D 1.0 mg L^{-1} + BA 0.5 mg L^{-1} resulted in callus initiation (14–18 days) followed by callus proliferation, and then by formation of embryogenic callus under dark conditions. Embryogenic callus regenerated in MS media supplemented with BA 3.0 mg L^{-1} + 2,4-D 0.5 mg.

Kanamycin (100 mg L^{-1}) completely inhibited growth of callus and the explants turned brown after 8–10 weeks. To prevent *A. tumefaciens* growth, cefotaxime 300 mg L^{-1} and carbenicillin 200 mg L^{-1} were used. Induction of callusing and regeneration were not affected in the presence of cefotaxime whereas carbenicillin inhibited callus growth and the tissue turned brown; hence we used cefotaxime alone for further studies.

In species where low transformation efficiencies are expected, the study of the effect of several factors by comparing the percentage of recovered transformed plants may prove unsuccessful because limited numbers of transformants are produced. Therefore, as a first approximation to evaluate the influence of diverse factors on the efficiency of T-DNA transfer, experiments were conducted to observe the levels of transient GUS expression in inoculated buds. It is known that results of transient GUS expression, originating mainly from non-integrated T-DNA copies, may not necessarily correlate with stable transformation events. However, these studies can be used as a guide, and only major differences between tested parameters were taken into consideration for the establishment of transformation protocol in ginger.

Preculture period and bacterial concentration

Preculture of young buds of ginger on callus induction medium for three days increased the transformation frequency to almost eight times compared to explants without preculturing (Fig. 1). It had a positive effect on the induction of competent cells for transformation. Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in many plants. However, extended preculture may be deleterious for transformation in some species (De Bondt et al., 1994).

β-glucuronidase activity was used to monitor trans-formation efficiency as affected by preculturing and
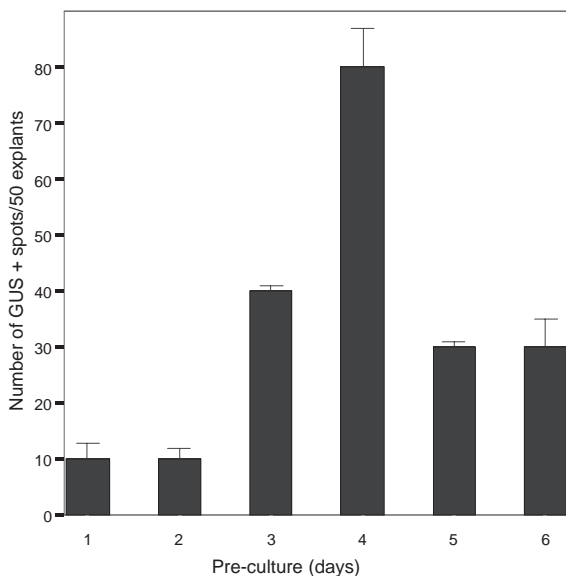
Figure 1. Influence of preculturing explants on transformation of ginger. Transformation is represented as the total number of *GUS+* in a set of 50 explants. Vertical bars indicate the SE from two experiments.

bacterial dilution. Blue staining was evident as soon as two days after co-cultivation of explants with EHA 105. Number of blue sectors/100 mg cell clumps ranged from 1 to 6.5. GUS activity was highest in cells precultured for three days and with a bacterial dilution of 1:20.

Co-cultivation period on transformation of ginger bud explants

Histochemical GUS assays indicated that transformation had occurred at specific zones, and each spot represented an independent transformation event. When the explants were transferred to selection medium immediately after inoculation with *Agrobacterium*, no transformation was observed. GUS activity was observed from the explants co-cultivated for 2 to 3 days. Although explants which had undergone co-cultivation for 4 to 5 days showed GUS activity, the tissues were adversely affected due to the overgrowth of bacteria. It is clear from the result that the co-cultivation time with *Agrobacterium* needed was 2 to 3 days to obtain efficient expression of GUS in ginger. In rice also it was reported that co-cultivation for 2 to 3 days with *Agrobacterium* was required to obtain efficient

expression of GUS (Rashid et al., 1996). With an infection time of 10 min, 80% of the explants showed bacterial overgrowth and a time of 5 min was found optimum since it showed no overgrowth in the selection media. Although prolonged co-cultivation periods more than three days have been successfully used for certain plants (Mourgues et al., 1996), 2 to 3 days co-cultivation has been routinely used in most reported transformation protocols, since longer co-cultivation periods frequently result in *Agrobacterium* overgrowth (Cervera et al., 1998).

