

A transient assay to evaluate the expression of polyhydroxybutyrate genes regulated by oil palm mesocarp-specific promoter

V. Omidvar · A. Siti Nor Akmar · M. Marziah ·
A. A. Maheran

Received: 18 March 2008 / Revised: 20 May 2008 / Accepted: 26 May 2008 / Published online: 19 June 2008
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Abstract The promoter of the oil palm metallothionein-like gene (*MT3-A*) demonstrated mesocarp-specific activity in functional analysis using transient expression assay of reporter gene in bombarded oil palm tissue slices. In order to investigate the tissue-specific expression of polyhydroxybutyrate (PHB) biosynthetic pathway genes, a multi-gene construct carrying PHB genes fused to the oil palm *MT3-A* promoter was co-transferred with a construct carrying GFP reporter gene using microprojectile bombardment targeting the mesocarp and leaf tissues of the oil palm. Transcriptional analysis using RT-PCR revealed successful transcription of all the three *phbA*, *phbB*, and *phbC* genes in transiently transformed mesocarp but not in transiently transformed leaf tissues. Furthermore, all the three expected sizes of PHB-encoded protein products were only detected in transiently transformed mesocarp tissues on a silver stained polyacrylamide gel. Western blot analysis using polyclonal antibody specific for *phbB* product confirmed successful translation of *phbB* mRNA transcript into protein product. This study provided valuable information, supporting the future engineering of PHB-producing transgenic palms.

Keywords Transient expression assay · Microprojectile bombardment · Transgenic plant · Polyhydroxybutyrate · Tissue-specific promoter

Introduction

Polyhydroxyalkanoates (PHA) are polyesters of various hydroxyalkanoates that are synthesized by many gram-positive and gram-negative bacteria. Two important types of PHA are the homopolymer polyhydroxybutyrate (PHB) and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). These polymers are accumulated by bacteria up to the level of 90% of dry weight under conditions of nutrient stress and are utilized as a carbon and energy reserve. Commercial production of PHA is hampered due to the high expenses of the fermentation process and polymer substrates (Poirier 2002). Production of PHA on a large agronomic scale in plant systems is seen as the only potential way for large scale production at a lower cost (Houmiel et al. 1999).

Production of PHB homopolymer has been initially reported in transgenic *Arabidopsis thaliana* (Poirier et al. 1992; Nawrath et al. 1994) and later on was reported in other plants (Slater et al. 1999; Saruul et al. 2002; Lössl et al. 2003), however production of PHBV copolymer has been limited primarily due to the unavailability of the metabolic precursors other than acetyl-CoA (Slater et al. 1999). PHB is synthesized from acetyl-CoA by the sequential action of three enzymes namely, β -ketothiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*), and PHB polymerase (*phbC*) (Fig. 1). Production of PHB in plants is based on the consumption of acetyl-CoA as an initial substrate, therefore oil crops, such as oil palm are potential targets for production of PHB due to their high flux of

Communicated by M. Jordan.

V. Omidvar · A. Siti Nor Akmar (✉) · A. A. Maheran
Department of Agriculture Technology, Faculty of Agriculture,
University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
e-mail: sakmar@agri.upm.edu.my

M. Marziah
Department of Biochemistry, Faculty of Biotechnology
and Biomolecular Science, University Putra Malaysia,
43400 Serdang, Selangor, Malaysia

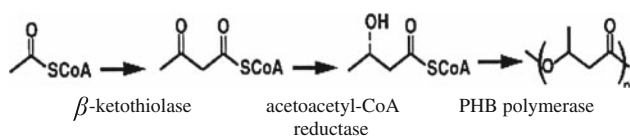


Fig. 1 Polyhydroxybutyrate (PHB) biosynthetic pathway. PHB is synthesized by the sequential action of β -ketothiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*), and PHB polymerase (*phbC*) in a three-step pathway (Madison and Huisman 1992)

acetyl-CoA during the oil synthesis stage (Reddy et al. 2003). The oil palm mesocarp is the site for storage oil synthesis, making it an ideal tissue for production of PHB. Introducing the PHB biosynthetic pathway genes into oil palm and mesocarp-specific expression of these genes under the regulation of mesocarp-specific promoters would be desirable and may create a high potential co-production system to produce PHB with much lower cost. The mesocarp-specific promoter (so-called MSP1; accession no: EU499363) corresponding to the oil palm *MT3-A* gene that is specifically and abundantly expressed in mesocarp (Siti Nor Akmar et al. 2002; Siti Nor Akmar and Zubaidah 2007) is being used to target the specific expression of PHB genes in mesocarp tissue. Construction of multi-genes vectors carrying PHB and PHBV genes under the regulation of MSP1 has been carried out (Masani and Parveez 2003), and transformation of these vectors into oil palm is still in progress.

