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REAGENTS FOR GENERATION AND ANALYSIS OF BIOPLASTIC PRODUCING PLANTS

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are a class of biodegradable polyoxoesters synthesized from acetyl-CoA that naturally accumulate as intracellular granules in a diverse range of bacteria. Biosynthesis of the simplest PHA (PHB, poly-3-hydroxybutyrate) can be accomplished though the action of three enzymes, beta-ketothiolase, acetoacetyl-CoA reductase and PHA polymerase. We constructed plasmids that contain genetic elements for the production of PHB, or the closely related copolymer PHBV (poly-3-hydroxybutyrate-co-3-hydroxyvalerate), in oil palm, which produces abundant levels of acetyl-CoA. These bacterial PHA biosynthetic genes have been engineered to include plant plastid targeting signals, in order to direct biosynthesis of the polymer inside the plastids. In addition, we generated antibodies for the detection of PHA biosynthetic enzymes. The plasmids and antibodies reported in this work should be suitable as tools and reagents for the construction and analysis of PHA-producing oil palm.

Keyword: polyhydroxyalkanoate, PHB, PHBV, metabolic engineering, plastid targeting.

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyoxoesters that are produced by a diverse range of bacteria as storage for carbon and reducing equivalents. PHAs accumulate when the bacteria are grown under limitation for a nutrients, such as nitrogen, while in the presence of an abundant carbon source such as glucose. Palm oil, or palm oil mill effluent are also attractive carbon sources for bacterial PHA production (Tan *et al*., 1997; Majid *et al*., 1999; Hassan *et al*., 2002; Loo *et al*., 2005; Annuar *et al*., 2007). PHAs occur intraceullarly as an amorphous phase within granules and can accumulate to 90% of the total cell mass of the bacteria (Lawrence *et al*., 2005). Intracellular stores of PHAs contribute to the survival of cells during nutrient deprivation (Willis and Walker, 1998; James *et al*., 1999). PHAs are biodegradable, and can be utilized (degraded) not only by PHA-producing bacteria, but by many non-PHA-producing bacteria and fungi (Jendrossek and Handrick, 2002; Lim *et al*., 2005; Sridewi *et al*., 2006).

PHAs are semi-crystalline thermoplastics that can be used to manufacture biodegradable plastics for use in consumer and medical applications (Boswell, 2001; Martin and Williams, 2003; Siew *et al*., 2007). The general structure of PHAs is shown in *Figure 1*. Depending on the chemical structure and length of the side chain in the polymer, PHAs can exhibit properties ranging from thermoplastics thermoplastic to elastomers (Hazer and Steinbuchel, 2007). More than 100 structurally distinct forms of PHA have been produced through genetic and metabolic engineering (Lenz and Marchessault, 2005).

Polyhydroxybutyrate (PHB) is the simplest PHA. PHB is a homopolymer of *R*-3-hydroxybutyrate and a useful model compound for studying the synthesis and properties of short-chain-length PHAs. Poly-(hydroxybutyrate-co-hydroxyvalerate) (PHBV) is a copolymer of *R*-3-hydroxybutyrate and *R*-3-

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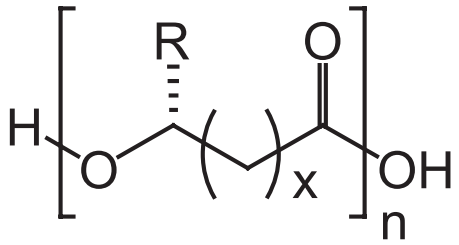

Figure 1. General structure of polyhydroxalkanoates. The chain length (x) and chemical structure of the side chain'R' affect the physical and mechanical properties of the polymer, including melting temperature, elasticity, and hydrophobicity.

hydroxyvalerate. The physical properties of PHBV depend on the precise ratio of HB to HV. PHBV is useful for the production of biodegradable plastics, due to its relatively low melting temperature and relatively high flexibility and toughness.

The biosynthesis of PHB from acetyl-CoA can be accomplished in the model bacterium *Ralstonia eutropha* by the action of three enzymes: beta-ketothiolase (encoded by *phaA*), acetoacetyl-CoA reductase (encoded by *phaB*) and PHA polymerase (encoded by *phaC*) (Peoples and Sinskey, 1989a, b). The pathway for PHB synthesis is diagrammed in *Figure 2a*. Biosynthesis of PHBV requires the use of an additional gene, *bktB*, which encodes a thiolase that can act either on acetyl-CoA or propionyl-CoA (Slater *et al*., 1998) and that can substitute for *phaA* in PHB biosynthesis. The pathway for PHBV synthesis is diagrammed in *Figure 2b*.

