Plant Cell Reports (2001) 20:157–162

GENETIC TRANSFORMATION AND HYBRIDIZATION

T.R. Ganapathi · N.S. Higgs · P.J. Balint-Kurti
C.J. Arntzen · G.D May · J.M. Van Eck

Agrobacterium-mediated transformation of embryogenic cell suspensions of the banana cultivar Rasthali (AAB)

Received: 22 February 2000 / Revision received: 2 October 2000 / Accepted: 5 October 2000

Abstract A protocol was developed for establishing embryogenic suspension cultures from in vitro-grown, thin shoot-tip sections of the banana cultivar Rasthali. The best medium for callus induction was an MS-based medium supplemented with 2 mg/l 2,4-D and 0.2 mg/l zeatin. The callus was transferred to liquid medium to establish embryogenic cell suspensions. These cultures were subsequently used for *Agrobacterium*-mediated transformation. The *Agrobacterium tumefaciens* strain EHA105 containing the binary vector pVGSUN with the *als* gene as a selectable marker and an intron-containing the *gusA* gene as a reporter gene was used for transformations. The herbicide Glean was used as a selection agent. Two hundred putative transformants were recovered, of which a set of 16 was tested by histochemical analysis for GUS expression and by Southern blot analysis with a probe for the *gusA* gene. The plants were positive for GUS expression and integration of the *gusA* gene. Two of the transformants were grown to maturity under greenhouse conditions. Bananas were harvested to test GUS expression by histochemical analysis. The fruit from both transgenics tested positive for GUS expression.

Keywords Banana · Agrobacterium · Rasthali

Abbreviations *BA* N^6-Benzyladenine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · GA_3 Gibberellic acid · *GUS* β-Glucuronidase · *IAA* Indole-3-acetic acid · *MS* Murashige and Skoog (1962) · *NAA* α-Naphthaleneacetic acid · *PCV* Packed cell volume · *SH* Schenk and Hildebrandt (1972)

Communicated by W. Parrott

T.R. Ganapathi · N.S. Higgs · P.J. Balint-Kurti
C.J. Arntzen · G.D May · J.M. Van Eck (✉)
The Boyce Thompson Institute for Plant Research,
Tower Road, Ithaca, NY 14853-1801, USA
e-mail: jv27@cornell.edu
Fax: +607-254-1242

Present address: T.R. Ganapathi, Nuclear, Agriculture, and Biotechnology Division, Bhabha Atomic Research Center, Trombay, Mumbai 400 085, India

Introduction

Banana (*Musa* spp.) is the fourth most important food crop in the world (Moffat 1999). The application of biotechnology for the improvement of this crop would be a useful tool to breeders for the introduction of traits of interest. Conventional breeding is complicated by the facts that only a few diploid clones produce viable pollen and that the germplasm of the most important commercial clones is both male- and female-sterile (Novak et al. 1989).

Plant regeneration of banana has been reported from various explant sources and from a variety of cultivars. Using cv. Grand Nain, which has a genomic constitution of AAA, Novak et al. (1989) cultured the bases of leaf sheaths and corm sections from in vitro-grown plants. For the cultivar Bluggoe, which has an ABB genomic composition, Dheda et al. (1991) cultured thin sections from highly proliferate shoot-tip cultures to produce embryogenic cell suspension cultures. Marroquin et al. (1993) established embryogenic suspensions from immature zygotic embryos of *Musa acuminata*. However, male flowers are the most responsive starting material for initiating embryogenic cultures of cv. Grand Nain (Escalant et al. 1994; Cote et al. 1996; Navarro et al. 1997; Sagi et al. 1998; Becker et al. 2000).

The recovery of transgenic plants of banana obtained by means of *Agrobacterium tumefaciens*-mediated transformation has been reported. May et al. (1995) recovered transformants from infected meristem and corm slices of banana. Hernandez et al. (in press) used embryogenic cell suspensions as material for *Agrobacterium*-mediated transformation.

