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Agrobacterium rhizogenes-mediated genetic transformation of *Musa acuminata* cv. Vaibalhla (AAA)

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Abstract

Agrobacterium rhizogenes-mediated genetic transformation of *Musa acuminata* cv. Vaibalhla (AAA) was successfully carried out using *A. rhizogenes* strain A4 harboring the binary vector pCAMBIA2301with *VrNHX1*gene. In the study, male flowers of *M. acuminata* cv. Vaibalhla were used as explants to obtain white bud-like structures by culturing on MS basal medium supplemented with 2 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l 1-Naphthaleneacetic acid (NAA). The subsequent shoot induction and plantlet regeneration were carried out in MS medium supplemented with Kinetin (2 mg/l) and NAA (0.5 mg/l). For the genetic transformation, *in vitro* raised plantlets were inoculated with *A. rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 for 30 min followed by 2 days of co-cultivation in the dark in a semi solid MS basal medium. The treated plants were then transferred and maintained in MS basal medium supplemented with ascorbic acid (75 mg/l), kanamycin (150 mg/l) and cefotaxime (400 mg/l). Initiation of hairy roots were observed within 2 days of transfer which were evaluated for GUS activity and the subsequent GUS^{+} roots were assayed for transient transformation by PCR using *npt*II and NHX1 gene specific primers. The transfer of the plasmid as well as the gene was confirmed with positive bands observed at 540 bp and 1.6 kb respectively.

Keywords

Agrobacterium rhizogenes, genetic transformation, *Musa acuminata*, Mizoram

Introduction

The capability of gene transfer to plants by various species of bacteria is now a widely known process (1). *Agrobacterium* species are used extensively for genetic modification in plants because they have the capacity to integrate genes to the genome of the host. But their utilization in banana (*Musa* spp.) is limited as they are highly recalcitrant resulting in no or very low regenerants (2). However, *A. rhizogenes*-mediated transformation can be used as a substitute to this limiting step of successful regeneration of the transformed plants, by isolation of transformed hairy roots which has untransformed aerial part (3). *A. rhizogenes* harbour T-DNA segment of the Ri plasmid within the nuclear genome (4) and the transformed roots has the ability to regenerate as adventitious hairy roots, without phytohormones, from the cells induced by Ri plasmid (5). Genetic transformations with *A. rhizogenes* have been achieved in coffee (6) and broccoli (7). Other studies on successful *A. rhizogenes*-mediated genetic transformation have also been reported for *Lithospermum erythrorhizon* (8), *Brassica oleracea* (9), *Arachis hypogaea*

(10), *Hypericum sinaicum* (11), *Phtheirospermum japonicum* (12), *Pisum sativum* (13) and *Glycine max* (14). However, in banana few reports are available such as in 'Silk' Banana (2). Hence this study was conducted with NHX1 (Na+/H+ antiport) gene (isolated from *Vigna radiata*) in *A. rhizogenes*-mediated transformation of *M. acuminata* cv. Vaibalhla (AAA) to evaluate the transformation efficiency.

Materials and Methods

Explant preparation

In vitro shoots of *M. acuminata* cv. Vaibalhla (AAA) were regenerated from immature male flowers of the same cultivar following the standardized protocol (15). *In vitro* raised shoots were then maintained in basal MS (Murashige & Skoog) solid medium supplemented with BAP (2 mg/l), NAA (0.5 mg/l) and ascorbic acid (75 mg/l) along with 3% sucrose and 100 mg/l myo-inositol and pH maintained at 5.6. All cultures were kept at 25±1 °C under 16:8 hr light and dark photoperiod with light intensity of 55 µM $m^{-2}s^{-1}$ using white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). Roots which appeared along with shoots were cut off prior to transformation experiment. Such clusters of shoot buds and regenerating tissues served as starting explants for the transformation experiment.

Agrobacterium strain

For the genetic transformation studies, A. *rhizogenes* strain A4 was used (Courtesy: Prof. L. Sahoo, Indian Institute of Technology Guwahati, Guwahati, India). NHX1 (Na+/H+ antiport) gene isolated from *Vigna radiata* was chosen as the gene of interest (16). The A4 strain was mobilized with pCAMBIA2301:VrNHX1 plant binary construct using a modified protocol of Freeze-thaw method (17). *npt*II (neomycin phosphotransferase) served as the selection marker and β-glucuronidase (GUS) gene (*uid*A) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter (Fig.1).

Fig. 1. Schematic construction of salt resistance gene VrNHX1 mobilized with *Agrobacterium rhizogenes* strain A4 harboring the binary vector pCAMBIA2301 containing *npt*II (neomycin phosphotransferase) as selectable marker and the β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter. LB and RB: left border and right border of T-DNA region

Mobilization of pCAMBIA2301:VRNHX1 plant binary construct to strain A4 of Agrobacterium rhizogenes

Preparation of competent cells: For the preparation of the competent cells, A4 strain of *A. rhizogenes* was cultured overnight followed by 100 ml dilution with YMA (Yeast Mannitol Agar) liquid medium. The logarithmically growing cells were analysed after 3 or 4 hrs in which the optical density (OD) was 0.4 – 0.6 at 600 nm. The cells were centrifuged at 3000 g for 20 min at 4°C and the pellet was washed once in 5 ml precooled TE (10 mMTris-HCl, pH 7.5; 1mM EDTA). This step was followed by re-suspension in fresh YMA medium (10 ml). For direct use, aliquots of 500 µl were used for transformation storage whilst for storage purpose, addition of glycerol was done in 20% final concentration which was frozen in liquid nitrogen and stored at -80°C (16).

