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BIOLISTIC-MEDIATED DNA DELIVERY AND ISOLATION OF TRANSGENIC OIL PALM (*Elaeis guineensis* Jacq.) EMBRYOGENIC CALLUS CULTURES

Keywords: Particle bombardment, transgenic embryogenic callus, oil palm, stable transformation

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Oil palm (Elaeis guineensis Jacq.) embryogenic calli were transformed withplasmids containing various combinations of genes encoding β-glucuronidase, phosphinotricin acetyltransferase and hygromycin phosphotransferase, using an electric discharge particle bombardment device. Experiments to optimize the accelerating force for DNA delivery into embryogenic tissue of oil palm were carried out. Selection and culture conditions to allow recovery of transgenic tissue are reported. Stable integration of transgenes was confirmed by molecular analysis. Due to the long time required for regeneration of transgenic plants from oil palm embryogenic callus, this report is significant as it describes, for the first time, conditions leading to the recovery of transgenic embryogenic callus cultures carrying and expressing transformed marker genes.

INTRODUCTION

Oil palm, a monocot, is an important economic crop for Malaysia, the major producer of palm oil in the world. Modification of oil quality and increasing yield via genetic engineering are important targets for research. Development of an efficient gene transfer system for oil palm remains one of the major bottlenecks in oil palm genetic engineering (Parveez *et al.*, 1994). In this report, we describe the successful recovery of genetically-engineered embryogenic oil palm callus tissues by particle bombardment. This is the first step in the development of transgenic oil palm expressing important genes controlling agronomic and industrial characteristics.

Particle bombardment is the preferred method for the genetic engineering of monocots (Christou, 1995; 1996). Transgenic monocots,

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including maize (Fromm et al., 1990; Gordan-Kamm et al., 1990), rice (Christou et al., 1991), wheat (Vasil et al., 1992), oat (Somers et al., 1992), sugar cane (Bower and Birch, 1992) and banana (Sagi et al., 19951, have been obtained successfully via particle bombardment. Agrobacterium-mediated transformation is also being used in increasing frequency (Hiei et al., 1994; Ishida et al., 1996). Optimization of DNA delivery parameters and determining effective selection procedures are key initial steps in making oil palm amenable to genetic manipulation.

In this report, we describe experiments addressing these two important steps. Due to the long time required for the recovery of plants from dedifferentiated oil palm tissues, this report is significant as it demonstrates, for the first time, that DNA bombardment can transform embryogenic callus.

MATERIALS AND METHODS

Induction of Callus

Samples of leaflets from unopened (-6) frond were aseptically transferred to sterile petri dishes containing solid callus initiation medium [MS salts (Murashige and Skoog, 1962)+Y3 vitamins (Eeuwans, 1976)+0.1g/l myo-Inositol and L-glutamine + 3% sucrose + 11mg/l 2,4-D+2.5 g/l activated charcoal + 7 g/l agar (Sigma Type II) adjusted to pH 5.7 with KOH] and cultured at 28°C in the dark. The petri dishes were sealed with Whatman parafilm. The callus tissue was subcultured every four weeks on the same medium until embryogenic callus could be selected.

Maintenance of Embryogenic Callus

Embryogenic callus was cultured on agar-solidified medium containing MS macro and micronutrients supplemented with 2.2mg/l 2,4-D and 30gm/l sucrose (adjusted to pH 5.7 with KOH). Embryogenic callus was maintained at 28°C, in the dark, and subcultured every 30 days onto fresh medium.

Bombardment Using Electric Discharge Device

Three plasmids, pAHC25 (Christensen *et* al., 1992) carrying *gusA* and *bar* genes driven by the maize ubiquitin promoter, pGH24 (Bates, Pers. Comm.) carrying *gusA* and *hpt* genes driven by CaMV 35S promoter and a third plasmid carrying *gusA*, *hpt* and ***bar genes,*** all driven by the CaMV 35S promoter, were used. Plasmid DNAs used in the experiments were isolated and purified using the Qiagen Maxi Prep Kit. DNA-gold coating and bombardment were carried out as described previously (McCabe and Christou, 1993). Transformation was carried out at accelerating voltages of 12, 16 and 22kV, using an electric discharge apparatus (Christou et al., 19911. The optimal timing of DNA delivery into embryogenic callus was determined by bombarding using the pAHC25 plasmid at one or five days after subculture.

