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CONSTRUCTION OF PHB AND PHBV TRANSFORMATION VECTORS FOR BIOPLASTICS PRODUCTION IN OIL PALM

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ABSTRACT

The construction of transformation vectors carrying bioplastic biosynthetic genes driven by constitutive and oil palm mesocarp-specific promoters was completed. Four planned transformation vectors were produced. The poly-3-hydroxybutyrate (PHB) producing constructs carried the phbA, phbB *and* phbC *genes, while the polyhyroxybutyrate-co-valerate (PHBV) producing constructs carried the* bktB, phbB, phbC *and* tdcB *genes. Each of these genes was fused with the transit peptide (*Tp*) of the oil palm acyl-carrier-protein (*ACP*) for targeting into the plastids of plant cells. All vectors carry the phosphinothricin acetyltransferase gene (*bar*) driven by an ubiquitin promoter as plant selectable marker. The matrix attachment region from tobacco (RB7MAR) was also included for stabilization of the transgene expression and to minimize the gene silencing due to positional effects. All constructs were verified by restriction analysis, polymerase chain reaction (PCR) and DNA sequencing.*

Keywords: poly-3-hydroxybutyrate (PHB), polyhyroxybutyrate-co-valerate (PHBV), constitutive promoters, tissue-specific promoter, plastid, transformation vectors.

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INTRODUCTION

Poly-3-hydroxybutyrate (PHB) is the most common polyhydroxyalkanoate (PHA) produced as a storage material by some bacteria under restricted growth condition (Senior and Dawes, 1973). Biotechnological production of PHA was first made in the 1980s by Imperial Chemical Industries (ICI) using a polymer-accumulating bacterium, *Ralstonia eutropha* (Somerville *et al*., 1994). The cost of production was high because the most useful polymer, polyhyroxybutyrate-co-valerate (PHBV), could only be effectively synthesized by supplementing the fermentation medium with propionate. The commercially produced PHBV is known as Biopol™.

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However, its commercial applications are limited due to the high production cost.

Plants offer an alternative large-scale factory for the production of complex products. A variety of PHA having different physical properties are now being synthesized in a number of transgenic plants, including *Arabidopsis* (Poirier *et al*., 1992; Nawrath, *et al*., 1994; Bohmert *et al*., 2000), tobacco (Arai *et al*., 2001; Bohmert *et al*., 2002), rape (Houmiel *et al*., 1999), cotton (John and Keller, 1996), maize (Hahn *et al*., 1997) alfalfa (Saruul *et al*., 2002) and flax (Wrobel *et al*., 2004). These were accomplished by introducing bioplastic biosynthetic genes into the cytoplasm or specific compartments such as the plastids. The production of PHB in the cytoplasm was only 0.1% dry weight (dwt) (Poirier *et al*., 1992), approximately 900 times lower than PHB production in the bacterium *R. eutropha*. Furthermore, growth of the transgenic plants producing PHB was reduced compared to the untransformed plants. This may due to the limited supply of acetyl-CoA (Nawrath *et al*., 1994).

The expression of PHB genes in plant compartments, such as the plastids, was thought to be a potential solution for PHB production in

transgenic plants. It was hypothesized that the large flux of acetyl-CoA in the plastids can be redirected towards higher production of PHB. The accumulation of PHB in the plastids can also minimize the potential deleterious effects on the plant growth. Using this strategy, Nawrath *et al.* (1994) generated transgenic plants that produced up to 14% dwt of PHB. Later, the yield of PHB was further improved to up to 40% dwt by putting all the PHB genes in a single transformation vector (Bohmert *et al*., 2000). The increase in PHB content in the latter case could be due to better coordination of the PHB expression (Poirier, 2002). Several copies of the promoter might lead to high levels of the transcription factors locally, allowing higher expression of the genes. It was also possible that the tightly linked genes reduced the level of gene silencing (Bohmert *et al*., 2000). However, growth retardation and low transformation efficiencies were also observed in this study. This could be due to the high transgene expression, which subsequently affected the plant metabolism. These problems were eliminated by using tissue-specific promoters, as demonstrated in transgenic plants such as *Arabidopsis* (Bohmert *et al*., 2002), rape (Houmiel *et al*., 1999), cotton (John and Keller, 1996) and flax (Wrobel *et al*., 2004).

Thus, the use of tissue-specific promoters was able to reduce the impact of transgene expression on the normal growth and development of plants while enabling the production of PHA in easily harvested tissues. However, the use of a specific promoter for all genes was only studied in rape, whereas in *Arabidopsis*, cotton and flax, only *phbA* was attached to a tissue-specific promoter. The low efficiency of transformation obtained from Bohmert *et al.* (2002) was probably associated with the constitutive expression of 3-ketothiolase (*phbA* or *bktB*), which was driven by the CaMV35S promoter. Bohmert *et al.* (2002) showed that the transformation rate dropped to 0.01% whenever the 3-ketothiolase gene (*phbA* or *bktB*) was used in a single-gene or multiple-gene transformation vector. Their results showed that the use of vectors expressing the plastid-targeted *phbB* and *phbC* genes led to normal transformation efficiencies of 1% and 3%, respectively. Slater *et al*, (1999) also showed that the low transformation efficiency (0.02%) of the PHBV vector was possibly caused by constitutive expression of the *ilvA* gene that subsequently placed a metabolic burden on the plants.

Based on these reports, for the successful production of PHB and PHBV in transgenic plants, it is important to follow these recommendations: (i) all the genes needed for the synthesis pathways of PHB and PHBV have to be introduced to the plant tissues as a new pathway in a single locus, (ii) all the PHB and PHBV genes have to be targeted into a subcellular compartment of the plant tissue, and (iii) all the PHB and PHBV genes have to be expressed in specific tissues in order to minimize possible negative effects on the transgenic plants. This study aimed to construct PHB and PHBV transformation vectors by following the above recommendations and using the vectors to transform oil palm for bioplastic production. This work is part of the Malaysia-Massachusetts Institute of Technology (MIT) Biotechnology Partnership Programme [MMBPP] funded by the Government of Malaysia.

MATERIALS AND METHODS

DNA Manipulation

The plasmids used for DNA manipulation and PCR amplification are listed in *Table 1.* All of the restriction enzyme digestions were carried out under the conditions recommended by the suppliers of the specific enzymes (Research Biolabs and Promega). The DNA inserts was isolated using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (QIAGEN). To allow ligation between the DNA inserts or vectors which had been digested with restriction enzymes that created the heterologous ends in some of the cloning experiments, the cohesive ends were first rendered blunt. The cohesive ends were filled in a reaction volume using 2.5 units of Platinum *Pfx* DNA polymerase (Invitrogen), 1 mM dNTP mixture, 1X *Pfx* amplification buffer and 1 mM $MgSO_4$, followed by incubation at 72°C for 30 min. To prevent self-ligation of the vector DNA, the 5' phosphate group was removed from the vector DNA by treatment with 10 units of Calf Intestinal Phosphatase (CIP) (Promega) in a 60 µl reaction volume. The mixture was incubated for 1 hr at 37°C after which the reaction was terminated and the mixture was purified using the QIAquick PCR Purification Kit (QIAGEN). The DNA inserts were ligated into vectors in a 20 µl reaction volume with 1X ligation buffer (Research Biolabs) at 16°C overnight. The DNA insert was used in an equal or up to three-fold molar concentration over the DNA vector. For ligation of the DNA with sticky ends, one unit of T4 DNA ligase (Research Biolabs) was used. For the DNA with blunt ends, the reaction was supplemented with 2 µl 40% PEG 4000 and two units T4 DNA ligase instead of one unit. The ligation mixtures, or plasmid DNA, were transformed into either *Escherichia coli* competent cells, strain DH5α or STBL4 (LifeTechnologies).

Polymerase Chain Reaction (PCR)

A list of all the primers used for PCR amplification is shown in *Table 2*. PCR amplification was performed on the MJ Research Inc. Programmable Thermal Controller (PTC-100™ or PTC-200™). Reactions were carried out in 25 µl

TABLE 1. PLASMIDS USED IN THIS STUDY

Plasmid	Description	Reference/source
pBluescript SK-	Commercial cloning vector	Stratagene
PCR 2.1TOPO	Commercial cloning vector	Invitrogen
pGreenII0000	Binary Ti vector for *Agrobacterium*-mediated plant transformation	John Innes Centre
pBI221	Contain *GUS* gene driven by the CaMV35S promoter	Clontech
pACP3	Carrying the full length oil palm *ACP* gene with the transit peptide	MPOB
pME12	Contains the *bktB* gene in pBluescript SK-	MIT
pAHC20	Carrying the *bar* gene driven by the maize ubiquitin promoter	USDA
pACT-F4	Carrying the *GUS* gene under the control of the rice Actin 1 promoter and Actin 1 intron, first exon and a portion of second exon (Act1)	Zhang *et al*. (1991)
pGHNC05	Carrying direct repeat RB7 MAR flanked by multiple cloning sites	Matzke *et al*. (1994)
pAeT41	Carrying the phbCAB genes in pBluescript SK-	MIT
pMP2	Carrying the oil palm mesocarp-specific promoter and *Nos* sequences	MPOB
pFC2	Cloning vector with multiple cloning sites of pBluescript SK- flanked by unique rare cutter enzymes sites for cloning useful genes	David *et al*. (1995)

mixtures containing plasmid DNA (10 ng), 1X PCR buffer, 200 nM of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5-1.0 unit *Taq* DNA polymerase (Promega). The reactions were initially performed using *Taq* DNA polymerase (Promega). Then, a *Taq* DNA polymerase with a proof reading activity, such as *Pfu* DNA polymerase (Promega), *Pwo* DNA polymerase or Expand High Fidelity PCR System (Roche), was used. A total of 1.5 units of enzyme together with 2 mM Mg_2SO_4 were used. The PCR programme was: 94°C for 3 min to denature the DNA template and then 25-35 cycles of 1 min at 94°C (denaturation), 1 min at an appropriate annealing temperature for the primers used to hybridize to the target sequence, and the extension step for polymerization at 72°C for 1 min. The extension time was roughly set as 1 min for 1 kb DNA. Thus, a longer DNA fragment would require a longer time. After the final cycle, the mixtures were further incubated for 5-10 min at 72°C. This was to ensure complete extension of the products. The PCR products were purified using either a QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (QIAGEN), and then transformed into One Shot® chemically competent *E. coli* cells according to the manufacturer's description (Invitrogen).

CONSTRUCTION OF PHB AND PHBV TRANSFORMATION VECTORS

Constitutive Vectors

This work was aimed at the construction of transformation vectors containing PHB and PHBV genes driven by three constitutive promoters (Ubiquitin, CaMV35S and Actin). The two vectors were designed in such a way that the promoter was only used once in a given vector.

