

TRANSFORMATION OF PHB AND PHBV GENES DRIVEN BY MAIZE UBIQUITIN PROMOTER INTO OIL PALM FOR THE PRODUCTION OF BIODEGRADABLE PLASTICS

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ABSTRACT

Three bacterial genes coding for the enzymes, 3-ketothiolase (bktB), acetoacetyl-CoA reductase (phaB) and PHB synthase (phaC), required for the synthesis of PHB from acetyl-CoA in bacteria were transformed into oil palm embryogenic calli. For the production of copolymer polyhydroxybutyrate-co-valerate (PHBV), the threonine dehydratase (tdcB) gene from Escherichia coli was also transformed into oil palm embryogenic calli for producing propionyl-CoA, the substrate for hydroxyvalerate. These genes were under the control of the maize ubiquitin promoter. Currently, many transformed embryogenic lines resistant to the herbicide Basta have already been produced. These transformed calli were later regenerated to produce a few hundred plantlets which are now growing in a biosafety greenhouse. Molecular analyses have demonstrated stable integration of the transgenes in their genome.

Keywords: biodegradable plastics, oil palm, transgenic, biolistics.

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INTRODUCTION

The world's first transgenic plant, tobacco, was reported almost 25 years ago (Fraley *et al.*, 1983). Since then the number of transgenic plants has increased exponentially. It was recently reported by the International Service for Acquisition of Agri-biotech Applications (ISAAA) that the area commercially planted with transgenic plants worldwide has increased 60-fold, from 1.7 million hectares in 1996 to 102 million hectares in 2006 (James, 2006). This is contributed by 22 countries and

involves the following crops soyabean, maize, cotton, rice, papaya, squash, canola and alfalfa.

Genetic engineering has been proven to be a useful tool, to produce more environmental-adaptive crops and novel high value products in their yield. One of the products in great demand is thermoplastics, or biodegradable plastics. Polyhydroxybutyrate (PHB), a biodegradable plastic, is the most common polyhydroxyalkanoate (PHA) produced as storage material by bacteria under restricted growth conditions (Senior and Dawes, 1973). PHA was first discovered almost 80 years ago but only in the last few decades have its thermoplastic and elastomeric properties been recognized (Poirier, 2002). Biodegradable plastics can be degraded completely in relatively quick time to CO₂ and H₂O under optimal conditions (Lössl *et al.*, 2003). PHA can be used to produce a wide range of environmental-friendly industrial polymers. PHB is stiff and brittle in nature (Holmes, 1988) but has similar chemical and physical properties as polypropylene (Steinbüchel, 1991). Moreover, its melting point is only 10°C lower than its degrading

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point, making it a difficult raw material to use. On the other hand, the co-polymer PHBV is more crystalline and has a much higher difference between its melting points. PHBV is also less stiff than PHB and tougher (Holmes, 1988).

PHB is derived from acetyl-CoA following three enzymatic reactions. The first enzyme, 3-ketothiolase, catalyses the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase subsequently reduces the acetoacetyl-CoA to D-(-)-3-hydroxybutyryl-CoA, which is then polymerized by PHB synthase to PHB (Anderson and Dawes, 1990) (Figure 1). On the other hand, propionyl-CoA is required for the synthesis of PHBV. The threonine deaminase (*ilvA*) or threonine dehydratase (*tdcB*) gene can convert the plant's threonine pool to propionyl-CoA by catalyzing the conversion of threonine to 2-ketobutyrate, and then to propionyl-CoA by the native plant pyruvate dehydrogenase complex (PDC).

An initial effort to produce PHA through bacterial (*Ralstonia eutropha*) fermentation was made by Imperial Chemical Industries (ICI) in the 1980s (Anderson and Dawes, 1990). However, the cost for producing the more useful PHBV was very high due to the need to supply propionate into the fermentation medium. Therefore, the commercial production PHBV (known as Biopol™) was only limited as it cost 5-10 times more than to produce it from petroleum-based polymers [about RM 0.25-0.5 kg⁻¹] (Poirier *et al.*, 1995). Therefore, cheaper alternative was sought. Plants have been proposed as a potential system as they produce oils and starch at RM 0.25-1.0 kg⁻¹ (Poirier, 2002).

The first production of PHB in plant cells was demonstrated in *Arabidopsis* cytoplasm using only the reductase and synthase genes (Poirier *et al.*, 1992). The ketothiolase gene was not transformed because it is endogenous in all plants. The highest amount of PHB synthesized was only 0.1% of the plant dwt. Later, in another report, three PHB genes were transformed into cotton using a fibre-specific promoter, up to 0.3% dwt PHB was reported to be synthesized in the cytoplasm (John and Keller, 1996).

Judging from the low amount of PHB synthesised in cytoplasm, targeting the genes into the plastid was proposed to increase yield of polymer (Nawrath *et al.*, 1994a). This was based on the observation that fatty acid synthesis in plants occurs in the plastid which therefore have the highest flux of acetyl-CoA for synthesis into PHB. It was also proposed that as the high level of starch in the plastid does not affect its function, the accumulation of PHB should not also interfere with the plastid function (Nawrath *et al.*, 1994a). Thus, when all the PHB genes were targeted into the plastid of *Arabidopsis*, PHB accumulation was increased up to 14% dwt (Nawrath *et al.*, 1994b). It was reported that all the PHB granules were contained in the plastids and they had similar properties to PHB produced in bacterial cells. As all the plants reported above were obtained after crossing three transgenic lines carrying the different PHB genes, a low yield of PHB was obtained as the results of the differential expression level and position effects of the three genes. Later, all the three genes were put onto a single plasmid and the resulting transgenic *Arabidopsis* synthesized PHB up to ~40% dwt (Bohmert *et al.*, 2000), a yield postulated to be the maximum for the plant.