Mobilization: Prior to mobilization, stored cells were thawed on ice. 0.5-1.0 µg of construct plasmid DNA were mixed with the competent A4 strains of *A. rhizogenes*. After this step, they were incubated successively for 5 min on ice followed by 5 min in liquid nitrogen and then 5 min at 37°C water-bath. After dilution in 1 ml of YMA medium the cells were shaken 2-4 hours at 28°C or room temperature. Aliquots of 200 µl were plated on solid YMA plates and incubated at 28°C for 24 hrs. Bacterial colonies were then picked and confirmed through colony PCR (Polymerase Chain Reaction).

Molecular confirmation of the plasmid

The combined region of *npt*II and VrNHX1 within the *Agrobacterium* cells were proved through colony PCR using respective *npt*II and VrNHX1 gene specific oligonucleotide primers. A small amount of freshly plated *A. rhizogenes* strain was picked from a colony using sterile toothpick. A sample of two was arranged; one served as the positive control while the other served as the negative control. 30 µl of sterile MilliQ water in sterile eppendorf tubes was mixed with the controls and thorough mixing was done by vortexing for 1-2 min. Incubation was done at 100 °C water -bath for 10 min after which centrifugation at 10000 rpm for 10 minutes was conducted. The supernatant serves as the template. The isolated DNA per milligram from each sample was calculated by measuring the absorbance at 260 nm and 280 nm using Bio-photometer plus (Eppendorf, Germany) (following the manufacturer's instruction). DNA purity was ascertained by calculating the absorbance ratio at 260 nm/280 nm. A 50 ng/µl DNA stock was arranged from the isolated plasmid DNA which was kept for further experiments.

A 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.4 µM of gene specific primers nptII (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and VrNHX1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG'), 1× Taq DNA polymerase buffer which contained 15 mMMgCl_2 (Sigma-Aldrich Pvt. Ltd.,) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore) was set up. The PCR was conducted in a PTC-100TM thermal cycler (MJ research Inc, Waltham, Mass, USA) which was set with an initial denaturation of DNA at 95 °C (4 mins), 35 cycles of 95 °C (0.30 mins), 58°C (0.30 mins) and 72°C (1:30 mins) with 10 min of final extension at 72°C. The amplified products obtained were resolved by electrophoresis on a 1.2% (w/v) agarose gel run in 1× TBE buffer and detected by ethidium bromide staining. The resulting fragments were scored in UV light using a gel documentation system

(Bio-rad, Australia). Analysis was done with Quantity one-1D software (Bio-rad, Australia). Double-digested (EcoRI and HindIII) 1Kb λ DNA ladder (Fermentas, USA) served as the molecular marker. To check the reproducibility, all the PCR reactions were conducted twice.

Kanamycin sensitivity assay of the explants

Kanamycin sensitivity assay was conducted prior to the genetic transformation studies to evaluate the effect of kanamycin concentration on the explants and to select the transformed plants from the non-transformed ones. It was performed by culturing non-transformed (control) *in vitro* shoot explants of *M. acuminata* cv. Vaibalhla (AAA) on MS medium supplemented with BAP (2 mg/l), NAA (0.5 mg/l) and ascorbic acid (75 mg/l) along with different concentrations (50, 100, 150 and 200 mg/l) of kanamycin.

Cefotaxime assay for control of Agrobacterium overgrowth

The antibiotic cefotaxime was employed for the prevention of over-development of *Agrobacterium*. For assessment of the optimum cefotaxime concentration which can remove the *Agrobacterium*, 5-month-old *in vitro* raised shoots derived from the floral parts of the male bud of *M. acuminata* cv Vaibalhla (AAA) was inoculated and co-cultivated with *A. rhizogenes* strain A4 harbouring pCAMBIA2301VrNHX1 plasmid. This was then subsequently transferred to MS medium supplemented with BAP (2 mg/l), NAA (0.5 mg/l) along with ascorbic acid (75 mg/l) containing different concentrations (100, 200, 300, 400 and 500 mg/l) of cefotaxime.

Preparation of the A. rhizogenes strain for transformation

A single bacterial colony of the A4 strain of *A. rhizogenes* harboring a binary vector pCAMBIA2301VrNHX1 containing nptII (neomycin phosphotransferase) gene and β-glucuronidase (GUS) gene (uidA) with an intron in the coding region both driven by CaMV 35S promoter was inoculated into 25 ml of YMA medium (liquid). This was grown overnight on a rotary shaker (180 rpm) at 28°C, until the OD (600 nm) reached to 0.8 which was followed by centrifugation (5000 rpm) for 5 min. The pellets were resuspended in liquid MS basal medium (pH adjusted to 5.5) with acetosyringone (100 µM) and was finally used for inoculation.