Promoter cassettes of pMB3, pMB9 and pMB12. Three promoter cassettes (*CaMV35S-NOS*, *UbiPro-NOS* and *Actin-NOS*) were required in an attempt to engineer both the vectors as follows: three pairs of primers, CaMV-F2 and CaMV-R2, UBI-F and UBI-R, ACT-F and ACT-R2, were used to amplify the CaMV35S, ubiquitin (*UbiPro*) and Actin promoter sequences, using template DNA of pBI221, pAHC20 and pACT-F4, respectively. The synthesized fragments, 0.8 kb CaMV35S and 1.9 kb UbiPro, were digested with *Bam*HI and *Spe*I, and the 1.3 kb Actin with *Spe*I; all were gel purified and cloned into pBluescript SK- (SK-) to create pMB1, pMB8 and pMB11, respectively. On the other hand, plasmid pBI221 was used as DNA template for the amplification of the 0.3 kb NOS-terminator sequence using the primers, NOS-F and NOS-R. The PCR product was gel purified and cloned into the PCR2.1TOPO to create pMB2. The NOS fragment was excised from pMB2 using the restriction enzymes *Spe*I and *Avr*II, and cloned into the *Avr*II site of pMB1, pMB8 and pMB11. The clones containing the DNA fragments, *CaMV35S-NOS* (1.1 kb) and *UbiPro-NOS* (2.1 kb), were identified by *Bam*HI digestion, while *Actin-NOS* (1.5 kb) was selected by *Spe*I digestion to create pMB3, pMB9 and pMB12, respectively. A unique *Avr*II site between the promoter and NOS region in pMB3, pMB9 and pMB12 was used as the cloning site for insertion of the genes of interest with *Spe*I overhangs.

TABLE 2. PRIMERS (5′ to 3′) USED IN PCR AMPLIFICATION. THE RESTRICTION ENZYMES SITES ARE UNDERLINED

Primers		Restriction site
CaMV-F2	CGGGATCC AGATCTAGCTTGCATGCCTGCAGG	*Bam*HI, *Bgl*II
CAMV-R2	GGACTAGT CCTAGGGAGTCCCCCGTGTTCTCTC	*Spe*I, *Avr*II
UBI-F	CGGGATCCCTGCAGTGCAGCGTGACC	*Bam*HI
UBI-R	GGACTAGT CCTAGGCTGCAGAAGTAACACC	*Spe*I, *Avr*II
BAR-F	GGACTAGTGCCATGCCGGCGGTCTGC	*Spe*I
BAR-R	GGACTAGTTCAGATCTCGGTGACGGGC	*Spe*I
ACT-F	GGACTAGTTCGAGGTCATTCATATGCT	*Spe*I
ACT-R2	GGACTAGT CCTAGGCTACAAAAAAGCTCCGC	*Spe*I, *Avr*II
NOS-F	GGACTAGT CCTAGGGATCGTTCAAACATTTGG	*Spe*I, *Avr*II
NOS-R	GGACTAGT GGATCCGATCTAGTAACATAGATGA	*Spe*I, *Bam*HI
TP-F	GGACTAGTTCCATGGCTTCGATCTCGG	*Spe*I
A1-R	GACAACGTCAGTCTCTGGTTTTGCAGCACAGG	
A2-F	CAAAACCAGAGACTGACGTTGTCATCGTATCC	
A3-R	GGACTAGTCCTTATTTGCGCTCGACTGC	*Spe*I
BKT1-R	CACTTCACGCGTCTCTGGTTTTGCAGC	
BKT2-F	CAAAACCAGAGACGCGTGAAGTGGTAG	
BKT3-R	GGACTAGTCCTCAGATACGCTCGAAG	*Spe*I
B1-R	AATGCGCTGAGTCTCTGGTTTTGCAGCAC	
B2-F	CAAAACCAGAGACTCAGCGCATTGCGTA	
B3-R	GGACTAGTCAGGTCAGCCCATATGCAG	*Spe*I
C1-R	CTTTGCCGGTCGCCTCTGGTTTTGCAGCAC	
C2-F	CAAAACCAGAGGCGACCGGCAAAGGC	
C3-R	GGACTAGTCGTCATGCCTTGGCTTTG	*Spe*I
TDC1-R	CGTATGTAATATGCTCTGGTTTTGCAGC	
TDC2-F	CAAAACCAGAGCATATTACATACGATCTGC	
TDC3-F	GGACTAGTCCTTAAGCGTCAACGAAACC	*Spe*I
MSP1-F	5′-GGCCACTAGTGCCCTTACTATAGGGCACGC -3	*Spe*I
MSP1-R	5-GCGCACTAGTCCTAGGCAGGAAACCAGAGAC-3	*Spe*I, *Avr*II
NOS2-F	5'-GGACTAGTGGCGCGCCGATCGTTCAAACATT-3'	*Spe*I, *Asc*I
NOS2-R	5'-GGACTAGTGCTAGCGATCTAGTAACATAGAT-3'	*Spe*I, *Avr*II
TP2-F	5′-GGGGCGCGCCT TCCATGGCTTC-3′	*Asc*I
PHBA-R	5′-GCGGCGCGCCTTATTTGCGCTC-3′	*Asc*I
PHBB-R	5′-GCGGCGCGCCTCAGCCCATA-3′	*Asc*I
PHBC-R	5′-GCGGCGCGCCTCATGCCTTG-3′	*Asc*I
BKTB-R	5′-GCGGCGCGCCTCAGATACGCT-3′	*Asc*I
TDCB-R	5′-GCGGCGCGCCTTAAGCGTCAAC-3′	*Asc*I

Mesocarp-Specific Vectors

Oil palm fruits are the main target for the production of PHB and PHBV in transgenic oil palm because the mesocarp contains a high flux of acetyl-CoA, the substrate for PHB and PHBV biosynthesis. The oil palm mesocarp-specific promoter (*MSP1*) (Siti Nor Akmar *et al*., 2003) was used to drive either the PHB or PHBV gene in a single transformation vector.

Mesocarp expression vector, pMB41. The primers MSP1-F and MSP1-R were used to amplify the 1.1 kb mesocarp-specific promoter (*MSP1*) using pMP2 as the template. The synthesized fragment was digested with *Spe*I, gel purified and cloned into the *Spe*I site of SK- to yield pMS1. The pBI221 was used as template to amplify the 0.3 kb *Nos*-terminator using the primers, NOS2-F and NOS2-R. The PCR product was digested with *Spe*I and cloned into SK- to create pMS2. The *Spe*I-*Avr*II DNA fragment of

pMS2 was cloned into the *Avr*II site of pMS1 to yield pMS3. The sense orientation of MSP-Nos in pMS3 was confirmed by PCR using the primers, MSP-F and NOS2-R. On the other hand, the *UbiPro-bar-Nos* expression cassette was released from pMB16 by *Bam*HI digestion and cloned into the *Bam*HI site of pMB21 to generate pMB35-46. The 1.4 kb *MSP1-Nos* fragment of pMS3 was cleaved by *Spe*I digestion, purified and cloned into the *Spe*I site of pMB35-46 to create pMB41.

RESULTS

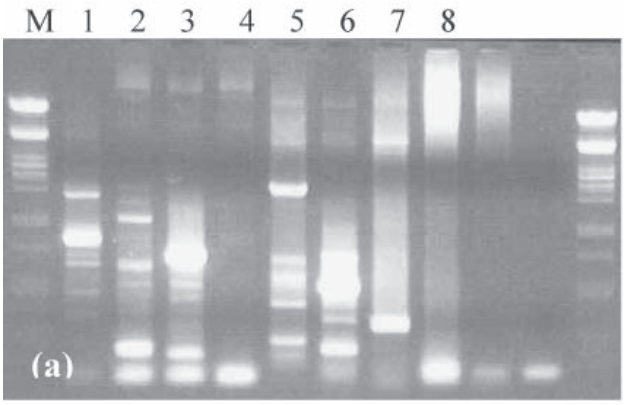
PCR Amplification

Due to no suitable restriction enzymes sites being available, PCR-based cloning was used to amplify and clone the promoters and genes of interest. The combinations of primers and DNA templates to amplify the specific PCR products are listed in *Table 3*. Based on the melting temperature (Tm) of the primers, the annealing temperature was initially set at 50°C. Then Tm was increased by 3ºC in each subsequent reaction until the products were obtained. After optimizing the temperature with *Taq* DNA polymerase, the proofreading *DNA polymerase* was used to amplify the target genes using the same annealing temperature. This was because the proofreading DNA polymerase only introduced a smaller error, if any at all, into PCR products than *Taq* DNA polymerase (Janice *et al*., 1996). *Figure 1a* shows the PCR products for each of the target genes, which were amplified using a different annealing temperature and extension time. The yields of the PCR products were later increased by the addition 1~4 mM $MgSO_4$ and 1%~4% DMSO as shown in *Figure 1b*.

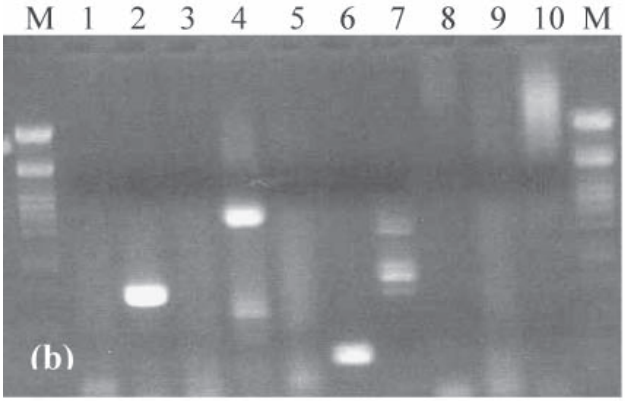
The choice of restriction sites is important to ensure that the vectors can be constructed in a