Production of PHA polymer in plants is a goal of the bioplastics industry, since it promises significant cost savings over bacterial fermentation (Snell and Peoples, 2002; Bohlmann, 2006). PHA biosynthesis can be accomplished by the introduction of the bacterial open reading frames with the appropriate plant transcription and translation signals (Snell and Peoples, 2002; Suriyamongkol *et al*., 2007). Poirier *et al*. (1992) were the first to report the production of PHB in the cytoplasm and vacuoles of transgenic *Arabidopsis*; the growth and seed production of these transgenic plants was greatly reduced (Poirier *et al*., 1992). The group later redirected the heterologous proteins into the plastids; PHB production in shoots was increased to 14% dry weight and furthermore, no obvious deleterious effect was observed (Nawrath *et al*., 1994). The PHB yield was further increased to 40% dry weight in transgenic *Arabidopsis* transformed with a single gene construct carrying *phaA*, *phaB*, and *phaC* (Bohmert *et al*., 2000). However, the plant growth was inversely proportional to the PHB yield and stunted growth and loss of fertility were observed in the high-producing lines. PHA production in transgenic plants has now been reported for several species, including *Arabidopsis*, *Brassica*, tobacco, cotton, corn, potato, alfalfa, sugarbeet, flax, sugarcane and switchgrass (Snell and Peoples, 2002). Transgenic rape expressing PHB genes in the seed leucoplast were shown to accumulate PHB up to 8% of the fresh seed weight (Houmiel *et al*., 1999), the results suggesting that accumulation of PHB in an oilseed system may be better tolerated.

Oil palm has the highest productivity of any oil crop, and produces abundant levels of acetyl-CoA, a key precursor in the biosynthesis of both fatty acids

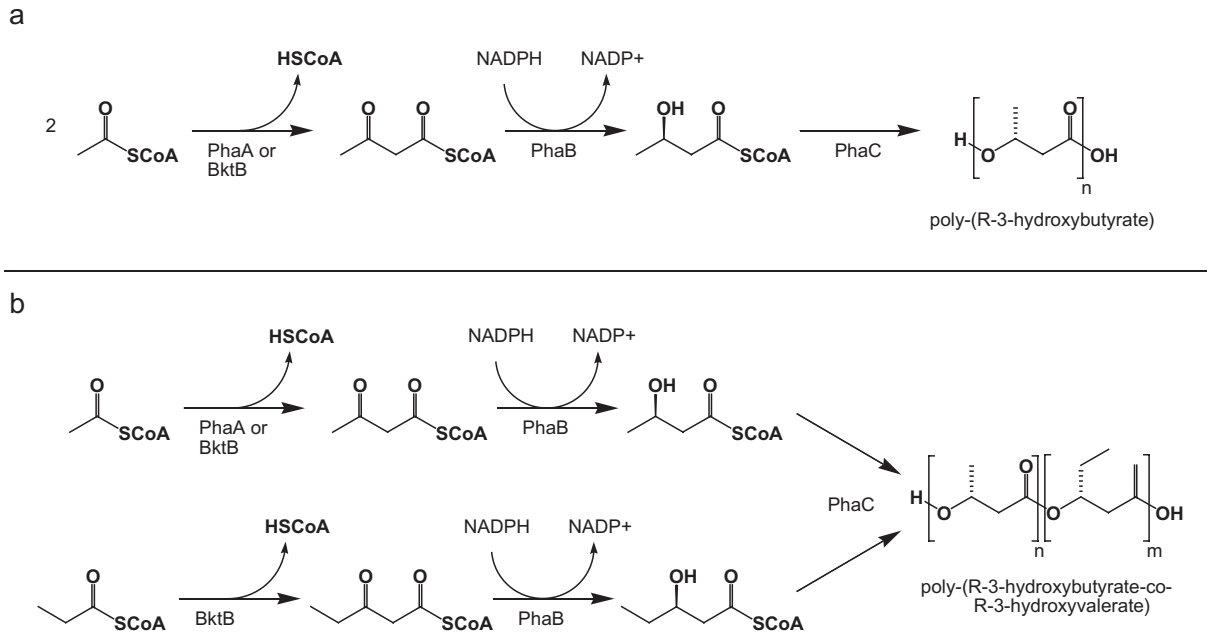

Figure 2. a. Metabolic pathway for biosynthesis of polyhydroxybutyrate (PHB) in R. eutropha. *b. Metabolic pathway for biosynthesis of poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) in* R. eutropha.

and PHAs (Oo *et al*., 1985; Ramli *et al*., 2002). Biolistic and *Agrobacterium*-mediated transformation methods have been developed as tools for engineering this important crop plant (Parveez *et al*., 2000; Abdullah *et al*., 2005). In this work, we describe the development of tools and reagents for the generation and analysis of bioplastic producing oil palm. We describe the construction of two vectors designed to direct the production of PHB and poly-hydroxybutyrate-co-hydroxyvalerate PHBV, respectively, in the plastids of plants. In addition, we describe the generation of antibodies that bind to PHA biosynthetic enzymes and that can be used in the analysis of PHA-producing oil palm.