DNA delivery conditions were optimized by transient GUS expression. Each blue spot arising from the histochemical localization of GUS activity, whether in a single cell or a group of cells was considered as one expression unit as defined by Klein et al. (1988). Optimization was not based on the number of blue spots alone, but also on shot-to-shot variability and the physical impact on embryogenic callus. There are three main advantages of transient expression. i) The highest expression will result in a higher chance of getting stable transformation as there are lower numbers of stable transformants over transients. ii) Low variability will result in a consistent performance of bombardment. iii) Finally, it is expected that bombardment which resulted in massive tissue dislocation may result in high injury to the cells, which lowers the chances of regenerating stable transgenic plants.

Selection of Transformed Embryogenic Callus

The minimal inhibitory concentrations of selection agents for oil palm were determined previously (Parveez et al., 1996). Embryogenic callus was exposed to a medium containing

either Basta or hygromycin at concentrations of 40mg/l or 80mg/l, at one or three weeks after bombardment. Tissues were subcultured to fresh medium under selection at monthly intervals.

Preparation of Total DNA from Embryogenic Callus

Resistant embryogenic callus was selected randomly after a minimum of five subcultures. The total DNA was isolated according to the method of Ellis (1993). Embryogenic callus clumps (10-50mg) were placed in a 1.5ml microfuge tube and immersed in liquid nitrogen. Frozen embryogenic callus was ground to a fine powder in the presence of 400µl EB2 buffer (500mM NaCl, 100mM Tris-Cl {pH 8.0} and 50mM EDTA {pH 8.0)) and 20µl 20% SDS. Four hundred µl of phenol mix (1:1. phenol: chlorofom) were then added, and the mixture thoroughly mixed and centrifuged (12 000 rpm, 2 min, RT). The aqueous phase was transferred to a new tube and mixed with 800µl absolute ethanol. DNA was recovered by centrifugation (12000rpm, 5min, RT). The pellet was washed with 70% ethanol and dissolved in 50µl TE (10 mM Tris-Cl and 1mM EDTA, pH 8.0).

Polymerase Chain Reaction (PCR)

Amplification of the *gusA*, ***bar*** and *hpt* genes was carried out using standard and touch-down PCR protocols (Sambrook et *al.*, 19891. The following primers were used to amplify the transgenes and part of the regulatory sequences. 35F1 5' TACAGTCTCAGAAGACCAAA3' and GUSR1 5'GGGGAGG CTACAGATGCTTTGC3' will amplify the *gusA* gene and 35S promoter resulting in a band size of 2321bp. 35F1 and HMRR1 5'GATCTCCAATCTGCGG GATC3' will amplify the *hmr* gene and 35S promoter resulting in a band size of 1684bp. BARF1 5'GGTCTGCACCATCGTCAACC3' and HMR2 5'ACTCCACGCGACGTCTGTCG3' will amplify the ***bar gene,*** 35S promoter and part of the ***hmr*** gene resulting in a band size of 1550bp.

Fifty ng of oil palm DNA and one ng of transforming plasmid DNA were used in PCR reactions. In the standard procedure, the following condition was used: 30 cycles at 92°C (50 sec), 60°C (50 sec) and 72°C (60 sec). For the touch down procedure, 10 cycles 92°C (45 sec), *70°C* (45 sec; -0.5°C per cycle), 72°C (60 sec) and 20 cycles 92°C (45 sec), 65°C (45 sec) and 72°C (60 sec) were used. Amplified DNA fragments were checked by electrophoresis on 1.4% agarose gels in 0.5X TBE (45mM Tris-Borate; 1mM EDTA, pH 8.0) buffer.