Post-cultivation in darkness

Culturing in darkness clearly improved callus induction (Figs. 2a and b). Callus formation and consequent regeneration were progressively increased by maintaining both transformed and untransformed explants for two weeks in darkness and then transferring them to light. When the explants were kept in dark for four and eight weeks, the callus became white and failed to regain green colour. In transformed explants, the GUS positive explants as well as callus formation increased from 2 to 4 weeks, but excessive exposure to darkness resulted in the occurrence of etiolated calli.

Acetosyringone as transformation enhancer

Our results show that 200 μM acetosyringone treated *Agrobacterium* and a two-step co-cultivation method were effective for obtaining transient GUS expression of cells. Ginger explants responded positively to the presence of acetosyringone in the co-cultivation medium. For example, GUS activity scored as number of GUS spots per 100 mg of cells was the highest in cells co-cultivated with 200 μm acetosyringone treated *A. tumefaciens* using the two step co-cultivation method (Tables 1 and 2). The *vir* gene activity, however, must be sustained at a high level during the 8 h inoculation and two day co-cultivation period. The beneficial effect of acetosyringone during co-cultivation to increase *Agrobacterium* mediated transformation frequencies was reported earlier too (e.g., Sheikholeslam and Weeks, 1987).
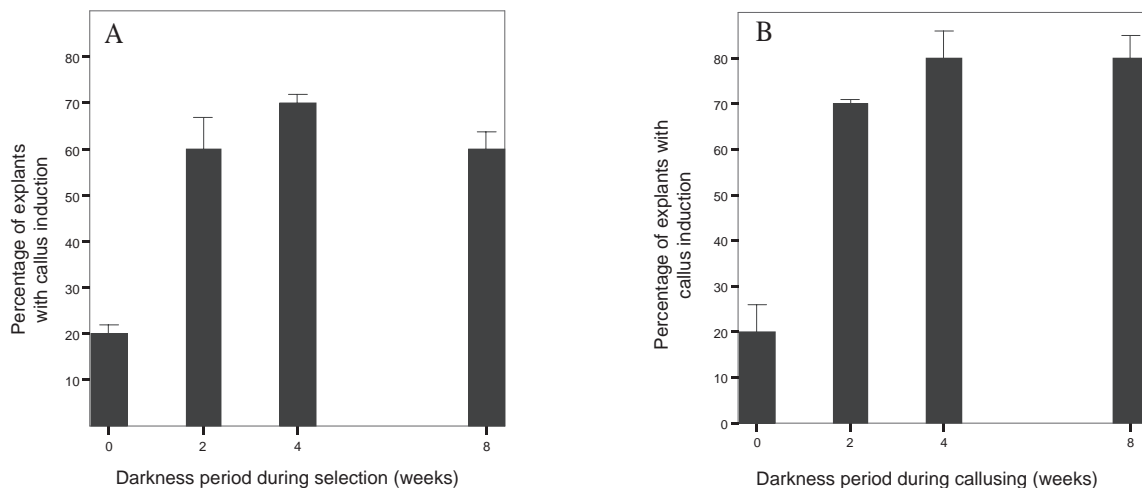
Figure 2. Influence of exposure to darkness on the regeneration potential of *Agrobacterium* inoculated (A) and non-inoculated control ginger explants (B). Explants were exposed to darkness for 0, 2, 4, or 8 weeks and then transferred to a 16 h photoperiod. Error bars denote standard errors.

