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ORIGINAL ARTICLE

An easy and efficient protocol in the production of *pflp* transgenic banana against Fusarium wilt

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Abstract This study describes an efficient protocol for *Agrobacterium tumefaciens*-mediated transformation of two subgroups of genotype AAA bananas (*Musa acuminata* cv. Pei Chiao and *Musa acuminata* cv. Gros Michel). Instead of using suspension cells, cauliflower-like bud clumps, also known as multiple bud clumps (MBC), were induced from sucker buds on MS medium containing N^6-Benzylaminopurine (BA), Thidiazuron (TDZ), and Paclobutrazol (PP333). Bud slices were co-cultivated with *A. tumefaciens* C58C1 or EHA105 that carry a plasmid containing *Arabidopsis* root-type ferredoxin gene (*Atfd3*) and a plant ferredoxin-like protein (*pflp*) gene, respectively. These two strains showed differences in transformation efficiency. The EHA105 strain was more sensitive in Pei Chiao, 51.3% bud slices were *pflp*-transformed, and 12.6% slices were *Atfd3*-transformed. Gros Michel was susceptible to C58C1 and the transformation efficiency is 4.4% for *pflp* and 13.1% for *Atfd3*. Additionally, gene integration of the putative *pflp* was confirmed by Southern blot. Resulting from the pathogen inoculation assay, we found that the *pflp* transgenic banana exhibited resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4. This protocol is highly advantageous to banana cultivars that have difficulties in setting up suspension cultures for the purpose of quality improvement through genetic transformation. In addition, this protocol would save at least 6 months in obtaining explants for transformation and reduce labor for weekly subculture in embryogenic cell suspension culture systems.

Keywords *Agrobacterium* · Transformation · Banana · Cauliflower-like bud clumps · Plant ferredoxin-like protein

Abbreviations

ANOVA	One-way analysis of variance
AS	Acetosyringone
Atfd3	*Arabidopsis* root-type ferredoxin gene
BA	N^6-benzylaminopurine
BI	Bud induction medium
CaMV 35S	Cauliflower mosaic virus 35S promoter
CC	Co-cultivation medium
ECS	Embryogenic cell suspension
FOC	*Fusarium oxysporoum* f. sp. *cubense*
HR	Hypersensitive response
IAA	Indole-3-acetic acid
MBC	Multiple bud clumps
MS	Murashige and Skoog
NOS	Nopaline synthase promoter
nptII	Neomycin phosphotransferase gene
PCR	Polymerase chain reaction
PFLP	Plant ferredoxin-like protein
PP333	Paclobutrazol
TDZ	Thidiazuron
YEB	Yeast extract broth

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Introduction

Bananas (*Musa* spp.) are the fourth most important food crop in the tropical and sub-tropical zones of the world. An

annual production of 8.5 tons of bananas was reported in 2007 (FAO 2007). Disease and pests, such as the banana bunchy top virus (Harding et al. 1993), the banana Xanthomonas wilt (Tushemereirwe et al. 2003) and Fusarium wilt (Tripathi et al. 2005), seriously limit banana production. Long generation times, triploidy, and sterility of most edible cultivars hamper the development of disease-resistant bananas through conventional breeding (Sági et al. 1995a, c). Transgenic manipulation therefore offers a great alternative approach for improvement. Introduction of foreign genes into different cultivars of bananas has been successful by *Agrobacterium* (May et al. 1995; Ganapathi et al. 2001; Tripathi et al. 2005, 2008), particle bombardment (Sági et al. 1995a; Schenk et al. 1999; Becker et al. 2000), and electroporation (Sági et al. 1994, 1995b; De García and Villarroel 2007). Most transformation protocols for bananas utilize embryogenic cell suspensions (Novak et al. 1989; Arinaitwe et al. 2004; Ganapathi et al. 2001). However, establishing viable cell suspensions is highly time consuming and cultivar-dependent. Transgenic plants of bananas, obtained from *A. tumefaciens*-mediated transformation through meristem and corm slices of banana, have been previously reported (May et al. 1995). Thus, meristemic tissue could be a substitution for plants where suspension culture is difficult to establish in protocols leading to genetic engineering.

Fusarium wilt (also known as Panama disease), caused by fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (FOC), is one of the destructive diseases in banana. In the tropics and subtropics, Cavendish banana varieties are highly susceptible to Foc race 4, known as 'tropical race 4' (TR4) in Asia (Berg et al. 2007; Molina et al. 2009). Fusarium wilt of banana can be described as a "classic" vascular wilt disease. It invades the vascular tissue (xylem) through the roots causing the external symptoms of yellowing of infected old leaves and then spreading to younger leaves. Inside the infected plants, typical discoloration was found in vascular tissues, including roots, corm, and pseudostem from pale to dark red. The destruction of conducting elements eventually causes death of infected plants (Ploetz 2006). The Fusarium wilt disease collapsed the banana production in Taiwan, Indonesia, Malaysia and Philippines (Hwang and Ko 2004; Berg et al. 2007) and nowadays endangers the banana cultivation in South China and South Africa (Viljoen 2002; Molina et al. 2009). Management approaches for *Fusarium* control have been developed, including implementation of quarantine policies to prevent disease introduced from other countries, and use of disease-free tissue culture planting materials (Molina et al. 2009). Recently, production of Fusarium wilt-resistant banana through selection of genetic variability from tissue culture has been developed in Taiwan (Hwang 2002). Despite the certain success in the use of somaclonal resistant varieties, Fusarium wilt is still a main choke point on banana production.