Genetic transformation

For the genetic transformation experiment, 5-month-old *in vitro* regenerated shoots of *M. acuminata* cv. Vaibalhla (AAA) obtained from the floral parts of the male bud were utilized. The roots of the obtained shoots were cut off using sterile blade and the remaining shoots were immersed into the resuspended pellets of the *Agrobacterium* with periodic shaking. Inoculated explants were blotted on a filter paper (sterile) to remove additional pellets and were then co-cultured in a solid basal medium of MS added with acetosyringone (100 µM). Maintenance of the co-cultures was accomplished by keeping them in dark at 25±1 °C for 3 days. Washing of the explants 2-4 times with liquid basal medium (MS) was done after co-cultivation and blotted dry on a sterile filter paper. The washed explants were finally inoculated on a basal medium (MS) in 25±1 °C under 16:8 hr light and dark photoperiod with light intensity of 55µMol $m^{-2}s^{-1}$ using white (cool) fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). The transformation experiment efficacy was measured by conducting several experiments independently such as (1) inoculation of 36 *in vitro* developed shoots for 0, 10, 20, 30 and 40 minutes with *Agrobacterium* suspension to find out the optimum duration of inoculation period; (2) 33 explants were co-cultivated in dark with *Agrobacterium* for 0, 1, 2, 3 and 4 days to determine the ideal duration for co-culture.

Regeneration and histochemical GUS assay of putatively transformed plants

GUS activity was visualized using the histochemical assay (18). Transient expression was examined after roots arise in the inoculated shoots of *M. acuminata* cv. Vaibalhla (AAA). The roots were cut off and immersed in GUS substrate solution for a period of 24 hrs at 37 °C. After incubation, roots were observed under stereomicroscope. The efficiency was determined by taking GUS positive roots (in %) for measuring the optimal condition of transformation. Transient expression of GUS was scored on a per explant basis by counting blue foci visibility on the axillary region of each root. The blue foci determined the distinct areas of cells with GUS activity.

For the assay of transformation, the standardized transformation conditions were followed where 3 separate experiments were done with 15, 20 and 25 explants. The co -cultured explants were inoculated on a semi solid basal medium (MS) containing 150 mg/l kanamycin and 400 mg/l cefotaxime. Maintenance of the explants was achieved by transferring them with an interval of 14 days onto a new medium containing the same amount of antibiotics until roots developed and attained the length of 5-7 cm. Analysis of histochemical GUS activity was done by cutting 1-2 cm basal sections of the regenerated roots. Putatively transformed plants obtained were selected according to the frequency of numbers and percentages of roots showing GUS activity (GUS^+).

Molecular analysis of the putative transformants

Screening of the putative transformants was performed by running PCR. The total genomic DNA was obtained by extraction from fresh roots of putatively transformed and control (non-transformed) plants of *M. acuminata* cv. Vaibalhla (AAA) using DNA isolation kit (Himedia, HiPurA™ Plant Genomic DNA Miniprep Purification Kit) and the presence of *npt*II gene was screened by PCR. The 540 bp region of *npt*II was amplified using 20 mers (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and 1.6 kb region of the *NHX*1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG) gene specific oligonucleotide primers. The amplification reaction was carried out under the following conditions: 95°C for 3 min (1cycle), 95°C for 1 minute (denaturation), 58°C for 0.30 min (annealing), 72°C for 1 min (extension) for 35 cycles followed by the final extension (10 min) at 72°C for (1 cycle). PCR was accomplished in a 20 µl reaction mixture volume containing ~100 ng

of purified genomic DNA, 100 µM dNTP mix (Himedia, India), 0.4 µM of gene specific primer *npt*II, 1× Taq DNA polymerase buffer containing 15 mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd., Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd., Bangalore). To ensure that the reagents were not contaminated, DNA from control (non-transformed) plants were extracted and included in the PCR. The amplified products were resolved by agarose gel electrophoresis (1%) and visualized by ethidium bromide staining. The resulting fragments were scored as mentioned earlier. To check the reproducibility, all the PCR reactions were repeated at least twice.

Data Analysis

Statistical analysis of the resulting data was analysed with Duncan's new multiple range test ($P<0.05$) following ANOVA using SPSS statistical software package version 16.0.

Results

Confirmation of presence of nptII and VRNHX1 gene in the A. rhizogenes strain A4

For confirmation of the *npt*II and Vr*NHX*1 genes in the T-DNA of pCAMBIA plasmid, a colony PCR was performed with the *npt*II (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and *NHX*1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG') gene specific primers. The plant binary construct pCAMBIA2301:VrNHX1 was successfully mobilized to the A4 strain of *A. rhizogenes* by modified Freeze-thaw method and for the confirmation of mobilization, a colony PCR was made use. Amplification in 540 bp and 1.6 kb fragments from A4pCAMBIA2301VrNHX1 clones guaranteed that *npt*II and *NHX*1 genes were present respectively (Fig. 2). The schematic construction of Vr*NHX*1 mobilized with *A. rhizogenes* harboring a binary vector pCAMBIA2301VrNHX1 containing *npt*II (neomycin phosphotransferase) as selectable marker and the β-glucuronidase (GUS) gene (*uid*A) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter.