Analysis of transformation

The *UidA (β-glucuronidase, GUS) intron* containing gene used to prevent bacterial GUS expression, enabled testing of GUS activity at early stages of transformation. In the optimization experiments, GUS assays were conducted two days after co-cultivation with *A. tumefaciens*. Stable expression was detected in transformed calli 40 days after co-cultivation (Fig. 3). All of the green regenerated shoots were checked for GUS activity, one of the shoots always gave consistent GUS expression while others did not consistently show the characteristic blue staining pattern

Table 1. Effect of acetosyringone on co-cultivation period for *vir* induction (*A. tumefaciens* strain EHA 105 were pelleted and resuspended in YEM liquid medium (1:20 v/v) containing 200 μM acetosyringone, incubated for different period and 2 days of further co-cultivation on 0.8% agar solidified callus induction medium).

Co-cultivation period in liquid medium (h)	Mean	Standard error
0.0	2.0	0.0
0.5	2.0	0.0
1.0	2.5	0.5
2.0	3.0	0.0
4.0	3.3	0.25
6.0	3.5	0.5
8.0	5.3	0.25

in repeated assays. All shoots from one clone showed strong GUS expression, while shoots from the other clones and control did not show any blue staining (Fig. 3). GUS expression in GUS positive shoots was clearly visible in leaves, due to easier penetration of substrate into the tissue.

In the PCR assays used to amplify *npt* II coding sequences in DNA extracted from kanamycin resistant shoots expressing GUS activity, a 600 bp fragment amplicon was obtained in the case of putative transformants, confirming transformation. No amplification was obtained in non-transformed (control) plantlets (Fig. 4).

Table 2. Effect of acetosyringone on transient GUS expression in ginger (200 μM acetosyringone treated *Agrobacterium tumefaciens* strain EHA 105 was co-cultivated for 8 h in 1:20 (v/v) diluted bacterial suspension with YEM, 2 days of further co-cultivation on agar solidified medium (0.8%) with different concentrations of acetosyringone).

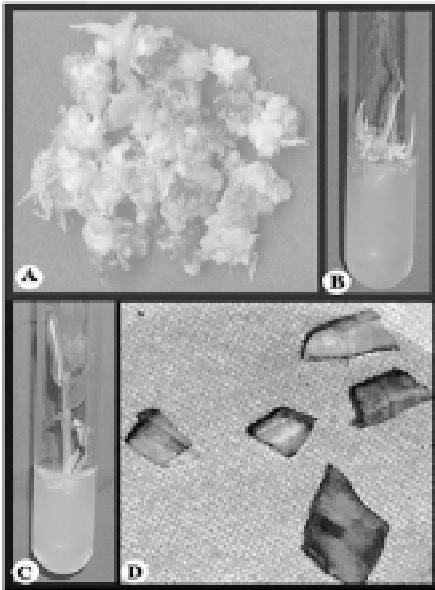| Acetosyringone (μM)	Mean	Standard error
100	3.5	0.5
200	11.5	0.5
300	5.5	0.5
400	4.5	0.5
500	3.5	0.5
1000	3.0	1.0
Without acetosyringone treated strain	3.5	0.5