To the best of our knowledge this is the first report on *Agrobacterium*-mediated transformation of the banana cultivar Rasthali. We describe herein the development of embryogenic suspension cultures, *Agrobacterium*-mediated transformation, and plant regeneration of Rasthali (genome AAB). Rasthali is a preferred Indian cultivar because it has a very sweet pulp and commands a higher price in Indian markets than the Cavendish cultivars.

Materials and methods

Plant material

In vitro-grown Rasthali plants were established from shoot tips of field-grown plants (Ganapathi et al. 1992). The in vitro plants were maintained on medium containing MS salts supplemented with: nicotinic acid (0.4 mg/l), thiamine HCl (0.5 mg/l), pyridoxine HCl (0.4 mg/l), cysteine (40 mg/l), glycine (2 mg/l), BA (4.5 mg/l), IAA (0.2 mg/l), sucrose (30 g/l), and Gelrite (2 g/l) (Sigma, St. Louis, Mo.). This medium was designated RM. The pH of the medium was adjusted to 5.7 prior to autoclaving.

Plants were maintained in GA7 Magenta boxes (Sigma, St. Louis, Mo.) with one plant per box. They were transferred to fresh medium every 4 weeks. Cultures were maintained at 26°±1 °C under a photoperiod of 16 h (light)/8 h (dark) at 45 μmol photons m^{-2} s^{-1}. The source of light for these cultures and those described throughout this report was from a mixture of cool and warm fluorescent bulbs (F40CW and F40WW) (Philips Lighting Co., http://www.lighting.philips.com/index.htm).

Callus initiation

Thin (400–500 μm) longitudinal sections from 1-cm-long shoot tips (4–6 whorls of leaves) were excised from invitro-grown plants after removal of the leaves and roots from the plantlets. A drop of sterile water was put on the shoot to avoid desiccation of the sections. Approximately five sections were cut from each shoot. To determine the best medium for callus initiation, we transferred the sections to MS- or SH-based media as described in Table 1. These basal media were chosen based on reports by Escalant et al. (1994), Dheda et al. (1991), and Novak et al. (1989). The four media listed in Table 1 were also supplemented with MS vitamins, biotin (1 mg/l), and solidified with Gelrite (2 g/l). The pH of the media was adjusted to 5.8 prior to autoclaving. Callus initiation medium was designated CI.

Ten shoot-tip explants were cultured in petri plates (100×20 mm) sealed with Nescofilm (Karlan Research Products, Santa Rosa, Calif.). Ten plates were cultured per media treatment (Table 1). The media test was carried out twice, which amounted to a total of 200 explants per treatment. Cultures were maintained in the dark at 26°±1 °C.

Suspension cultures

Suspension cultures were established from 6-month-old, shoot-tip-derived callus. To initiate suspensions, we transferred 100 mg of callus to 25-ml Erlenmeyer flasks containing 8 ml of a medium designated M2 (Cote et al. 1996), which is an MS-based liquid medium supplemented with 2,4-D (1 mg/l), biotin (1 mg/l), l-glutamine (100 mg/l), malt extract (100 mg/l), and sucrose (45 g/l). The pH of the medium was adjusted to 5.3 prior to autoclaving.

Each culture was sealed with a cotton plug and covered with aluminum foil. They were maintained on a gyratory shaker at 80 rpm under a photoperiod of 16 h (light)/8 h (dark) at 45 μmol photons m^{-2} s^{-1} and a temperature of 26°±1 °C.