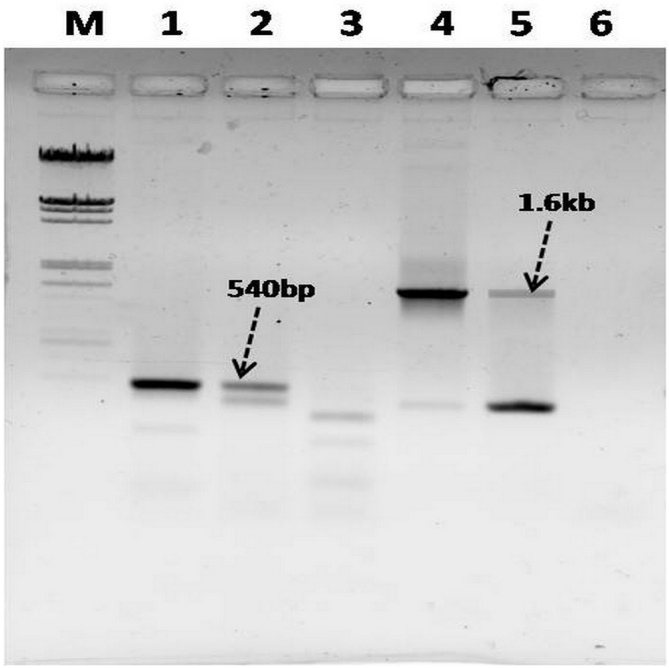

Fig. 2. Confirmation of the presence of *npt*II and VrNHX1 gene in the T-DNA of the plasmid construct 35SpCAMBIA2301VrNHX1. M-Marker, 1- *npt*II positive control, 2- Sample, 3- *npt*II negative control, 4- Vr*NHX*1 positive control, 5- Sample, 6- Vr*NHX*1 negative control.

Effect of kanamycin

Kanamycin sensitivity assay on the 5 months old *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) on MS + BAP (2 mg/l) + NAA (0.5 m/l) + ascorbic acid (75 mg/l) after 5 weeks of culture were carried out using different concentrations of kanamycin (50, 100, 150, 150 and 200 mg/l) with absence of antibiotic as the control. 200 mg/l of kanamycin concentration caused complete necrosis. It caused inhibition of growth and hindered the explant's capability to regenerate and simultaneously led to death by 6 weeks of culture (Table 1). In contrast, the explants in the kanamycin free media as well as 50 mg/l kanamycin concentration produced 100% root induction with 7.46 and 7.00 roots per explant after 5 weeks of culture respectively, while 100 mg/l and 150 mg/l concentration of kanamycin resulted in 53.33 and 33.33 roots (average) per explant. This result demonstrated that 150 mg/l kanamycin is an effective selection marker for root development from *in vitro* shoots of the selected banana variety as the sub-lethal concentration.

Table 1. Kanamycin assay on 5 months old *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) on MS + BAP (2 mg/l) + NAA (0.5 m/l) + ascorbic acid (75 mg/l) after 5 weeks of culture

Concentration (mg/L)	No. of shoots inoculated	Survival (%) (Mean ± SE)	No. of roots/shoot (Mean ± SE)*
0.0	15	100±0.00a	7.46±0.17a
50.0	15	100±0.00a	7.00±0.11a
100.0	15	53.33±3.84b	3.66±0.40b
150.0	15	33.33±4.22c	0.53±0.17c
200.0	15	0.00±0.00d	0.00±0.00d

*Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Effect of cefotaxime

The effect of cefatoxime in the induction of roots was checked by culturing *in vitro* shoot explants of *M. acuminata* cv. Vaibalhla (AAA) on Murashige and Skoog medium with phytohormones BAP (2 mg/l), NAA (0.5 mg/l) along with ascorbic acid (75 mg/l) containing discrete amount of cefotaxime (100, 200, 300, 400 and 500 mg/l) (Table 2). Of the several concentrations analysed, cefotaxime 400 mg/l had lesser damaging effect on root formation from *in vitro* shoots while it was effective in controlling the increase of *Agrobacterium* growth resulting in an average survival % of 93.33.

Optimization of inoculation period and co-culture duration

The period of explant inoculation efficiency varied from placing the drops of bacterial suspension to different duration of incubation period (within 10 to 30 min). In this study, the longer the inoculation time, the lesser is the % of GUS^+ plants. Thus, the inoculation time of 30 min is the

optimum for getting good % of GUS$^+$ roots (Table 3). When the co-culture period of the explants with *A. rhizogenes* harboring a binary vector pCAMBIA2301VrNHX1 was extended to 2 days, there was an increase in the putative transformation frequencies. When the co-cultivation time was increased further, it minimized the incidence of transformation resulting in overgrowth of the bacteria (Table 4).