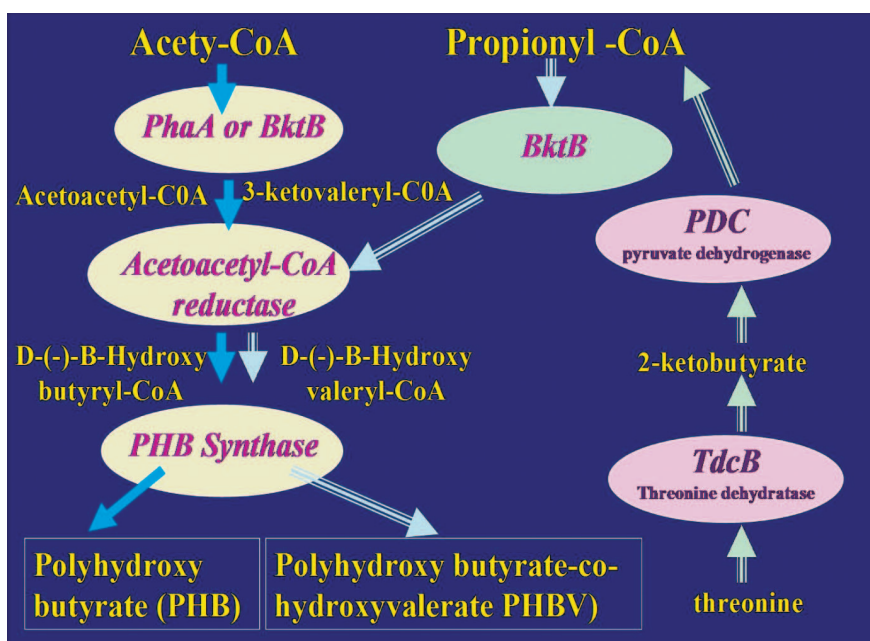


Figure 1. Simplified pathway for biosynthesis of polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHBV) in oil palm.

It was reported that besides cytoplasm, PHB is also produced in other compartments, such as the leukoplasts and peroxisomes. In rape, targeting PHB genes into the seeds peroxisomes resulted in PHB accumulating to 3%-7% dwt (Houmiel *et al.*, 1999). Concurrently, Hahn and colleagues (1999) reported that targeting PHB genes to the leucoplasts of Black Mexican Sweet maize, resulted in accumulation of PHB up to 2% dwt.

Besides producing the common PHB, synthesis of the copolymer, PHBV, was also demonstrated in transgenic rape and *Arabidopsis* (Slater *et al.*, 1999). Using the *bktB*, *phaB*, *phaC* and *ilvA* genes driven by the constitutive promoter (CaMV 35S) and targeted to the plastid, the researchers synthesized PHBV up to 1.6% dwt in transgenic *Arabidopsis*. Similarly, using a seed-specific promoter and targeting the genes to the plastid in rape resulted in accumulation of up to 2.3% dwt of PHBV.

This article will elaborate on transforming of two constructs for the PHB and PHBV genes driven by maize ubiquitin promoters into embryogenic calli of oil palm. Three genes for synthesizing PHB from the bacterium *Alcaligenes eutrophus* H16 (Peoples and Sinskey, 1989a,b,c) were used with addition of the *tdcB* gene from *E. coli* (Guillouet *et al.*, 1999) for PHBV. This research was carried out as part of Malaysia-Massachusetts Institute of Technology Biotechnology Partnership Programme or MMBPP (Parveez *et al.*, 1999). It is important to clarify at this point that oil palm genetic engineering is mainly for research. The research is carried out in laboratories and the transgenic oil palms are planted in a fully contained biosafety greenhouse for evaluation. The eventual commercial planting of transgenic oil palm will be dictated by the economic demands and public sentiment on genetically modified organisms (GMOs) (Parveez, 2003).

MATERIALS AND METHODS

Large Scale Plasmid Isolation

One millilitre overnight *E. coli* culture containing the desired PHB or PHBV gene constructs was inoculated into 500 ml LB medium (5 g NaCl, 5 g tryptone and 2.5 g yeast extract) containing 75 µg/ml antibiotic (ampicillin). The overnight culture was transferred into large centrifuge bottles and the cells were pelleted by centrifugation (4000 rpm, 10 min and 4°C). DNA isolation was carried out using the QIAGEN Maxiprep kit. The plasmid DNA was dissolved in 1 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The concentration and purity of the plasmid were determined using a spectrophotometer. About 300 - 500 µg per 100 ml DNA with good purity (A260/280 > 1.8 - 2.0) were obtained. The DNA quality was further verified by restriction digests followed by electrophoresis on 1% agarose gel.

DNA-microcarrier Preparation

DNA precipitation onto gold microcarriers was carried out according to the manufacturer's instructions for the Biolistics PDS/He 1000 (Bio-Rad) device. Five microlitres of DNA solution (1 µg ml⁻¹), 50 µl CaCl₂ (2.5 M) and 20 µl spermidine (0.1 M, free base form) were added sequentially to the 50 µl gold microcarrier suspension. The mixture was vortexed for 3 min, spun for 10 s in a microfuge and the supernatant discarded. The pellet was washed with 250 µl absolute ethanol. The final pellet was resuspended in 60 µl absolute ethanol. Six microlitres of the solution were loaded into the centre of the macrocarrier and air dried. Bombardment was carried out once at the following conditions: 1100 psi rupture disc pressure, 6 mm rupture disc to macrocarrier distance, 11 mm macrocarrier to stopping plate distance, 75 mm stopping plate to target tissue distance and 67.5 mmHg vacuum pressure.