Suspension cultures containing actively dividing and densely packed cytoplasmic cells were recovered after 3–4 months. During this time, the suspensions were subcultured every 10 days by removing 4 ml of medium, then adding 4 ml of fresh M2 medium. Thereafter, the suspensions were cultured in 125-ml Erlenmeyer flasks containing 25 ml medium on a gyratory shaker at 80 rpm. At each subculture, 0.5 ml PCV of cells from a suspension plus 4.5 ml of the suspension culture medium were added to 25 ml of M2 medium. Suspensions were subcultured once every 10 days. Cultures were maintained at 26°±1 °C under a photoperiod of 16 h (light)/8 h (dark) at 45 μmol photons m^{-2} s^{-1}.

Table 1 The effect of various media on callus initiation from shoot-tip sections of Rasthali

Media	Number of shoot-tip sections	Percentage of sections with callus
MS basal with 2,4-D (2 mg/l) and zeatin (0.2 mg/l)	200	42±3.26[a]
MS basal with picloram (2 mg/l)	200	24.5±7.76
SH basal with 2,4-D (2 mg/l) and zeatin (0.2 mg/l)	200	39.5±5.72
SH basal with picloram (2 mg/l)	200	1.5±0.82

[a] Standard error

Embryo development and plantlet regeneration

For embryo development, 0.5 ml PCV of suspension culture was aspirated onto glass fiber discs (Fisher Scientific, Pittsburgh, Pa.), then transferred to either of the following two media to determine the best medium for embryo development. The first medium, designated SHP, was an SH-based medium supplemented with MS vitamins, l-glutamine (100 mg/l), malt extract (100 mg/l), biotin (1 mg/l), picloram (1 mg/l), and sucrose (45 g/l). The second medium, designated RR, contained 1/2-strength MS salts, MS vitamins in addition to zeatin (2 mg/l), sucrose (30 g/l), and Gelrite (2 g/l). The pH of the medium was adjusted to 5.3 prior to autoclaving. Four plates were cultured per medium treatment, and the experiment was performed twice. Cultures were transferred to fresh medium every 4 weeks and maintained in the dark at 26°±1 °C.

For the development of small plantlets, germinated embryos with coleoptiles 3 mm long were transferred to medium designated MM4, which is a modification of the M4 medium reported by Cote et al. (1996). MM4 contains 1/2-strength MS salts, MS vitamins, L-glutamine (100 mg/l), malt extract (100 mg/l), biotin (1 mg/l), BA (0.5 mg/l), GA_3 (0 or 1 mg/l), and sucrose (45 g/l). The pH of the medium was adjusted to 6.0 prior to autoclaving. Cultures were maintained at 26°±1 °C under a photoperiod of 16 h (light)/8 h (dark) at 70 μmol photons m^{-2} s^{-1}.

Embryos that germinated were transferred to a medium designated PD for continued plantlet development. PD medium contains MS salts and vitamins, NAA (1 mg/l), sucrose (30 g/l), and Gelrite (2 g/l). The pH of the medium was adjusted to 5.3 prior to autoclaving. Cultures were maintained at 26°±1 °C under a photoperiod of 16 h (light)/8 h (dark)at 70 μmol photons m^{-2} s^{-1}. Complete plantlets (shoots and roots) developed in 4–6 weeks. At this stage, the plantlets were transferred to soil and were maintained under mist for 2–3 weeks in the greenhouse for acclimatization to greenhouse conditions.

Agrobacterium and plasmid

Agrobacterium tumefaciens strain EHA 105 (Hood et al. 1993) harboring pVGSUN (Zeneca Plant Science, UK) (Fig. 1) was used for transformations. The plasmid pVGSUN contains both a reporter and selectable marker gene. The reporter gene is an intron-containing *gusA* gene under the control of the Gelvin promoter (Ni et al. 1995). The selectable marker gene, *als* (Lee et