EXPERIMENTAL

DNA Preparation and Manipulation

Standard approaches were used for preparation and manipulation of DNA, for transformation of *E. coli*, and for PCR (Ausubel *et al*., 2007). Genomic DNA was prepared from *Ralstonia eutropha* as described previously (York *et al*., 2001). The strains and plasmids used in this study are listed in *Table 1*. oligonucleotide The oligonucleotide primers are listed in *Table 2*. All constructs containing PCR products were confirmed by sequencing at the MIT Biopolymers Laboratory.

TABLE 1. STRAINS AND KEY PLASMIDS USED IN THIS STUDY

Strain or plasmid	Description	Source or reference
Escherichia coli strains		
DH5a	Strain for ligation/cloning experiments	New England Biolabs, Ipswich MA
TOP10	Strain for ligation/cloning experiments	Invitrogen
STBL-4	Restriction-deficient strain. *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *gyr*A96 *gal*$^{-}$ *thi*-1 *sup*E44λ^{-} *rel*A1 Δ (*lac-pro*AB)/ F′*pro*AB^{+} *lac*I^{q}Z ΔM15 Tn*10* Tet^{R}	Invitrogen
BL21(DE3) pLysS	Strain for T7 expression system	Studier and Moffatt (1986)
Ralstonia eutropha strain		
AeH16	Wild-type, gentamicin resistant, also termed DSM 428 and ATCC17699	ATCC17699
Plasmids		
pBluescript II KS	High copy number vector used for cloning, confers ampicillin resistance. Nucleotide accession number X52327.	Stratagene, (Alting-Mees and Short, 1989)
pCR2.1-TOPO	High copy number vector used for cloning, confers resistance to ampicillin and kanamycin.	Invitrogen
pAHC20	Source of maize ubiquitin promoter and *Streptomyces hygroscopicus bar* gene	Peter Quail, (Christensen and Quail, 1996)
pGUS:7S3′ end	Source of soyabean beta conglycinin 7S 3′ transcriptional termination sequence	Philip Lessard, (Fujiwara *et al*., 1992)
pME22	Expression vector for directing PHB production in plants	This study
pME26	Expression vector for directing PHBV production in plants	This study
pET14b	Expression plasmid for the production of N-terminal histidine-tagged proteins	Novagen (EMD Biosciences)
pET14b-BktB	pET14b containing *R. eutropha bktB*	This study
pETE14b-PhaB	pET14b containing *R. eutropha phaB*	This study

Note: The construction of additional plasmids is described in the experimental section.

TABLE 2. OLIGONUCLEOTIDE PRIMERS USED IN THIS STUDY

Name of primer	Sequence 5′-3′	Notes
ME1-bktB1	GGCCGGATCCTGACGCGTGAAGTGGTAGTGG	nt 4311 in Accession AF026544 (forward)
ME1-bktB2	GCGCGGATCCGTTCTTCGTCAGCGAAGCAAGG	nt 5529 in Accession AF026544 (reverse)
ME1-bktB3	GGCCGGCATATGACGCGTGAAGTGGTAGTG	-
ME1-phaB1	CGGCGGATCCTGACTCAGCGCATTGCGTATG	nt 3953 in Accession J04987 (forward)
ME1-phaB2	GGCCGGATCCGCTGGCTGCACCGCAATAC	nt 4758 in Accession J04987 (reverse)
ME1-phaB3	GGCCGGCATATGACTCAGCGCATTGCGTATG	-
ME1-phaC1	GCGGCGGATCCTGGCGACCGGCAAAGGCG	nt 843 in Accession J05003 (forward)
ME1-phaC2	GCGCGGGATCCGGCACTCATGCAAGCGTC	nt 2629 in Accession J05003 (reverse)
ME1-pUBI1	CCGGACTAGTCTGCAGTGCAGCGTGACCC	nt 1 in Accession I06936 (forward)
ME1-pUBI3	GCGCGGATCCTGTATCTGCAGAAGTAACACC	nt 1994 in Accession I06936 (reverse)
ME1-rbcS1	GGCCAGATCTCACCATGGCTTCTATGATATCCTCTTCC	nt 1086 in Accession X00806 (forward)
ME1-rbcS2	GCGCGGATCCGATCTCTGGTCAATGGTGGC	nt 1408 in Accession X00806 (reverse)
ME1-rbcS3	gcatgcagGTGTGGCCTCCAATTGGAA	nt 1342 in Accession X00806 (forward)
ME1-rbcS4	ggaggccacacCTGCATGCACTTTACTCTTCCA	nt 1262 in Accession X00806 (reverse)
7S3′B	GGGATCCCGTCCTTTGTCTTCA	nt 3470 in Accession M13759 (forward)
7S3′AS	CCACTAGTTCCTAGGATGCAATTTCTTTTCTGGT	-
ME1-tdcB1	GCGGCGGATCCTGCATATTACATACGATCTGCC	nt 45793 in Accession U18997 (forward)
ME1-tdcB2	GCGCGCGGATCCTTAAGCGTCAACGAAACCGGTC	nt 46782 in Accession U18997 (reverse)

Note: The accession numbers are from the GenBank database at the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov).