Previously, we isolated a ferredoxin-like protein (PFLP, formerly called AP1) from sweet pepper (Lin et al. 1997). This peptide has been reported to enhance resistance against bacterial pathogens such as *Xanthomonas oryzae* pv *oryzae* in rice (Tang et al. 2001); *Erwinia carotovora* subsp. *carotovora* in transgenic *Oncidium* orchid (You et al. 2003; Liau et al. 2003); tobacco (Huang et al. 2006) and calla lily (Yip et al. 2007); *Pseudomonas* spp. in tobacco (Lin et al. 1997; Huang et al. 2004) and *Ralstonia solanacearum* in tomato (Huang et al. 2007). Especially, in *pflp* transgenic tomato, high expression of PFLP in root tissue exhibited resistance to *Ralstonia solanacearum*. Thus, we inferred that this antimicrobial peptide might have the same bioactivity as that in banana plant and whether the transgenic plants were resistant to fungal pathogens.

In this paper, we report the successful production of transgenic banana through *Agrobacterium tumefaciens*-mediated transformation, and the introduction of resistance to Fusarium wilt disease by the expression of the ferredoxin-like protein gene.

Materials and methods

Culture initiation from sucker

Two cultivated banana cultivars of genotype AAA were used in this study. *Musa accuminata* cv. Pei Chiao belongs to the Cavendish sub-group, and *Musa accuminata* cv. Gros Michel belongs to the Gros Michel sub-group. Stocks of MBC were supplied by the Taiwan Banana Research Institute (TBRI). Young suckers were selected from healthy and true-to-type mother plants. The outer layers of leaves and corm tissue of the suckers were removed to obtain a block measuring 10 cm long, containing the shoot apex. The block was surface-sterilized with 75% (v/v) ethanol. Under aseptic conditions, leaf sheaths and bases were trimmed to expose the meristematic region in the axial of the concentric leaves. The shoot tip was decapitated and a block of tissue measuring 1.5 × 1.5 × 1.5 cm was excised and inoculated onto a multiplication medium containing BA. The multiplication medium consisted of MS macro and micro salts supplemented with 0.4 mg/l thiamin HCl, 100 mg/l both of myo-inositol and L-tyrosine, 30 g/l sugar, and 5.5 g/l agar. Growth regulators included 4 mg/l N^6-benzylaminopurine (BA), 1.6 mg/l indole-3-acetic acid (IAA) and 80 mg/l adenine sulfate. The pH was adjusted to 5.8 before autoclaving. Glass cylindrical culture vessels were 7 cm in diameter and 10 cm high, containing 50 ml of medium. Each glass vessel contained one piece of

explant (sucker), and two to three pieces of propagule in later subculture cycles. The explant was incubated at 26–28°C with a 14-h light/dark cycle (daylight fluorescent tubes).

Induction of MBC

Small buds induced from the explant (sucker) were subcultured onto MBC induction medium, containing 2.0 mg/l TDZ (Phytotechnology Laboratories) and 2.0 mg/l PP333. To induce clusters of small buds (mixture of buds with tiny green leaf sheath and naked white meristerms), 2 mg/l each of TDZ and PP333 were added to the basic multiplication medium.

MBC stocks were cut vertically into pieces (5 × 5 mm) and placed on bud induction medium (BI) containing MS basal salts (Murashige and Skoog 1962) supplemented with MS vitamins, 2 mg/l BA, 2 mg/l TDZ and 3% (w/v) sucrose, and was then solidified with 6.8% Bacto Agar (Sigma, USA) for plant material multiplication. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were incubated in the dark with a temperature of 26 ± 2°C before *Agrobacterium* inoculation.

MBC cultures were maintained through the subculture of small pieces excised from swelled MBC on the BI medium at 3-week time intervals. For the transformation test, MBC (1 × 1 cm) were cut vertically into 1-mm-thick slices and used as explants. These bud clumps formed shoots during plant development.

Binary vectors, and *Agrobacterium* strains and infection

Agrobacterium tumefaciens strain C58C1 (Deblaere et al. 1985) or EHA105 harboring a binary vector pBI121SPFLP (Dayakar et al. 2003) or pBI121Atfd3 was used (Fig. 1). The T-DNA region of these plasmids contains two genes. The nopaline synthase (NOS) promoter drives the neomycin phosphotransferase gene (*npt*II). A cauliflower mosaic virus 35S (CaMV 35S) promoter drives *pflp* gene, including the signal peptide to target the transcript to the chloroplast (SPFLP) or *Atfd3* gene. These bacterial strains were cultured in liquid yeast extract broth (YEB) medium containing 5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 0.5 g/l $MgSO_4$, and 5 g/l mannitol, supplemented with 50 mg/l kanamycin and 50 mg/l rifamycin (Lichtenstein and Draper 1985).