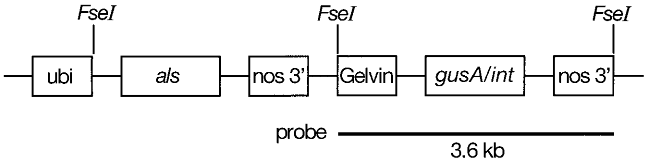


Fig. 1 Diagram of the T-DNA region of pVGSUN. *ubi1* Maize polyubiquitin-1 promoter, *als* acetolactate synthase gene, *nos 3′* nopaline synthase terminator, *Gelvin* Gelvin promoter, *gusA/int* (GUS) β-glucuronidase gene containing its intron. *FseI* is the restriction enzyme used to digest the plasmid DNA, which generated a 3.6-kb probe for Southern analysis

al. 1988), which confers resistance to sulfonylurea herbicides, is driven by the *ubi1* promoter from maize (Christensen et al. 1992). *Agrobacterium* was grown in yeast extract medium (Sigma, St. Louis, Mo.) supplemented with appropriate antibiotics to an OD_{660} of 0.4–0.5, then centrifuged at 8,000 rpm for 10 min. The pellet was resuspended in MS liquid medium and diluted to a density of 5×10^7 cells/ml.

Transformation of embryogenic suspensions

Suspension culture cells in M2 medium 7 days post-subculture were used for the transformations. The cells were passed through an 85-μm sieve to remove the large cell clumps. The sieved embryogenic cells (0.5 ml PCV) were incubated with *Agrobacterium* in the presence of 10 ml of M2 medium supplemented with 100 μ*M* acetosyringone (Aldrich, Milwaukee, Wis.) for 30 min. Suspension cells were then aspirated onto glass filter discs with a Büchner apparatus and transferred to solidified M2 medium containing 2 g/l Gelrite and 100 μ*M* acetosyringone. Three days post-co-culture in the dark, filter disks with cells were transferred to solidified M2 medium containing carbenicillin (500 mg/l) (INALCO, San Luis Obispo, Calif.). One week later, the cells were transferred to solidified M2 medium containing carbenicillin (500 mg/l) and Glean (5 or 10 μg/l of active ingredient) (DuPont, Wilmington, Del.). After 3 weeks, the cultures were transferred to solidified M2 medium containing 10 μg/l Glean. Glean at a concentration of 10 μg/l was incorporated into all subsequent media.

Histological GUS assays

Several samples were tested for histochemical localization of GUS (Gallagher 1992). These samples included plated suspension cultures 6 days after cocultivation, leaf sections from 6-month-old greenhouse-grown plants, and fruit slices from bananas at three different ripening stages (green, green with a trace of yellow, all yellow). All samples were incubated overnight at 37 °C in an assay buffer [100 m*M* Na PO_4 (pH 7.0), 50 m*M* ascorbic acid, 1 μ*M* X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt)]. Leaf samples were then washed three times with sterile deionized water followed by 70% ethanol for clearing.