Maintenance of Embryogenic Callus

Embryogenic calli were maintained on agar-solidified medium containing MS macro and micronutrients supplemented with 2.2 mg litre⁻¹ 2,4-D and 30 g litre⁻¹ sucrose. The medium was adjusted to pH 5.7 with KOH prior to autoclaving. Embryogenic calli were cultured at 28°C in the dark, and subcultured every 30 days onto fresh medium.

Selection and Regeneration of Transformed Embryogenic Callus

The minimal inhibitory concentrations of the selection agents for oil palm had been determined previously (Parveez *et al.*, 1996). The bombarded embryogenic calli were exposed to a medium containing 50 mg litre⁻¹ Basta after one week. The tissues were subcultured onto fresh medium under selection at monthly intervals. The embryogenic cultures were transferred onto media containing MS macro and micronutrients and Y₃ vitamins supplemented with 100 mg litre⁻¹ each of myo-inositol, L-glutamine, L-arginine and L-asparagine, 5 µM IBA, 0.7% agar and 30 g litre⁻¹ sucrose (pH 5.7) to form polyembryogenic cultures. The embryogenic calli were incubated at 28°C in light and subcultured every 30 days onto fresh medium. Small plantlets were produced from the polyembryogenic cultures on a medium containing MS macro and micronutrients and Y₃ vitamins supplemented with 100 mg litre⁻¹ each of myo-inositol, L-glutamine, L-arginine and L-asparagine, 0.1 µM NAA, 0.4% agar and 30 g litre⁻¹ sucrose (pH 5.7). Polyembryogenic calli were incubated at 28°C in light until sufficient shoots were produced. Roots were initiated from small plantlets on a medium containing MS macro and micronutrients and Y₃ vitamins supplemented

with 300 mg litre⁻¹ L-glutamine, 100 mg litre⁻¹ myo-inositol, 10 µM 2,4-D, 70 µM NAA, 0.15% activated charcoal and 60 g litre⁻¹ sucrose (pH 5.7). The small plantlets were incubated at 28°C in light until roots were formed. The full regenerated plantlets were later transferred into polybags and grown in a biosafety screenhouse.

Small-scale Preparation of Total Plant DNA

Resistant embryoids and leaflets were selected randomly and subjected to total DNA isolation according to the method of Ellis (1993). Tissues (10-50 mg) were placed in a 1.5 ml microfuge tube and immersed in liquid nitrogen. The frozen tissues were ground to a fine powder in the presence of 400 µl EB2 buffer (500 mM NaCl, 100 mM Tris-HCl {pH 8.0} and 50 mM EDTA {pH 8.0}) and 20 µl 20% SDS. Four hundred µl phenol mix (1:1; phenol:chloroform) were then added, thoroughly mixed and centrifuged (12 000 rpm, 2 min). The aqueous phase was transferred into a new tube and mixed with 800 µl absolute ethanol. DNA was recovered by centrifugation (12 000 rpm, 5 min). The pellet was washed with 70% ethanol and dissolved in 50 µl TE buffer.

Large-scale Preparation of Total Plant DNA

Total DNA extraction was carried out on plantlets according to the modified CTAB method (Doyle and Doyle, 1987). Samples (1-10 g) were chilled and grounded in liquid nitrogen. Once the sample was properly grounded, 10 ml CTAB extraction buffer (100 mM Tris-HCl; pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% CTAB; 1% PVP and 0.2% 2-mercaptoethanol) were added. The sample was then incubated at 65°C for 1 hr. After 20 min at room temperature, 10 ml chloroform: isoamyl alcohol (24:1) were added and the sample centrifuged at 4°C, 13 000 rpm for 5 min. The aqueous phase was transferred into a new tube. The sample was extracted twice with 5 ml chloroform: isoamyl alcohol (24:1). DNA was precipitated by the addition of 6 ml chilled isopropanol. After 20 min at room temperature, the sample was centrifuged at 13 000 rpm for 5 min. The supernatant was discarded and pellet was air-dried at room temperature. The pellet was washed using Washing Buffer (76% ethanol, 1 mM ammonium acetate) and incubated at room temperature for 20 min before being centrifuged at 13 000 rpm for 5 min. The pellet obtained was dried and dissolved in 500 µl TE Buffer.

Polymerase Chain Reaction (PCR)

Amplification of the *bar*, PHB and PHBV genes was carried out using standard or touch-down PCR

protocols (Sambrook *et al.*, 1989). Fifty ng oil palm DNA and 1 ng plasmid DNA were used in PCR reactions. The following amplification conditions were used in the standard procedure: 30 cycles at 92°C (50 s), 60°C (50 s) and 72°C (60 s). For the touch down procedure, 10 cycles at 92°C (45 s), 70°C (45 s; -0.5°C per cycle), 72°C (60 s) and 20 cycles 92°C (45 s), 65°C (45 s) and 72°C (60 s) were used. The amplified DNA fragments were resolved by electrophoresis on 1.4% agarose gel in 0.5X TBE (45 mM Tris-Borate; 1 mM EDTA, pH 8.0) buffer.