Commercial production of PHA in plants requires the development of a well-established transformation technique in order to produce transgenic plants that strongly express the transgene and more importantly in the correct site. Furthermore, production of certain foreign molecules especially pharmaceutical products in plants has recently raised many bio-safety concerns (Mascia and Flavell 2004). Transient expression assays are the rapidly expanding techniques, which have opened up new strategies for transgenic studies without delay and environmental risks associated with the production of stable transgenic plants. In many cases, transient expression systems have been shown to provide ultimately the same information as approaches using transgenic plants (Lu et al. 2007).

In this study, the tissue-specificity and strength of the oil palm mesocarp-specific promoter was initially evaluated via transient assay of GUS reporter gene in different oil palm tissues, including mesocarp, leaf, and root. Subsequently, the expression of PHB biosynthetic pathway genes, driven by the activity of MSP1 was evaluated in transiently transformed mesocarp tissue at both transcriptional and translational levels. This is the first report of using the oil palm mesocarp-specific promoter to regulate expression of foreign genes, and the results showed successful tissue-specific engineering of the entire PHB

pathway genes using transiently transformed oil palm tissues as a model system.

Materials and methods

Preparation of target materials for bombardment

Oil palm (*Elaeis guineensis*, D × P) fruits at 12 weeks after anthesis, young leaves, and roots from 3-month-old germinated seedlings were sterilized in 20% Clorox and rinsed thoroughly with sterile distilled water. Mesocarp part of the fruits and leaves were excised to 1 cm × 1 cm slices. Roots were cut into slices of 1 cm in length. The tissue slices were arranged in the center (approximately 3 cm in diameter) of Petri dishes containing Murashige and Skoog medium (Murashige and Skoog 1962). The tissues were kept at 28°C in a growth chamber 1 day prior to the bombardment.

Plasmid constructs

Plasmid constructs were used as follows: *pMS29* plasmid carrying PHB genes (*phbA*, *phbB*, and *phbC*) in fusion to MSP1, constructed by Masani and Parveez (2003), *35SpEGFP* and *pBI221* plasmids carrying GFP and GUS reporter genes, respectively, in fusion to CaMV35S promoter (Clontech) (Fig. 2). The *MSP1pBI221* was constructed by removing the CaMV35S promoter in *pBI221* and replacing with MSP1 using *HindIII* and *SmaI* restriction enzymes. Plasmid DNA constructs were purified using QIAprep[®] Miniprep Plasmid Purification Kit (Qiagen).

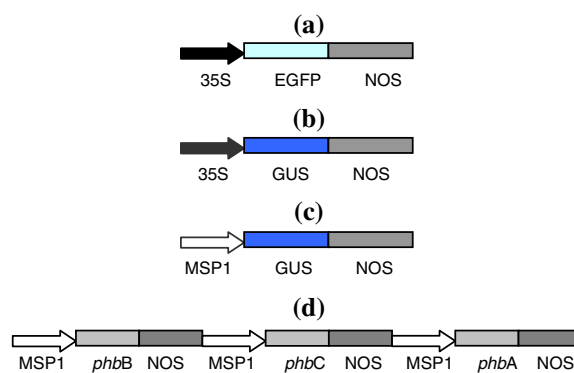


Fig. 2 Schematic diagram of *35SpEGFP* (a), *pBI221* (b), *MSP1pBI221* (c), and *pMS29* (d) plasmids. The *35SpEGFP* vector was constructed by inserting an 800 bp *HindIII*-*SmaI* fragment containing CaMV35S promoter into the MCS of *pEGFP-1* (Clontech). The *MSP1pBI221* was constructed by replacing the CaMV35S promoter with MSP1. The *pMS29* construct was reported by Masani and Parveez (2003); β -ketothiolase: *phbA*; acetoacetyl-CoA reductase: *phbB*; PHB polymerase: *phbC*

DNA-microcarrier preparation

Preparation of gold microcarriers was carried out following the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, CA, USA). Sixty milligram of 1- μ m diameter gold microcarriers was resuspended in 1 ml of 100% ethanol and vigorously vortexed for 2 min, and then centrifuged at 10,000 rpm for 1 min. The recovered pellet was washed twice with sterile distilled water, and finally the microcarriers were resuspended in 1 ml of sterile distilled water and kept at 4°C.

For each co-bombardment, into an aliquot of 50 μ l of microcarriers, 2.5 μ l of each DNA constructs (1 μ g/ μ l), 50 μ l of CaCl₂ (2.5 M) and 20 μ l of spermidine (0.1 M) were added one by one followed by continuous vortexing. The mixture was vortexed for an additional 3 min, and then the DNA-microcarriers were recovered by centrifugation at 10,000 rpm for 10 s. The pellet was washed twice with 250 μ l of 100% ethanol, and was finally resuspended in 60 μ l of 100% ethanol.