Individual genetic elements were amplified by PCR and engineered to contain restriction sites as appropriate. The maize ubiquitin 1 promoter was amplified from pAHC20, using primers ME1-UBI1 and ME1-UBI3 which introduce SpeI and BamHI sites and remove the maize start codon, and cloned as a blunt-ended fragment into the EcoRV site of pBluescript II KS, such that the SpeI site in the PCR product is adjacent to the SpeI site in the multicloning site of the vector. This plasmid was cut with SpeI and religated to eliminate the small SpeI fragment. The resulting plasmid was digested with NotI and HindIII to release the insert, the ends of the insert filled by treatment with DNA Polymerase I large (Klenow) fragment, and the blunt-ended fragment cloned into the EcoRV site of pBluescript II KS such that the BamHI site is adjacent to the BamHI site in the multicloning site of the vector, yielding pME1. The pea rubisco small subunit plastid targeting sequence was amplified from pea genomic DNA, using primers ME1-rbcS1 and ME1-rbcS2, which introduce BglII and BamHI sites, then used as template for removal of the intron by splicing by the overlap extension (Horton *et al*., 1989), using primers ME1-rbcS1, ME1-rbcS2, ME1-rbcS3, and ME1-rbcS4. The resulting PCR product (*i.e.* the plastid plastid-targeting sequence lacking the intron) was cloned as per the manufacturer's instructions into PCR2pCR2.1-TOPO, yielding plasmid pME2. The soyabean beta conglycinin 7S 3′ transcriptional

termination sequence was amplified from the pGUS:7S3′ end (Lessard *et al*., 1991; Fujiwara *et al*., 1992) with primers 7S3′B and 7S3′AS, which introduce a BamHI site at the 5' end and AvrII-SpeI sites at the 3' end, and the PCR product cloned as per the manufacturer's instructions into pCR2.1-TOPO, yielding plasmid pME3.

R. eutropha phaC, *bktB* and *phaB* were amplified from *R. eutropha* genomic DNA using primers ME1-phaC1 and ME1-phaC2; ME1-bktB1 and ME1-bktB2; and ME1-phaB1 and ME1-phaB2, respectively, which introduce BamHI sites at both ends of the fragment. The *phaC* and *bktB* PCR products were cloned directly into the EcoRV site of pBluescript II KS, yielding pME4 and pME5, respectively. The *phaB* PCR product was digested with BamHI and cloned into the BamHI site of pBluescript II KS, to yield pME6. The *E. coli tdcB* gene was amplified from *tdcB*-pCRscript (Guillouet *et al*., 1999) using primers ME1-tdcB1 and ME1-tdcB2 and cloned per the manufacturer's instructions into pCR2.1-TOPO, yielding plasmid pME23.

The maize ubiquitin promoter and transcriptional termination signal were joined as follows: pME1 was partially digested with SpeI (cleaving the SpeI site in the multiple cloning site to yield linearized plasmid) and digested to completion with BamHI, then ligated with the BamHI-SpeI fragment of pME3 (containing the transcriptional termination signal) to yield pME8. The plastid targeting sequence was cloned between the promoter and transcriptional termination signal by ligating the BglII-BamHI fragment of pME2 into the BamHI site of pME8, such that the plastid sequence was in correct orientation, to yield pME9. The pME8 was subjected to partial digestion with PstI to yield a linearized plasmid, and ligated with the 0.6 kb PstI fragment from pAHC20 containing the *Streptomyces hygroscopicus bar* gene such that the *bar* gene was inserted in the correct orientation in the PstI site located between the ubiquitin promoter and transcriptional termination signal, yielding pME10.

The *phaC*, *bktB*, *phaB* and *tdcB* genes were each subcloned as BamHI fragments from pME4, pME5, pME6 and pME23 into the BamHI site of pME9, such that each gene was in the correct orientation between the plastid targeting signal and transcriptional termination signal, and under the control of the ubiquitin promoter, to yield pME11, pME12, pME13 and pME24, respectively. The SpeI fragment of pME11 was cloned into the AvrII site of pME10 to yield pME20. The SpeI fragment of pME13 was cloned into the AvrII site of pME12 to yield pME21. The SpeI fragment of pME21 was cloned into the AvrII site of pME20 to yield pME22. The SpeI fragment of pME24 was cloned into the AvrII site of pME22 to yield pME26. Recombination-deficient *E. coli* strain STBL4 was used as the host for construction of pME20, pME21, pME22, pME24 and pME26. All plasmids were confirmed by restriction analysis.