Southern analysis

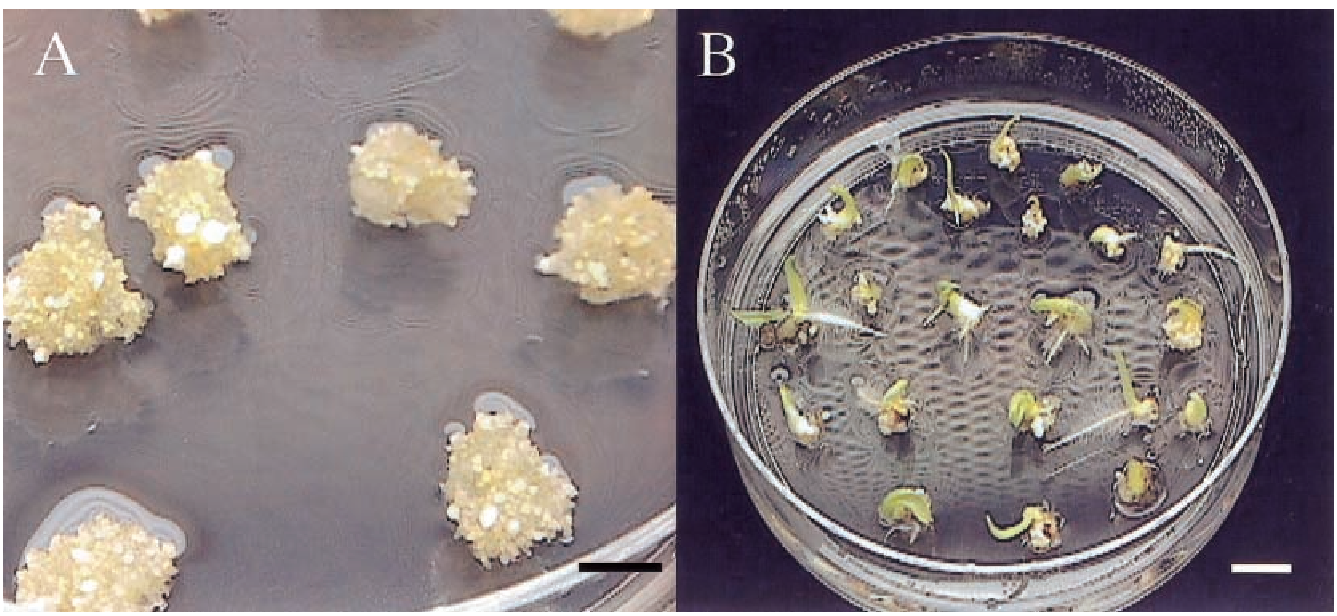
Genomic DNA was isolated from 16 putative transgenic lines of in vitro-grown plants according to a modified CTAB protocol (Stewart and Via 1993). The restriction enzyme *Nco*I was used to digest 15-μg aliquots of DNA from each sample. The digested DNA was separated on a 0.8% agarose gel. and subsequently transferred to Nytron (Schleicher and Schuell, Keene, N.H.) according to the manufacturer's instructions. The *gusA* probe was a 3.6-kb *Fse*I fragment internal to the T-DNA from pVGSUN (Fig. 1) that was radiolabeled (α-[^{32}P]dCTP-labeled) by random priming. Membranes were hybridized and washed as recommended by the membrane supplier.

Results and discussion

Somatic embryogenesis

Occasionally, an endogenous bacterial contaminant was observed on the shoot-tip sections. The contaminated sections were removed from the plates and discarded. The shoot-tip cultures showed callus development on

Fig. 2A, B Regeneration from embryogenic suspensions of *Musa acuminata* cv. Rasthali. **A** Development of embryos on RR medium 4 weeks after plating the suspensions. **B** Germinating embryos on MM4 medium (*bars*: 0.5 cm)