Biolistic transformation

Biolistic transformation was carried out by co-bombardment of *MSP1pBI221* and *35SpEGFP* plasmids into mesocarp, leaf, and root tissues and co-bombardment of *pMS29* and *35SpEGFP* plasmids into mesocarp and leaf tissues. In a parallel experiment, co-bombardment of *pBI221* and *35SpEGFP* plasmids was carried out in order to provide a qualitative analysis system for comparison of the CaMV35S and MSP1 promoters. For each co-bombardment, 10 μ l of DNA-coated microcarriers was loaded onto the center of macrocarrier and left to be air-dried for almost 2 min. The system was set up based on the optimized parameters for transient transformation of oil palm tissues described by Zubaidah and Siti Nor Akmar (2003). The distances between rupture disk and macrocarrier, macrocarrier and stopping screen, and stopping screen to target were adjusted to 6 mm, 11 mm, and 6 cm, respectively. The bombardment was carried out using rupture disks of 1,550 psi for mesocarp and 1,100 psi for leaf and root tissues while the vacuum was maintained at 27 Hg. The bombarded tissues were kept at 28°C prior to reporter assay.

Histochemical GUS assay in tissues co-bombarded with MSP1pBI221/35SpEGFP gene constructs

The GFP spots were observed in mesocarp, leaf, and root tissue 2 days after co-bombardment, using Nikon SMZ1000 microscope equipped with U.V source and GFP filter (excitation at 360–480 nm and emission at 480–500 nm). Transient expression of GFP reporter gene provided an internal reference for identification of successfully

transformed tissues. The GFP-expressing tissue slices were selected for histochemical GUS assay following the procedure described by Jefferson (1987), with some modifications. The transiently transformed mesocarp, leaf, and root tissue slices from each co-bombardment were placed separately in sufficient amount of GUS staining solution (0.1 M NaPO₄ buffer, pH 7.0, 10 mM EDTA, pH 7.0, 0.5 mM K Ferricyanide, pH 7.0, 0.5 mM K Ferrocyanide, pH 7.0, 10 mM X-Gluc, and 0.1% Triton-X) and incubated at 37°C for 12 h. Following GUS staining, the blue spots were observed under the white spectrum using Nikon SMZ1000 microscope.

RT-PCR analysis of PHB genes

GFP analysis was carried out at 12 h and 2 days after co-bombardment of tissues with *pMS29* and *35SpEGFP* gene constructs. The transiently transformed tissues expressing GFP were used for RT-PCR analysis. The total RNA from non-transformed and transformed mesocarp and leaf tissue slices was extracted according to the protocol described by Gao et al. (2001), and were subjected to RT-PCR analysis. Three sets of primers were designed based on the sequences of the three PHB bacterial genes from the bacterial source of *Alcaligenes eutrophus* (accession no. J04987) in order to prime the specific amplification of *phbA*, *phbB*, and *phbC* transcripts in RT-PCR reactions (Table 1). In addition gene-specific primers were designed for *MT3-A* and GFP reporter genes. RT-PCR analysis of *MT3-A* and GFP reporter genes was also carried out in order to provide internal and positive controls, respectively.

RT-PCR was carried out using the QIAGEN[®] OneStep RT-PCR Kit following the manufacturer's instructions. The reactions were set up for each gene separately in a 50- μ l reaction mixture, containing 10 μ l of 5 \times QIAGEN OneStep RT-PCR Buffer, 2 μ l of 25 mM dNTP, 1.5 μ l of each gene-specific forward and reverse primers, 2 μ l of QIAGEN OneStep RT-PCR Enzyme Mix, 1 μ l of total RNA, and 32 μ l of RNase-free water. The RT-PCR cycling was performed as follows: reverse transcription step for 30 min at 50°C, initial PCR activation step for 15 min at 95°C, followed by 30 cycles of denaturation, annealing, and extension steps for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, respectively. The final extension step was programmed for 10 min at 72°C. Electrophoresis analysis was carried out on a 1.2% agarose gel at the constant voltage of 80 V for an hour.

TOPO[®] TA cloning of RT-PCR products and sequence analysis

Cloning of the RT-PCR products was carried out using TOPO TA Cloning Kits (Invitrogen[™] life technologies)

Table 1 List of the primers used in RT-PCR reactions

Gene	Primer sequence	T _m (°C)	GC content (%)
<i>phbA</i>	Forward: 5'-ccatgaccatcaacaaggtg-3'	60.4	50
	Reverse: 5'-tattccttgccacgttctc-3'	60.4	50
<i>phbB</i>	Forward: 5'-aatgtggctgactgggactc-3'	62.4	55
	Reverse: 5'-gttgaacagcaggtcaggt-3'	62.4	55
<i>phbC</i>	Forward: 5'-tgacgattcccagtttctc-3'	60.4	50
	Reverse: 5'-ggagatgctatccgaatgga-3'	60.4	50
<i>MT3-A</i>	Forward: 5'-cacgagtacagattacaac-3'	58.9	40.9
	Reverse: 5'-gtgcgaaactggataaggaag-3'	60.6	47.6
GFP	Forward: 5'-acgtaaacggccacaagttc-3'	60.4	50
	Reverse: 5'-aagtcgtgctctcatgtg-3'	60.4	50

following the instructions of the manufacturer. Restriction analysis of white colonies (~ten colonies) was carried out using the *EcoRI* restriction enzyme in order to confirm the presence of the inserts. The recombinant clones were sent for sequencing and the obtained sequences were compared with the sequence of the bacterial PHB genes using the workbench software (<http://workbench.sdsc.edu>).