Total RNA Extraction

RNA from the transgenic samples was extracted using a method modified from Prescott and Martin (1987). The 10 g frozen leaf tissue were powdered in a mortar in liquid nitrogen and then homogenized with the extraction buffer [0.05 M Tris-HCl (pH 8.5), 0.15 M LiCl, 5 mM EDTA, 5% SDS, 0.1 M aurintricarboxylic acid, 0.4% β-mercaptoethanol] in a GSA tube (Sorvall). The buffer was added to the sample at a ratio of 1:3 (w/v). A half volume of phenol (pH 8.0) and a half volume of chloroform were added to the homogenate and the phases were separated by centrifugation at 13 000 rpm (25°C) for 30 min. The aqueous layer was removed to a new centrifuge tube and re-extracted with another half volume of phenol (pH 8.0) and half volume of chloroform. The aqueous layer was transferred to a new centrifuge tube and an equal volume of chloroform: isoamylalcohol (24:1) added before the mixture was vortexed and centrifuged for 30 min at 13 000 rpm (25°C). The aqueous layer was transferred to a new centrifuge tube and 8 M LiCl added to a final concentration of 2 M. The mixture was mixed by inversion and stood overnight at 4°C to precipitate the RNA. The RNA was pelleted by centrifugation at 13 000 (4°C) for 30 min. The supernatant was discarded and the pellet resuspended in 1.5 ml 2 M LiCl. The mixture was vortexed and spun again at 13 000 rpm for 30 min. The supernatant was discarded and the pellet dissolved in 5ml RNase-free water. The 8 M LiCl was added to the mixture to a final concentration of 2 M, mixed and stored at 4°C overnight to precipitate the RNA. The RNA was pelleted by centrifugation at 13 000 rpm (4°C) for 30 min. The pellet was then rinsed with 4 ml 2 M LiCl. The supernatant was discarded and 1 ml RNase-free water added to resuspend the pellet. The mixture was centrifuged at 10 000 rpm for 5 min to pelletize the insoluble materials. The supernatant was removed to a new SS34 tube and 1/19 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol added to the mixture. The mixture was mixed and stored at -80°C at least 2 hr to precipitate the RNA. The RNA was pelleted by centrifugation at 13 000 rpm (4°C) for 10 min. The supernatant was discarded and

the pellet rinsed two times in 1 ml 70% cold ethanol and dried under vacuum. The RNA was dissolved in RNase-free water and stored at -80°C until use.

Total RNA Extraction from *Ralstonia eutropha*

A single colony of *R. eutropha* from a Tryptic Soy Broth (TSB) plate containing 10 µg ml⁻¹ gentamycin was cultivated in 5 ml TSB broth, containing 10 µg ml⁻¹ gentamycin, at 30°C for 24 hr. Then, 100 µl of the culture were transferred into 5 ml new TSB broth and grown for another 16 hr. The cells were pelleted by centrifugation, washed with 0.9% saline and transferred into 5 ml PHB production medium (PHA-high medium) to yield cultures with an initial OD₆₀₀ of 1.0. The cells were cultured for another 48 hr.

Extraction was carried out according to the modified method of Ausubel *et al.* (1987), suitable for gram negative bacteria. Ten millilitres bacterial cultures were centrifuged at 10000 rpm for 10 min. The pellet was resuspended in 10 ml protoplasting buffer [15 mM Tris-HCl (pH 8.0), 0.45 M sucrose and 8 mM EDTA (pH 8.0)]. Then, 80 µl 50 mg ml⁻¹ lysozyme were added and the mixture incubated for 15 min on ice. The protoplast was collected by centrifugation at 7000 rpm (5 min) and resuspended in 0.5 ml gram negative lysing buffer [10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM sodium citrate and 1.5% sodium dodecyl sulphate]. Fifteen µl aurintricarboxylic acid were added, mixed gently and incubated at 37°C for 5 min. Then, the mixture was chilled on ice and 0.5 ml saturated NaCl added to the mixture. The tube was upturned several times, incubated on ice for 10 min and centrifuged for 10 min at 10000 rpm (4°C). The supernatant was removed to a new tube and precipitated with 1 ml 100% ethanol overnight at -20°C. The RNA was pelleted by centrifugation for 30 min at 9000 rpm (4°C), washed twice with 1 ml 70% ethanol, air-dried and resuspended in RNase-free dH₂O.

Northern Hybridization

The 30 µg total RNA were transferred from 1% agarose-formaldehyde gel to Hybond-N membrane using 20X SSC as the transfer buffer and UV crosslink. Specific probes were randomly prime labelled with α-32P using a klenow fragment and hybridized to the membrane. Hybridization was carried out overnight at 65°C in hybridization buffer (5X SSC, 5X Denhardt, 0.5% SDS). The membranes were washed at 65°C in 2X SSC and 0.1% SDS for 15 min and once in 1X SSC and 0.1% SDS for 10 min, then exposed to X-ray film at -80°C and developed.

RESULTS

Regeneration of Transgenic Oil Palm Plantlets

The optimized conditions for DNA delivery into oil palm embryogenic calli, via microprojectile bombardment, have been determined earlier (Parveez *et al.*, 1997; 1998). Using the conditions, oil palm embryogenic calli were bombarded with two plasmids carrying the *bar* and PHB or PHBV genes (Willis *et al.*, 2008). Embryogenic calli bombarded without DNA were used as the controls. In this study, all the three PHB genes (*bktB*, *phaB* and *phaC*) or four genes for PHBV (*bktB*, *phaB*, *phaC* and *tdcB*) were individually attached to a transit peptide of a small subunit of rubisco from pea (Nawrath *et al.*, 1994b). All the PHB, PHBV genes and selectable marker gene (*bar*) were driven by the maize ubiquitin promoter and its intron (Christensen *et al.*, 1992). Schematic diagrams of the PHB and PHBV constructs are given in Figure 2.