TABLE 3. PRIMERS AND TEMPLATE DNA COMBINATIONS

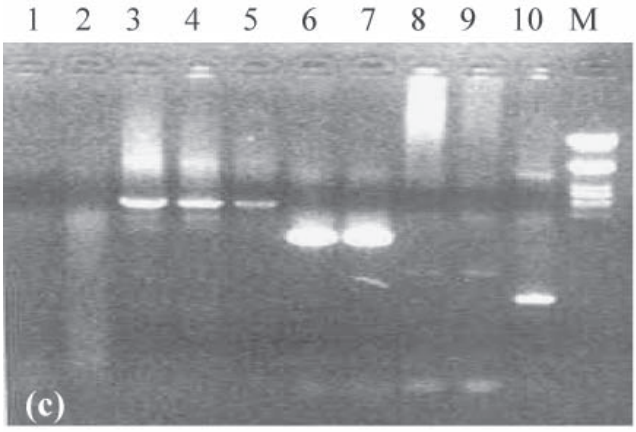
Target fragment	Product size (kb)	Forward primer	Reverse primer	Template DNA
CaMV35S	0.8	CaMV-F2	CaMV-R2	pBI221
ubiquitin	1.9	UBI-F	UBI-R	pAHC20
Actin	1.3	ACT-F	ACT-R2	pACT-F4
NOS	0.3	NOS-F	NOS-F	pB1221
bar	0.5	BAR-F	BAR-R	pAHC20
Tp	0.2	TP-F	A1-R	pACP3
Tp	0.2	TP-F	BKT1-R	pACP3
Tp	0.2	TP-F	B1-R	pACP3
Tp	0.2	TP-F	C1-R	pACP3
Tp	0.2	TP-F	TDC1-R	pACP3
Tp	0.2	TP-F	ACCT1-R	pACP3
phbA	1.1	A2-F	A3-R	pAeT41
bktB	1.1	BKT2-F	BKT3-R	pME12
phbB	0.7	B2-F	B3-R	pAeT41
phbC	1.7	C2-F	C3-R	pAeT41
tdcB	0.9	TDC2-F	TDC3-R	pME24
TpphbA	1.3	TP-F	A3-R	PCR Tp & phbA
TpbktB	1.3	TP-F	BKT3-R	PCR Tp & bktB
TpphbB	0.9	TP-F	B3-R	PCR Tp & phbB
TpphbC	1.9	TP-F	C3-R	PCR Tp & phbC
TptdcB	1.1	TP-F	TDC3-R	PCR Tp & tdcB
MSP1	1.1	MSP1-F	MSP1-R	pMP2
Nos	0.3	NOS2-F	NOS2-R	pBI221
TpphbA	1.3	TP-F	PHBA-R	pMB4
TpphbB	0.9	TP-F	PHBB-R	pMB5
TpphbC	1.9	TP-F	PHBC-R	pMB6
TpbktB	1.3	TP-F	BKTB-R	pMB7
TptdcB	1.1	TP-F	TDCB-R	pTDCB



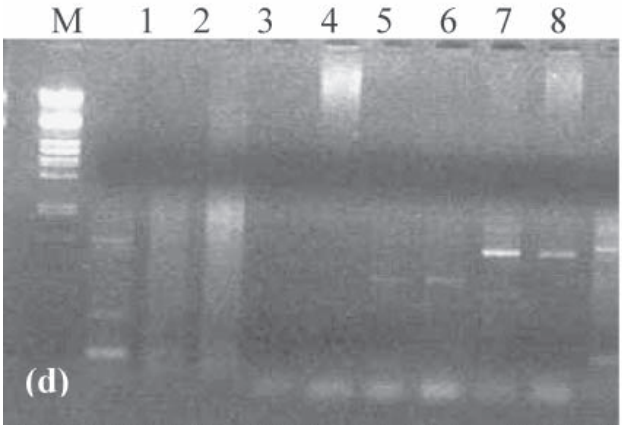
1: ACT-F+ACT-R2 (Actin)
2: A2-F+A3-R (phbA)
3: B2-F+B3-R (phbB)
4: C2-F+C3-R (phbC)
5: UBI-F+UBI-R (UbiPro)
6: BAR-F+BAR-R (bar)
7: NOS-F+NOS-R (Nos)
8: CaMV-F2+CaMV-R2 (CaMV35S)



1: A2-F+A3-R (phbA)
2: B2-F+B3-R (phbB)
3: C2-F+C3-R (phbC)
4: UBI-F+UBI-R (UbiPro)
5: BAR-F+BAR-R (bar)
6: NOS-F+NOS-R (Nos)
7: ACT-F+ACT-R2 (Actin)
8: CaMV-F2+CaMV-R2 (CaMV35S)



C2-F+C3-R (phbC)
1: 4% 2: 5%
UBI-F+UBI-R (UbiPro)
3: 4% 5: 6%
4: 5%
A2-F+A3-R(phbA)
6: 4% 7: 5%
ACT-F+ACT-R2 (Actin)
8: 4% 9: 5%
BAR-F+BAR-R (bar)
10: 4%



C2-F+C3-R (phbC)
1: 1% 2: 2%
CaMV-F2+CaMV-R2 (CaMV35S)
4: 1% 5: 2%
A2-F+A3-R (phbA)
6: 1% 7: 2%
ACT-F+ACT-R2 (Actin)
8: 1% 9: 2%

Figure 1. PCR amplification of genes of interest using different annealing temperatures (TA) and extension times (ET). a. (TA: 55°C, ET: 2 min), b. (TA: 60°C, ET: 3 min), and using different concentrations of $MgSO_4$ (c) and DMSO (d).

modular manner, such that the genetic elements can be exchanged in and out of the vectors in a relatively easy manner. As listed in *Table 2*, the primers were designed to contain unique restriction sites such as *Bam*HI-*Bgl*II and *Spe*I-*Avr*II at the 5′ ends of CaMV-F2 and CaMV-R2, respectively. The 5′ ends of the NOS-F and NOS-R primers for amplification of the *Nos*-terminator were also designed by introducing the *Spe*I-*Avr*II and *Spe*I-*Bam*HI sites, respectively. These sites were chosen because they were not present in the sequences of CaMV35S promoter, Nos-terminator or other PHB or PHBV genes. The uses of these sites are to enable the cloning of multiple genes in tandem. These enzymes recognize different hexanucleotide sequences that generate restriction fragments with identical protruding termini. Ligation of these fragments generates junction sequences that cannot be cleaved by the restriction enzymes used to prepare the DNA fragments. The reverse primers for amplification of the promoter genes were also designed to remove the translation start codon of the promoter genes. This is important for inserts which contain their own start codons to avoid undesired translational initiation by promoters.

The transit peptide sequence was fused to the 5′ end of the open reading frame of each gene by a two-step PCR amplification. The primers used were designed without additional restriction sites or linker. For example, the DNA sequence encoding for the transit peptide (*Tp*) was fused to the 5′end of the threonine dehydratase gene (*tdcB*) by PCR amplification using the primers TP-F, TDCB1-R, TDCB2-F and TDCB3-R (*Table 2*). The primers TDCB2-F and TDCB3-R were designed to recognize the 5′end and 3′end of the *tdcB* gene, and primer TP-F was synthesized based on the sequences at the 5′ends of the transit peptide sequences. Primer TDCB1-R was designed to contain sequences complementary to the 3′end of the transit peptide and 5′end of the *tdcB* genes. PCR amplification was performed as outlined in Materials and Methods Section, except that the primers TP-F, TDCB1-R, TDCB2-F and TDCB3-R were used at concentrations of 200, 50, 50 and 200 mM, respectively.

CONSTRUCTION OF TRANSFORMATION VECTORS

All the PCR products were cloned into cloning vectors as shown in *Figure 2*, then sequenced for confirmation of their identity (data not shown). A list of all the promoter cassettes and genes of interest is shown in *Table 4*. The steps taken to generate PHB and PHBV transformation vectors driven by the constitutive and MSP1 promoters using these

TABLE 4. LIST OF CONSTRUCTED PROMOTER CASSETTES AND GENES OF INTEREST

Ligation	Vector	Description
PCRCaMV35S/BamHI+SK/BamHI	pMB1	CaMV35S-SK
PCRNOS+ PCR2.1	pMB2	NOS-PCR2.1
pMB2/SpeI+pMB1/AvrII	pMB3	CaMV35S-NOS-SK
PCRTpphbA/SpeI+SK/SpeI	pMB4	TpphbA-SK
PCRTpphbB/SpeI+ PCR2.1	pMB5	TpphbB-SK
PCRTpphbC/SpeI+SK/SpeI	pMB6	TpphbC-SK
PCRTpbktB/SpeI+SK/SpeI	pMB7	TpbktB-SK
PCRUbiPro/BamHI+SK/BamHI	pMB8	UbiPro-SK
pMB2/SpeI+pMB10/AvrII	pMB9	UbiPro-NOS-SK
PCRbar/SpeI+SK/SpeI	pMB10	bar-SK
PCRActin/SpeI+SK/SpeI	pMB11	Actin-SK
pMB2/SpeI+pMB17/AvrII	pMB12	Actin-NOS-SK
PCRTptdcB+PCR2.1	pTDCB	TptdcB-PCR2.1
PCRMSP/SpeI+SK/SpeI	pMS1	MSP1-SK
PCRNos/SpeI+ SK/SpeI	pMS2	Nos-SK
pMS2/SpeI/AvrII + pMS1/AvrII	pMS3	MSP1-Nos-SK
PCRTpphbA/AscI +pFC2/AscI	pMS4	TpphbA-pFC2
PCRTpphbB/AscI +pFC2/AscI	pMS5	TpphbB-pFC2
PCRTpbktB/AscI +pFC2/AscI	pMS6	TpbktB-pFC2
PCRTptdcB/AscI +pFC2/AscI	pMS7	TptdcB-pFC2
PCRTpphbC/AscI+pFC2/AscI	pMS8	TpphbC-pFC2

plasmids are described below.