Southern Blot Hybridization

DNA from transformed and untransformed embryogenic callus were digested with Sac1 restriction enzymes at 37°C for 16 hours. Digested and undigested DNA were separated on 0.8% agarose gel in 1 X TBE buffer and was later capillary-transferred onto nylon membranes (Southern, 1975). Agarose gel containing digested (5µg) and undigested (3µg) DNA was soaked in depurinating buffer (0.2M HCl) for 10 minutes, denaturing buffer (1.5M NaCl and 0.5M NaOH) for 45 minutes and transferred into neutralization buffer (1M Tris; pH 8.0 and 1.5M NaCl) for one hour. The gel was subsequently transferred onto 3MM paper with the end of paper soaked into 20 X SSC (175.3gm NaCl and 88.2g sodium citrate/litre, pH adjusted to 7.0 with 10M NaOH). Nylon membranes (Amersham) were blotted overnight. After transfer, membranes were washed with 2 X SSC and baked at 80°C for 2-4 hours.

Oligolabelling of DNA fragments (prepared by PCR) for use as probes was carried out using the method of Feinberg and Vogelstein (1983). DNA (6µl;~10ng) was added to 20µl 5 X OLB [0.25M Tris-HCl; pH 8.0; 25mM $MgCl_2$, 0.36% (v/v) 2-mercaptoethanol, 1M HEPES; pH 6.6; 30% hexadeoxyribonucleotides (90 O.D. units/ml)], boiled for five minutes and chilled on ice. Two µl 0.1M dNTPs (except dCTP), 5µl $\propto^{32}$P (dCTP; 370 KBq/µl), 2µl 10mg/ml BSA, 1µl Klenow (6U/µl) and 14µl distilled water were then added. The labelling reaction was carried out by incubating at 37°C for 30 minutes. The probe was denatured by the addition of 50µl 1M NaOH for one minute, 50µl 1M HCl for one minute and 50µl Tris-HCl (pH 7.5) for one minute. The denatured probe was stored on ice.

Pre-hybridization and hybridization were carried out in the same buffer. Membranes were pre-hybridized [40% Pipes/NaCl, pH 6.8; 1.5% Pipes, 8.7% NaCl and 0.37% EDTA; pH 8.0; 20% Denhardt's 50X (1% BSA, 1% Ficoll, 1% PVP and 10% SDS); 0.5% SS-DNA 10mg/ml and 39.5% distilled water] for 90 minutes at 65°C. The denatured probe was added and hybridized for 20 hours at 65°C. After hybridization, the membranes were washed once with 2 X SSC for one minute and then twice with 0.1 X SSC and 0.1% SDS, the first washing for 30 minutes and the second for 45 minutes (65°C). The washed membranes were wrapped with Saran Wrap and exposed to X-ray film with an intensifying screen at -70°C for two days.

RESULTS AND DISCUSSION

Transformation

Optimization of DNA delivery conditions was carried out by bombarding established oil palm embryogenic callus at different voltages and at different times. Two days after the bombardment, transient expression of the marker gene *gusA* was determined. We observed that the technique was capable of delivering biologically-active DNA into oil palm embryogenic callus. Significantly higher transient *gusA* gene expression was obtained when the embryogenic callus was bombarded one day after subculture *(Table 1)*. The optimum voltage for efficient delivery of plasmid DNA into embryogenic callus was determined by bombarding at 12, 16 (once or twice) and 22kV. We observed that increasing the accelerating voltage resulted in significantly higher transient *gusA* gene expression. Bombardment at the highest voltage (22kV) resulted in significant tissue damage. We were not able at this stage, to evaluate the impact of higher voltage on stable transformation. Therefore, in all subsequent experiments, embryogenic callus was bombarded at the three voltages, one day after subculture.

Selection of Transformed Embryogenic Callus

Selection of transformed embryogenic callus was carried out using either Basta or hygromycin at concentrations of 40mg/l or 80mg/l. Bombarded tissues were first cultured on medium in the absence of selection agent for one or three weeks. Upon transfer to fresh medium containing selection agent, untransformed embryogenic callus began to die, allowing only resistant embryogenic callus to proliferate. As expected, no callus was recovered from control plates bombarded only with gold particles after selection on Basta or hygromycin. Resistant callus appeared after two to three months culturing on Basta and three to four months on hygromycin-containing medium. Both selection agents were found to be suitable. However, Basta was more convenient because it resulted in a phenotype easier to score. This result, however, contradicts observations in other systems [e.g. rice] (Christou et al., 1991) in which hygromycin selection was more effective.