Regeneration and confirmation of putatively transformed roots

Using the standardized conditions of co-culture, *in vitro* shoots of *M. acuminata* cv. Vaibalhla (AAA) which were regenerated previously from the floral parts of the male bud were used as the starting material. They were inoculated on MS medium with no addition of phytohormones containing ascorbic acid (75 mg/l), kanamycin (150 mg/l) and 400 mg/l cefotaxime for root regeneration. New roots started to appear after 2 days. The resistant roots formed were having lateral roots of uniform length (Fig. 3). Within 5 weeks of culture, an average of 11.33 kanamycin resistant roots were attained from the 3 independent experiments (Table 5). The *in vitro* explants were maintained by transferring them to new medium having the same amount of antibiotics

Table 2. Antibiotic (Cefotaxime) assay on 5 months old *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) inoculated with *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 on MS + BAP (2 mg/l) + NAA (0.5 mg/l) + ascorbic acid (75 mg/l)

Concentration (mg/L)	No. of shoots inoculated	Time (days) taken to show bacterial overgrowth	No. of explants showing bacterial overgrowth (Mean ± SE)	Survival (%) after 30 days (Mean ± SE)*
0.0	15	1	5.00±0.00a	0.00±0.00a
100.0	15	1	5.00±0.00a	0.00±0.00a
200.0	15	3-5	4.33±0.33a	13.33±8.85a
300.0	15	3-5	2.33±0.33b	53.33±3.84b
400.0	15	7-10	0.33±0.33ce	93.33±8.85ce
500.0	15	7-10	0.33±0.33de	93.33±8.85de

*Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 3. Effect of inoculation time on the efficiency of *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 transformation of *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA).

Inoculation period (min)	No. of explants	Putative transformation (%)*
0	36	0.00±0.00a
10	36	19.44±1.97b
20	36	30.55±1.75cg
30	36	44.44±1.60dfg
40	36	36.10±1.64efg

* Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 4. Effect of co-cultivation duration on the efficiency of *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 transformation of *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA)

Co-cultivation duration (days)	No. of explants	Putative transformation (%)*
0	33	0.00±0.00a
1	33	12.12±2.56bfg
2	33	39.39±1.76c
3	33	15.15±2.56dfg
4	33	18.18±4.03eg

* Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 usin g Duncan's new multiple range test.