SDS-PAGE and western blot analysis of PHB genes

The total protein was extracted from non-transformed and transformed mesocarp and leaf tissue slices according to the protocol described by Teong et al. (2003), and analyzed by separation on a denaturing polyacrylamide gel. Silver staining of the gel was carried using Silver Stain Plus Kit from Bio-Rad. Concentrations of protein samples were determined using Quick Start™ Bradford Protein Assay Kit (Bio-Rad). Blotting of electrophoretically separated protein samples onto Hybond™-C nitrocellulose membrane was carried out using Tris–glycine transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine) at constant current of 400 mA for 2 h using Transphor apparatus (Amersham Biosciences). Blocking of the membrane was carried out for 1 h by soaking the membrane in 1× TBS (20 mM Tris, 500 mM NaCl, pH 7.4) containing 0.1% Tween and 5% dried skimmed milk. Then, the membrane was washed twice for 1 min with fresh changes of washing buffer (0.1% Tween in TBS). Probing of protein blots was carried out using polyclonal primary antibody against *phbB* and monoclonal primary antibody (Clontech) specific for GFP gene with recommended serial dilutions in TBS. After probing, membrane was washed twice in washing buffer and then the secondary detection was carried out for 1 h by diluting the Goat Anti-Rabbit IgG-AP secondary antibody (Bio-Rad) in TBS. Detection of the signals was carried out using Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).

Results

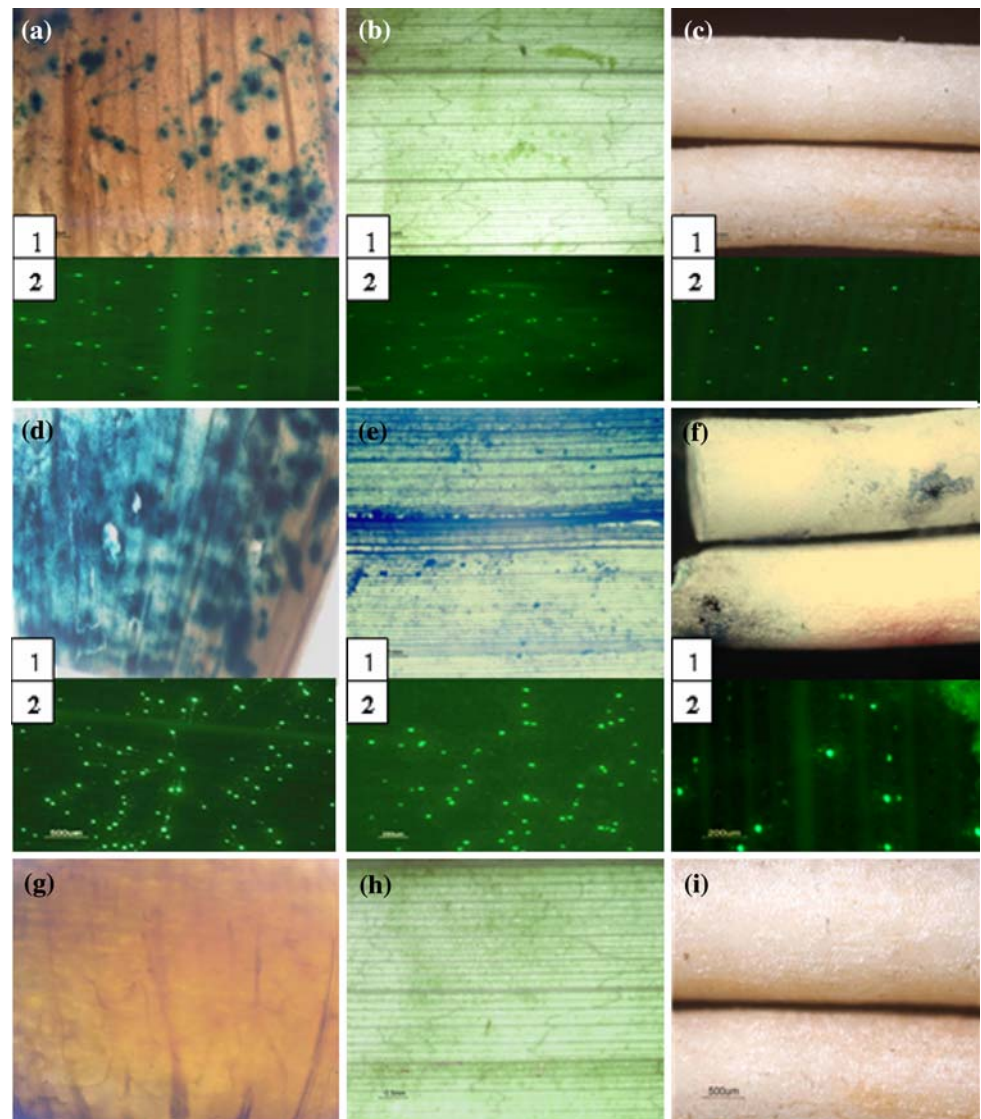
Analysis of tissue-specificity of the MSP1 via histochemical GUS assay