TABLE 1. EFFECT OF ACCELERATING VOLTAGE AND TIMING OF BOMBARDMENT ON TRANSIENT *gusA* GENE EXPRESSION IN OIL PALM EMBRYOGENIC CALLUS

Voltage (kV)	1 Day'	5 Day
22	140.7 (±21.70)[a]#	43.3 (±9.03)[cd]
16	86.3 (±26.74)[b]	32.7 (±11.08)[d]
16[@]	77.3 (±10.62)[bc]	35.7 (±9.74)[d]
12	47.3 (±19.91)[cd]	13.3 (±4.64)[d]

*Mean number of transient *gusA* expression units two days after bombardment. Results were obtained from three independent bombardments. Values in parentheses indicate standard error.
*Bombardment was carried out one day after subculture to fresh medium. "Bombardment was done twice. Values followed by different letters are significantly different at $P=0.05$ by t-test

The mean number of resistant embryogenic callus clumps obtained after five subcultures, in replicated experiments (three replicates), are summarized in ***Table*** 2. In this experiment, three replications were performed to obtain a reliable and statistically acceptable comparison. Analysis of variance (ANOVA) revealed no significant difference in the number of resistant embryogenic callus obtained after each treatment. This suggested that any combination of selection procedures and accelerating voltages would result in the same efficiency of resistant embryogenic callus produced. Stable transformation data show that increased transient *gusA* gene expression resulting from bombardment of higher voltages did not significantly increase stable transformation efficiency. Koprek et al. (1996) reported a similar observation in barley. Selection at three weeks after bombardment was preferred as it allows transformed cells to divide several times. The amplification process will result in a critical mass of transformed cells which is important for maintaining the survival of cells under selection pressure (Ozias-Akins et al., 1993). Bombardment at a moderate voltage (16kV) was preferred as it resulted in less tissue dislocation after bombardment. Selection using a lower concentration of Basta or hygromycin was preferred as there is some evidence in other systems suggesting that the regeneration capacity of stably transformed embryogenic callus increases under this condition. Selection at a lower concentration of hygromycin (50mg/l compared to 100 mg/l) in rice resulted in higher numbers of transformed callus and transgenic plants (Christou and Ford, 1995). Similar result has also been reported for wheat using Basta (Vasil et *al.*, 1992).

Polymerase Chain Reaction (PCR)

Recovering resistant embryogenic callus surviving on selection is not adequate proof of stable integration of transgenes into the plant genome. Molecular analysis is required to confirm stable integration of transgenes. A number of resistant embryogenic callus clumps obtained after five subcultures were selected randomly from each treatment and subjected to a preliminary PCR analysis. Initially, to obtain reliable PCR results, amplification of an internal control was used. The internal control was a pair of primers which always specifically amplified a 1Kb size band in oil palm (Cheah, pers. comm.). All the samples (including untransformed control) were subjected to amplification of the internal control, which

TABLE 2. EFFECT OF SELECTIVE AGENTS CONCENTRATION, ACCELERATION VOLTAGE AND TIME FOR SELECTION EXPOSURE ON THE RECOVERY OF RESISTANT EMBRYOGENIC CALLUS CLUMPS

Treatment	Basta 40mg/l	Basta 80mg/l	Hygromycin 40mg/l	Hygromycin 80mg/l
12V-1W[b]	6.7 (f1.25)	4.0 (f1.63)	4.3 (f1.25)	3.0 (f0.81)
12V-3W	7.3 (f1.70)	5.0 (±2.16)	6.7 (f1.24)	3.3 (f1.25)
16V-1W	5.0 (±2.10)	3.3 (±0.47)	4.0 (k1.63)	3.7 (±0.47)
16V-3W	5.7 (f1.24)	4.7 (f1.20)	5.0 (f1.41)	4.7 (f1.24)
16V-1W2X[c]	4.3 (k1.25)	4.0 (±1.63)	5.0 (±0.82)	4.0 (±1.41)
16V-3W2X	5.3 (f1.24)	5.7 (f2.05)	7.3 (±0.94)	6.0 (±1.63)
22V-1W	5.3 (f1.70)	4.7 (±0.94)	3.7 (±0.92)	5.0 (f1.41)
22V-3W	6.7 (f1.24)	5.0 (±1.41)	6.3 (f1.24)	5.0 (f2.16)

[a]Mean number of resistant callus obtained on selection medium after 5 subcultures. Results were obtained from three independent bombardments (replicates). Values in parentheses indicates standard errors. [b]Bombardment at 12kV and selection was performed one week after bombardment. [c]Bombardment was done twice.

should show amplification of a control band and, if not, were further purified until the control band could be amplified. The internal control is important to ensure that any sample which failed to amplify transgenes were actually untransformed and not due to unpure DNA or the amount of DNA used. In addition, an untransformed control was also used as a negative control.

