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Efficient regeneration and genetic transformation platform applicable to five *Musa* varieties

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ABSTRACT

Background: Banana (*Musa* spp.) is an important staple food, economic crop, and nutritional fruit worldwide. Conventional breeding has been seriously hampered by their long generation time, polyploidy, and sterility of most cultivated varieties. Establishment of an efficient regeneration and transformation system for banana is critical to its genetic improvement and functional genomics.
Results: In this study, a vigorous and repeatable transformation system for banana using direct organogenesis was developed. The greatest number of shoots per explant for all five *Musa* varieties was obtained using Murashige and Skoog medium supplemented with 8.9 μM benzylaminopurine and 9.1 μM thidiazuron. One immature male flower could regenerate 380–456, 310–372, 200–240, 130–156, and 100–130 well-developed shoots in only 240–270 d for Gongjiao, Red banana, Rose banana, Baxi, and Xinglongnaijiao, respectively. Longitudinal sections of buds were transformed through particle bombardment combined with *Agrobacterium*-mediated transformation using a promoterless β-glucuronidase (GUS) reporter gene; the highest transformation efficiency was 9.81% in regenerated Gongjiao plantlets in an optimized selection medium. Transgenic plants were confirmed by a histochemical assay of GUS, polymerase chain reaction, and Southern blot.
Conclusions: Our robust transformation platform successfully generated hundreds of transgenic plants. Such a platform will facilitate molecular breeding and functional genomics of banana.

1. Introduction

Bananas (*Musa* spp.) are one of the most important foods and commercial crops in tropical and subtropical developing countries. However, those tropical and subtropical environments that are ideal for growing bananas are also regions with high rates of plant disease, pest pressure, and abiotic stress, which heavily affect the yield and quality of crops. Therefore, the development of new banana varieties with greater disease-resistance and stress-tolerance is critical. Furthermore, conventional breeding is seriously hampered by the complex and polyploid nature of the *Musa* genome and its high degree of sterility [1,2,3]. Recent developments in transgenics have provided an effective means for banana breeding, and the integration of such tools, including high efficiency regeneration and genetic transformation, into banana improvement programs is imperative.

Most previous transgenic research on banana mainly involved embryogenic cell suspension systems [4,5,6,7,8,9,10,11,12,13]. However, the induction and regeneration of embryogenic cells is time-consuming and has a low frequency of spontaneous mutation and regeneration. Moreover, embryogenic cells are heavily restricted by genotype [14]. Thin cell layers originating from the shoot tip represent newly developed receptor material for the genetic transformation of banana. Compared with embryogenic cell suspension systems, thin cell layer explants have a high regeneration rate [15], making them ideal for genetic engineering of banana [16]. However, thin cell layer regeneration systems require the use of different media at different culture stages, which complicates the process.

Genetic transformation methods used by previous studies on banana included electric shock [17], *Agrobacterium*-mediated transformation [7,18,19,20,21,22], and particle bombardment [23,24,25], all of which have very low transformation efficiencies. Khanna et al. [8] reported a

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method to improve the efficiency of banana transformation by the inhibition of *Agrobacterium*-induced cell death using animal antiapoptotic gene expression. However, the potential toxicity of these genes in food is another problem, and further studies are ongoing to restrict the expression of these genes to an early culture phase. Moreover, few reports have combined multiple transgenic methods to study banana. In this present study, we report the development of a simple and efficient regeneration system that combines two transgenic methods and uses only one kind of medium suitable for use with five *Musa* varieties.

2. Materials and methods

2.1. Plant material

Five kinds of immature, floral apices from Baxi (*Musa* AAA group, cv. Brazilian), Gongjiao (*Musa acuminata* L. AA group, cv. Mas), Red banana (*Musa corniculata* L. AAA group), Rose banana (*M. acuminata* L. AA group), and Xinglongnaijiao (*Musa* AAB group) were obtained from the Institute of Tropical Bioscience and Biotechnology banana plantation (20N, 110E, Chengmai county, Hainan, China). Floral apices were peeled layer by layer until to 10 cm in length and then put into a clean bench to continue peeling until 3 cm in length before being cross-cut into 1–2-mm-thick slices (one flower could be cut into 10–12 slices).