Histochemical GUS assay revealed mesocarp-specific activity of the MSP1 which has directed the expression of GUS reporter gene specifically in mesocarp tissue slices. Panels a₁, b₁, and c₁ in Fig. 3 show the results of GUS assay in transiently transformed GFP-expressing mesocarp, leaf, and root tissue slices (a₂, b₂, and c₂, respectively) 2 days after co-bombardment with *MSP1pBI221* and *35SpEGFP* gene constructs. As illustrated GUS expression was only detected in mesocarp tissue slices, while no expression level of GUS gene was detected in leaf and root tissue slices. Panels d₁, e₁, and f₁ of the figure show the expression of GUS in GFP-expressing mesocarp, leaf, and root tissue slices (d₂, e₂, and f₂, respectively) co-bombardment with *pBI221* and *35SpEGFP* gene constructs. As expected, expression of GUS gene was detected in all different tissues, which was driven by the activity of the constitutive CaMV35S promoter. Furthermore no endogenous GUS activity was detected in non-transformed tissues (panels g, h, and i).

Identification of transiently transformed tissues for analysis of PHB gene expression

The main difficulty of using biolistic-based expression systems is the variability in the number of individual bombardment events between independent experiments. This demands a very large number of assays that need to be performed to obtain statistically meaningful comparisons. However, the use of an internal control, such as GFP reporter gene has been shown to provide a reliable reference for quantitative analysis of transgenes (Schenk et al. 1998). Using GFP reporter gene in co-bombardment with PHB genes provided an estimate of the efficiency of the biolistic delivery and more importantly allowed the

Fig. 3 Histochemical GUS assay in oil palm mesocarp, leaf, and root tissue slices. Panels **a**₁, **b**₁, and **c**₁: GUS assay in GFP-expressing mesocarp, leaf, and root tissue slices co-bombarded with *MSP1pBI221* and *35SpEGFP*, respectively. Panels **a**₂, **b**₂, and **c**₂: GFP-expressing mesocarp, leaf, and root tissue slices co-bombarded with *MSP1pBI221* and *35SpEGFP*, respectively used for GUS assay. Panels **d**₁, **e**₁, and **f**₁: GUS assay in GFP-expressing mesocarp, leaf, and root tissue slices co-bombarded with *pBI221* and *35SpEGFP*, respectively. Panels **d**₂, **e**₂, and **f**₂: GFP-expressing mesocarp, leaf, and root tissue slices co-bombarded with *pBI221* and *35SpEGFP*, respectively used for GUS assay. Panels **g**, **h**, and **i**: GUS assay in intact mesocarp, leaf, and root tissue slices, respectively



selection of transiently transformed tissues which presumably have received both reporter and PHB genes. Furthermore GFP assay being a non-destructive assay allowed subsequent use of the transformed tissues for RT-PCR, SDS-PAGE, and western blot analysis. However, transient expression of GFP gene does not necessarily guaranty the successful expression of PHB genes.

An important consideration was to determine how long after bombardment the gene expression analysis should be carried out in the transiently transformed tissues. This is firstly because that cellular mRNA will be degraded very shortly after translation (Gao et al. 2001) and secondly in certain cases the foreign gene products will be identified as a pathogen or undesirable compounds by the cell defense system which causes the halting of transcription and further exclusion from the metabolic pathways (Lyer et al. 2000). Figure 4 illustrates the transient expression of GFP reporter gene in mesocarp and leaf tissue slices after 12 h and

2 days of co-bombardment with *pMS29* and *35SpEGFP* gene constructs. Two different time intervals were used for RT-PCR analysis in order to have an indication on the stability and durability of expression of the introduced metabolic pathway.