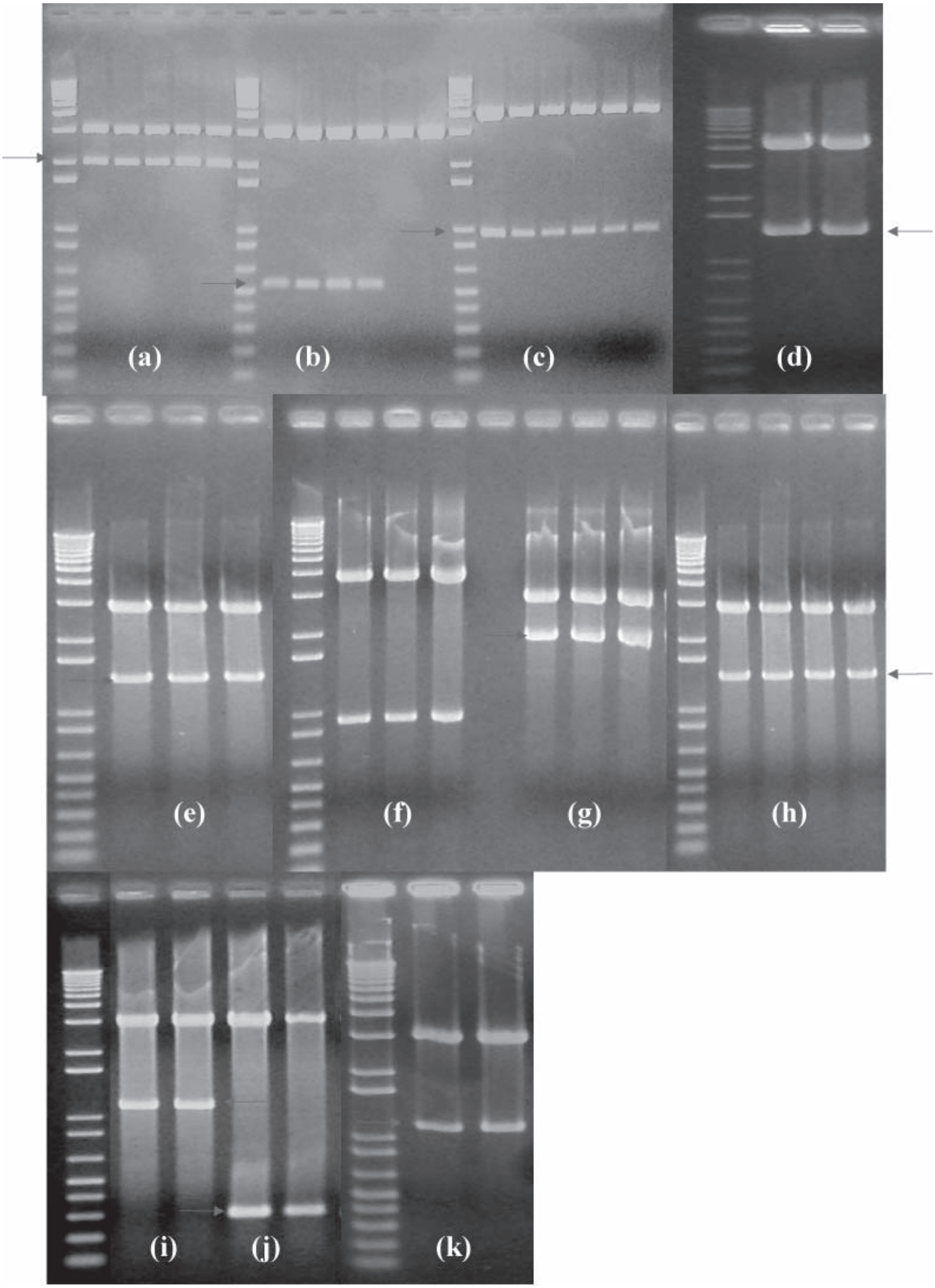


Figure 2. Cloning of PCR products (arrows) into cloning vectors. a. UbiPro (1.9 kb), b. bar (0.5 kb), c. CaMV35S (0.8 kb), d. Actin (1.3 kb), e. TpphbA (1.3 kb), f. TpphbB (0.9 kb), g. TpphbC (1.9 kb), h. TpbktB (1.3 kb), i. MSP1 (1.1 kb), j. Nos (0.3 kb) and k. TptdcB (1.1 kb).

Construction of PHB and PHBV Transformation Vectors Driven by Constitutive Promoters

Construction of* phbA *(pMB13) and* bktB *(pMB14) expression vectors. The pMB13 expression vector was constructed by cloning the 1.3 kb *Spe*I fragment of pMB4 (*TpphbA*) into the *Avr*II site of pMB3. Selected clones were screened by *Bam*HI digestion to cleave an approximately 2.6 kb *CaMV35S-TpphbA-NOS* fragment (*Figure 3a*). The sense orientation of the sequence was confirmed by PCR amplification with primers A2-F and NOS-R to give an approximately 1.5 kb PCR product (*Figure 3b*).

The pMB14 expression vector was constructed by cloning the 1.3 kb *Spe*I fragment of pMB7 (*TpbktB*) into the *Avr*II site of pMB3. As shown in *Figure 3a*, the positive clones were selected by screening with *Bam*HI to cleave an approximately 2.6 kb *CaMV35S-TpbktB-NOS* fragment. The sense orientation of the *TpbktB* sequence was confirmed by PCR amplification using primers BKT2-F and NOS-R to give an approximately 1.5 kb PCR product (*Figure 3b*).

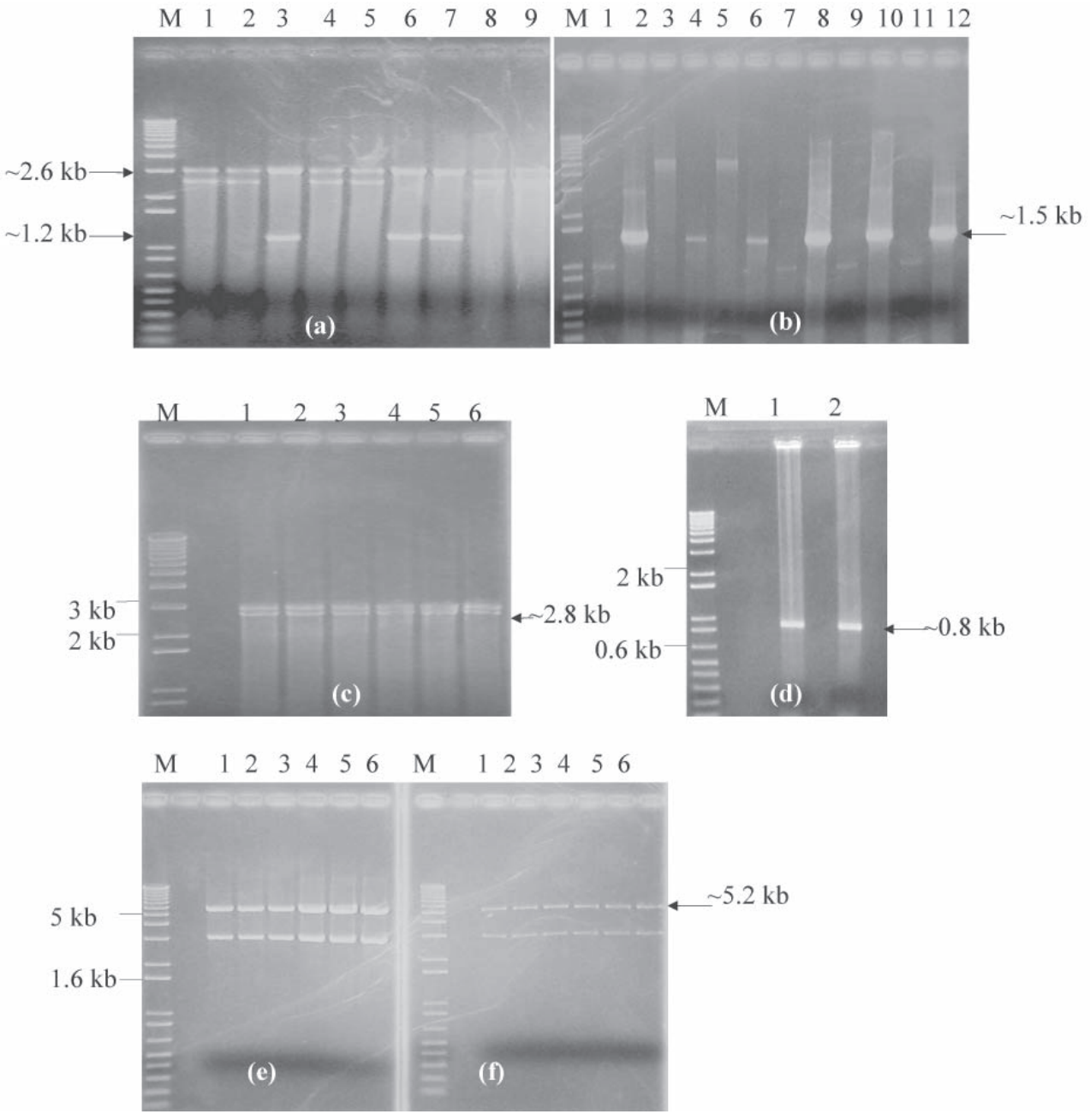


Figure 3. Selection of pMB13, pMB14, pMB16, pMB17 and pMB18. a. Restriction analysis of pMB13 (1~4) and pMB14 (5~9), b. PCR analysis of pMB13 (1~6) and pMB14 (7~12). c. Restriction analysis of pMB16, d. PCR analysis of pMB16. e. Restriction analysis of pMB17. f. Restriction analysis of pMB18. Arrows and size in kb indicate the size of fragment, 2.6 kb: CaMV35S-TpphbA-NOS and CaMV35S-TpbktB-NOS, 1.2 kb: CaMV35S-NOS, 1.5 kb: PCR product of phbA-NOS and bktB-NOS, 2.8 kb: UbiPro-bar-NOS, 0.8 kb: PCR product of bar-NOS and 5.2 kb: CaMV35S-TpphbA-NOS-UbiPro-TpphbB-NOS and CaMV35S-TpbktB-NOS-Ubiquitin-TpphbB-NOS.

Construction of* phbB *(pMB15) expression vector and* bar *(pMB16) selection vector. The *UbiPro-TpphbB-NOS* expression cassette in pMB15 was constructed by cloning the 0.9 kb *Spe*I fragment of pMB5 (*TpphbB*) into the *Avr*II site of pMB9, and then screened by digesting the resulting clones with *Bam*HI to cleave an approximately 3.0 kb *UbiPro-TpphbB-NOS* fragment. The sense orientation of the *TpphbB* sequence was confirmed by PCR amplification with B2-F and NOS-R primers to give an approximately 1.2 kb PCR product.

The *UbiPro-bar-NOS* (pMB16) gene expression fragment was constructed by cloning the 0.5 kb *Spe*I fragment of pMB10 (*bar*) into the *Avr*II site of pMB9. Selected clones were screened by digesting with *Bam*HI to cleave an approximately 2.8 kb *UbiPro-bar-NOS* fragment (*Figure 3c*). The sense orientation of the *bar* gene was confirmed by PCR amplification using BAR-F and NOS-R primers to give an approximately 0.8 kb PCR product (*Figure 3d*).