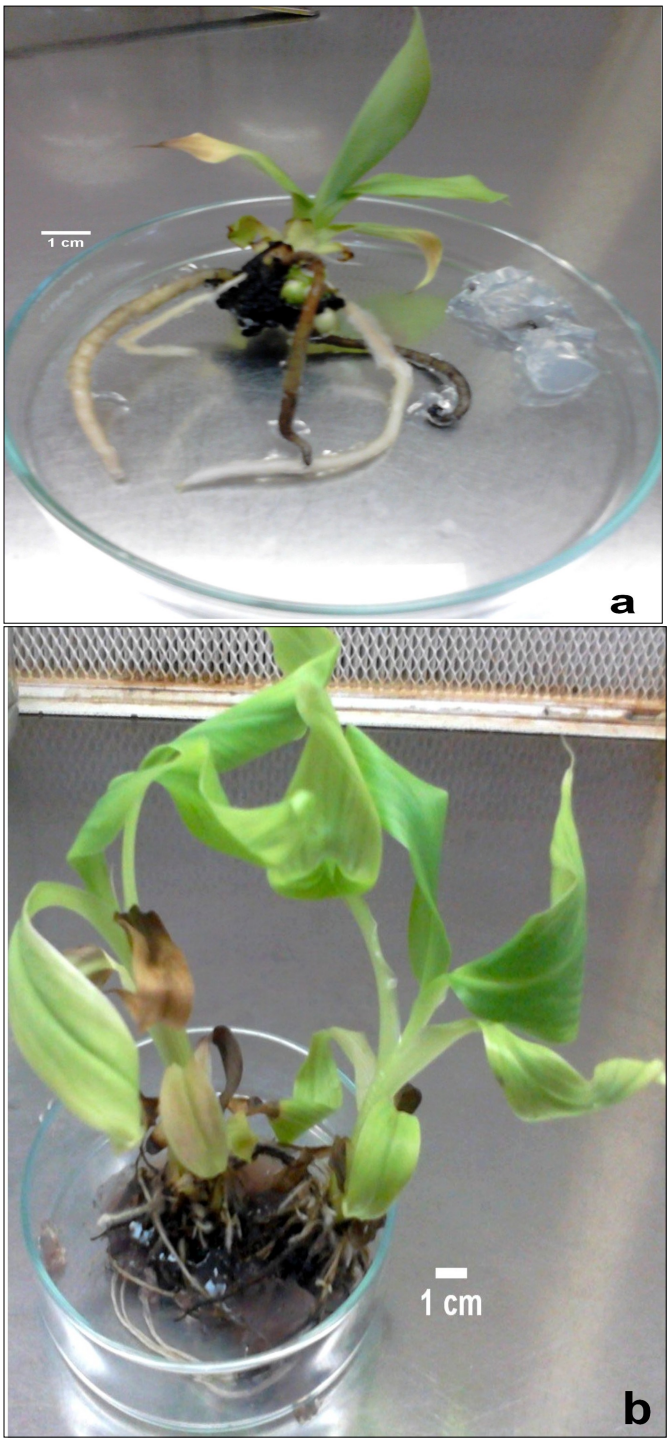

Fig. 3. Hairy roots of *M. acuminata* cv. Vaibalhla (AAA): **a** – Control plants showing normal roots after 10 days of culture; **b** –Treated plants cultured with *Agrobacterium rhizogenes* strain A4 harbouring binary vector pCAMBIA2301VrNHX1 plasmid on MS basal medium + ascorbic acid (75 mg/l) + kanamycin (150 mg/l) + cefotaxime (400 mg/l) showing hairy roots after 10 days of culture.

with 2 weeks interval until roots attained the length of 5-7 cm. Presence of a blue colour at the root tips of the regenerated roots indicated histochemical GUS activity. Putatively transformed root showed pale blue colour GUS expression in a 30 min inoculated explant (Fig. 4). This blue coloration was also observed in roots which were co-cultivated for 2 days (Fig. 5). When both of 30 min inoculation and 2 days co-cultivation were combined, there was an intense blue GUS expression (Fig. 6). From the 3 different independent transformation experiments, a total of 17 GUS$^+$ roots were obtained overall. The PCR performed on the regenerated kanamycin resistant GUS$^+$ roots showed amplification of the 540 bp fragments of the *npt*II gene indicating the presence of transgenes. There was no amplification in the non-transformed control roots (Fig. 7). However, not all the 34 regenerated roots showed fragments. The putatively transformation rate revealed by the percentage of PCR$^+$ roots of the explants varied from 12.00 to 20.00%.

Table 5. Summary of transformation of *in vitro* regenerated shoot explants derived from immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) for hairy root formation co-cultivated with *Agrobacterium rhizogenes* strain A4 harboring binary vector pCAMBIA2301VrNHX1 plasmid on MS basal medium + ascorbic acid (75 mg/l) + kanamycin (150 mg/l) + cefotaxime (400 mg/l)

Experiment	No. of explants inoculated	No. of explants showing resistant roots	Gus$^+$analysed roots (%)	PCR$^+$(%)
1	15	9	7/9 (77.77)	3/15 (20.00)
2	20	12	4/12 (33.33)	2/20 (10.00)
3	25	13	6/13 (46.15)	3/25 (12.00)
Total	60	34	17	8

Fig. 4. Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) in 30 min inoculated experiment: Putatively transformed root showing GUS expression.

Fig. 5. Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated for 2 day: **a**– Putatively transformed root showing GUS expression; **b** – Control.

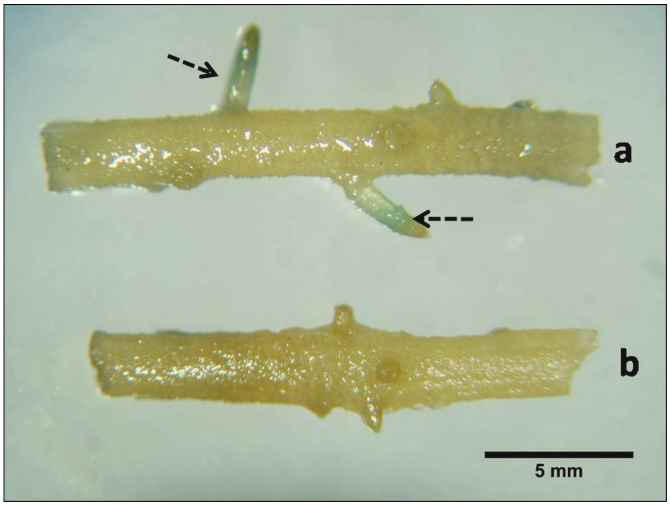

Fig. 6. Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) inoculated for 30 minutes and co-cultivated for 2 days: **a** – Putatively transformed root showing GUS expression; **b** – Control.