Agrobacterium cultures were grown overnight (220 rpm, 28°C, in the dark) in 20 ml liquid YEB medium supplemented with 50 mg/l kanamycin and 50 mg/l rifamycin. The next day, the bacterial culture (A_{600} = 0.8–1.0) was centrifuged at 6,000 rpm or 3,030*g* for 20 min. The pellet was then resuspended in 20 ml autoclaved liquid cocultivation (CC) medium (BI medium lacking agar and

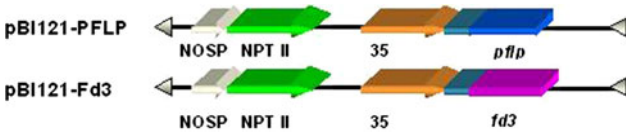

Fig. 1 Gene construction for transgenic bananas. NOSP The promoter region of the *Agrobacterium tumefaciens nopaline synthase* gene, *NPTII* the coding sequence of the *neomycin phosphotransferase* II gene, *35* the CaMV 35S promoter sequence, *pflp* the coding sequence for the sweet pepper PFLP containing its signal peptide, *Atfd3* the coding sequence of *Arabidopsis* root-type ferredoxin gene

supplemented with 200 μM acetosyringone, AS (Aldrich, St. Louis, MO, USA) to improve subsequent transformation frequency as described by Belarmino and Mii (2000). This suspension was used as the bacterial inoculum for infection. Bud slices from bud clumps were co-cultured in a petri dish containing 20 ml of *Agrobacterium* suspension and were shaken gently for 20 min at room temperature. Then, the slices were placed on a sterilized paper towel to remove the remaining liquid, and were cultured on the CC medium in the dark for 2 days. The control discs were similarly treated in a CC medium without inoculation.

Growth and recovery of the transgenic plants

Cefotaxime concentration used to inhibit *Agrobacterium tumefaciens* growth in this study was the same as reported in a previous study (Yip et al. 2007). After 2 days of cocultivation, the discs were then transferred onto the regeneration medium (BI medium containing 500 mg/l cefotaxime) and incubated at 26 ± 2°C with a 16-h photoperiod (60 μmol m^{-2} s^{-2}) under cool white fluorescent lamps for 3 weeks. Except for those specifically mentioned, all plants were cultured under the same conditions. The slices were subcultured for another 3 weeks on the BI medium supplemented with 250 mg/l cefotaxime. Subsequently, the greenish shoots from the bud tissue were transferred to the shooting medium (MS medium containing 250 mg/l cefotaxime). Root formation was then induced from the newly elongated shoots on the rooting medium (MS medium containing 0.1 mg/l NAA). Transgenic plantlets were rinsed with water to remove medium, and then transplanted to pots and grown under greenhouse conditions (16 h light, 100 μmol m^{-2} s^{-2}). After 2 months growing in pots, the plants were transferred to field in a net house for further studies.

PCR amplification, Southern blot, and western blot analysis

DNA isolated from transformed and control plants were analyzed for the presence of the *pflp* gene by means of polymerase chain reaction (PCR). Total genomic DNA was

isolated from young leaf tissue as described by Dayakar et al. (2003). For the amplification of the *pflp* gene, the upstream primer **35S**: 5′-AAGGGATGACGCACAATCCC ACTATCCTTC-3′ and the downstream primer **Ag3**: 5′-CG AGCTCGTTAGCCCACGAGTTCTGCTTCT-3′) was used to amplify a 650 base pairs (bp) fragment. And for the amplification of the *Atfd3* gene, the **35S** upstream primer and the downstream primer **arafd3:** 5′-CCGTAGAACG AAACTGATAACC-3′ which amplifies a 700-bp fragment was used.

Each 30-µl PCR reaction mixture contained 3 µl Taq plus precision 10× buffer (Protech, Taiwan), 10 mM dNTP, 10 µM of each primer and 1.25 units Taq DNA polymerase (Protech, Taiwan), and 500 ng of plant DNA or 20 ng of plasmid DNA. The PCR reaction included 5 min at 94°C, followed by 30 cycles of 60 s at 94°C, 45 s at 62°C, and 60 s at 72°C with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 1.0% agarose gels and were visualized with ethidium bromide staining under UV illumination.

To confirm the integration of the *pflp* gene in transgenic banana plants, Southern blot analysis was performed. Ten putative transgenic lines were selected for Southern blot. Genomic DNA was extracted from the leaves of in vitro-grown plantlets. DNA samples (20 µg each) were digested with *Eco*RI, electrophoretically separated on agarose gel, and were then transferred to nylon membranes (Boehringer Mannheim, Germany) using standard procedures (Sambrook et al. 1989). The membranes were then hybridized at 65°C with the full-length *pflp* marker probe labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) by PCR using the forward primer 5′-AAGGGATGAC GCACAATCCCACTATCCTTC-3′ and the downstream primer 5′-CGAGCTCGTTAGCCCACGAGTTCTGCTT CT-3′. The PCR reaction lasted for 5 min at 94°C, followed by 30 cycles of 80 s at 94°C, 60 s at 60°C, and 60 s at 72°C with a final extension at 72°C for 10 min. After hybridization, the membranes were washed under high stringency conditions (2× SSC, 0.1% SDS) and were developed using the DIG luminescent detection kit (Boehringer Mannheim).

Western blot analysis was performed as previously described (Huang et al. 2004). Total protein was extracted by homogenizing 0.2 g of fresh leaf tissue of transgenic and untransformed plants in 0.5 ml Tris buffer (150 mM NaCl, 50 mM Tris pH 7.5) using a plastic pestle fitted to a 1.5-ml centrifuge tube. The protein concentrations of the samples were determined with Coomassie brilliant blue dye using the microassay method as recommended by the manufacturer (BioRad). Six micrograms of each protein sample were fractionated in 12.5% polyacrylamide plus SDS (SDS–PAGE) gels. These gels were then either stained with Coomassie blue or were electro-transferred onto nitrocellulose membranes using the BioRad blue tank method. PFLP proteins were detected on western blots using anti-PFLP antibodies (Dayakar et al. 2003) and a mouse anti-rabbit IgG-peroxidase conjugate.