This analysis demonstrated that all resistant callus samples are able to amplify the genes used for selection *(bar* or *hpt)*. No bands were detected in untransformed control *(Figure 1)*. Successful amplification of the *bar* gene was performed by touch down PCR. The expected size of the *bar* gene insert (1550 bp) was amplified for all the resistant embryogenic callus lines selected on basta *(Figure 1a)*. Standard PCR was used to amplify the *gusA* and *hpt* genes. The expected band size for the *hpt* gene insert (1684 bp) was amplified from all resistant embryogenic callus selected on hygromycin *(Figure 1b)*. However, only 88% of the resistant embryogenic callus lines we sampled showed amplification of the *gusA* gene *(Figure 1c)*. Co-integration of unselected gene(s)

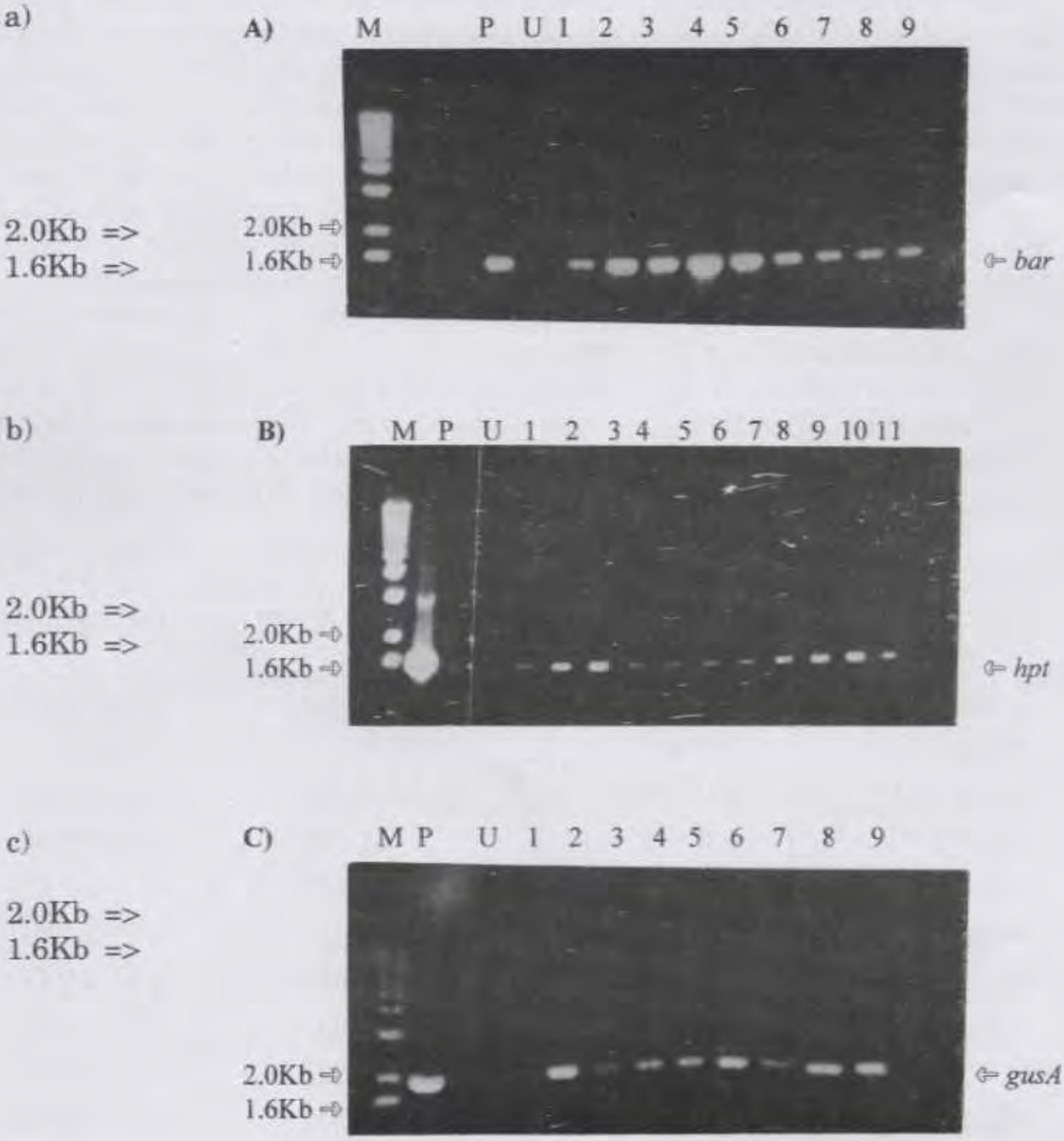

Figure 1. PCR analysis of transgenic oil palm embryogenic callus. Lane M:1Kb DNA marker (BRL); P:transforming plasmid; U:untransformed callus; lanes 1-11:putative transformed callus

(gusA and *bar* or *hpt)* was observed in over 80% of the resistant embryogenic callus lines we analyzed through PCR. This high frequency of co-integration was not surprising as all the genes were linked on the same transforming plasmid. Similar observation were made in other systems, including soybean and wheat (Christou and Swain, 1990; Vasil *et al.*, 1991).

Southern Blot Hybridization

Amplification of transgenes using PCR is not definitive evidence of stable integration of transgenes into the host genome. Potrykus (1990) has stated that a number of factors need to be determined before stable integration of a transgene can be confirmed. Among the main requirements are : effective controls for treatment and analysis, tight correlation between treatment and predicted results and complete Southern blot analysis with positive signals on high molecular weight DNA hybrids between the transgene and host genomic DNA.

Southern blot analysis of total DNA from five resistant embryogenic callus clumps and one untransformed control were carried out. PCR amplified *bar* and *hpt* genes were used as probes. Hybridization in high molecular weight, undigested DNA in all resistant embryogenic callus lines we tested confirmed stable integration of the transgenes into the oil palm genome *(Figures 2A,* lane 1-5). No hybridization was seen in digested or undigested total DNA from untransformed embryogenic callus *(Figure 2,* lane U). In these experiments, all DNAs were digested with SacI which cuts once in each plasmid used, except for pGH24 (three times). Digested DNA from resistant embryogenic callus bombarded with pAHC25, selected on Basta *(Figure 2B,* lanes 1 and 3) exhibited banding patterns consistent with the integration of *bar*