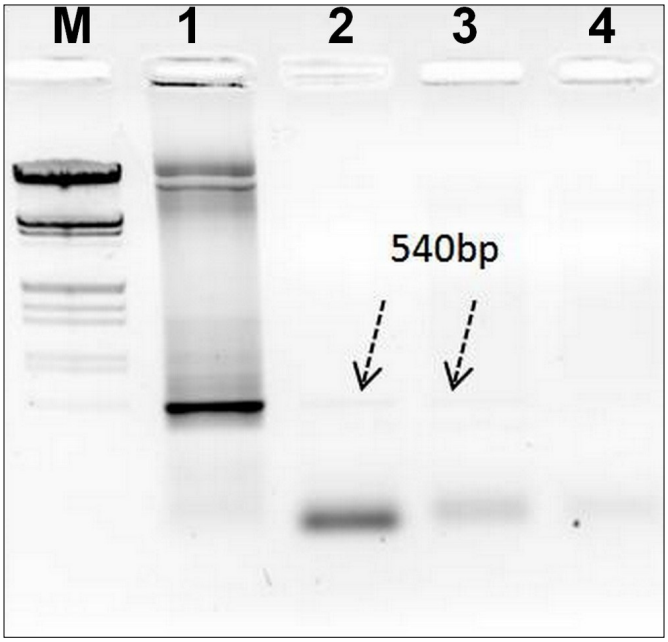

Fig. 7. Molecular analysis of putatively transformed roots of *M. acuminata* cv. Vaibalhla (AAA): PCR amplification of the 540 bp fragment of the *npt*II gene. M-marker, lane 1- *npt*II positive control, lane 2,3- putatively transformed roots, lane 4 - *npt*II negative control.

Discussion

In the present study, *A. rhizogenes*-mediated genetic transformation of *M. acuminata* cv. Vaibalhla (AAA) was carried out using *A. rhizogenes* strain A4 harboring the binary vector pCAMBIA2301with VrNHX1gene. The subsequent shoot induction and plantlet regeneration were carried out in MS medium supplemented with Kinetin (2 mg/l) and NAA (0.5 mg/l) following the earlier report (14). The 5 month-old *in vitro* raised plantlets were inoculated with *A. rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 for 30 min followed by 2 days of co-cultivation in the dark in a semi solid MS basal medium. The treated plants were then transferred and maintained in MS basal medium supplemented with ascorbic acid (75 mg/l), kanamycin (150 mg/l) and cefotaxime (400 mg/l). Successful *A. tumefaciens*-mediated genetic transformation of *Parkia timoriana* has also been demonstrated with the use of 500 mg/L cefotaxime as the sub-lethal concentration to check the bacterial growth (19). The putatively transformed roots were confirmed by positive GUS expression and PCR amplification by nptII and NHX1 gene specific primers. Stable integration of the genes needs to be confirmed by further gene copies and expression analysis. The usage of organized cultures for transformation is appealing because of its relatively short regeneration time. Reports are on the production of transgenic Cavendish cv. *Grand Naine* by co-cultivating wounded meristems with *A. tumefaciens* (20). However, some workers consider this technique to be of limited value because in the selection medium there was possible generation of plants with genetic chimera (21) probably occurring as `escapes'. Nevertheless, in this investigation, the repeated culture of the putative transformants caused the survival of only complete transformants and no escapes were encountered indicating the viability of organized structures such as shoot buds and reap the benefit of faster results. In previous reports of *A. rhizogenes*-mediated genetic transformation of Silk banana (AAB), kanamycin with 200 mg/l concentration controlled the *Agrobacterium* overgrowth (2) whereas in this investigation, cefotaxime of 400 mg/l served the purpose indicating that this cultivar requires less cefotaxime concentration to control the overgrowth. Similarly, *Agrobacterium tumefaciens*-mediated transformation using 100 mg/l kanamycin and 300 mg/l cefotaxime *M. acuminata* cv. Vaibalhla (AAA) has also been reported (22).

Conclusion

This is also the first report on the *A. rhizogenes*-mediated transient genetic transformation of *M. acuminata* cv. Vaibalhla (AAA) using the binary vector pCAMBIA2301with VrNHX1gene by inducing hairy roots resulting in formation of composite banana plants. The genetic transformation protocol described here will facilitate further genetic characterization and functional studies in this banana cultivar for advancement of research programs for genetic improvement.

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Authors contributions

Both the authors contribute equally in the design and conduct of the experiments, data analysis and preparation of the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