The bombarded tissues were first cultured on embryogenic medium in the absence of selection agent for three to four weeks. Upon transfer to fresh medium containing the selection agent (50 mg litre⁻¹

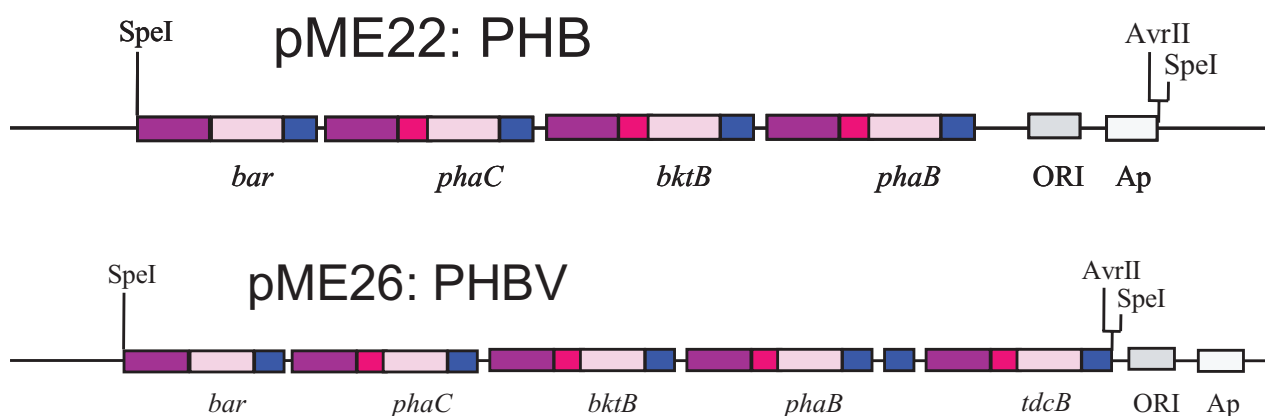


Figure 2. Schematic diagram of plasmids used for transformation. Plasmid pME22 (top) carries the *bar*, *phaC*, *bktB* and *phaB* genes, all driven by the maize ubiquitin promoter for synthesizing PHB. Plasmid pME26 (bottom) carries the *bar*, *phaC*, *bktB*, *phaB* and *tdcB* genes, all driven by maize ubiquitin promoter for synthesizing PHBV. All the *phaC*, *bktB*, *phaB* and *tdcB* genes are fused at the 5' end with a transit peptide of a small subunit of rubisco from pea.

herbicide Basta), the untransformed embryogenic calli began to die, allowing only the resistant embryogenic calli to proliferate. The transformed embryogenic calli were subcultured onto fresh medium containing the selection agent, once a month. The Basta-resistant embryogenic callus colonies normally appeared after five to six months on selection medium. The resistant colonies were later proliferated and regenerated to produce transgenic oil palms.

In this study, the bombardment of oil palm embryogenic calli resulted in 100 and 17 transformation events for the PHB and PHBV plasmids, respectively. Similarly, 265 and 20 plantlets were produced from the bombardment using the PHB and PHBV plasmids, respectively. The plantlets were transferred into soil in small polybags and grown in a biosafety greenhouse (Figure 3).

Molecular Analyses on Transgenic Oil Palm Cultures and Plantlets

Polymerase chain reaction (PCR). Recovering resistant embryogenic callus surviving on selection medium is not adequate proof of stable integration of transgenes into the plant genome. Molecular analyses are required to confirm this. Genomic DNA was extracted from transgenic cultures and leaves using either the Ellis (1993) mini preparation method or CTAB method (Doyle and Doyle, 1987). DNA from a number of embryoids or plantlets (originating from a few differently resistant embryogenic calli clumps) were isolated and subjected to PCR analysis. DNA from untransformed plants was also isolated and used as negative controls. Initially, to obtain reliable

PCR results, amplification of an internal control fragment was carried out, using a pair of primers which specifically amplified a 1 Kb size fragment of oil palm genomic DNA (Cheah, personal communication). All the samples (including the untransformed control) were subjected to amplification using the internal control, which should also amplify a control fragment. If the amplification failed to show the PCR product, the DNA samples were further purified until the control fragment could be amplified. The internal control is important to ensure that any sample which failed to amplify the transgenes was actually untransformed and not due to impure DNA or an inadequate amount of DNA used. Samples with positive internal control were later subjected to PCR analysis using the *bar* gene that confers resistance against Basta used as the selection agent.

The samples showing positive amplification of the *bar* gene were later subjected to amplification of their PHB and PHBV genes. Amplification of the *bktB*, *phaB*, *phaC* and *tdcB* genes was carried out using the following primer sets: *bktB*-F/*bktB*-R, *phaB*-F/*phaB*-R, *phaC*-F/*phaC*-R and *tdcB*-F/*tdcB*-R, respectively. The primers were designed to contain almost the whole sequence of the genes used for transformation. PCR amplification of the *bktB* gene using the *bktB*-F/*bktB*-R primers was expected to result in an 1185 bp fragment, which was obtained when the transforming plasmid was used as template. No amplification of the gene was observed for the negative control. It was shown that 41 transformed lines were positive for the *bktB* gene out of the 59 samples analysed, or 69%.