Fusarium resistance assay and disease rating

Meristems of newly-formed suckers were excised from transgenic plants, subjected to tissue culture for multiplication, and rooted in vitro. The regenerated plantlets were transplanted to pots in the greenhouse. Four-week-old plants were used for inoculation. Plants were planted into peat moss mixed with FOC, race 4 suspension (final inoculation 1×10^5 conidia/ml). After inoculation, plants were incubated in a growth chamber at 32°C for further observation. In the growth chamber, disease development was evaluated 9 weeks after inoculation. The rating scale ranged from 0 to 3, with plants exhibiting no internal symptoms scoring 0 and plants showing 100% vascular discoloration scoring 3. Percentage of disease severity was calculated using the formula of Sherwood and Hagedorn (1958): Disease severity (%) = [$\sum$ (no. of plants in a disease scale category) × (specific disease scale category)/(total no. of plants in the trial) × (maximum disease scale category)] × 100. Experiments were analyzed using one-way analysis of variance (ANOVA) and the Duncan test. Significance was evaluated at $P < 0.05$.

Results

Establishment and growth of multiple bud clumps

Small pieces of tissue with naked white meristems were excised from sucker meristems and subcultured every 50–60 days to obtain large globules of MBC containing numerous naked white meristems (Fig. 2). Shoot tips formed new and tiny buds after 2–3 months on BI medium in the dark. These buds formed clumps after 7–10 months. The morphology of the MBC formed from Pei Chiao and Gros Michel were obviously different. MBC of Pei Chiao formed tiny bud clusters, while the MBC of Gros Michel formed tuber-like structures (Fig. 3). Both buds and tuber-like structures resemble are a kind of primordial shoot. As with buds, the whole tuber-like structure, when cut in slices, could still develop into shoots on a shooting medium.

Transformation and recovery of MBC on Pei Chiao and Gros Michel

After *Agrobacterium* infection, the slices were co-cultivated with the bacteria plus 200 µM AS on the BI medium. After 2 days, slices were cultured in BI medium containing

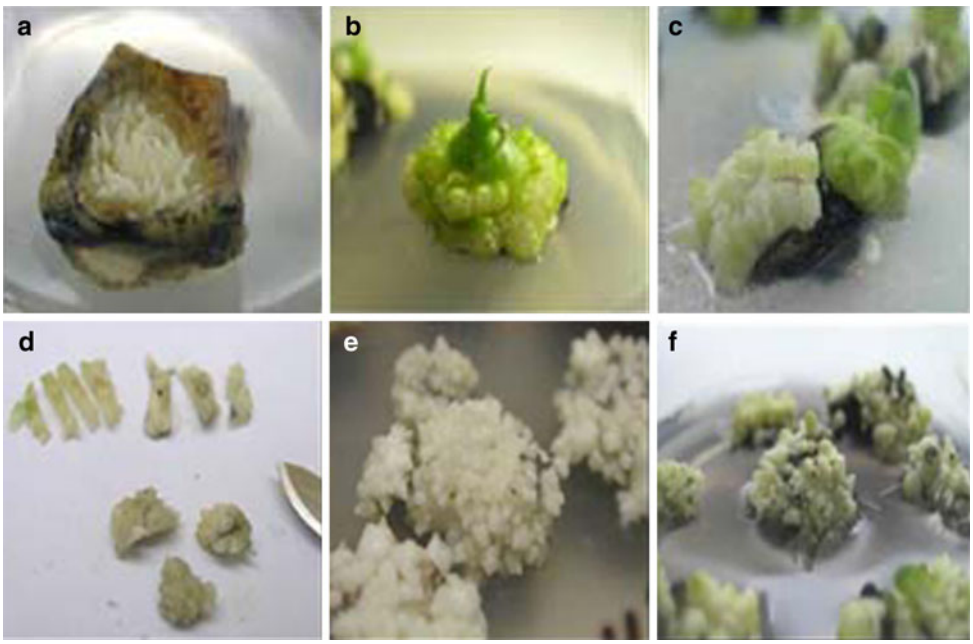

Fig. 2 Induction of multiple bud clumps (MBC). **a** Meristem of sucker in BA medium. **b** Small buds from panel a subcultured in Thidiazuron + Paclobutrazol medium. **c** Intermediate stages in Thidiazuron + Paclobutrazol medium for the induction of MBC. **d–f** MBC propagated in MS medium containing Thidiazuron and Paclobutrazol