Construction of* phbA-phbB *(pMB17) and* bktB-phbB *(pMB18) expression vectors. A plasmid carrying the *CaMV35S-TpphbA-NOS* and *UbiPro-TpphbB-NOS* expression cassettes, designated pMB17, was constructed by cloning the 3.0 kb *Bam*HI fragment of pMB15 into the *Bgl*II site of pMB13. The ligation destroyed both the enzyme sites at which the generated fragment could be cleaved by *Bam*HI digestion for further recombination. The positive clones were selected by screening with *Bam*HI to cleave an approximately 5.2 kb *CaMV35S-TpphbA-NOS-UbiPro-TpphbB-NOS* fragment (*Figure 3e*). The orientation of the sequence was confirmed by *Apa*I digestion to cleave DNA fragments of approximately 3.4 kb, 3.0 kb and 2.2 kb. The same approach was used to produce the pMB18 expression vector, which contained the expression cassettes of *CaMV35S-TpbktB-NOS* and *UbiPro-TpphbB-NOS* from pMB14 and pMB15 (*Figure 3f*).

Construction of the* phbC *(pMB19) and* phbC-tdcB *(pMB20) expression vectors. An *Actin-TpphbC-NOS* expression cassette in pMB19 was constructed by cloning the 1.9 kb *Spe*I fragment of pMB6 (*TpphbC*) into the *Avr*II site of pMB12. The positive clones were selected by screening with *Spe*I to cleave an approximately 3.9 kb *Actin-TpphbC-NOS* fragment. The sense orientation of the *TpphbC* sequence was confirmed by PCR amplification using the C2-F and NOS-R primers to give an approximately 2.2 kb PCR product.

The construction of pMB20 was performed using the plasmids pTDCB, pMB9 and pMB19. The 1.1 kb *Spe*I fragment of pTDCB (*TptdcB*) was cloned into the *Avr*II site of pMB9 to produce the *UbiPro-TptdcB-NOS* expression cassette. A DNA fragment of this cassette was then digested with *Bam*HI and cloned into the *Bam*HI site of pMB19 to produce pMB20. The positive clones for pMB20 were selected by *Spe*I digestion to cleave an approximately 7.1 kb *Actin-TpphbC-NOS-UbiPro-TptdcB-NOS* fragment.

Construction of pMB21 backbone vector. The binary transformation vector, pGreenII0000, which can be used in *Agrobacterium*-mediated transformation protocol (Hellens *et al.*, 2000), was used as the parent plasmid for the construction of pMB21. The pMB21 backbone vector was constructed as detailed in *Figure 4* using two DNA fragments of RB7MAR (1.1 kb) from plasmid pGHNC05, which were digested with *Kpn*I-*Pst*I and *Spe*I-*Sac*I, and then cloned into the *Kpn*I-*Pst*I and *Spe*I-*Sac*I sites of pGreenII0000, respectively. The pMB21 vector was used as a backbone vector as it contains multiple cloning sites that are flanked by RB7MAR and synthetic Left-border (LB) and Right-border (RB) sequences.

Construction of pMB22 and pMB23 expression vectors. The *phbA-phbB* (pMB22) and *bktB-phbB* (pMB23) flanked with RB7MAR expression vectors were constructed as follows. The pMB22 was constructed by cloning the *Bam*HI fragment of pMB17 into the *Bam*HI site of pMB21. The positive clones were selected by digestion with *Bam*HI to cleave an approximately 5.2 kb *CaMV35S-TpphbA-NOS-UbiPro-TpphbB-NOS* fragment. The orientation of the sequence was confirmed by digestion with *Bgl*II to cleave DNA fragments of approximately 4.9 kb, 3.8 kb and 2.4 kb.

The pMB23 was constructed by cloning the *Bam*HI fragment of pMB18 into the *Bam*HI site of pMB21. The positive clones were selected by digestion with *Bam*HI to cleave an approximately 5.3 kb *CaMV35S-TpbktB-NOS-UbiPro-TpphbB-NOS* fragment. The orientation of the sequence was confirmed by digestion with *Bgl*II to cleave DNA fragments of approximately 5.0 kb, 3.7 kb and 2.4 kb (*Figure 4c*).

Construction of pMB24 and pMB25 expression vectors. The *bar-phbA-phbB* (pMB24) and *bar-bktB-phbB* (pMB25) gene expression fragments flanked by RB7MAR expression vectors were constructed as described in *Figure 5*. The 2.8 kb *Spe*I and *Eco*RV restricted *UbiPro-bar-NOS* from pMB16 were cloned into the *Sma*I site of pMB22 and pMB23 by blunt-end ligation to generate pMB24 and pMB25, respectively. The positive clones were screened by PCR amplification using primers BAR-F and BAR-R. The amplification should produce an approximately 0.5 kb PCR product (*Figure 6a*).

Construction of PHB (pMB26) and PHBV (pMB27) transformation vectors. The *Spe*I fragments of pMB19 (*Actin-TpphbC-NOS*) and pMB20 (*Actin-TpphbC-NOS-UbiPro-TptdcB-NOS*) were cloned into the *Spe*I site of pMB24 and pMB25, to produce

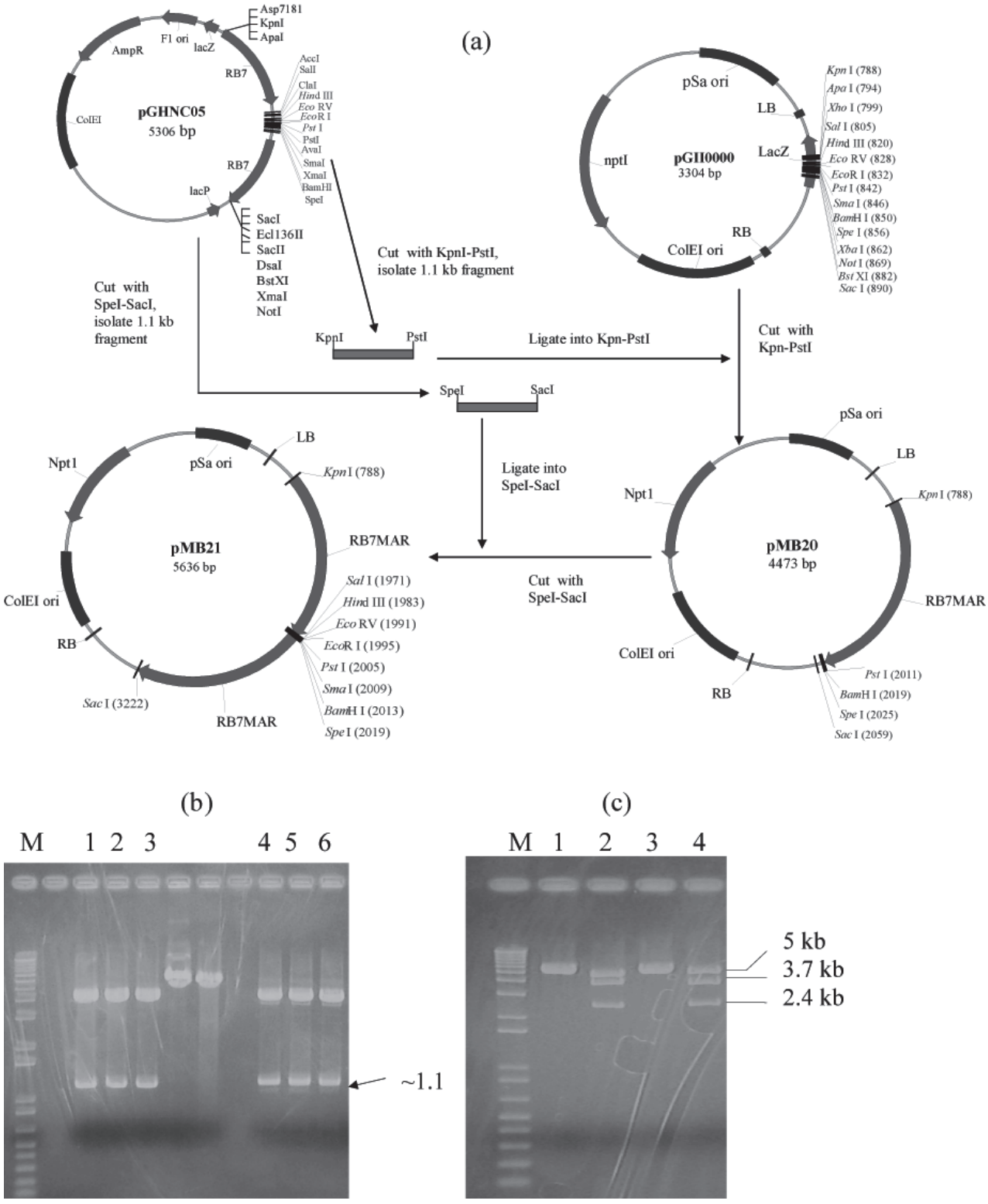


Figure 4. a. Construction of pMB21. b. Restriction enzymes of pGHNC05 to cleave 1.1 kb RB7MAR with KpnI-PstI (lanes 1~3) and SpeI-SacI (lanes 4~6). c. Confirmation of the orientation DNA fragments of pMB22 with BglII (lane 2) and pMB23 (lane 4).