2.2. Regeneration

The cross-cut banana floral apices slices were used as explants and cultured on Murashige and Skoog (MS) medium in Petri dishes (diameter, 9 cm) to induce shoot growth. MS media recipes supplemented with 8.9 μM benzylaminopurine (BA), and one plant growth regulator, 9.3 μM kinetin, 9.1 μM zeatin, 9.1 μM thidiazuron (TDZ), or 8.9 μM 1-naphthaleneacetic acid (NAA), were used. Explants were transferred to fresh media every 15 d. After 4 months of growth, the number and fresh weight of regenerated strong shoots from each recipe were calculated to determine the optimal growth recipe. When shoot lengths reached 3–5 cm, they were cut and transferred to MS medium without growth regulators to regenerate roots for 1 month. Subsequently, plantlets with roots of 5–8 cm in length were transplanted into coconut coir medium. All plant tissue culture media used in the present study included MS basal medium with 7 g/L agar (Solarbio, Japan) and 40 g/L sucrose adjusted to pH 5.8 before autoclaving at 120°C for 15 min. All cultures were incubated at 28 ± 1°C.

2.3. Genetic transformation

2.3.1. Plasmids and bacteria

Agrobacterium tumefaciens strain pCAS04/AGL1 (kindly provided by Professor Chengcai Chu, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) was used in the present study. The transferred DNA (T-DNA) region of the binary vector pCAS04 contained neomycin phosphotransferase II driven by a promoter from the maize ubiquitin gene, a promoterless β-glucuronidase (*gus*) gene at the right border of the T-DNA for gene trapping, and a strong rice actin promoter (GB accession: S44221) at the left border of the T-DNA for activation tagging. The bacterial strain containing the transformation vector was inoculated into liquid yeast extract broth medium [26] containing 0.1 mM spectinomycin and 60 μM rifampicin and incubated for 24 h at 28°C with reciprocal shaking (200 cycles/min).

2.3.2. Particle bombardment

The sturdy shoots induced from Gongjiao floral apices slices were longitudinally excised into 1–2-mm-thick slices. These slices were closely arranged in the center of a Petri dish with the optimal medium before being placed in a Biolistic PDS-1000/He™ gene gun, and a 28″ Hg vacuum was created. Shoots were bombarded thrice with 0.6-μm gold particles packaged with 5 μg of the pCAS04 plasmid under a 4-cm effective firing range and 1300 psi He pressure. The bombarded shoots were placed at 28 ± 1°C for 2 d in the dark to recover growth.

2.3.3. Agrobacterium-mediated transformation

Cultured AGL1 *Agrobacterium* cells with the pCAS04 plasmid were collected by centrifugation at 5000 × *g* for 10 min at room temperature and then suspended to a final OD_{600} of 0.3–0.5 in the inoculation medium. Bombarded shoots that recovered growth were immersed in the bacterial suspension with 250 μM acetosyringone for 30 min, cocultivated with *Agrobacterium* in the optimal medium with 250 μM acetosyringone at 28 ± 1°C in the dark for 2 d, and then transferred to fresh optimal medium without acetosyringone. After cocultivation, tissue segments were transferred onto recovery medium containing the same ingredients as inoculation media but supplemented with 0.6 mM carbenicillin (Amresco, USA) and up to 72 μM geneticin (G418) (Gibco, USA). Tissues were cultured at 28 ± 1°C in the dark and subcultured every 2 weeks with fresh recovery medium. After 1 month, tissue segments formed many small white shoots that grew into strong shoot clusters after the second month. Shoot clusters were transferred to new dishes and cultured for 2 months under a 16/8 h light/dark photoperiod using cool-white fluorescent lights [50 μmol/m^2·s]. Then, green shoots of 3–5 cm in length were excised from the shoot clusters and cultured separately to induce roots on MS media containing 36 μM G418. The plantlets were transferred to coconut coir after 1 month on the rooting medium. One month later, the putative transformed plantlets were obtained.