RT-PCR analysis

Figure 5 shows the result of RT-PCR analysis of PHB genes in mesocarp and leaf tissues, 12 h after co-bombardment with *pMS29* and *35SpEGFP* gene constructs. RT-PCR analysis showed that the engineering of PHB biosynthetic pathway genes under the regulation of *MSP1* in transformed mesocarp tissue has resulted in successful transcription of *phbA*, *phbB*, and *phbC* genes, based on the presence of the expected size bands. The expected sizes of *phbA*, *phbB*, and *phbC* transcripts based on the primers were 261, 173, and 178 bp,

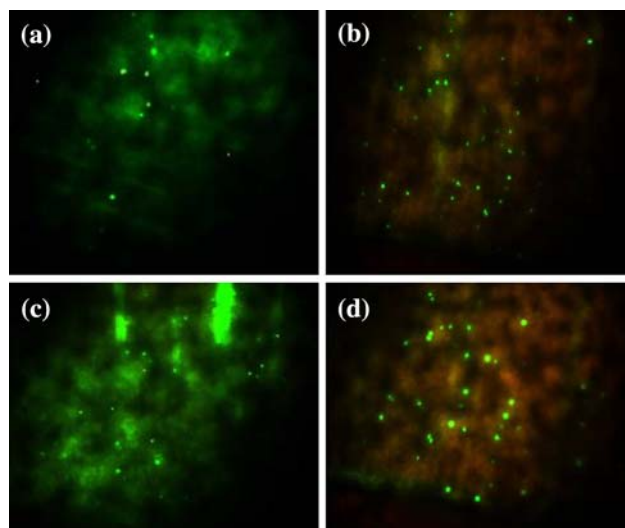


Fig. 4 GFP reporter assay in oil palm mesocarp and leaf tissue slices at different times after co-bombardment with MSP1: PHB and 35S: GFP gene constructs. Panels **a** and **b**: GFP expression in mesocarp and leaf tissues, respectively 12 h after co-bombardment. Panels **c** and **d**: GFP expression in mesocarp and leaf tissues, respectively 2 days after co-bombardment

respectively. No expression level of PHB genes was detected in both non-transformed and transformed leaf tissues; however RT-PCR analysis of GFP gene (positive control) showed the successful transcription of this gene in transformed leaf tissues, which showed the efficiency and accuracy of both biolistic delivery and RT-PCR techniques.

RT-PCR was repeated using total RNA extracted from GFP positive mesocarp and leaf tissues 2 days after bombardment and interestingly produced the same results (data not shown), which indicates that expression of the introduced PHB pathway was stable within 2 days and most likely did not interfere with other metabolic pathways in transiently transformed mesocarp tissues.

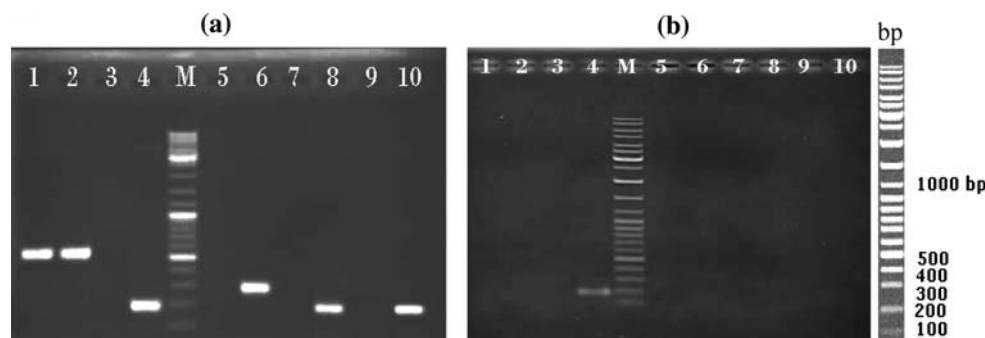


Fig. 5 RT-PCR analysis of PHB genes in non-transformed and transformed mesocarp (**a**) and leaf tissues (**b**). Lanes 1 and 2 (RT-PCR of *MT3-A* in non-transformed and transformed tissues, respectively), lanes 3 and 4 (RT-PCR of GFP in non-transformed and transformed tissues, respectively), lanes 5 and 6 (RT-PCR of *phbA* in

Using *MT3-A* gene as an internal control provided a reliable reference for RT-PCR analysis of PHB genes. It was shown that while RT-PCR was able to detect and amplify the *MT3-A* mRNA transcript in non-transformed mesocarp tissues, no expression level of PHB genes was detected; indicating that there is no endogenous activity of PHB genes in non-transformed mesocarp tissue and in fact the expression of PHB genes in mesocarp tissue after bombardment is the absolute result of the PHB pathway engineering. All the three PHB transcripts from the RT-PCR step were subjected to sequence analysis in order to confirm their identity. Sequence alignment of the PHB transcripts and the bacterial PHB genes from *A. eutrophus* showed 100% homologies between sequences.