Figure 3. Transgenic oil palm plantlets carrying the polyhydroxybutyrate (PHB) or polyhydroxybuturate (PHBV) gene in a biosafety nursery.

PCR amplification of the *phaB* gene using the *phaB*-F/*phaB*-R primers was expected to result in a 741 bp fragment. PCR analysis using the primers indicated that 51 out of 59 transgenic oil palm samples were positive for the *phaB* gene. Similarly, the expected 741 bp band of the *phaB* gene was amplified when the transforming plasmids were used. In this case, 86% of the *bar* gene positive samples were also positive for the *phaB* gene. When *phaC*-F/*phaC*-R primers were used to amplify the *phaC* gene, a 1770 bp fragment was expected, and 49 out of 59 of the transgenic oil palm samples were positive for it (Figure 4) or 83%.

Finally, for the samples transformed with the PHBV gene construct, the *tdcB*-F/*tdcB*-R primers were used to amplify a 990 bp fragment of the *tdcB* gene. It was demonstrated that beside the positive control, two out of seven, the PHBV-transformed samples tested showed the amplification or 29%. Overall, with the exception of the *tdcB* gene, 70% to 85% of the unselected PHB genes were co-integrated with the *bar* gene used for selection. This high frequency of co-integration was not surprising since all the genes were linked on the same transforming plasmid.

Southern Blot Hybridization

PCR analysis of the *bar*, *bktB*, *phaB*, *phaC* and *tdcB* genes indicated their presence in the DNA samples from the transgenic oil palms tested. However, the PCR results were not definitive evidence of stable integration of the transgenes in the host genome.

Potrykus (1990) stated that a number of factors needs be determined before a 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. This will be carried out later.

Total RNA Isolation and Northern Blot Analysis

Total RNA from oil palm green leaves was initially isolated using five isolation methods [modified Hosein (2001); Ausubel *et al.*, (1987); modified Prescott and Martin (1987), López-Gómez and Gómez-Lim (1992) and, Zeng and Yang (2002)]. Based on the consistency of the RNA yield and cleanliness, the modified method of Zeng and Yang (2002) was the most suitable for oil palm. Using this method, 60-120 µg total RNA were consistently obtained from 1 g leaf. The purity of the RNA samples (A_{260}/A_{280} ratio) was usually 1.8-2.0 with the ratio always > 1.7. Similarly, for comparison, total RNA isolation from a wild type PHB producing bacterium (*Ralstonia eutropha*) was also carried out using the method by Ausubel *et al.* (1987).

Northern hybridization for the *phaB*, *phaC* and *bktB* genes did not show any positive signal although there was a signal for Ribosomal probe indicating that the hybridization had been successful (data not shown). The negative results for the PHB gene hybridization may be due to the low abundance of

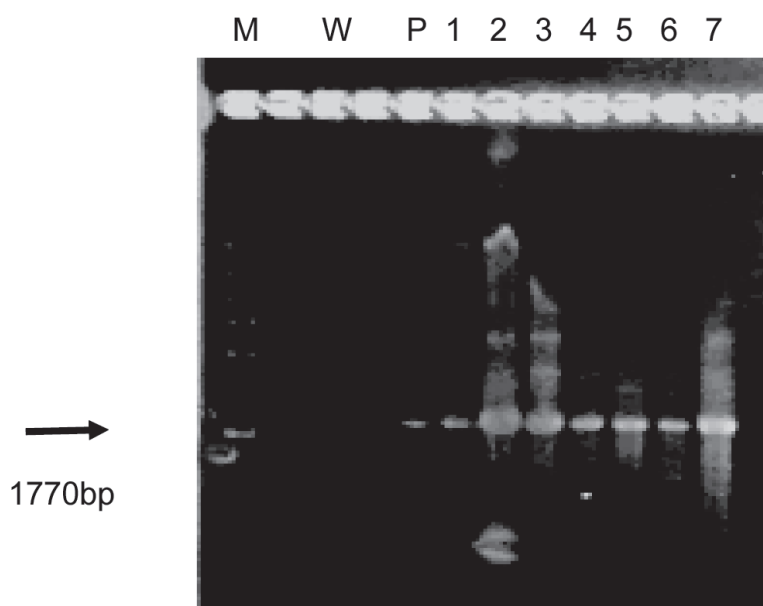


Figure 4. PCR analysis on DNA from transformed oil palm plantlets using primer pairs to amplify the *phaC* gene. The expected band size – 1770 bp for *phaC* is indicated by the arrows. Lane M = 1 kb plus DNA ladder marker; W = water (negative control); P = plasmid control (positive control pME22 or pME26); 1-7 = oil palm samples transformed with PHB or PHBV genes.

the target gene, or that the gene was not expressed. In order to confirm the expression of the transgenes, a more sensitive method, namely, real-time PCR, was proposed to be carried out later.

DISCUSSION

Resistant embryogenic calli obtained after Basta selection were regenerated to plantlets. The plantlets were transferred to soil and grown in a biosafety greenhouse and all were normal phenotypically with normal growth. None of them have so far showed any negative effect. Molecular analyses of the transgenic plantlets, using PCR has demonstrated the integration and expression of the transgene in some of the transformants tested.

Evaluation in the biosafety nursery demonstrated that none of the plantlets showed any negative effect from the accumulation of PHB. It is suggested here that the normal appearance of the transgenic oil palm may be due to the low amount of the PHB synthesized in oil palm samples tested. This could be answered after HPLC analysis being carried out on the plantlets. It was reported in several plants that high PHB is detrimental to their growth and morphology.