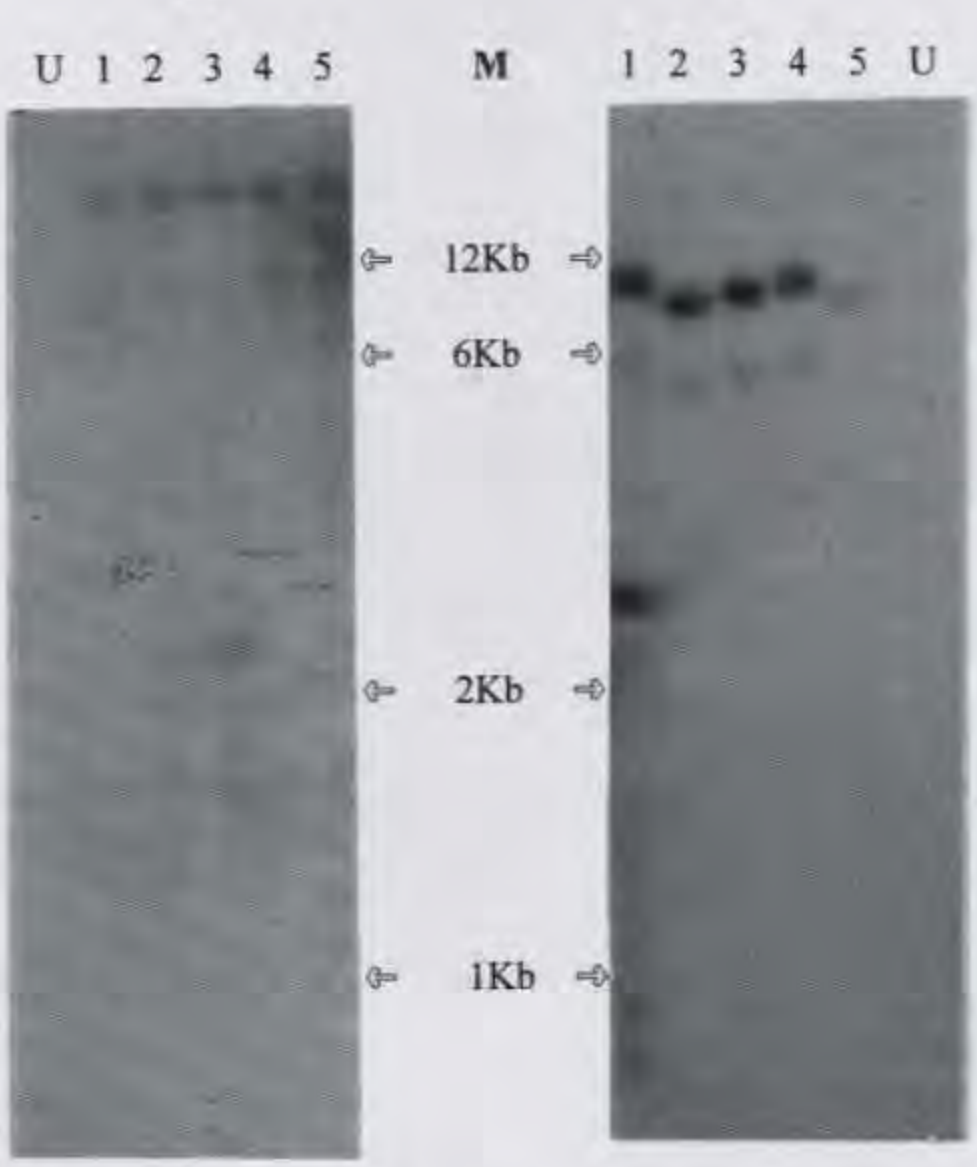

Figure 2 : Southern blots containing (A) undigested DNA and (B) SacI digested DNA were hybridized with α ^{32}*P-dCTP labelled probe of PCR amplified* bar *and* hpt *genes. Hybridization bands were detected in all 5 transgenic oil palm embryogenic callus lines bombarded with different plasmids (lanes 1-5). Untransformed control embryogenic callus (lane U). M:1 Kb DNA marker (BRL).*

gene in different genomic locations. Similar observation were made for DNA isolated from resistant embryogenic callus bombarded with the triple gene construct selected with Basta (3.9kb) or hygromycin (2kb) *(Figure 2B,* lanes 2 and 5, respectively) and also for tissues bombarded with pGH24 and selected on hygromycin (4kb; *Figure 2B,* lane 4).

In a separate analysis, digested and undigested DNA from three resistant embryogenic callus clumps were probed with *bar* and *gusA* genes. The three clumps were bombarded with the plasmid, pAHC25, and selected on Basta. Hybridization to high molecular weight undigested DNA and *EcoR1* digested DNA from all three clumps demonstrated the stable integration of transgenes into the oil palm genome *(Figure 3,* Lane 1-6). No hybridization was observed for *EcoR1* digested DNA from untransformed control *(Figure 3,* Lane U). *EcoR1* digested DNA hybridized to two bands of the expected size of 3.1kb and 6.5kb. The 3.1kb band corresponded to the ubiquitin promoter, intron, *bar* gene and the nos terminator. The 6.5kb band corresponded to the ubiquitin promoter, intron, *gusA* gene, nos terminator and part of the plasmid vector (pUC8). The results obtained indicated co-transformation of both transgenes into the oil palm genome.