SDS-PAGE analysis

Comparison of the protein profiles in non-transformed and transformed mesocarp tissues at 2 days after bombardment (Fig. 6) showed the presence of three distinguishable additional bands (nearly 40, 30, and 60 kDa) corresponding most probably to *phbA*, *phbB*, and *phbC* gene products, respectively. The molecular weights of the encoded products of PHB genes are 40, 26, and 60 kDa for *phbA*, *phbB*, and *phbC*, respectively. Furthermore, no distinct changes were observed in the protein profiles from non-transformed and transformed leaf tissues. In order to confirm the above findings western blot analysis was performed using polyclonal antibody specific for *phbB* gene product.

Western blot analysis

Due to unavailability of the specific antibody for *phbA* and *phbC* gene products, western analysis was only carried out for *phbB* product and it revealed the successful translation of *phbB* transcript into protein product in transiently transformed mesocarp tissues 2 days after bombardment

non-transformed and transformed tissues, respectively), lanes 7 and 8 (RT-PCR of *phbB* in non-transformed and transformed tissues, respectively), and lanes 9 and 10 (RT-PCR of *phbC* in non-transformed and transformed tissues, respectively)

(Fig. 7, panel a). Expectedly, due to the mesocarp-specific activity of the MSP1, regulation of *phbB* gene by this promoter did not result in translation into protein product in transformed leaf tissues (panel c). As illustrated in panels b and d, a band of the expected size of GFP product (55 kDa) was detected in protein blots from transformed mesocarp and leaf tissues challenged with monoclonal antibody specific for GFP product (The GFP protein was not distinguishable in the silver-stained polyacrylamide gel). The reason of including the GFP gene in western analysis was

to provide an elite positive control to determine the accuracy and efficiency of blotting and probing procedures.

Discussion

In order to establish a safe strategy for production of transgenic plants, it would be desirable to survey different aspects of the transformation process as well as the expression pattern of the foreign genes prior to stable

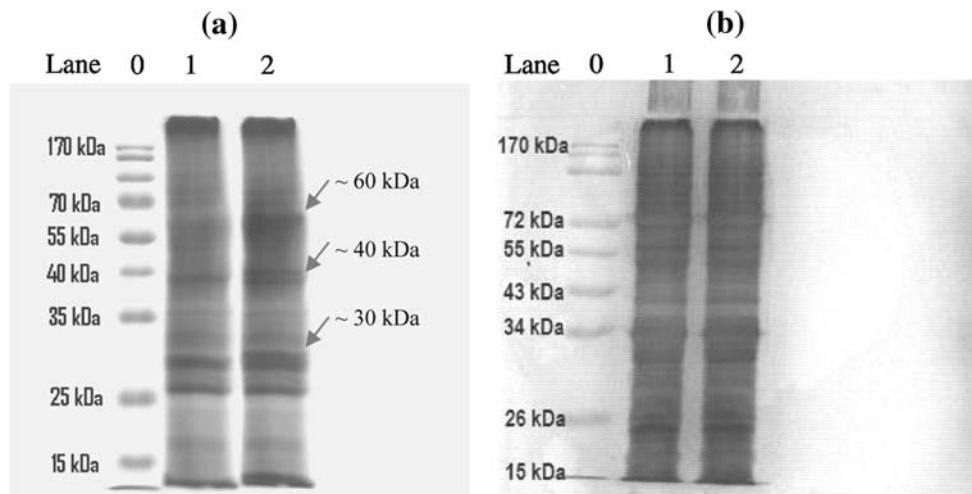


Fig. 6 Analysis of the protein profiles in non-transformed and transformed mesocarp and leaf tissues by denaturing polyacrylamide gel electrophoresis. Panel **a**: protein profiles in non-transformed (*lane 1*) and transformed mesocarp tissues (*lane 2*).

Panel **b**: protein profiles in non-transformed (*lane 1*) and transformed leaf tissues (*lane 2*). The additional bands observed with sizes of 40, 30, and 60 kDa, corresponding to *phbA*, *phbB*, and *phbC* protein products