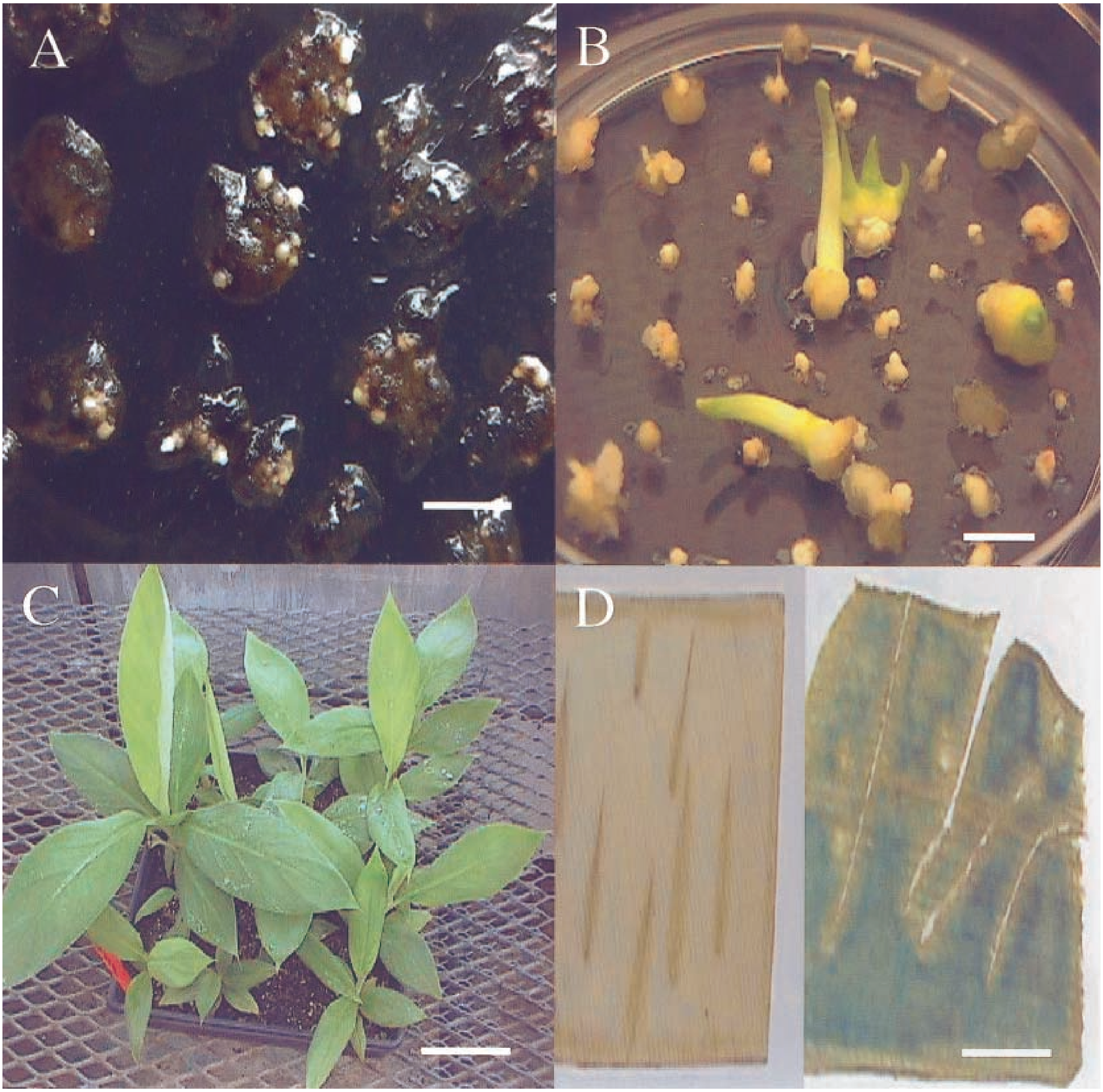


Fig. 3A–D Regeneration of transgenic plants from embryogenic suspensions of *Musa acuminata* cv. Rasthali transformed with pVGSUN which contains the *gusA* gene. **A** Developing transformed embryos on RR medium containing Glean (10 μg/l) (*bar*: 0.5 cm). **B** Development of shoots from transformed embryos on MM4 medium (*bar*: 0.5 cm). **C** Transformants acclimatized to greenhouse conditions (*bar*: 5 cm). **D** Histological GUS assay with leaf segments from greenhouse-grown plants. The segment on the *left* is from a control (non-transformed) plant, and the segment on the *right* is from a transformant (*bar*: 0.5 cm)

both the MS- and SH-based medium (Table 1). Maximum frequency of callusing was noted on medium supplemented with 2,4-D and zeatin, regardless of the basal salts used. Initially, the callus that formed was yellowish and compact, with white friable embryogenic tissues developing within 4–5 months after initiation. This callus along with the explants were transferred to solidified M2 medium. Embryogenic tissues were maintained up to 1 year by subculturing once every 4–6 weeks. The whitish embryogenic tissues were used to initiate the embryogenic suspensions.