In this study, all the three PHB genes or four PHBV genes were fused together into one vector, attached with a plastid-targeting sequence and transit peptide of the pea rubisco small subunit (Nawrath *et al.*, 1994b; Zhong *et al.*, 2003), and driven by the same maize ubiquitin promoter (Christiansen *et al.*, 1992). This strategy was chosen because in other plants, it could cause the accumulation of PHB. Using multi-gene vectors (all genes linked on a single vector) raised the level of PHB over that by a single-gene vector which required crossing to bring together the genes (Bohmert *et al.*, 2000, Valentin *et al.*, 1999; Mitsky *et al.*, 2000). Having all the genes closely linked in the vector will likely result in them being more closely integrated into the genome. This is postulated to allow for their higher expression and reduce their silencing (Mitsky *et al.*, 2000). However, in transgenic alfalfa, fusing all the genes on the same plasmid did not produce the high amounts of PHB as reported in *Arabidopsis* (Saruul *et al.*, 2002).

Further, targeting the genes into the plastid of oil palm would help to synthesize more PHB as the substrate, acetyl-CoA, is abundant in the organelle. It was postulated that as fatty acids in plants are synthesized in the plastid, it should be the site with a large flux of carbon (acetyl-CoA), stored as lipids (Poirier, 2002).

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REFERENCES

- ANDERSON, A J and DAWES, E A (1990). Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.*, 54: 450-472.
- AUSUBEL, F M; BRENT, R; KINGSTON, R E; MOORE, D D; SEIDMAN, J G; SMITH, J A and STRUHL, K (1987). *Current Protocols in Molecular Biology*. Wiley, New York.
- BOHMERT, K; BALBO, I; KOPKA, J; MITTENDORF, V; NAWRATH, C; POIRIER, Y; TISCHENDORF, G; TRETHEWEY, R N and WILLMITZER, L (2000). Transgenic *Arabidopsis* plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. *Planta*, 211: 841-845.
- CHRISTIENSEN, A H; SHARROCK, R A and QUAIL, P H (1992). Maize polyubiquitine genes: thermal perturbation of expression and transcript splicing and promoter activity following transfer to protoplast by electroporation. *Plant Mol. Biol.*, 18: 675-689.
- DOYLE, J J and DOYLE, J L (1987). Isolation of plant DNA from fresh tissue. *Focus*, 12(1): 13-15.
- ELLIS, T H N (1993). Approaches to the genetic mapping of pea. Modern methods of plant analysis. *Vegetables and Vegetable Prod.*, 16: 117-160.
- FRALEY, R T; ROGERS, S G; HORSCH, R B; SANDERS, P R and FLICK, J S (1983). Expression of bacterial genes in plant cell. *Proc. Natl. Acad. Sci. USA*, 80: 4803-4807.

- GUILLOUET, S; RODAL, A A; AN, G H; LESSARD, P A and SINSKEY, A J (1999). Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. *Applied and Environmental Microbiology*, 65: 3100-3107.
- HAHN, J J; ESCHENLAUER, A C; SLEYTR, U B; SOMERS, D A and SRIENC, F (1999). Peroxisomes as sites for synthesis of polyhydroxyalkanoates in transgenic plants. *Biotechnology Progress*, 15: 1053-1057.
- HOLMES, P A (1988). Biologically produced (R)-3-hydroxyalkanoate polymers and co-polymers. *Development in Crystalline Polymers-2* (Basset, D C ed.). Elsevier, London. p. 1-65.
- HOSEIN, F (2001). Isolation of high quality RNA from seeds and tubers of the mexican yam bean, *Pachyrhizus erosus*. *Plant Mol. Biol. Reporter*, 19: 65a-65e.
- HOUMIEL, K L; SLATER, S; BROYLES, D; CASAGRANDE, L; COLBURN, S; GONZALEZ, K; MITSKY, T A; REISER, S E; SHAH, D; TAYLOR, N B; TRAN, M; VALENTIN, H E and GRUYS, K J (1999). Polyhydroxybutyrate production in oilseed leukoplasts of *Brassicca napus*. *Planta*, 209: 547-550.
- JAMES, C (2006). Global status of commercialized biotech/GM crops: 2006. *ISAAA Briefs No. 35-2006*. ISAAA, Ithaca, NY, USA.
- JOHN, M E and KELLER, G (1996). Metabolic pathway engineering in cotton. Biosynthesis of polyhydroxybutyrate in fiber cells. *Proc. Natl. Acad. Sci. USA*, 93: 12768-12773.
- LÓPEZ-GÓMEZ, R and GÓMEZ-LIM, M A (1992). A method for extracting intact RNA from fruit rich in polysaccharides using ripe mango mesocarp. *HortScience*, 27 (5): 440-442.
- LÖSSL, A; EIBL, C; HARLOFF, H J; JUNG, C and KOOP, H H (2003). Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. *Plant Cell Rep*, 21: 891-899.
- MITSKY, T A; SLATER, S C; REISER, S E; HAO, M and HOUMIEL, K L (2000). Multigene expression vectors for the biosynthesis of products via multienzyme biological pathways. PTC Publication Number: WO/2000/052183.
- NAWRATH, C; POIRIER, Y and SOMERVILLE, C R (1994a). Plastid targeting of the enzymes required for the production of polyhydroxybutyrate in higher plants. *Biodegradable Plastics and Polymers* (Doi, Y and Fukuda, K ed.). Elsevier, Amsterdam, p. 136-149.
- NAWRATH, C; POIRIER, Y and SOMERVILLE, C (1994b). Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proc. Nalt. Acad. Sci. USA*, 91: 12760-2764.
- PARVEEZ, G K A; CHOWDHURY, M K U and SALEH, N M (1996). Determination of minimal inhibitory concentration of selection agents for oil palm (*Elaeis guineensis* Jacq.) transformation. *Asia-Pacific J. Molecular Biology and Biotechnology*, 4: 219-228.
- PARVEEZ, G K A; CHOWDHURY, M K U and SALEH, N M (1997). Physical parameters affecting transient GUS gene expression in oil palm using the biolistics device. *Industrial Crops and Product*, 6: 41-50.
- PARVEEZ, G K A; CHOWDHURY, M K U and SALEH, N M (1998). Biological parameters affecting transient GUS gene expression in oil palm embryogenic calli via microprojectile bombardment. *Industrial Crops and Product*, 8: 17-27.
- PARVEEZ, G K A; SAMBANTHAMURTHI, R; SITI NOR AKMAR, A; RASID, O, MASRI, M M and CHEAH, S C (1999). Production of transgenic oil palm – current success and future considerations. *Proc. of the 1999 PORIM International Palm Oil Congress – Agriculture Conference* (Ariffin, D; Chan, K W and Sharifah, S R S A eds.). p. 3-13.
- PARVEEZ, G K A (2003). Novel products from transgenic oil palm. *AgBiotechNet*, 5 (ABN 113): 1-8.
- PEOPLES, O P and SINSKEY, A J (1989a). Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem*, 264: 15293-15297.
- PEOPLES, O P and SINSKEY, A J (1989b). Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). *J. Biol. Chem*, 264: 15298-15303.
- PEOPLES, O P and SINSKEY, A J (1989c). International patent, WO 89/ 00202.