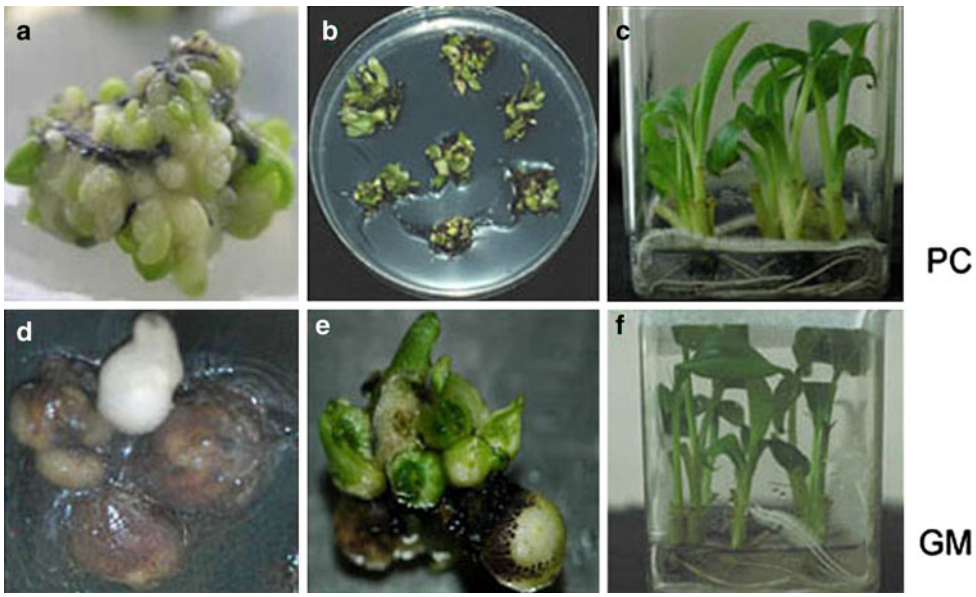

Fig. 3 Production of initial multiple buds and shoots of Pei Chiao (PC, *upper panel*) and Gros Michel (GM, *lower panel*) tissue cultures. **a** Multiple buds formed after 14 days on 3B medium in the dark. **b** More than one shoot sprouting on MS medium after 8 weeks under light. **c** Rooting of single shoot after 14 days on rooting medium. **d** Tuber-like structure formed on basal shoot basal of Gros Michel after 21 days on 3B medium under light. **e** New shoots sprouting from the tuber-like structure after 8 weeks. **f** Rooting of shoots after 14 days on rooting medium

500 μM cefotaxime to remove *Agrobacterium*. All explants started browning on the first 2 days, and new green buds sprouted at the periphery within 14 days (Fig. 4b). Single shoots were excised from the shoot cluster and subsequently transferred to a shooting medium (MS basal medium containing 250 μM cefotaxime) after 3 months (Fig. 4c). The newly expanded leaves were used for PCR analysis to confirm transgene existence. The PCR-positive plant was designated as the putative transgenic plant. These plants were transferred to the rooting medium. The plantlets started elongating and rooting within 2–3 weeks (Fig. 4d).

The transformation of Pei Chiao was done 1 year earlier than Gros Michel. Therefore, data collected from the field show Pei Chiao only. The putative transgenic plants of Pei Chiao were then transplanted to pots in the greenhouse for 2 months and subsequently transferred to the field (Fig. 4e). The *pflp*-transgenic plants grew and flowered after 8–10 months (Fig. 4f, g). These plants formed normal banana fruit 1–2 months after flowering.

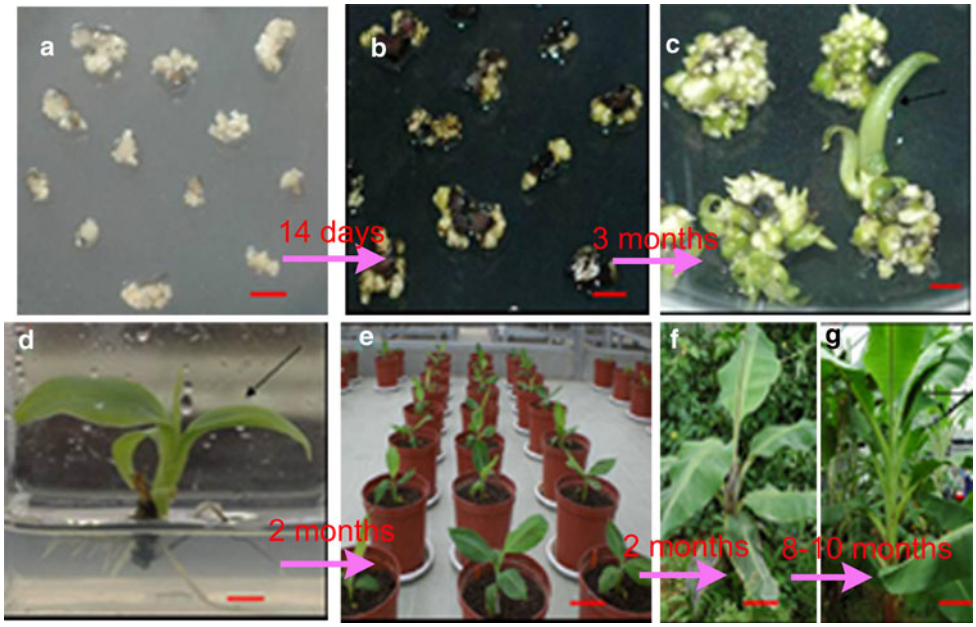

Fig. 4 Production of transgenic banana Pei Chiao using *Agrobacterium tumefaciens*-mediated transformation. **a** Initial materials for transformation (MBC were cut into slices 1-mm thick (*bar* 1 cm). **b** Green buds from browning explants after 14 days on BI medium (*bar* 1 cm). **c** Shooting (*arrow*) after 3 months (*bar* 1 cm). **d** Rooting of single shoot after 14 days on rooting medium (*arrow* leaf ready for PCR analysis, *bar* 1 cm). **e** Growing plants after transferred to pots in the greenhouse (*bar* 1 cm). **f** Plants transferred to field after 2 months from pots (*bar* 10 cm). **g** Growing plants in field after 8–10 months (*bar* 10 cm)

Comparison of *Agrobacterium* strains on transformation efficiency on Pei Chiao and Gros Michel