Expression of Histidine-tagged BktB and PhaB

The *E. coli* strains DH5α and BL21(DE3) pLysS were used for the cloning of genes and expression of proteins, respectively. Plasmids were constructed to accomplish expression of the N-terminal His-tagged versions of BktB and PhaB. The *bktB* gene was amplified from *R. eutropha* genomic DNA using primers ME1-bktB3 and ME1-bktB2. The resulting PCR product was cloned into pCR2.1-TOPO, and the BamHI-NdeI fragment containing *bktB* ligated into pET14b that had been digested with BamHI and NdeI, yielding pET14b-bktB. The *phaB* gene was amplified from *R. eutropha* genomic DNA using primers ME1-phaB3 and ME1-phaB2. The resulting PCR product was cloned into pCR2.1-TOPO, subjected to partial digestion with NdeI, followed by complete digestion with BamHI and SpeI. The BamHI-NdeI fragment containing *phaB* was ligated into pET14b that had been digested with BamHI and NdeI, yielding pET14b-phaB.

Proteins were expressed by use of the pET expression system (Novagen, www.novagen.com). Plasmids pET14b, pET14b-bktB and pET14b-phaB were transformed into the host strain BL21 (DE3) pLysS. Cultures were inoculated from single colonies into LB medium supplemented with 100 μg ml^{-1} ampicillin, grown overnight on a roller drum at 37°C, then subcultured into 300 ml of LB supplemented with 100 μg ml^{-1} ampicillin and cultured until the OD_{600} reached 0.5. Protein expression was induced by the addition of IPTG to 0.4 mM final concentration. Cells were harvested after 4 hr of induction, then disrupted by brief sonication for 5 min in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The cell suspension was centrifuged at 10 000 rpm for 20 min and the supernatant transferred to a new tube. The induced protein was further purified using a Histag affinity column (Qiagen) as per the manufacturer's instructions. Centricon columns (Amicon) were used to remove imidazole and concentrate the eluted his-tagged protein. Proteins were quantitated by the Lowry assay (bovine serum albumin used as protein standard) (Lowry *et al*., 1951). SDS-polyacrylamide gel electrophoresis was used to assess the protein expression and purification.

Polyclonal Antibodies Against His-BktB and His-PhaB

Rabbit polyclonal antibodies against His-BktB and His-PhaB were generated by use of standard protocols (Harlow and Lane, 1988) at Covance Antisera Services (Denver, PA). Rabbits HM3892 and HM3893 corresponded to anti-BktB and anti-PhaB

sera, respectively. The immunoglobulin G (IgG) fraction of the exsanguination bleeds of rabbit sera was purified by use of a 1 ml HiTrap Protein G column (GE Healthcare Life Sciences, Piscataway, NJ) as follows: the column was washed with 10 ml of 20 mM sodium phosphate, pH 7.0. The 10 ml of rabbit serum (filtered through a 0.45 μM filter) were loaded onto the column, and 1 ml fractions collected. The column was washed with 6 ml of 20 mM sodium phosphate, pH 7.0. The IgG fraction was eluted by applying 3 ml 0.1 M glycine (pH 2.7) and collecting 1 ml fractions in microfuge tubes containing 75 μl 1 M Tris HCl to neutralize the pH of the samples.

Detection of Protein by Immunoblotting

For detection of proteins by immunoblotting, whole bacterial cells were lysed and separated by SDS PAGE (10% SDS PAGE, DTT added to loading buffer) on mini gels, and the proteins transferred to an Immobilon P PVDF membrane (catalogue #IPVH20200, Millipore, Bedford MA) by electroblotting, according to the manufacturer's instructions. Electrotransfer was conducted at 100 volts for approximately 1 hr on ice. Protein detection was accomplished by use of the Western-Light Chemiluminescent Detection System kit (Applied Biosystems, Bedford MA, catalogue #WL10RC), according to the manufacturer's instructions. The anti-BktB and anti-PhaB antibody samples corresponded to IgG preparation elution fraction 2 in both cases. These samples were used at dilutions of 1/2500.

RESULTS AND DISCUSSION

Design and Construction of PHA Expression Vectors

We used the following criteria in the design of a plan for construction of PHA expression vectors: first, we chose a set of genetic elements that we deemed would be necessary or useful for construction of the vectors. Our choice of genetic elements took into account previously published work on construction and analysis of plant expression vectors (Lessard *et al*., 2002), particularly vectors for production of PHAs in plants. Second, we chose a series of restriction sites to engineer via PCR at the ends of the genetic elements to enable ligation (fusion) of the elements by cloning. When possible, we chose sites that are not naturally present in the given genetic elements. This allowed cleavage of the ends of the genetic elements without internal cleavage of the elements. In choosing sites that were used for ligation of complete genes into vectors, we aimed to use sites that occur once in the multicloning site of the vector, allowing for relatively straightforward ligation of genes into vectors. Third, we designed a cloning strategy to enable sequential cloning of particular elements such that we could construct modular vectors, so that the genetic elements can be swapped in and out of the vectors in a straightforward manner. The details of the choice and engineering of each genetic element are discussed below.