G418 was tested at different concentrations (0, 18, 36, 54, 72, and 90 μM) in both shoot formation and rooting medium to determine optimal concentrations for transgenic plantlet selection. There was little difference in shoot formation and growth at all G418 concentrations tested (data not shown), and 72 μM was selected for shoot formation. G418 concentrations had a great influence on rooting after shoots were transferred to selection medium, and 36 μM G418 was selected for the rooting medium.

2.4. GUS histochemical assay

Histochemical localization of stable *GUS* expression in the putatively transformed plantlets was investigated according to the protocol by Jefferson [27]. Plant materials were incubated in 100 mM sodium phosphate buffer (pH 7.0) containing 2 mM 5-bromo-4-chloro-3-indoyl glucuronide (Bebco, USA) at 37°C for 48 h.

2.5. Polymerase chain reaction and southern blot analysis

Total genomic DNA was isolated from leaves of control (untransformed) and putatively transformed plantlets using a DNeasy Plant Mini kit (Qiagen, USA). The polymerase chain reaction (PCR) amplification mixture contained 100 ng of each sample DNA as the template, 400 μM dNTPs, 2.5 mM $MgCl_2$ PCR buffer, 0.05 U/μL Taq (Shenergy Biocolor Bioscience and Technology, Shanghai, China), 0.2 μM primer P1 (5′-TCAGCGCGAAGTCTTTATAC-3′), and 0.2 μM primer P2 (5′-TTCAGTTCGTTGTTCACACA-3′) to amplify the fragments of inserted *GUS* DNA. Samples underwent 35 PCR cycles using a programmed temperature control system (Biometra, Germany) under the following conditions: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C. Amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel. Transformation efficiency was calculated as the number of PCR-positive transgenic lines regenerated on a G418-selective medium for each cultivar.

Genomic DNA prepared as described above was digested by the HindIII restriction enzyme under the conditions suggested by the enzyme's manufacturer. The DNA fragments were separated on a 1.2%

agarose gel and then blotted onto a nylon membrane (Amersham, USA). Ultraviolet crosslinking was performed using a CL-1000 ultraviolet crosslinker at 8000 μJ/cm^2 for 4 min. A hybridization probe was prepared using PCR-amplified DNA from the pCAS04 construct as a template and a Digoxigenin labeling kit (Cat. 1093657, Roche) according to the manufacturer's instructions. Hybridization was performed at 42°C for 20 h, and the membrane was washed twice in 2× saline sodium citrate/0.5% sodium dodecyl sulfate solution at room temperature for 5 min, twice in 2× saline sodium citrate/0.1% sodium dodecyl sulfate solution at 68°C for 15 min each, and then once in 2× maleic acid/0.6% Tween-20 for 5 min. The membrane was then soaked in detection buffer with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt before being exposed to a film for 4 h.

2.6. Statistical analysis

All experiments were repeated thrice, and data are presented as means ± standard error. One-way analysis of variance was performed, and interactions between means were separated by the least significant difference at $P = 0.05$.

3. Results

3.1. Efficient regeneration of five Musa varieties

With 8.9 μM BA in MS media as the base, four plant growth regulator media recipes supplemented with kinetin, zeatin, TDZ, or NAA were evaluated separately to determine which recipe most effectively optimized plant growth. After 6 months, shoots were induced from the wounded edges of explants of floral apices from five different *Musa* varieties, and then strong shoots were cross-cut into slices. Three to four months later, shoots were induced again from the wounded edges of slices. Then, the number of shoots in each explant and fresh weight of each shoot were determined (Table 1; B1–B3, G1–G3, H1–H3, R1–R3, and X1–X3 in Fig. 1). The greatest number of shoots per explant for all five *Musa* varieties was obtained using MS medium supplemented with BA and TDZ, whereas the fresh weight of each shoot showed little difference between recipes (Table 1). Furthermore, Gongjiao explants regenerated the most number of shoots, with BA and TDZ medium, followed by Red banana, Rose banana, Baxi, and Xinglongnaijiao (Table 1; Fig. 1).