In order to optimize the *Agrobacterium* strains used in transformation system, we introduced two transgenes (*pflp* and *Atfd3*) into 2 cultivars (Pei Chiao and Gros Michel), respectively, with two *A. tumefaciens* strains, C58C1 and EHA105. The transformation efficiency was determined by the results of PCR analyses. More than 200 explants were analyzed for each transformation. Table 1 indicates that strain preference for transformation is dependent on plant material and the transgene. In the case of Pei Chiao, the EHA105 strain exhibited more efficiency for transformation (51% for *pflp* and 23.7% for *Atfd3*) than C58C1 (23.7% for *pflp* and 4.3% for *Atfd3*). However, in Gros Michel transformation, there was no significant difference between strains of C58C1 and EHA105. We also found that transformation efficiency was transgene-dependent. The *pflp* gene was more effective for transfer into Pei Chiao than Gros Michel. However, regarding the *Atfd3* gene, there was no differences in variety preferences.

Molecular characterization of transgenic plants

Shoots with two newly expanded leaves were subjected to PCR analysis for the presence of the *pflp* gene. As a positive control, the *pflp* gene was amplified from the pBI121SPFLP plasmid (Fig. 5a, lane 1). The expected 650 bp *pflp* band was found in 13 lines out of 16 shoots, and was absent in the untransformed controls (Fig. 5).

The genomes of ten randomly selected putative transgenic lines (αPL-5, αPL-7, αPL-12, αPS-20, αPL39-2, γPCL-37, γPCL-31, γPEL-85, γPEL-50, and γPEL-29) were analyzed for *pflp* integration by Southern blot analysis. The cDNA of the *pflp* gene was chosen as the probe to characterize transgenic loci. Genomic DNA isolated from the leaves of the transformed and wild-type plants was digested with *Eco*RI and probed with the *pflp* gene. The number of bands displayed by Southern blot reflected the copy number of the transgene in the plant genome. Results indicated that 6 out of 10 lines obtained a single copy of the transgene, and 3 lines had more than one copy of the transgene (Fig. 5b), thereby confirming their transgenic nature. No signal was observed in the non-transformant genome (Fig. 5b, lane 11).

Four randomly selected putative transgenic lines (αPS20, αPL39-2, γPCL37 and γPEL85) were used to determine the accumulation of the translational product of *pflp*. Proteins were extracted from the leaves of untransformed and transgenic plants. These protein samples were fractionated using 12.5% SDS–PAGE, and were then blotted and probed with an anti-PFLP antibody followed by a mouse anti-rabbit IgG-peroxidase conjugate. Endogenous ferredoxin could be recognized by the anti-PFLP polyclonal antibody. Therefore, the signal could also be observed in the wild-types (Fig. 5c, lanes 1–2). The probing signals (22 kDa in molecular weight) of transgenic plant samples (Fig. 5c, lanes 3–6) were stronger than those from the untransformed plants. This result indicated that the transgene was expressed in transgenic plants.

Homogeny and stability of transgenes

In order to know the homogeneity of the transgene on vegetative propagated offspring, 5 transgenic lines were

Table 1 Comparison of transformation efficiency by *Agrobacterium-tumefaciens* on bananas

Banana cultivar	*Agrobacterium* strains	Transgene	Total no. of explants (*a*)	No. of plantlets for PCR analysis (*b*)	No. of plantlets showing transgene (*c*) (*c*/*b*,%)	Transformation efficiency (*c*/*a*, %)
Pei Chiao	C58C1	*pflp*	552	1,043	131 (12.6)	23.7
	EHA105	*pflp*	224	319	115 (36.1)	51.3
	C58C1	*Atfd3*	442	113	19 (16.8)	4.3
	EHA105	*Atfd3*	454	129	57 (44.2)	12.6
Gros Michel	C58C1	*pflp*	545	131	24 (18.3)	4.4
	EHA105	*pflp*	443	111	15 (13.5)	3.4
	C58C1	*Atfd3*	267	74	35 (47.3)	13.1
	EHA105	*Atfd3*	297	87	35 (40.2)	11.8