Engineering the Maize Ubiquitin Promoter

The maize ubiquitin 1 promoter has been shown effective at directing the expression of transgenes in oil palm (Chowdhury *et al*., 1997). We engineered this promoter, derived from the plasmid pAHC20 (Christensen and Quail, 1996), such that the 2 kb PstI fragment spanning the ubiquitin promoter and intron sequence is flanked by an SpeI site at the upstream end and by a BamHI site at the downstream end. In the ubiquitin promoter product derived from PCR with primers ME1-pUBI1 and ME1-pUBI3, the product does not include the normal start codon of ubiquitin (it was removed based on the sequence of primer ME1-pUBI3). Since the plastid targeting sequence and the *bar* gene each contains their own start codon, it was crucial to remove the start codon from the ubiquitin promoter in order to avoid undesired translational initiation from the ubiquitin start codon.

Engineering of Plastid Targeting Sequence

We engineered a plastid targeting sequence to be fused to the bacterial open reading frames, since transport of the heterologous proteins into the plastid has been shown to improve the health of PHA-producing plants (Nawrath *et al*., 1994). The pea rubisco small subunit plastid targeting sequence (Coruzzi *et al*., 1984) was engineered such that it contained a BglII site just upstream of the start codon and a BamHI site at the end of the sequence; this enabled construction of in-frame fusions of the plastid targeting sequence with other open reading frames. The plastid targeting sequence was also engineered such that the start codon was part of an NcoI site, which has been previously demonstrated to function as an effective translational initiation site (Kozak, 1987). The plastid targeting sequence of the rubisco small subunit of pea (*rbcS*) naturally contains an intron (Coruzzi *et al*., 1984). We utilized splicing by overlap extension (Horton *et al*., 1989) to remove the intron from the plastid targeting sequence used in the PHB and PHBV expression vectors. The translation of the plastid targeting sequence was: MASMISSSAVTTVSRASRGQSAAVAPFGGLKSM TGFPVKKVNTDITSITSNGGRVKCMQVWPPIGKKKF ETLSYLPPLTRD..(fusion to other open reading frame).

Engineering of Transcriptional Termination Signal

Since bacterial messenger RNAs are not polyadenylated, and plant transcripts require this modification, we engineered a transcriptional termination for use in the expression vectors. We engineered the soyabean beta conglycinin 7S 3' transcriptional termination signal (Doyle *et al*., 1986; Lessard *et al*., 1991) such that it contains a BamHI site at the 5' end and AvrII and SpeI sites at the 3' end. The BamHI site was used to enable ligation of the ubiquitin promoter and the transcriptional termination signal such that a BamHI site exists between those two genetic elements, permitting the subcloning of genes between the promoter and the transcriptional termination signal. We cloned the plastid targeting sequence as a BglII/BamHI fragment into the BamHI site, resulting in loss of the BglII/BamHI sites at one point of ligation and retention of the BamHI site at the other point of ligation. We then cloned the *phaC*, *bktB*, *phaB* or *tdcB* genes into the remaining BamHI site. In this way, we constructed gene modules in which each bacterial gene was prepared for expression in plant cells; that is, each bacterial gene was under the control of the ubiquitin promoter and included the necessary plastid targeting sequence and transcriptional termination signal.

Cloning the *bar* Gene for Use in Expression Plasmids

The *Streptomyces hygroscopicus bar* gene, encoding resistance to the herbicide Basta™, was chosen as the selectable marker for use in the PHB and PHBV expression vectors. Basta resistance has been shown to be a suitable marker for selection of transgenic oil palm (Parveez *et al*., 1996). We subcloned the *bar* gene as a 0.6 kb PstI fragment from pAHC20 into a PstI site of pME8 (an intermediate plasmid containing the ubiquitin promoter and a transcriptional termination signal) such that the *bar* gene was located between the ubiquitin promoter and the transcriptional termination signal and the *bar* gene is in the proper orientation for expression from the ubiquitin promoter.

Engineering of Open Reading Frames

For the open reading frames encoding the necessary biosynthetic enzymes, we selected the *phaB* and *phaC* genes of *R. eutropha* (Peoples and Sinskey, 1989a, b) and used the *bktB* gene of *R. eutropha* in place of the *phaA* gene of *R. eutropha* to accomplish expression of beta-ketothiolase activity. This reflects the demonstration that *bktB* is particularly useful for production of precursors for PHBV copolymer in bacteria and plants. Production of PHBV requires the introduction of an additional enzymatic activity: threonine dehydratase (E.C. 4.2.1.16). Threonine dehydratase converts threonine to 2-oxobutyrate, which can be converted to propionyl-CoA, a key precursor for PHBV biosynthesis. The *E. coli ilvA* gene has been expressed in plants as part of a strategy to accomplish PHBV production (Slater *et al*., 1999). We selected the catabolic threonine dehydratase of *E. coli*, encoded by the *tdcB* gene, since heterologous expression of this enzyme has proven more useful than *ilvA* in metabolic redirection of the carbon flow in the gram positive bacterium *Corynebacterium glutamicum* (Guillouet *et al*., 1999; 2001).