3.2. High efficiency genetic transformation

Longitudinal sections of banana buds were used as explants because they have the highest regeneration efficiency. After bombardment by gene gun, explants (M in Fig. 1) were immersed in bacterial suspensions for 30 min and then cocultivated with *Agrobacterium* on BA + TDZ growth medium for 2 d. The explants were sampled for histochemical analysis after being grown for 15 d (Fig. 2B, E), 2 months (Fig. 2C, F), and 4 months (Fig. 2D, G). In Gongjiao samples, *GUS* expression was detected in almost 10 out of every 100 explants (10%). Because the pCAS04 construct used in transformation experiments contained a promoterless *GUS*, *GUS* expression in the putative transformants indicated genomic insertion of the construct downstream from a functional promoter. Therefore, *GUS* staining could be used to identify transgenic plants (Fig. 2A). As shown in Fig. 2B–D, strong *GUS* expression was observed at different shoot developmental stages, indicating that the regenerated shoots were transgenic.

The putative transgenic plantlets shown in Fig. 3A were further analyzed at the DNA level by PCR. As shown in Fig. 3C and Table 2, 38 of 391 putative transgenic Gongjiao plantlets were found to be PCR-positive for *GUS* expression, with a transformation efficiency of 9.81% using our combined transformation method, which was 1.6- and 3.3-fold higher than that of gene gun and *Agrobacterium* methods, respectively. Seven of 158 putative transgenic Baxi plantlets were PCR-positive and had 4.61% transformation efficiency with the combined method, which was 2.4- and 4-fold higher than that of gene gun and *Agrobacterium* methods, respectively. For Red banana, 23 of 317 putative transgenic plantlets were PCR-positive and had a transformation efficiency of 7.28% using our combined method, which was 3.4- and 3.7-fold higher than that of gene gun and *Agrobacterium* methods, respectively. Nine of 199 putative transgenic Rose banana plantlets were PCR-positive, with a 4.33% transformation efficiency using the combined transformation method, which was 2.6- and 2.3-fold higher than that of gene gun and *Agrobacterium* methods, respectively. Finally, six of 90 putative Xinglongnaijiao transgenic plantlets were PCR-positive, and the transformation efficiency was 7% using our combined transformation, which was 2.9- and 3.9-fold higher than that of gene gun and *Agrobacterium* methods, respectively (Table 2).

PCR and *GUS* assay results indicated a relatively stable transformation efficiency. To test if any of the putative transformants had the transgene integrated into the host genome, we subjected seven randomly selected PCR-positive plants to Southern blot (Fig. 3B). Although the nontransgenic plants showed negative results, six of the seven putative transgenic plants showed that the transgene was present in the banana genome (Fig. 3D). The transgene was most likely inserted in a single genomic location in plants 1, 3, 4, and 5 and in three and four locations in plants 6 and 7, respectively.

4. Discussion

Genetic transformation of banana has been an important tool for molecular breeding and gene function identification in recent years [10,28]. However, the dependency of regeneration and genetic transformation on banana plant genotype has made it difficult to repeat published results. Here, we report the development of an efficient banana regeneration and genetic transformation platform that has four major advantages over previously reported techniques.

First, our regeneration platform is very simple, only using one medium (MS with 8.9 μM BA + 9.1 μM TDZ) for the induction and differentiation of adventitious shoots. High-throughput regeneration and transformation using embryogenic cell suspension as reported by Tripathi et al. [29] required six different media to derive well-developed shoots from the immature floral apices of banana. Moreover, Hrahsel

Table 1
Regeneration analysis of five *Musa* varieties using four types of media.