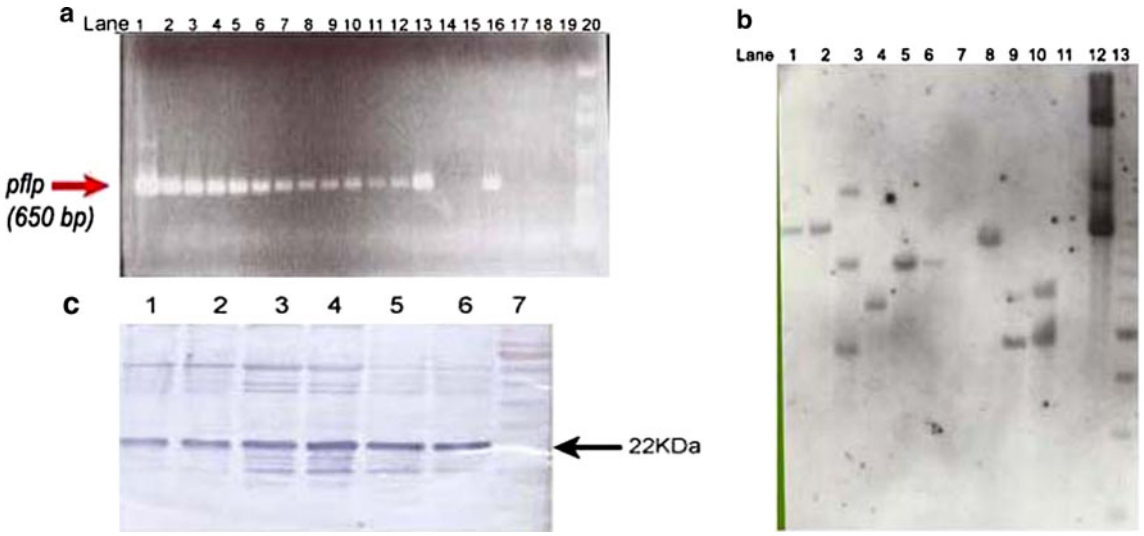

Fig. 5 Molecular characterization of *pflp*-transgenic bananas on Pei Chiao. **a** PCR analysis of transgenes on *pflp* putative transformants. *Lane 1* pBI121/SAP1 plasmid DNA were used as positive control. *Lanes 2–17 pflp* putative transformants, and *lanes 18, 19* wild-types, *lane 20* 100-bp marker. **b** Southern blot analysis of DNA from greenhouse-grown banana plantlets. Ten *pflp* transformants (*lanes 1–10*), and wild-types (*lane 11*) were shown. *Lane 12* pBI121/SAP1 plasmid DNA was used as positive control. 1-kb marker (*lane 13*). **c** Western blot analysis for protein expression of *pflp* in transgenic banana leaves. Four *pflp* transformants (αPS20, αPL39-2, γPCL37 and γPEL85; *lanes 3–6*), and 2 wild-types (*lanes 1* and *2*) were shown. *Lane 7* molecular marker

randomly selected for investigation. Genomic DNA was extracted from T_0 sucker leaves and subjected to PCR analysis. The results showed that the delivery of transgene was different among transgenic lines. More than 80% of the five lines contained the *pflp* transgene (Table 2). The homogeneity was also evaluated by PCR analyses of the leaf tissue. Eight leaves of each transgenic plant were chosen. The leaves containing the transgene could be as high as 100% (all eight leaves were PCR positive) or as low as 12.5% (one out of eight was PCR positive). The discrepancy in the homogeneity of the transgene appeared in the evaluation process. We also detected the protein expression of PFLP on these transgenic offsprings. The probing signals (22 kDa in molecular weight) of samples from the offsprings were stronger than those from the untransformed plants (Fig. 6). This result indicated that the transgene was expressed translationally in transgenic offsprings.

Transgenic banana exhibit enhanced resistance to Fusarium wilt disease

In order to clarify the resistance of transgenic banana to fungal pathogen, the biological effects of the *pflp* activity were examined. Two individual transgenic lines (αPS20, γPEL85) of plantlets regenerated from meristem culture of T_0 transgenic plants were challenged with the *Fusarium oxysporum* f. sp. *cubense* (FOC) race 4 suspension (final inoculation 1×10^5 conidia/ml). Yellowing of banana leaves and wilting appeared at 9 weeks after FOC

inoculation in the greenhouse. Wild types developed more severve internal symptom than transgenic lines. The percentage of severity in wild-type is 41.6% but in transgenic lines decreased to 14.2% in αPS20 and 20.8% in γPEL85 (Fig. 7f).

Discussion

This study demonstrates an easy and efficient protocol with no selection stress through *Agrobacterium*-mediated transformation on AAA genotype bananas. In this system,

Table 2 Homogeny of *pflp* genes in T_0 plants (mother plant) and T_0 suckers in Pei Chiao

Plant lines	No. of leaves of T_0 plants containing *pflp* transgene (PCR positive leaf)	Percentage of leaves of T_0 plants containing *pflp* transgene, %	No. of T_0 suckers produced from T_0 plants	Percentage of T_0 suckers containing *pflp* transgene, %
Wt	ND	ND	ND	ND
αPS20	8 (A–H)	100	5	83.3
αPL39-2	1 (G)	12.5	3	100
γPCL37	4 (A, E, F, G)	50	2	100
γPEL50	3 (A, F, H)	37.5	2	100
γPEL85	6 (A, B, E, F, G, H)	75	4	80

ND Not detected

Fig. 6 Western blot analysis of leaf protein (6 μg per lane) extracted from untransformed (*lanes 1* and *19*) and T_1 transgenic plants αPS20-1–αPS20-5 (*lanes 2–6*), αPL39-2-1–αPL39-2-3 (*lanes 7*, *8*, *10*), γPCL37-1–γPCL37-2 (*lanes 11–12*), γPEL50-1–γPEL50-2 (*lanes 13–14*), γPEL85-1–γPEL85-4 (*lanes 15–19*). The blot membrane was hybridized with anti-PFLP antibody. The apparent molecular weight of 22 kDa for PFLP is marked