Upon transfer into liquid M2 medium, these tissues released embryogenic cells and cell aggregates. Fine embryogenic cells with dense cytoplasm were obtained 3 months after initiation into liquid medium. Six suspension culture lines were established, and all had similar regeneration frequencies of approximately 200 regenerants per 0.5 ml PCV for each line. The suspensions were subcultured once every 10 days by adjusting to 3% of the packed cell volume. A multiplication ratio of 1:2 to 1:3 was noted every 10 days.

The suspensions (approx. 0.5 ml PCV) were aspirated onto glass fiber filters and maintained on solidified M2 medium for 1 week before being transferred to SHP and RR media. Embryo development was noted within 30–45 days. Globular-stage embryos were visible 4 weeks after transfer to the above media (Fig. 2A). The selection of media (SHP vs. RR) for embryo development was based upon the number of plantlets that were recovered after transfer to MM4 medium. The number of plantlets recovered from cultures initially maintained on SHP medium was 75 ± 0.87 (standard error), whereas 182 ± 0.97 plantlets were recovered from cultures initially cultured on RR.

To regenerate plantlets, we transferred the embryos to MM4 and MM4 + GA_3 (1 mg/l) (Fig. 2B). With the addition of GA_3, the number of plantlets recovered from cultures initially placed on SHP medium increased to 175 ± 1.21, and the number recovered from cultures originally on RR increased to 200 ± 0.5.

For the development of complete (shoots and roots) plantlets, the germinating embryos were transferred to PD medium. Plantlets were large enough to be transferred to the greenhouse within 4–6 weeks. Approximately 150–200 plants could be obtained from an initial starting material of 0.5 ml PCV of suspension culture in 6 months.

Agrobacterium-mediated transformation

Embryogenic cells co-cultivated with *Agrobacterium* EHA 105 (pVGSUN) were tested for GUS expression 6 days after cocultivation. Blue staining indicative of the presence of GUS was noted over the entire surface of the tissue. Non-transformed control cells did not stain blue.

The transformed cells multiplied and became more organized. A concentration of 5 μg/l Glean was chosen because in previous experiments when 10 μg/l Glean was incorporated, there was extensive browning of the cells.

After 3 weeks, globular-stage embryos could be seen on the surface of the embryogenic cells (Fig. 3A). Glean was increased to a concentration of 10 μg/l at this point to prevent the growth of any non-transformed material. These embryos could be easily transferred with forceps to MM4 medium containing 10 μg/l Glean for maturation. Coleoptile emergence was noted within 4–8 weeks (Fig. 3B).

Germinated embryos were transferred to PD medium containing 10 μg/l Glean for complete plantlet development. Approximately 100% of the germinated embryos obtained on MM4 medium rooted on PD medium containing Glean. A plantlet with profuse rooting and vigorous shoot growth could be obtained within 4–6 weeks.

The transformation protocol was performed four times, with two different cell lines. For each transformation, 0.5 ml PCV of suspension culture cells was incubated with the *Agrobacterium*. Approximately 200 putative transformants were recovered from these four transformations, and the recovery of plants was similar for each transformation. An average of 40 independently transformed plantlets could be obtained in 4 months from an initial 0.5 ml PCV of embryogenic suspension culture.

A subset of 16 putative transformants was selected for analysis. These plants were selected because they were the first plants to appear with vigorous root systems. All samples tested positive for GUS expression. Confirmed transformants were transferred to the greenhouse (Fig. 3C), and GUS expression was subsequently observed in leaf segments from the greenhouse-grown plants (Fig. 3D).

Two of the confirmed transformants, 5(11) and 6(3), were grown to maturity. GUS expression was observed in the fruit of both transgenic lines at each ripening stage, but not from the control (non-transformed) plant (Fig. 4).