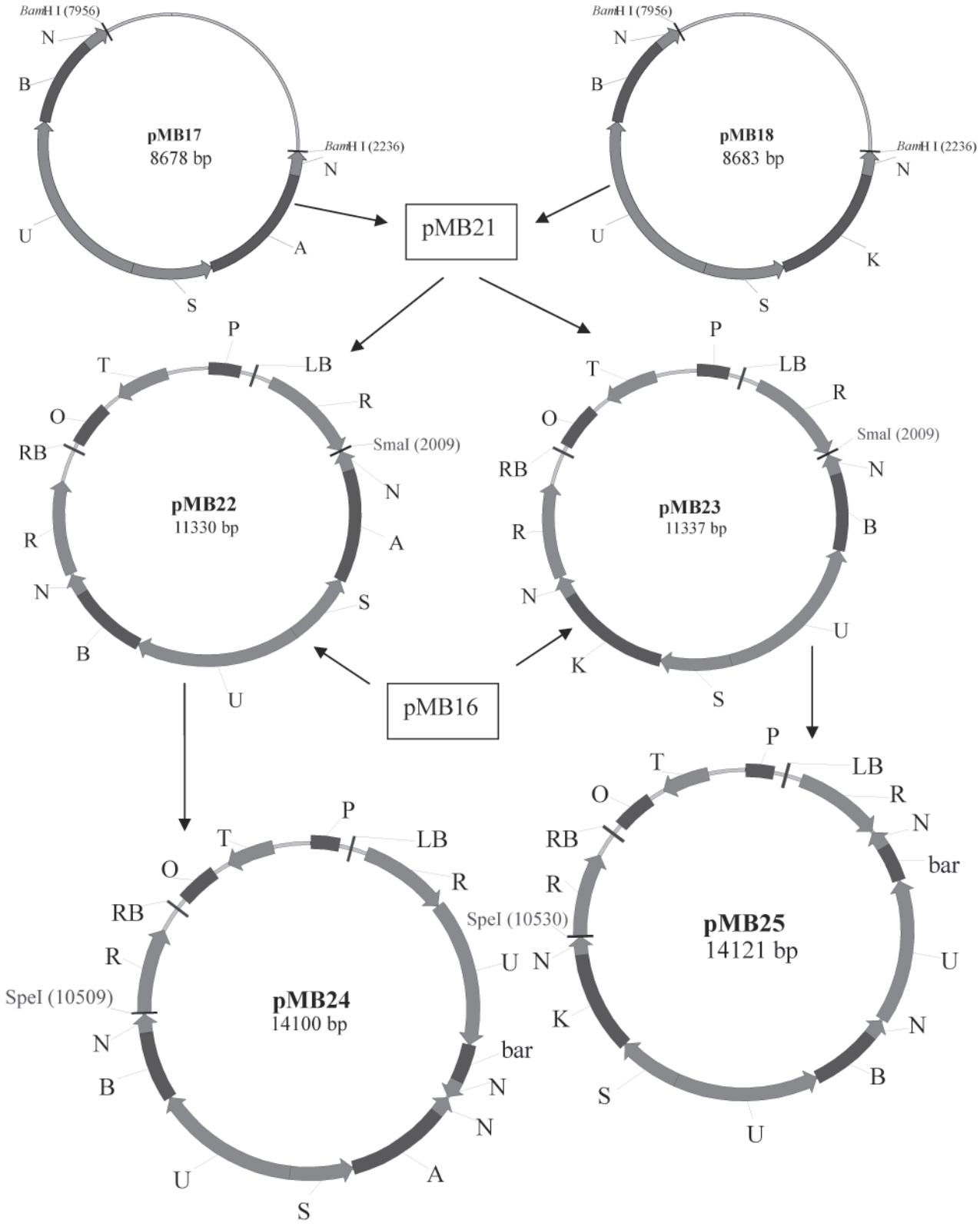


Figure 5. Construction of pMB22, pMB23, pMB24 and pMB25. The restriction sites (cloning sites) and numbers indicate the approximate position in the vectors. A: TpphbA, B: TpphbB, K: TpbktB, N: NOS, O: CoIE1 ori: P: pSa Ori: R: RB7MAR, S: CaMV35S, T: npt1, U: UbiPro.

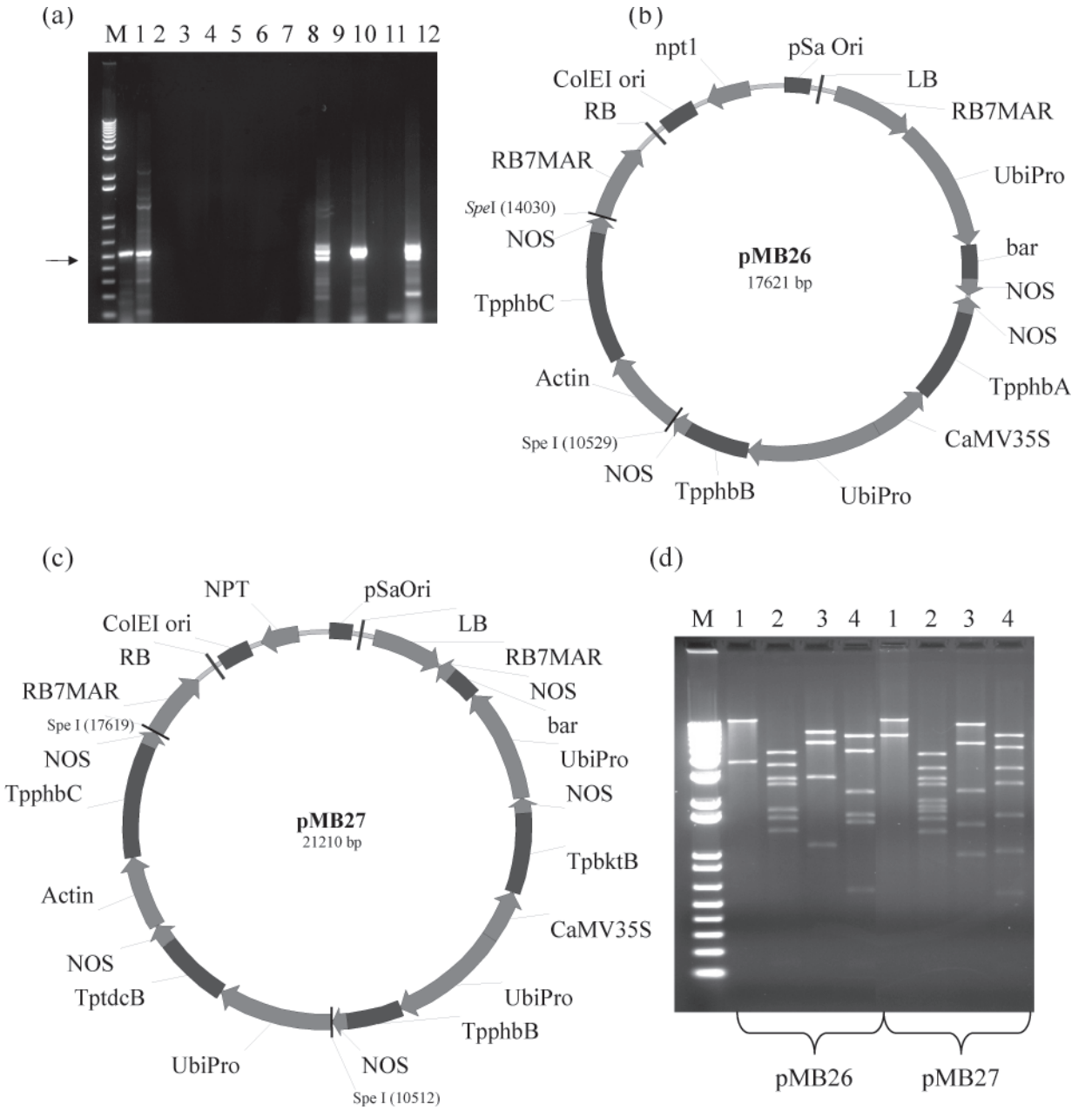


Figure 6. a. Selection of pMB24 and pMB25 (lanes 1 and 2: positive clones for pMB24, lanes 8, 10 and 12: positive clones for pMB25. Arrow indicates PCR products of ~0.5 kb), b. physical map of pMB26, c. physical map of pMB27, d. restriction enzyme analysis of pMB26 and 27 (lane 1: SpeI; lane 2: BglII; lane 3: BamHI; lane 4: SalI).

pMB26 and pMB27 as shown in *Figures 6b* and *6c*, respectively. The positive clones were selected by digestion with *Spe*I to cleave an approximately 4.2 kb *Actin-TpphbC-Nos* fragment for pMB26 and 7.1 kb *Actin-TpphbC-NOS-UbiPro-TptdcB-NOS* for pMB27 (*Figure 6d*).

Construction of PHB and PHBV Transformation Vectors Driven by Oil Palm Mesocarp-Specific Promoter (MSP1)

Construction of mesocarp expression vector, pMB41. The construction of pMB41 vector was performed using plasmids pMB35-46 and pMS3. The mesocarp-specific promoter (*MSP1*)-*Asc*I-*Nos*-terminator (*Nos*) cassette of pMS3 was cloned into pMB41 for insertion of the genes of interest using the *Asc*I site. The sense orientation of the MSP1 promoter sequence was confirmed by PCR (MSP1-F and NOS2-R) to give approximately 1.4 kb PCR product. In order to control the expression of genes in the oil palm mesocarp, the MSP1 promoter (1.1 kb) was used to drive the expression of transgenes during the period of oil synthesis (15-20 weeks after anthesis). This promoter will ensure that most of the effects on lipid metabolism are confined to the storage lipids without greatly affecting the lipid metabolism in leaves or other tissues which can otherwise lead to deleterious effects in the transgenic plants.

Construction of pMS22, pMS23, pMS24, pMS25 and pMS26. Five intermediate plasmids that consisted of the MSP1 promoter, coding regions of the PHB or PHBV gene and *Nos* sequences, were prepared for the construction of the final transformation vectors. The plastid-targeted genes, *TpphbA*, *TpphbB*, *TpphbC*, *TpbktB* and *TptdcB*, were individually amplified from pMB4, pMB5, pMB6, pMB7 and pTDCB, respectively, using combinations of two primers as listed in *Table 3*. The PCR products were digested with *Asc*I, gel purified and cloned into the *Asc*I site of pFC2 to create plasmids, pMS4, pMS5, pMS6, pMS7 and pMS8. The *Asc*I fragments of *TpphbA* (1.3 kb), *TpphbB* (0.9 kb), *TpphbC* (1.9 kb), *TpbktB* (1.3 kb) and *TptdcB* (1.1 kb) were inserted individually into the *Asc*I site of pMB41. The insertions of these fragments resulted in the production of intermediate plasmids designated as pMS22 (11.1 kb), pMS23 (10.7 kb), pMB24 (11.7 kb), pMS25 (11.1 kb) and pMS26 (10.9 kb). The orientation of the inserted genes was confirmed by PCR amplification using primers, TP-F and NOS2-R. The amplifications resulted in PCR products of approximately 1.6 kb *TpphbA-Nos* from pMS22, 1.2 kb *TpphbB-Nos* from pMS23, 2.2 kb *TpphbC-Nos* from pMS24, 1.6 kb *TpbktB-Nos* from pMS25 and 1.4 kb *TptdcB-Nos* from pMS26.