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References

1. Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LMA et al. Gene transfer to plants by diverse species of bacteria. Nature. 2005; 433: 629-33. https://doi.org/10.1038/nature03309
2. Venkatachalam L, Lokesh V, Bhagyalakshmi N. A rare event of *Agrobacterium rhizogenes*-assisted genetic transformation of 'Silk' banana (genotype-AAB). Journal of Microbial and Biochemical Technology. 2011; 3:1. https://doi.org/10.4172/1948-5948.1000043
3. Bosselut N, Ghelder CV, Claverie M, Voisin R, Onesto JP, Rosso MN, Esmenjaud D. *Agrobacterium rhizogenes*-mediated transformation of Prunus as an alternative for gene functional analysis in hairy-roots and composite plants. Plant Cell Reports. 2011; 30: 1313-26. https://doi.org/10.1007/s00299-011-1043-9
4. Chilton MD, Tepfer DA, Petit A, David C, Cassedelpart F, Tempe J. *Agrobacterium rhizogenes* inserts T DNA into the genomes of the host plants root cells. Nature. 1982; 295:432-34. https://doi.org/10.1038/295432a0
5. Moore L, Warren G, Strobel G. Involvement of a plasmid in hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid. 1979; 2: 617-26. https://doi.org/10.1016/0147-619X(79)90059-3
6. Kumar V, Satyanarayana KV, Sarala Itty S, Indu EP, Giridhar P et al. Stable transformation and direct regeneration of *Coffea canephora* P ex. Fr. by *Agrobacterium rhizogenes* mediated transformation without hairy-root phenotype. Plant Cell Reports. 2005;25:214-22. https://doi.org/10.1007/s00299-005-0045-x
7. Henzi MX, Christey MC, McNeil DL, Davis KM. *Agrobacterium rhizogenes* mediated transformation of broccoli (*Brassica oleracea* L. var. *Italica*) with an antisense 1- aminocyclopropane -1-carboxylic acid oxidase gene. Plant Science. 1999; 143:55-62. https://doi.org/10.1016/S0168-9452(99)00024-2
8. Yazaki K, Tanaka S, Matsuoka H, Sato F. Stable transformation of Lithospermumerythrorhizon by *Agrobacterium rhizogenes* and shikonin production of the transformants. Plant Cell Reports. 1998; 18: 214-19. https://doi.org/10.1007/s002990050559
9. Christey MC, Sinclair BK, Braun RH, Wyke L. Regeneration of transgenic vegetable brassicas (*Brassica oleracea* and *B. campestris*) via Ri mediated transformation. Plant Cell Reports. 1997;16:587-93. https://doi.org/10.1007/BF01275497
10. Liu S, Su L, Liu S, Zeng X, Zheng D, Hong L, Li L. *Agrobacterium rhizogenes*-mediated transformation of *Arachis hypogaea*: an efficient tool for functional study of genes. Biotechnology and Biotechnological Equipment. 30:869-78. https://

doi.org/10.1080/13102818.2016.1191972

11. Khlifa HD, Klimek-Chodacka M, Baranski R, Combik M, Taha HS. *Agrobacterium rhizogenes*-mediated transformation of *Hypericum sinaicum* L. for the development of hairy roots containing hypericin. Brazilian Journal of Pharmaceutical Sciences. 2020; 56:e18327. http://dx.doi.org/10.1590/s2175-97902020000118327
12. Ishida JK, Yoshida S, Ito M, Namba S, Shirasu K. *Agrobacterium rhizogenes*-Mediated Transformation of the Parasitic Plant *Phtheirospermum japonicum*. PLoS ONE. 2011;6:e25802. https://doi.org/10.1371/journal.pone.0025802
13. Leppyanen IV, Kirienko AN, Dolgikh EA. *Agrobacterium rhizogenes*-mediated transformation of *Pisum sativum* L. roots as a tool for studying the mycorrhizal and root nodule symbioses. PeerJ. 2019; 7: e6552. http://doi.org/10.7717/peerj.6552
14. Cheng Y, Wang X, Cao L, Ji J, Liu T, Duan K. Highly efficient *Agrobacterium rhizogenes*mediated hairy root transformation for gene functional and gene editing analysis in soybean. Plant Methods, 2021; 17:73. https://doi.org/10.1186/s13007-021-00778-7
15. Hrahsel L, Basu A, Sahoo L, Thangjam R. *In vitro* propagation and assessment of the genetic fidelity of *Musa acuminata* (AAA) cv. Vaibalhla derived from immature male flowers. Applied Biochemistry and Biotechnology. 2014; 172: 1530-39. https://doi.org/10.1007/s12010-013-0637-9
16. Mishra S, Alavilli H, Lee B, Panda SK, Sahoo L. Cloning and Functional Characterization of a Vacuolar Na+/H+ Antiporter Gene from Mungbean(VrNHX1) and Its Ectopic Expression Enhanced Salt Tolerance in *Arabidopsis thaliana*. PLOS ONE; 2014; 9: e106678. https://doi.org/10.1371/journal.pone.0106678
17. Höfgen R, Willmitzer L. Storage of competent cells for *Agrobacterium* transformation. Nucleic Acid Research. 1988; 16 (20): 9877. https://doi.org/10.1093/nar/16.20.9877
18. Jefferson RA. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Molecular Biology. 1987;204:387-405. https://doi.org/10.1007/BF02667740
19. Thangjam R, Sahoo L. *In vitro* regeneration and *Agrobacterium* tumefaciens-mediated genetic transformation of *Parkia timoriana* (DC.) Merr.: a multipurpose tree legume. Acta Physiologiae Plantarum. 2012; 34: 1207-15. https://doi.org/10.1007/s11738-011-0917-3
20. May GD, Afza R, Mason HS, Wieko A, Novak, FJ, Arntzen CJ. Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. Nature Biotechnology. 1995; 13: 486-92. https://doi.org/10.1038/nbt0595-486
21. Becker BK, Dugdale B, Smith MK, Harding RM, Dale JL. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv. '*Grand Nain*' via micro projectile bombardment. Plant Cell Reports. 2000; 19: 229-34. https://doi.org/10.1007/s002990050004
22. Hrahsel L, Basu A, Sahoo L, Thangjam R. Genetic Transformation of *Musa acuminata* cv. Vaibalhla (AAA) using *Agrobacterium tumefaciens*. Science and Technology Journal. 2017;5:120-31. https://doi.org/10.22232/stj.2017.05.02.09

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