Southern analysis

In each sample, hybridization was observed to a common 3.4-kb *Nco*I fragment entirely comprised of T-DNA sequence (Fig. 5). Unique *Nco*I fragments were also detected that represented about 1.2 kb of DNA at the right border of the T-DNA plus flanking genomic DNA. In most transformants, the T-DNA copy number was between 1 and 4 with one individual [5(8)] containing about 12 copies. Transformants 5(4) and 5(5) had a similar pattern of bands as did 5(1) and 5(6), indicating that these plants originated from the same transformation events.

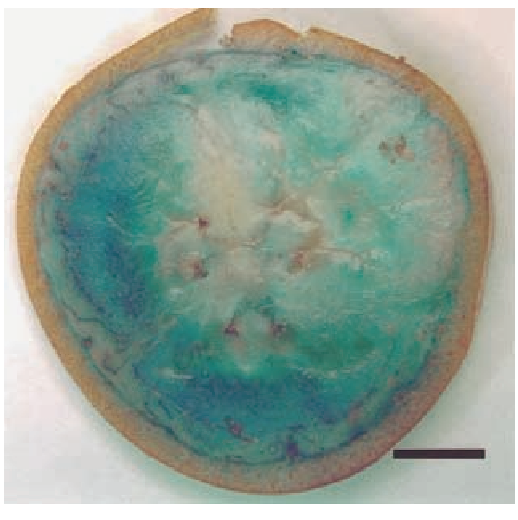


Fig. 4 Histological GUS assay of a fruit slice from a *Musa acuminata* cv. Rasthali transformant [6(3)]. The sample was taken from a banana that was completely yellow (*bar*: 1 cm)

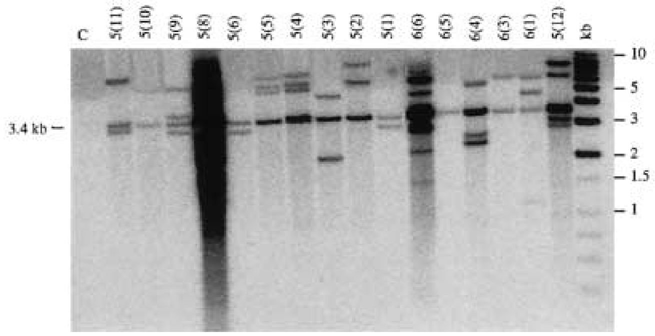


Fig. 5 Southern blot analysis of genomic DNA (15 μg per lane) of plants recovered after *Agrobacterium*-mediated transformation of embryogenic suspensions of *Musa acuminata* cv. Rasthali. DNA was digested with *Nco*I and hybridized to a probe from the right border of the pVGSUN T-DNA. A common 3.4-kb band is seen in each lane except for the control (*C*) (non-transformed plant). The number of bands reflects the number of T-DNA insertions

The method described is similar to the method described by Novak et al. (1989). For this method, cv. Grand Nain suspensions were established from callus initiated from corm slices, young leaves, and shoot-tip sections. Our method differs from that of Novak et al. in the establishment of embryogenic callus, the initiation of embryogenic suspensions, plant regeneration, and the cultivar. This regeneration scheme results in a greater number of regenerants in a shorter amount of time as compared to earlier published reports by Escalant and Teisson (1989).

May et al. (1995) reported *Agrobacterium*-mediated transformation of meristems and corm slices from cv. Grand Nain. However, the authors also noted that the system could give rise to chimeric plants containing both transformed and non-transformed cells. A field trial of the cv. Rasthali transformants described herein has been established in India at the Bhabha Atomic Research Center. Observations will be made on the chimeric nature of the transformants in addition to the occurrence of somaclonal variation and the expression of the *gusA* gene at various developmental stages.

Acknowledgements The authors would like to thank Zeneca Plant Science for supplying pVGSUN and Sara Abend for her technical assistance. This work was supported in part by the Department of Biotechnology, New Delhi, India, the Boyce Thompson Institute for Plant Research, Zeneca Plant Science, and Agritope, Inc.

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