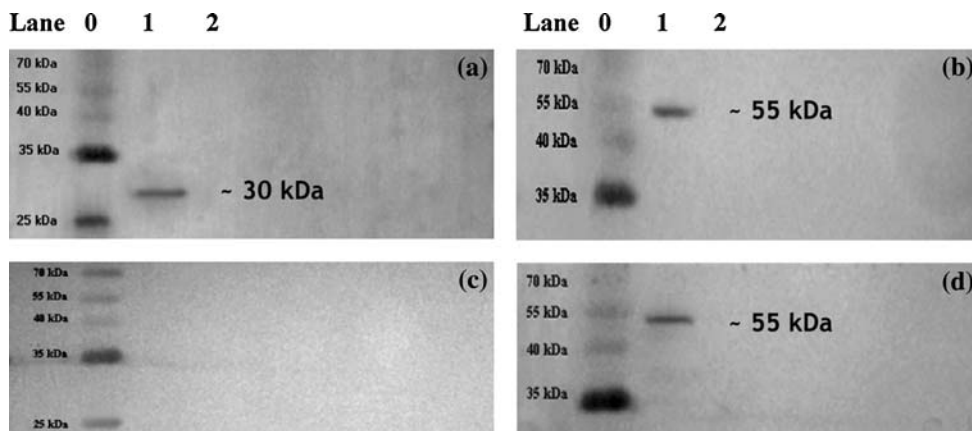


Fig. 7 Western blot analysis of *phbB* and GFP (positive control) genes in mesocarp and leaf tissues co-bombarded with MSP1: PHB and 35S: GFP. Panel **a**: western analysis of *phbB* in blotted protein extracted from transformed and non-transformed mesocarp tissue slices (*lanes 1 and 2*, respectively). Panel **b**: western analysis of GFP in blotted protein extracted from transformed and non-transformed mesocarp tissue slices (*lanes 1 and 2*, respectively). Panel **c**: western

analysis of *phbB* in blotted protein extracted from transformed and non-transformed leaf tissue slices (*lanes 1 and 2*, respectively). Panel **d**: western analysis of GFP in blotted protein extracted from transformed and non-transformed leaf tissue slices (*lanes 1 and 2*, respectively). The size of *phbB* and GFP products are 26 and 55 kDa, respectively

transformation. This is more crucial when we are dealing with tree crops, such as oil palm where their transgenic production takes several years. The adopted biolistic-based transient assay in this study provided fast and valuable information with respect to the strength and tissue-specificity of the novel oil palm promoter (MSP1), the viability and functionality of the chimeric PHB construct, and the tissue-specific engineering of the PHB biosynthetic pathway genes under the regulation of MSP1.

Constitutive expression of foreign gene products in transgenic plants may be less profitable in comparison to tissue-specific expression, due to the high energy required for such production and problems associated with disposal of the remaining parts (Halfon et al. 1997). Engineering of PHB genes under the regulation of the oil palm MSP1 allows the expression to be channeled specifically in mesocarp tissue.

A prerequisite prior to the use of any promoter in transgenic studies is to characterize the functional behavior of the promoter in the plant that is to express the foreign gene. However, *in vivo* characterization of plant promoters in perennial crops such as the oil palm through production of transgenic plants is time-consuming. Evaluation of promoter's activity via transient expression assays provides the possibility to test the functional behavior of the promoter in a short period of time (Schenk et al. 1998).

The present study acknowledges the tissue-specificity of the MSP1, which potentially regulated the expression of PHB genes exclusively in oil palm mesocarp tissue. Mesocarp-specific behavior of MSP1 is the result of the specific interaction between *cis*-acting promoter elements and mesocarp-specific transcription factors which are produced only in oil palm mesocarp. Apparently such transcription factors are not expressed in oil palm leaves and roots, resulting in failure of detecting GUS reporter gene and PHB transcripts in these tissues.

Several phenomena can effect the expression of the foreign bacterial genes in plant systems, including transcriptional and post-transcriptional gene silencing, DNA methylation of the promoter, codon usage frequency, and base composition of the DNA (Dale and Schantz 2003).

A major concern in using PHB construct for production of transgenic palms was the probability of the occurrence of transcriptional gene silencing due to the repeated use of the same promoter (MSP1) in gene construct. However, the matrix attachment region of tobacco (RB7-MAR) which has a stabilizing effect on transgene expression was incorporated into the PHB gene construct (Masani and Parveez 2003), which might overcome the problem leading to silencing of the transgenes (Allen et al. 1996; Yoshida and Shinmyo 2002). In addition, there was certain degree of uncertainty in transferring PHB genes from bacterial source into a eukaryotic system with different codon usage

and the likelihood of slowing down or even halt the transcription due to the codon bias phenomena. The occurrence of codon bias is more prevalent with respect to the genes which have a high expression level, such as genes encoding the enzymes of the metabolic pathways (in our case; PHB genes) (Fisk and Dandekar 1993; Miyasaka 2002).

All these difficulties and uncertainties associated with production of transgenic palms reinforced the need for initial evaluation of the engineering program in a rapid transient biolistic-based assay. The biolistic technique was shown to be a potential approach to test the functional behavior of the MSP1 as well as tissue-specific expression of PHB genes. This study showed the successful transcription of all three PHB genes into mRNA transcripts in transiently transformed oil palm mesocarp tissues. The transcripts remained stable up to 2 days and have successfully been translated into proteins without demonstrating any of the problems associated with expression of multiple foreign genes as discussed earlier. These results are very supportive on the possibility of engineering PHB production in the mesocarp of oil palm. Understanding these data prior to production of transgenic palms brought degrees of certainty on the prospects of long-term transgenic program for PHB-producing transgenic palms.

Acknowledgments The authors acknowledge the financial support from MOSTI. We thank En. Masani and Dr Ahmad Parveez from MPOB for *pMS29* vector construct and collaborators from MIT, USA, for the polyclonal antibody specific for *phbB*.

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