Construction of pMS27, pMS28, pMS29, pMS30 and pMS31. The transformation vectors, pMS29 (16.9 kb) bearing the three PHB genes (*phbA*, *phbB*, *phbC*) necessary for PHB production, and pMS31 (19.53 kb) bearing four genes (*bktB*, *phbB*, p*hbC*, *tdcB*) necessary for PHBV production, were generated from the *Spe*I DNA fragments of pMS22, pMS23, pMS24, pMS25 and pMS26 as outlined in *Figures 7* and *8*, respectively. All the vectors created from these

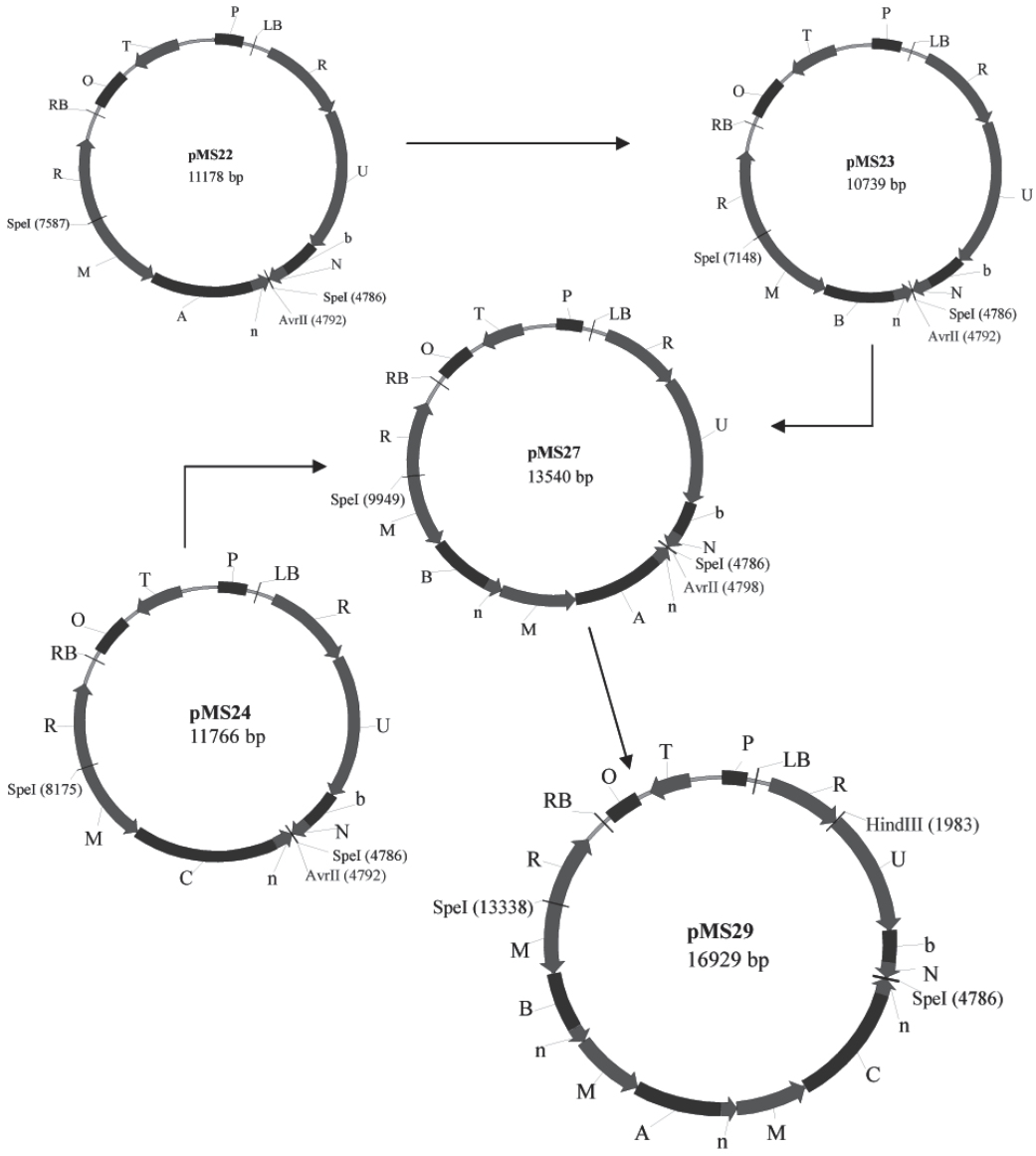


Figure 7. Strategy for the construction of plasmid pMS29 for production of PHB. The restriction sites (cloning sites) and the numbers indicate the approximate position in the vectors. A: TpphbA, B: TpphbB, K: TpbktB, C: TpphbC, N and n: Nos, O: CoIE1 ori: P: pSa Ori: R: RB7MAR, S: CaMV35S, T: npt1, U: UbiPro, b: bar.

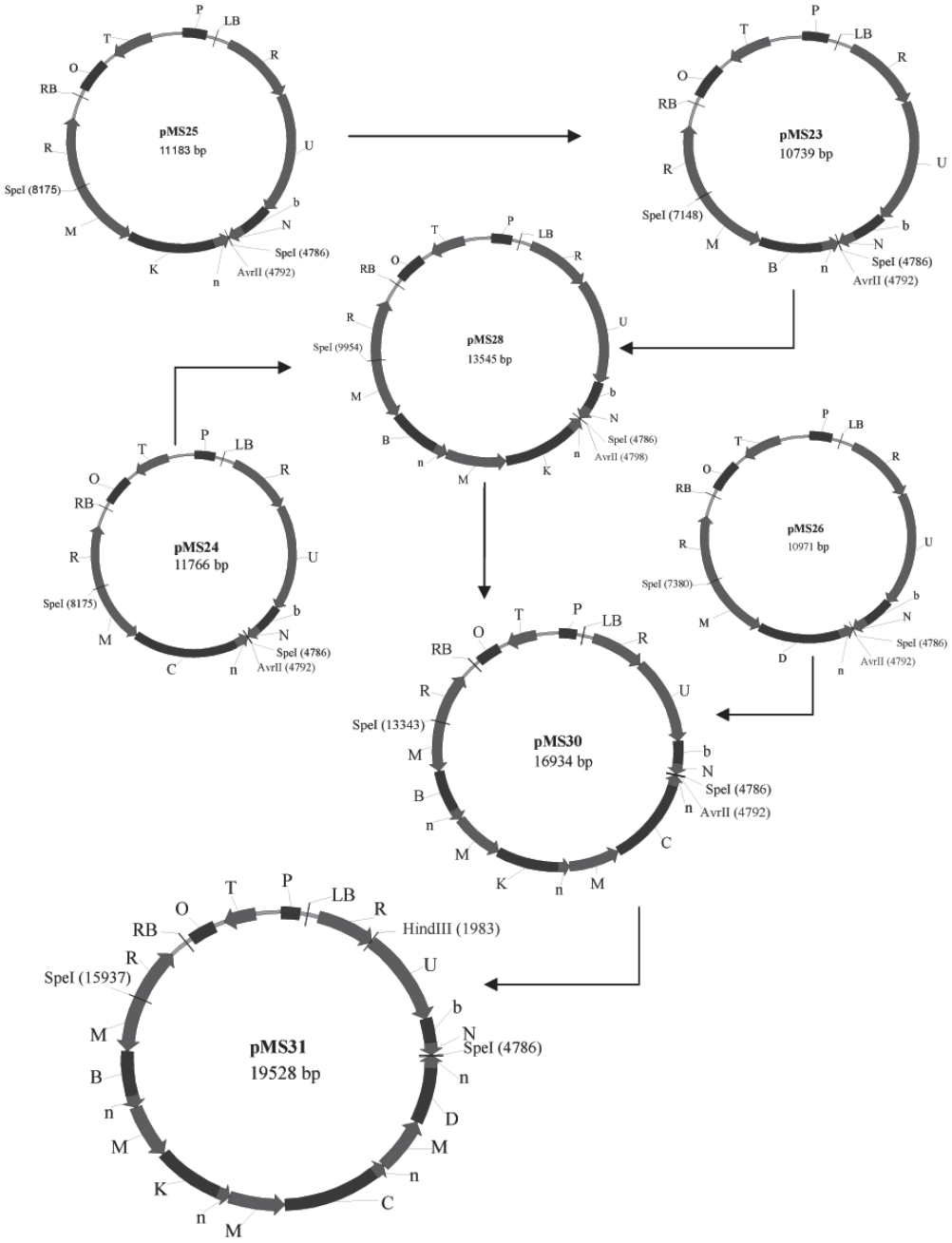


Figure 8. Strategy of the construction of pMS31 for production of PHBV. The restriction sites (cloning sites) and the numbers indicate the approximate position in the vectors. A: TpphbA, B: TpphbB, K: TpbktB, C: TpphbC, N and n: Nos, O: CoIE1 ori: P: pSa Ori: R: RB7MAR, S: CaMV35S, T: npt1, U: UbiPro, b: bar, D: TptdcB.

vectors were confirmed by digestion with *Spe*I, *Bgl*II, *Bam*HI and *Sal*I for the presence of DNA fragments with the expected size (*Figure 9*). The pMS27 was developed by inserting the 2.3 kb *Spe*I fragment of pMS22 (containing *MSP1-TpphbA-Nos*) into the *Avr*II site of pMS23 to produce a plasmid with the *phbA* and *phbB* expression cassettes. The 3.4 kb *Spe*I fragment of pMS24 (containing *MSP1-TpphbC-Nos*) was then cloned into the *Avr*II site of pMS27 to produce pMS29. The clone for pMS29 was selected by digestion with *Spe*I as shown in *Figure 7*. On the other hand, pMS28 was constructed by introducing the 2.7 kb *Spe*I fragment (*MSP1-TptdcB-Nos*) of pMS25 into the *Avr*II site of pMS23 to give a 13.3 kb expression vector containing the *bktB* and *phbB* expression cassettes. The 3.4 kb *Spe*I fragment of pMS24 (containing *MSP1-TpphbC-Nos*) was cloned into the *Avr*II site of pMS28 to generate pMS30 (16.9 kb). Then, pMS26 was digested with *Spe*I to cleave a 2.7 kb *MSP1-TptdcB-Nos* fragment. The fragment was cloned into the *Avr*II site of pMS30 to create pMS31.

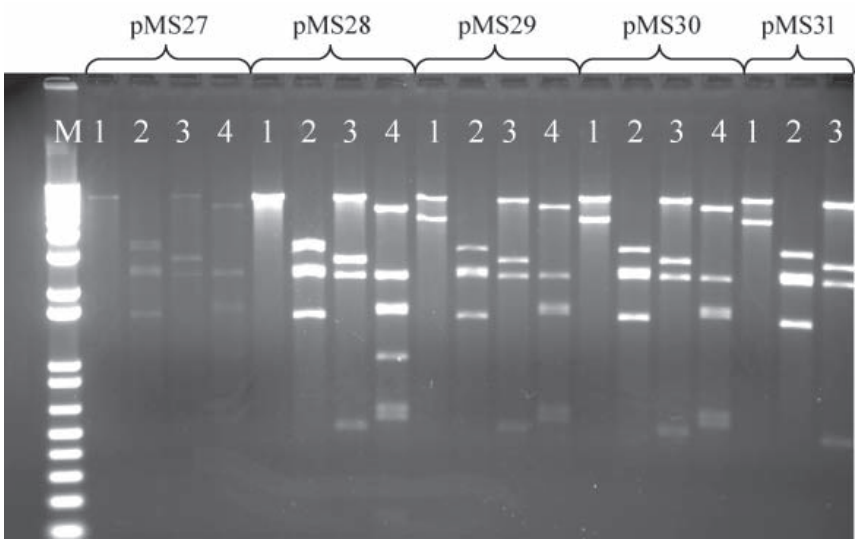


Figure 9. Restriction endonuclease analysis of pMS27~pMS31. Equal amounts of DNA of each plasmid were digested with different restriction endonucleases (lane 1: SpeI; lane 2: BglII; lane 3: BamHI; lane 4: SalI) and separated on 1% agarose gel in 1X TAE buffer. The sizes of the observed fragments in each digestion were as predicted. Lane M is 1 kb plus DNA ladder.