M	Baxi		Gongjiao		Red banana		Rose banana		Xinglongnaijiao	
	A	B(g)	A	B(g)	A	B(g)	A	B(g)	A	B(g)
I	4.00 ± 1.30^{c}	0.50 ± 0.03^{a}	16.00 ± 0.80^{c}	0.82 ± 0.03^{a}	12.00 ± 1.20^{c}	0.70 ± 0.03^{ab}	12.00 ± 1.20^{b}	0.22 ± 0.03^{b}	6.00 ± 1.20^{b}	0.59 ± 0.05^{bc}
II	9.00 ± 0.80^{b}	0.53 ± 0.10^{a}	22.00 ± 2.40^{b}	0.79 ± 0.06^{a}	23.00 ± 0.80^{b}	0.70 ± 0.01^{ab}	15.00 ± 2.10^{b}	0.27 ± 0.01^{a}	7.00 ± 0.80^{ab}	0.67 ± 0.03^{ab}
III	13.00 ± 0.80^{a}	0.43 ± 0.08^{a}	38.00 ± 2.10^{a}	0.74 ± 0.07^{ab}	31.00 ± 2.60^{a}	0.73 ± 0.10^{a}	20.00 ± 2.40^{a}	0.28 ± 0.01^{a}	10.00 ± 1.20^{a}	0.74 ± 0.09^{a}
IV	2.00 ± 0.80^{d}	0.35 ± 0.03^{a}	4.00 ± 0.80^{d}	0.63 ± 0.03^{b}	7.00 ± 1.70^{d}	0.54 ± 0.02^{b}	4.00 ± 1.20^{c}	0.14 ± 0.01^{c}	2.00 ± 0.80^{c}	0.46 ± 0.05^{c}

M, MS media supplemented with 8.9 μM BA and (i) 9.3 μM kinetin, (ii) 9.1 μM zeatin, (iii) 9.1 μM thidiazuron, or (iv) 8.9 μM NAA. A, mean number of shoots/explant; B, mean fresh weight of each shoot. Data are presented as means ± standard error of n = 3 biological replicates. Means denoted by the same letter do not significantly differ at $P < 0.05$ as determined by the least significant difference multiple range test.