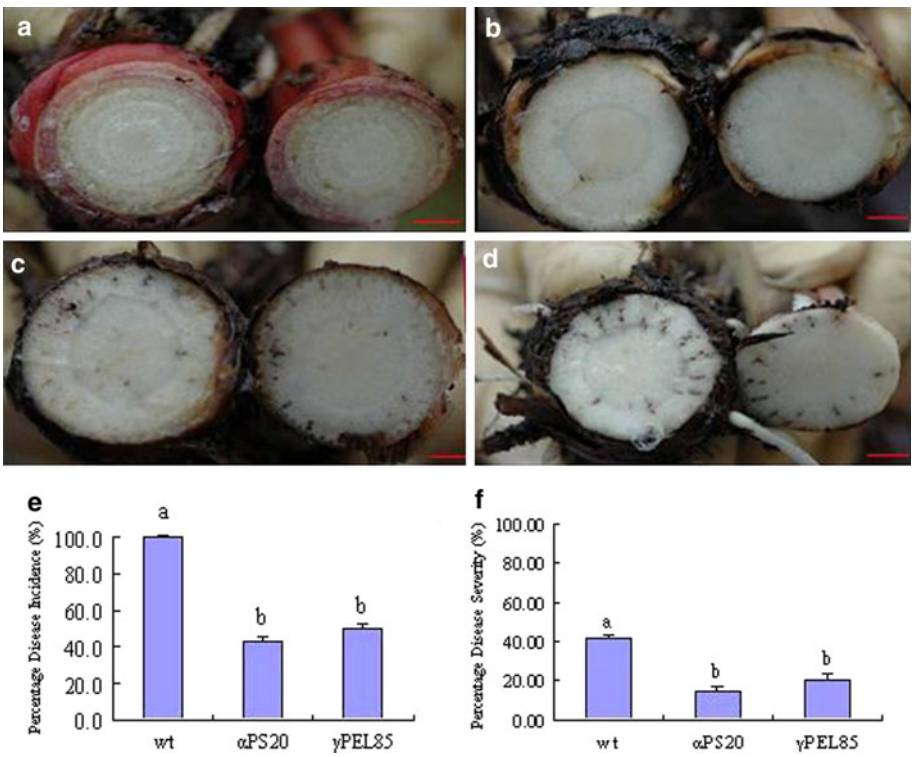

Fig. 7 Characterization of disease severity levels on *Fusarium* challenge test. Disease severity score of 0 observed in *pflp* transgenic plants 9 weeks after infection of *Fusarium* in growth chamber (**a**), compared with disease severity scores of 1 (**b**), 2 (**c**) and 3 (**d**) in wild-types observed on cross section of corm after the same treatment. *Scale bars* 1 cm. Percentage disease incidence (**e**) and disease severity (**f**) of transgenic banana compared with wild-types 9 weeks after inoculation. Data were analyzed using one-way analysis of variance (ANOVA) and the Duncan test. *Bars* represented with the *same letter* are not significantly different at $P < 0.05$

MBC were used as a transformation target instead of embryogenic cells. Establishing the MBC culture was easier and more time saving when compared with suspension cell cultures. Compared with the suspension cell culture system, the MBC system does not need to induce the friable "ideal" embryogenic callus that would diminish at least 6 months to get explants for further transformation. Additionally, curtailing the embryogenic cell suspension (ECS) culture procedure would dispense with much labor for weekly subculture. Moreover, plants regenerated through MBC would save about 2 months to get plantlets as compared with ECS. The other advantage of this procedure is easier and capable of reducing manpower compared to meristem preparations from tissue culture plants. Apical shoot tips are excised from in vitro regenerated shoots and successfully express the *β-glucuronidase* (*uid A*) gene in the plantain system (Tripathi et al. 2005). However, excising apical meristems from individual tissue culture plants requires a lot of skills. In our MBC system, meristem from suckers could be excised by naked eyes (instruments such as microscope is unnecessary) and numerous tiny and naked meristems could be induced directly from one single sucker collected from the field. In our observation, neglect of kanamycin throughout the entire culture processes diminishes somaclonal variation on tissue culture explants. Furthermore, roots form normally during MBC regeneration. The survival rate of these well-developed rooted plantlets may increase when transplanted to the greenhouse.

Several reports have shown differences in transformation efficiency when using different strains of *A. tumefaciens* (Tripathi et al. 2005). However, some controversial results for this view have previously been reported (reviewed by Smith and Hood 1995; Hiei et al. 1997; Liau et al. 2003). The *Agrobacterium* culture was pre-incubated with AS in the medium to increase transformation efficiency in different banana cultivars (Khanna et al. 2004). We found differences in transformation efficiency between the two strains here. This phenomenon is cultivar independent. EHA105 is the best strain for transformation of MBC on Pei Chiao while C58C1 is the best one on Gros Michel. This difference may be due to the higher susceptibility of the multiple bud explants to different strains in these two cultivars. The mechanism behind this observation is still unclear.

Production of mosaic plants is concerned on transformation through meristemic tissue. Also, the delivery of transgene to progeny is very important for successful transformation. Most bananas are sterile with no seeds and propagate through suckers. In order to understand the homogeny of the transgene, leaf samples were collected from the whole transgenic mother plant. The results indicated that not all leaves contain the *pflp* transgene, and the gene distributes randomly in plant. Our experiment implied that transformation through the MBC system may result in the formation of mosaic plants. Formation of mosaic plants may occur because multiple cells are involved in shoot development, while only a proportion of them may be transformed. Although the existence of mosaic plants was proven in this study, more than 80% of newly formed progeny suckers receive the transgene from their mosaic mothers. And the transgene could be expressed translationally on those transgenic offsprings. We also found that these transgenic bananas exhibited resistance in FOC. For plant protection in agricultural practice, resistance of less than 100% is allowed. This result shows that the strategy to produce mosaic transgenic bananas in this study can be applicable for agricultural use.

In conclusion, we have established a safe and efficient system for the production of transgenic bananas by using MBC through *Agrobacterium tumefaciens*-mediated transformation and these transgenic bananas exhibited resistance to FOC. These studies demonstrated the *pflp* gene not only exhibited resistance on bacterial pathogen but also on fungal pathogen. The whole system can be applied to banana cultivars that have difficulty in establishing embryogenic suspension cell cultures.

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