The genes *phaC*, *phaB*, *bktB* and *tdcB* were engineered such that BamHI sites were introduced at both the 5′ end and 3′ end of the open reading frame. The 5′ BamHI serves as the site for ligation of each of these genes to the plastid targeting sequence. The plastid targeting sequence, in turn, was engineered to contain a BamHI site; thus, each PHA biosynthesis fusion protein contained the plastid targeting sequences, a three amino acid linker consisting of Arg-Ile-Leu, and the PHA biosynthetic protein beginning with the amino acid normally encoded by the second codon of the native open reading frame (*Figure 3*). This approach is analogous to the approach used by other researchers to generate PHA biosynthesis fusion proteins, although the fusion proteins generated in their system included a different linker, consisting of Ser-Arg-Val (Nawrath *et al*., 1994).

The PHB and PHBV expression vectors were constructed by combining the necessary modules, engineered as described above, in the proper orientation. The restriction-deficient *E. coli* strain, STBL4, was utilized as the host for construction of the modular expression vectors. We used the AvrII and SpeI sites engineered at the 3′ end of the transcriptional termination signal to enable cloning of multiple genes in tandem. The AvrII and SpeI enzymes cleave sites to yield compatible cohesive ends. Ligation of an AvrII site with an SpeI site results in the loss of both sites. We introduced genes flanked by SpeI sites at the promoter end and the transcriptional termination end into other plasmids by digesting the genes with SpeI and cloning the SpeI fragment into an AvrII site (or an SpeI site) in the existing plasmid. In subcloning a gene module as an SpeI fragment into the AvrII site of the recipient plasmid, both the SpeI and AvrII sites will be lost. However, we designed and engineered the gene modules such that the transcriptional termination signal will introduce a new AvrII site in the new plasmid, thus providing a new site on which to clone the next gene into the plasmid. The pME22, the PHB expression vector, contains the *bar*, *phaC*, *bktB* and *phaB* open reading frames, each with a promoter, plastid targeting sequence and transcriptional

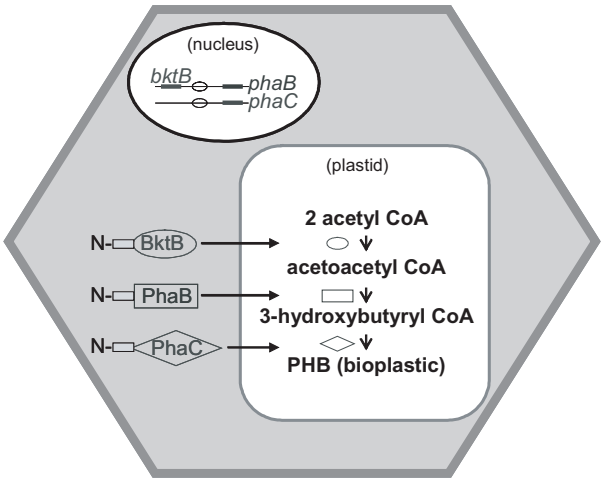

Figure 3. Schematic of polyhydroxyalkanotes (PHA) production in plants. Nuclear-encoded transgenes direct the expression of proteins that are targeted to the plastid, whereupon they catalyze the formation of bioplastic.

termination signal. These vectors are diagrammed in *Figure 4*.

Generation of Antibodies Against PHA Biosynthetic Enzymes

In addition to constructing vectors directing the expression of PHA biosynthetic genes, we generated antibodies that recognize PHA biosynthetic enzymes to facilitate analysis of plants expressing the heterologous genes. We utilized Novagen's pET expression system, specifically the expression plasmid pET14b. The expression plasmids are designed to express the proteins at high levels and to yield histidine-tagged versions of proteins, which facilitates purification of the protein of interest by use of a nickel column. The plasmids pET14b, pET14b-BktB and pET14b-PhaB served as the vector control, the His-BktB expression plasmid, and the His-PhaB expression plasmid, respectively. Thus, pET14b-BktB and pET14b-PhaB contained the *bktB* and *phaB* open reading frames (ORFs), respectively, cloned in frame with a sequence that added a N-terminal histidine tag to products of the cloned ORFs. Nucleotide sequence analyses confirmed the absence of errors in the *bktB* ORF as cloned in pET14b-BktB. Nucleotide sequence analyses revealed the presence of a single nucleotide change in the *phaB* ORF as cloned in pET14b-PhaB. This change results in a mutant protein, PhaB A128T. Since the process of polyclonal antibody production involves generating antibodies against many short amino acid sequences within the target protein, the presence of one amino acid change within the target protein will have little or no deleterious effect on the generation of effective antibodies.