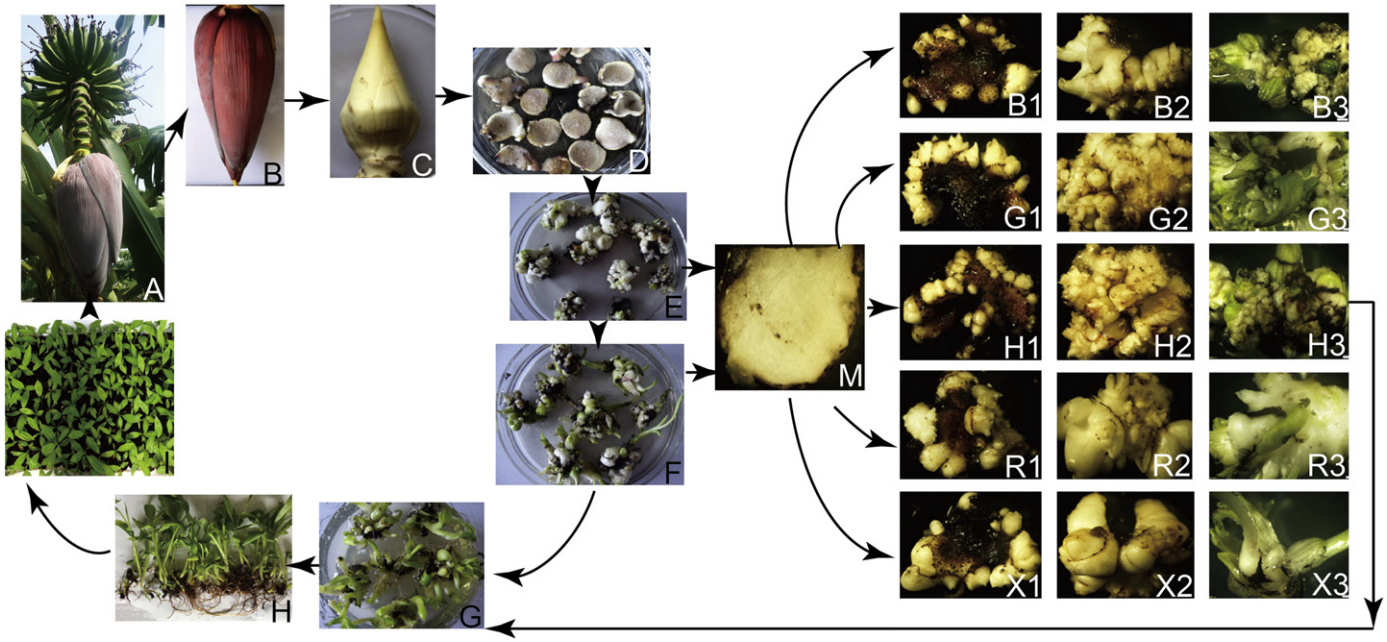

Fig. 1. Regeneration of five *Musa* genotypes. A, B: floral apex of banana; C: 3-cm long floral apex of banana; D: 1–2-mm-thick tissue slices; E, F: shoots regenerated from slices; G: regenerated banana roots; H: transplantation; I: plantlets; M: slices cut from E and F; B1–B3: different stages of Baxi regeneration; G1–G3: different stages of Gongjiao regeneration; H1–H3: different stages of Red banana regeneration; R1–R3: different stages of Rose banana regeneration; X1–X3: different stages of Xinglongnaijiao regeneration.

et al. [30] reported the *in vitro* propagation of *M. acuminate* (AAA) cv. Vaibalhla from the immature floral apices of banana through direct shoot regeneration that needed two different media. To our knowledge, all previously reported banana regeneration and transformation studies have required the use of more than two kinds of media for different culture stages, making ours the first to use only one kind of medium with favorable results.

Second, our regeneration platform is repeatable. Most problems with banana regeneration and transformation stem from poor repeatability due to variety dependency. In general, only one method

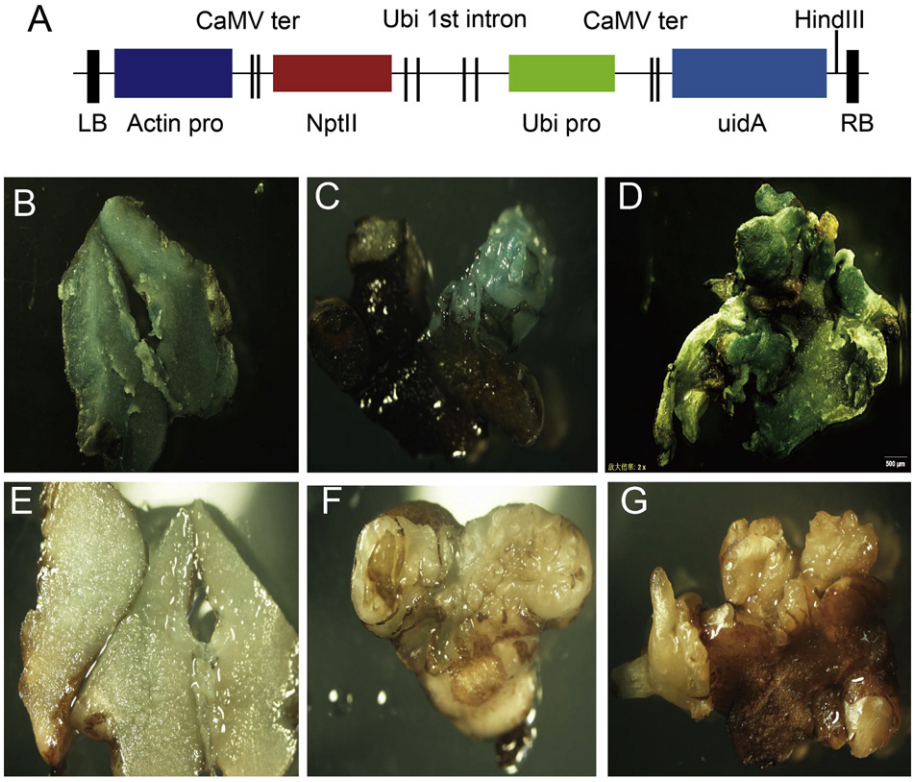

Fig. 2. *GUS* expression in transgenic Gongjiao banana tissues. A, Map of plasmid pCAS04 used to transform tissues. RB and LB represent the right and left borders of T-DNA. ubi pro, ubiquitin promoter; ubi 1st intron, the first intron of ubiquitin; nptII, neomycin phosphotransferase II-resistant gene; actin pro, promoter of act-GB; CaMVter, terminator of CaMV 35S; uidA, *GUS* without promoter; HindIII, restriction digestion site. Untransformed control (E, F, G) and transgenic plant tissues (B, C, D) were stained to detect *GUS* expression.