Figure 3. Transformation of *Zingiber officinale* bud explants by *Agrobacterium tumefaciens* strain *EHA 105*. A. Transformed buds showing stable GUS expression, B. Proliferation of transformed shoots (MS + 2,4-D 1.0 mg L^{-1} + BA 0.5 mg L^{-1})+ kanamycin 100 mg L^{-1} + cefotaxime 200 mg L^{-1}, C. Elongation of transformed shoots (MS + 2,4-D 1.0 mg L^{-1} + BA 0.5 mg L^{-1})+ kanamycin 100 mg L^{-1} + cefotaxime 200 mg L^{-1}, and D. *GUS* expression in leaves of the transformed shoots.
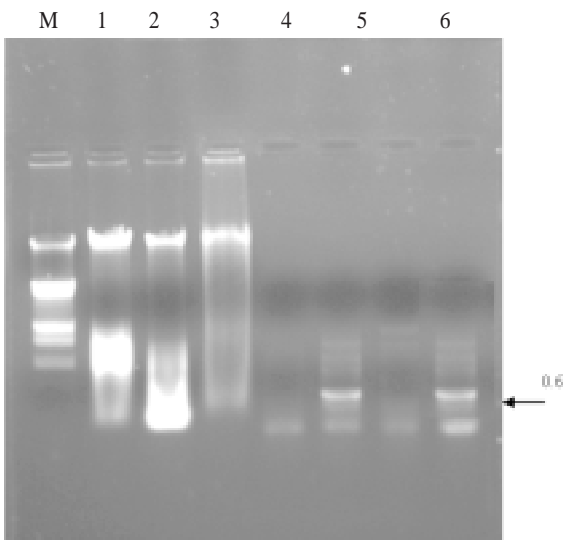

Figure 4. Detection of genes in transgenic ginger plants by PCR. M = Marker DNA (1 kb DNA ladder), Lane 1 & 2 = genomic DNA from Putative transgenic ginger shoots, Lane 3 = genomic DNA from untransformed ginger shoots, Lane 4 = untransformed control, Lane 5 = transformed, Lane 6 = Negative control, Lane 7 = Positive control. Arrow heads point to amplification of the 600 base pair fragment of the *npt* II gene
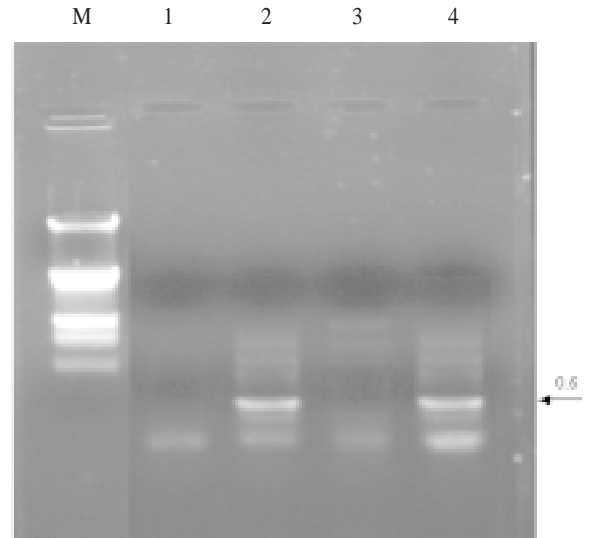

Figure 5. Electrophoretic analysis of the PCR products of transgenic ginger shoots with primers for *npt* II. M = Marker DNA (1 kb DNA ladder), Lane 1 = untransformed control, Lane 2 = transformed, Lane 3 = negative control, Lane 4 = positive control (*p35S GUS INTRON*). Arrow heads point to amplification of the 600 base pair fragment of the *npt* II gene.

On a final note, this is the first report on the genetic transformation of ginger and we present here a protocol for regeneration and genetic transformation of ginger buds through somatic embryogenesis. We found that young buds had very good embryogenic potential and were superior to other explants. A suitable transformation protocol for ginger would include three day preculture of explants on callus induction medium, bacterial dilution of 1:20 (v/v) as the initial inoculum, an infection time of 5 min, two day co-cultivation with *Agrobacterium* and post cultivation on callus induction medium with 100 mg L^{-1} kanamycin and 300 mg L^{-1} cefotaxime under darkness for two weeks, followed by a16/8 h photoperiod regime.

Acetosyringone was effective at a concentration of 200 μm for *vir* induction incubated for 8 h and co-cultivation on callus induction medium. The *vir* gene activity must be sustained at a high level during the 8 h inoculation and two day co-cultivation period. With young bud as explant, a transformation frequency of 1.1 to 2.2 % was noticed. The callus growth was very slow in the presence of antibiotics. Transformation protocol presented here

are potentially useful for the introduction of economically valuable genes into the ginger genome.

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