- POIRIER, Y; DENNIS, D E; KLOMPARENS, K and SOMERVILLE, C (1992). Polyhydroxybutyrate, a biodegradable thermoplastics, production in transgenic plants. *Science*, 256: 520-523.
- POIRIER, Y; NAWRATH, C and SOMERVILLE, C (1995). Production of Polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Bio/Technology*, 13: 142-150.
- POIRIER, Y (2002). Polyhydroxyalkanoate synthesis in plants as tool for biotechnology and basic studies of lipid metabolism. *Prog. Lipid Res.*, 41: 131-155.
- POTRYKUS, I (1990). Gene transfer to cereals: an assessment. *Bio/Technol.*, 8: 535-542.
- PRESCOTT, A and MARTIN, C (1987). A rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant Molecular Biology Reporter*, 4: 219-224.
- SAMBROOK, J; FRITSCH, E F and MANIATIS, T (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
- SARUUL, P; SRIENC, F; SOMERS, D A and SAMAC, D A (2002). Production of a biodegradable plastic polymer, poly- β -hydroxybutyrate, in transgenic alfalfa. *Crop Sci.*, 42: 919-927.
- SENIOR, P J and DAWES, E A (1973). The regulation of poly- β -hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochem. J.*, 134: 225-238.
- SLATER, S; MITSKY, T A; HOUMIEL, K L; HAO, M; REISER, S E; TAYLOR, N B; TRAN, M; VALENTIN, H E; RODRIGUEZ, D J; STONE, D A; PEDGATE, S R; KISHORE, G and GRUYS, K J (1999). Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. *Nature Biotechnology*, 17: 1011-1016.
- STEINBÜCHEL, A (1991). Recent advances in the knowledge of bacterial poly(hydroxy-alkanoic acid) metabolism and potential impacts on the production of biodegradable thermoplastics. *Acta Biotechnologica*, 11: 419-427.
- VALENTIN, H E; BROYLES, D L; CASAGRANDE, L A; COLBURN, S M; CREELY, W L; DELAQUIL, P A; FELTON, H M; GONZALEZ, K A; HOUMIEL, K L; LUTKE, K; MAHADEO, D A; MITSKY, T A; PADGETTE, S R; REISER, S E; SLATER, S; STARK, D M; STOCK, R T; STONE, D A; TAYLOR, N B; THORNE, G M; TRAN, M; and GRUYS, K J (1999). PHA production, from bacteria to plants. *Int. J. Biol. Macromol.*, 25:303-306.
- WILLIS, L B; YORK, G M; LESSARD, P L; JO, Y B; OMAR, R; MOHAMMD ARIF, A M; PARK, C and SINSKEY, A J (2008). Reagents for generation and analysis of bioplastic producing plants. *J. Oil Palm Research Special Issue on MMBPP Vol. 2 (July 2008)*: 56-68.
- WRÓBEL, M; ZEBROWSKI, J and SZOPA, J (2004). Polyhydroxybutyrate synthesis in transgenic flax. *J. of Biotechnology*, 107: 41-54.
- ZENG, Y and YANG, T (2002). RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter*, 20: 417a-417e.
- ZHONG, H; TEYMOURI, F; CHAPMAN, B; MAQBOOL, S B; SABZIKAR, R; EL-MAGHRABY, Y; DALE B and STICKLEN, M B (2003). The pea (*Pisum sativum* L.) rbcS transit peptide directs the *Alcaligenes eutrophus* polyhydroxybutyrate enzymes into the maize (*Zea mays* L.) chloroplasts. *Plant Science*, 165: 455-462.