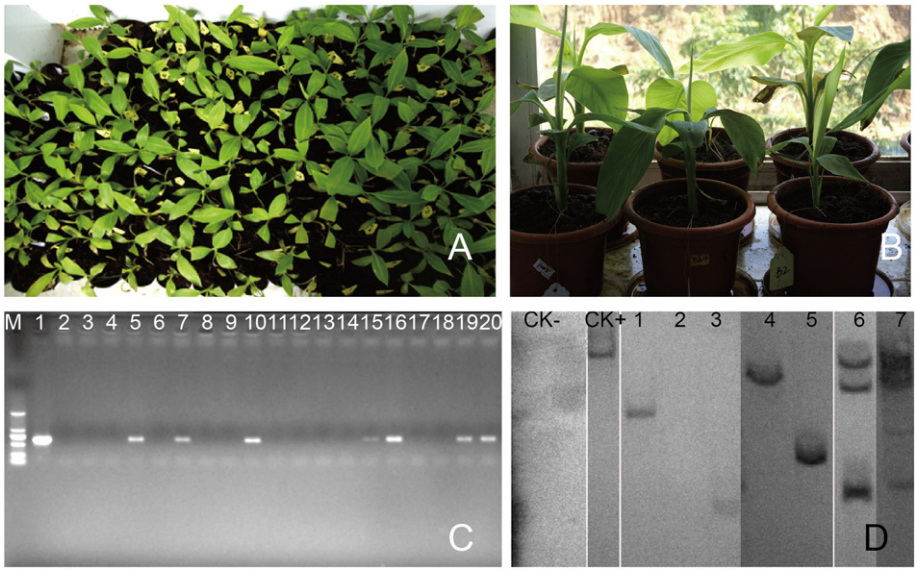

Fig. 3. Molecular assay of putative transgenic banana plants. A, Plantlets for PCR detection. B, Plantlets for Southern blot assay. C, PCR of plants derived using the direct shoot formation method. Plasmid pCAS04 DNA was used as a positive control (lane 1) and untransformed plants were used as a negative control (lane 2). D, Seven PCR-positive plants were randomly selected and hybridized with the *GUS* probe. Plants 1, 3, 4, and 5 indicated one insertion, whereas plants 6 and 7 indicated three and four possible insertions, respectively. CK^+, plasmid pCAS04 DNA; CK^-, untransformed control.

is usually suitable for use with one variety. For example, Santos et al. [3] established a method for the plantain-type banana (*Musa* sp.) cultivar Three Hand Planty (AAB genomic group, International Transit Center accession: ITC.0185). Paul et al. [28] established an effective method for 'Lady Finger' banana, Sreedharan et al. [10] for cv. Rasthali banana, and Wei et al. [6] for the edible banana *M. acuminata* cv. Mas (AA group). The most versatile method we found in the literature was reported by Tripathi et al. [29], which suited three banana cultivars, including Cavendish Williams, Gros Michel, and Sukali Ndizi. In contrast, we report a simple method that uses only one kind of growth medium (MS with 8.9 μM BA and 9.1 μM TDZ) for the regeneration of five kinds of immature floral apices that is repeatable and may overcome the problems associated with variety dependency.

The third major benefit to our method is that it substantially shortens the banana plant growth cycle. Another problem with banana regeneration is that it is time-consuming. Our method was able to produce well-developed plantlets from immature floral apex in only 240–270 d; the shortest growth cycle using other methods was 344–478 d [29]. Therefore, our method would serve to greatly improve regeneration efficiency.