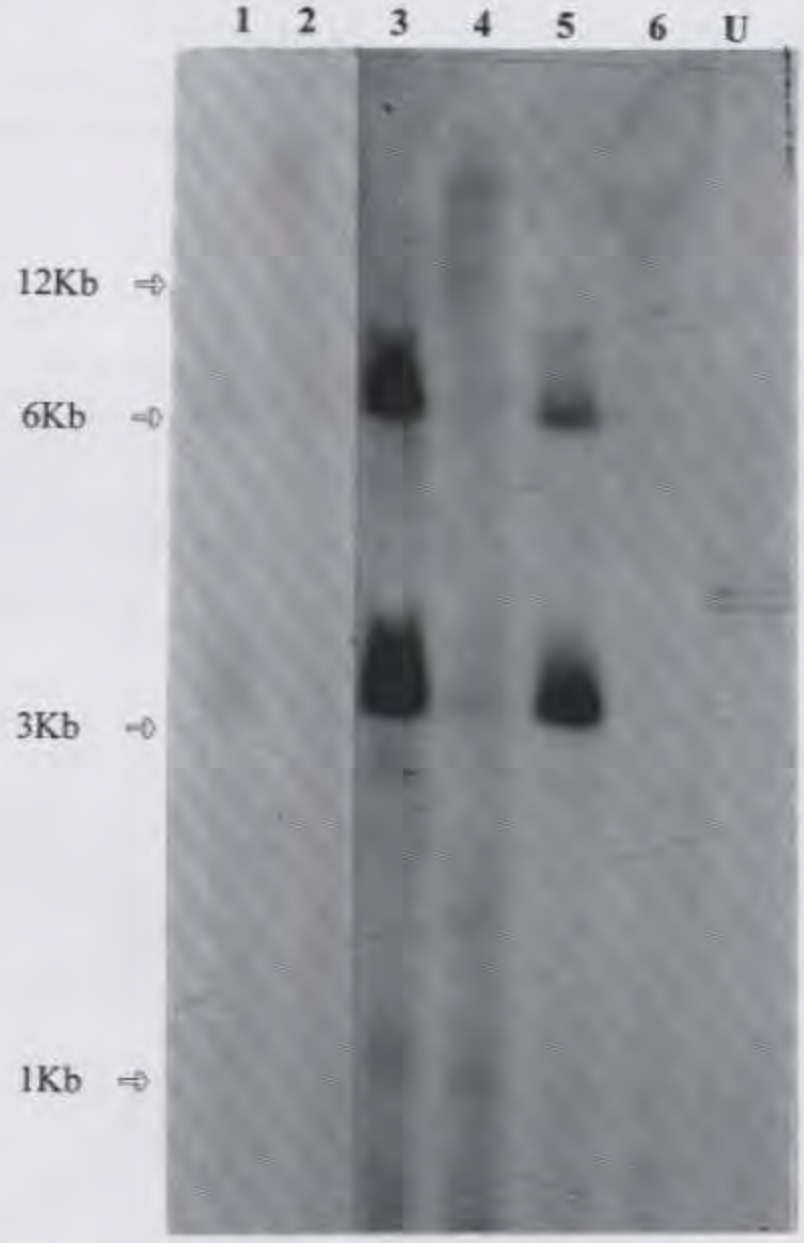

Figure 3. Southern blot hybridization of transgenic oil palm embryogenic callus. Southern blots containing EcoR1 digested DNA (Lane 1,3,5), undigested DNA (Lane 2,4,6) of transformed embryogenic callus and EcoR1 digested DNA of untransformed DNA (Lane U) were hybridized with α ^{32}P-dCTP labelled probe of PCR amplified bar *and* gusA *genes. Hybridization bands were detected in all three transgenic oil palm embryogenic callus lines bombarded with pAHC25.*

CONCLUSION

In this paper, we described the stable integration of foreign genes into the oil palm genome. PCR analysis, Southern blot hybridization and gene expression documented delivery, integration and expression of the transgenes. The production of transgenic oil palm embryogenic callus is a necessary first step towards the production of transgenic oil palm. Experiments to regenerate transgenic plants from these and subsequent experiments are ongoing. Recently, whole plantlets were regenerated from the transgenic embryogenic callus cultures. However, due to the slow growth of the cultures and availability of leaves samples for molecular and protein analysis, the progress will be published later.

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