DISCUSSION

The main objective of this part of the project, *Transformation of Oil Palm for the Synthesis of PHB and PHBV* is to develop PHB and PHBV transformation vectors. The work was largely based on previously published work on construction and analysis of plant transformation vectors, particularly transformation vectors for production of PHA in plants. The choice of genetic elements and the strategy for construction of PHB and PHBV transformation vectors are important factors that determine the success of this work.

The *phbA*, *phbB* and *phbC* genes of *R. eutropha* were identified and used for synthesis of PHB, whereas its *bktB*, *phbB*, *phbC* genes and *tdcB*, encoding for the enzyme, threonine dehydratase, of *E. coli* were used to synthesis PHBV. The translation start codon ATG was excluded from the promoters (ubiquitin, CaMV35S, actin and MSP1) and replaced with that from the transit peptide. The presence of multiple translation initiations may interfere with the expression of the transgenes. Targeting the PHB or PHBV production into specific compartments, such as the plastid has been demonstrated to be a more successful strategy with better yield. In order to target the PHB and PHBV proteins into the plastid of oil palm leaves, a 180 bp DNA fragment encoding a transit peptide (*Tp*) of the oil palm acyl-carrier-protein (ACP) (Rasid *et al*., 1999) was fused to the 5′ end of each gene. The transit peptide could facilitate transport of the proteins into the plastid (Somerville *et al*., 1994). The transit peptide sequence was fused to the 5′ end of the open reading frame of the PHB or PHBV gene by a two-step PCR amplification using two pairs of primers. The use of these primers eliminated the requirement of restriction sites or linkers. Nawrath *et al.* (1994) ligated the plastid targeting sequence to the PHB gene using a three amino acid linker consisting of Ser-Arg-Val, which reduced the expression of the transgenes causing differences in the PHB yield in several transgenic plants (Bohmert *et al*., 2002). The start codon of the PHB or PHBV gene was also removed to ensure that the translation initiated from the start codon of the transit peptide. The advantage of using the same transit peptide, *Tp*, for both the PHB and PHBV genes is to ensure that any variability in expression of the genes is not due to the transit peptide used.

The maximum PHB yield (40% dwt) was reported for transgenic *Arabidopsis* plants transformed using multiple-gene transformation vectors (Bohmert *et al*., 2000). There was no possible scientific explanation provided on why the use of multiple-gene transformation vectors produced a higher yield of PHB compared to the use of a single-gene transformation vector. The use of multiple-gene transformation vectors also eliminated the need for cross pollination which would require four to six generations to combine three or four transgenes in a plant. It was hypothesized that introduction of the entire of PHB and PHBV pathways into plants by using multiple-gene vectors resulted in better coordination of gene expression and a reduction in gene silencing (Bohmert *et al*., 2000). The PHB and PHBV genes being expressed and terminated by their own promoters and terminators linked in the same direction could result in better coordination of the gene expression (Nawrath *et al*., 1994). Cross-pollination of homozygous plants containing the same promoter, transit peptide and terminator was suggested to cause the homology-dependent gene

silencing mechanism among the genes in the transgenic plants. Based on these hypotheses, four multiple-gene vectors with the PHB and PHBV genes driven by constitutive promoters, namely, pMB26 and pMB27, as well as by the MSP1 promoter, namely, pMS29 and pMS31, were designed. Due to the complexity of the multiple-gene vectors, the choice of restriction sites is important to ensure that the vectors can be constructed in a modular manner, such that the genetic elements can be exchanged in and out of the vectors in a relatively easy manner. The unique sites (*Bam*HI, *Bgl*II, *Spe*I, *Avr*II *and Asc*I) were introduced using PCR amplification to facilitate the cloning of different DNA fragments. These sites were selected based on the sequence information of the genetic elements used. Cloning by using these sites generated intermediate vectors to produce the PHB and PHBV transformation vectors, in which all the PHB and PHBV genes were linked together.

All the vectors were engineered using the binary Ti vector pGreenII0000 for transformation into plants cells using *Agrobacterium*-mediated or micro-projectile bombardment methods. The pGreenII0000 is a mini binary vector which has the advantage of being relatively small (3.5 kb). It replicates to high copy numbers in *E. coli* and is stable in *A. tumefaciens*. The pGreenII0000 carries the *npt1* resistance gene that confers resistance to the antibiotic kanamycin both in *E. coli* and *A. tumefaciens*. Its small size makes it more suitable to carry the more than 10 kb multiple-gene DNA fragments in this study, because the efficiency of *in vitro* recombination is inversely proportional to the size of the plasmid (Sambrook *et al*., 1989). All the vectors carried the phosphinothricin acetyltransferase gene (*bar*), which confers resistance to the herbicide Basta™. Parveez *et al.* (1996) reported that Basta™ is one of the most suitable selection agent for oil palm as it can stop the growth of embryogenic calli at low concentration. The ubiquitin promoter was chosen to drive the *bar* gene in this study for selection of oil palm transformants. The ubiquitin promoter has been characterized as a suitable promoter for monocots. Oil palm transformation studies have also revealed the ubiquitin promoter to be efficient and stable (Chowdhury *et al*., 1997; Parveez, 1998). All the vectors also contained the matrix attachment region of tobacco (RB7MAR) at their left-border and right-border (Matzke *et al*., 1994). The RB7MAR was used to flank the PHB or PHBV gene inserted in the final vectors to stabilize the transgene expression and minimize gene silencing due to positional effects. Studies (Spiker *et al*., 1996; Allen *et al*., 2000) have demonstrated that when RB7MAR was attached to both sides of a transgene, the expression level in stably transfected cell lines was proportional to the transgene copy number. This indicates that the gene activity is independent of its position in the chromosome.

The uniqueness of these vectors was the use of different promoters to drive each PHB or PHBV gene, the use of *phbA* or *bktB* genes, and finally the use of the *tdcB* gene. The use of two 3-ketothiolase genes, *i.e*. *phbA* in pMB26 and pMS29 for PHB production, and *bktB* in pMB27 and pMS31 for the production of PHBV, was based on previous findings (Houmiel *et al*., 1999; Saruul *et al*., 2002). The *phbA* gene was more effective (*i.e*. two-fold higher) than *bktB* gene in producing PHB. On the other hand, *bktB* has a higher specificity for propionyl-CoA than *phbA* for PHBV production. The *tdcB* gene in pMB27 and pMS31 was used to convert the plant's threonine pool to propionyl-CoA by catalyzing threonine to 2-ketobutyrate, and then to propionyl-CoA by native plant pyruvate dehydrogenase complex (PDC). Although researchers have previously demonstrated that targeting of anabolic threonine deaminase (*IlvA* gene) to plastid (along with *bktB*, *phbB*, and *phbC*) can be used for PHBV production, it exhibits product inhibition by isoleucine (Slater *et al*., 1999). In contrast, the *tdcB* gene functions more effectively in producing the precursor of PHBV as it is not subject to product inhibition (Goillouet *et al*., 1999).

Most of the vectors constructed in this work have 3 ~ 4 sets of repeat sequences such as the promoter, transit peptide and *Nos*-terminator. The direct repeat sequences are unstable in the most commonly used strains of *E. coli* (Schmidt and Bloom, 1999). Since *E. coli* STBL4 was able to maintain pHS30 containing over 100 of 32 bp repeats (Strader and Howell, 1995), these PHB/PHBV vectors were transformed into STBL4 cells to avoid the loss of fragments due to recombination. This strain managed to retain the genetic property of these vectors for stable maintenance of direct repeat sequences. Furthermore, DNA sequencing of these vectors was performed to ensure that all sequences were identical to the corresponding original sequences. In addition, each vector was also confirmed by restriction analyses. The size of fragments generated are agreeable with the expected size of these fragments.

The orientation of each gene assembled in these transformation vectors may also affect the expression of transgenes (Ingelbrecht *et al*., 1991). For example, in pMS29, the orientation of all the PHB genes are in the same direction. This can eliminate transcriptional read-through into a downstream gene, and result in substantial reduction of the transgene expression in the transgenic plants. If the sequences of *MSP1-TpphbB-Nos* and *MSP1-TpphbA-Nos* are in opposite orientations, the MSP1 promoter of *phbB* gene may transcribe *phbA* in an antisense orientation. In addition, both sequences of RB7MAR at LB and RB were also in the same direction as the PHB, PHBV and *bar* genes. These RB7MAR sequences are expected to protect these genes from the influence of the flanking DNA sequences by binding the

nuclear matrix and causing the encapsulated genes to loop out. Thus, the RB7MAR may increase transgene expression and decrease the variability of PHB, PHBV and *bar* gene expression. If the direction of both RB7MAR is in opposite orientation to the flanking genes, the capability of RB7MAR to bind with the nuclear matrix of plant cells is expected to decrease and thus reduce the transgene expression (Allen *et al*., 2000).

CONCLUSION

The main goal of this work was to examine the possibility of producing high yields of PHB and PHBV by transgenic oil palms for commercial application. This was attempted by constructing two PHB (pMB26, pMS29) and PHBV (pMB27 and pMS31) transformation vectors driven by constitutive (ubiquitin, CaMV35S and actin) and tissue-specific (MSP1) promoters. The cloning process involved manipulation and generation of two backbone and nearly 50 intermediate vectors. The sequence integrity and orientation of each vector were verified by restriction enzymes, PCR and DNA sequencing. Each PHB and PHBV gene was fused with the transit peptide (*Tp*) of the oil palm acyl-carrier-protein (*ACP*) gene for plastid targeting in the plastids of the plant cells. These genes were linked together, adjacent to the selectable marker, *bar* gene, driven by the ubiquitin promoter for oil palm transformation. All the linked genes were flanked with RB7MAR to stabilize the transgene expression, minimize gene silencing from positional effects and increase the expression levels. In addition, the position and direction of the assembled genes were also considered to reduce transcriptional interference. All assembled genes were cloned in a binary Ti vector, pGreenII0000, for *Agrobacterium*-mediated transformation. It is hoped that the transgenic plants produced from these vectors will be stable and synthesize high levels of PHB or PHBV.

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