Finally, our regeneration and transformation platform was highly efficient using one medium (*i.e.*, MS with 8.9 μM BA + 9.1 μM TDZ). One immature floral apex could regenerate 380–456, 310–372, 200–240, 130–156, and 100–130 well-developed shoots for Gongjiao, Red banana, Rose banana, Baxi, and Xinglongnaijiao, respectively, (Table 1), which met the transformation demand well. Commonly, the auxin/cytokinin proportion determines the fate of explant morphogenesis [31]. TDZ, with its cytokinin- and auxin-like effects, was used as a substitute to phenylurea (Nphenyl-N′-1,2,3-thidiazol-5-ylurea) and is presently among the most active cytokinin-like substances for plant shoot organogenesis. Our result was consistent with the previous reports about several plant tissue cultures [32,33,34]. The 9.81% transformation efficiency in our study was stable and higher than that reported by Tripathi et al. [0.1–0.14%] [29] and higher than that of gene gun or *Agrobacterium*-mediated transformation (Table 2). Furthermore, although bananas are monocotyledons and not ideal *Agrobacterium* hosts, particle bombardment makes small wounds in the tissue, which are good channels for *Agrobacterium* invasion.

In conclusion, we have developed an efficient regeneration system suitable for five *Musa* varieties through direct organogenesis using only

Table 2
Transformation efficiency of five *Musa* varieties using different transformation methods.

Transformation efficiency	Method	No. of accepted materials used	No. of putative transgenic plants	No. of PCR positive lines	Transformation efficiency (%)
Baxi	A	11.00 ± 0.82	137.67 ± 6.02	2.67 ± 0.47	1.93 ± 0.30^b
	B	47.67 ± 2.05	605.67 ± 37.19	7.00 ± 0.82	1.16 ± 0.15^c
	C	13.33 ± 2.36	158.33 ± 16.99	5.00 ± 0.82	4.61 ± 0.39^a
Gongjiao	A	13.33 ± 2.36	465.67 ± 63.88	28.00 ± 2.45	6.06 ± 0.41^b
	B	47.00 ± 2.16	1596.00 ± 37.16	47.67 ± 6.94	2.99 ± 0.47^c
	C	11.00 ± 0.82	391.00 ± 20.54	38.33 ± 2.05	9.81 ± 0.29^a
Red banana	A	11.00 ± 0.82	310.33 ± 6.55	6.67 ± 0.94	2.15 ± 0.27^b
	B	50.00 ± 0.82	1117.00 ± 70.37	21.67 ± 2.87	1.95 ± 0.27^b
	C	11.00 ± 0.82	317.00 ± 12.68	23.00 ± 1.63	7.28 ± 0.74^a
Rose banana	A	11.00 ± 0.82	181.67 ± 16.78	3.00 ± 0.82	1.66 ± 0.44^b
	B	48.33 ± 1.25	819.67 ± 50.51	15.67 ± 1.25	1.92 ± 0.20^b
	C	11.00 ± 0.82	199.67 ± 13.02	8.67 ± 1.25	4.33 ± 0.49^a
Xinglongnaijiao	A	11.00 ± 0.82	95.00 ± 6.16	2.33 ± 0.47	2.44 ± 0.36^b
	B	49.33 ± 2.49	435.00 ± 21.35	7.67 ± 1.25	1.76 ± 0.28^b
	C	11.00 ± 0.82	89.67 ± 11.32	6.33 ± 1.25	7.00 ± 0.49^a

A, gene gun; B, *Agrobacterium*-mediated; C, gene gun + *Agrobacterium*. Data are collected after two times of subcultures and 2 months after which the putative transgenic shoots were obtained. Data are presented as means ± standard error of n = 3 biological replicates. Means denoted by the same letter do not significantly differ at $P < 0.05$ as determined by the least significant difference multiple range test.

one medium and an efficient system for the production of transgenic banana plants using gene gun combined with *Agrobacterium*-mediated genetic transformation.

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Conflicts of interest

The authors declare no conflicts of interest.

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