His-BktB and His-PhaB were expressed and purified based on use of the pET expression system. SDS PAGE analyses of purified His-BktB and His-PhaB are shown in *Figures 5a* and *6a*. The apparent

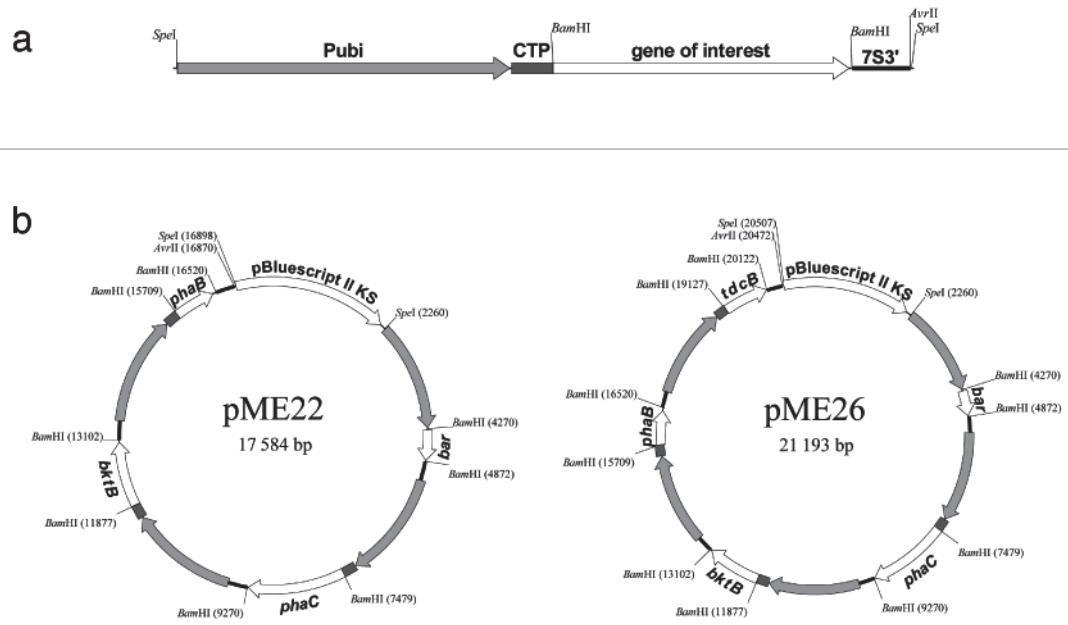

Figure 4. Schematic representation of modular gene expression vectors. a. Each module was engineered to contain the appropriate combination of maize ubiquitin promoter (Pubi), plastid targeting sequence (CTP), coding region, and termination signal (7S3'), using the restriction sites indicated. b. Diagrams of pME22 and pME26 showing the order and orientation of the engineered genetic elements. The maize ubiquitin promoter by the light grey arrows, the plastid targeting sequence by the dark grey boxes, and the termination signal by the thick black lines.

molecular weights of the purified proteins were consistent with His-BktB (expected sizes, BktB: 40878 Da, His-BktB: 43059 Da) and His-PhaB (expected sizes, PhaB: 26353 Da, His-PhaB: 28534 Da). Polyclonal rabbit antibodies were raised against His-BktB and His-PhaB. The IgG fractions of the anti-BktB and anti-PhaB sera were purified and the results of the IgG purification are described in *Table 3*. For each antibody, elution fraction '2' was tested for its ability to detect the target protein. The results for detection of His-BktB and His-PhaB are shown in *Figures 5b* and *6b*. The immunoblot results suggest that the antibody samples will be useful for detection of BktB and PhaB in general, and that they can be applied for the analysis of transgenic plants.

TABLE 3. QUANTITATION OF PROTEIN IN IgG FRACTIONS FOR THE ANTI-BktB AND ANTI-PhaB ANTIBODIES

Fraction	BktB antisera (mg ml^{-1})	PhaB antisera (mg ml^{-1})
elution 1	24	21
elution 2	79	81
elution 3	18	20
elution 4	9.7	10

Note: Protein concentrations are expressed as mg ml^{-1}
IgG elution fraction 2 was used for the immunoblot analyses.

Figure 5. Purification and immunoblot analyses of His-tagged BktB. a. SDS PAGE analysis of purified His-tagged BktB protein. The marker in Lane 1 corresponds to the Prestained Broad Range Marker (New England Biolabs, Ipswich Massachusetts, USA). Lanes 2-10 correspond to the elution fractions of 250 mM imidazole. b. Immunoblot analyses to test anti-BktB antibody. Antibody sample corresponds to rabbit HM3892 IgG elution 2 fraction, 1:2500 dilution. Lanes: 1, BL21 (DE3) pLysS pET14b cells (0.16 μl), pre-induction; 2, BL21(DE3) pLysS pET14b cells (0.8 μl), pre-induction; 3, BL21(DE3) pLysS pET14b cells (0.16 μl), 2 hr post-induction; 4, BL21(DE3) pLysS pET14b cells (0.8 μl), 2 hr post-induction; 5, blank; 6, BL21(DE3) pLysS pET14b-BktB cells (0.16 μl), pre-induction; 7, BL21(DE3) pLysS pET14b-BktB cells (0.8 μl), pre-induction; 8, BL21(DE3) pLysS pET14b-BktB cells (0.16 μl), 2 hr post-induction; 9, BL21(DE3) pLysS pET14b-BktB cells (0.8 μl), 2 hr post-induction.

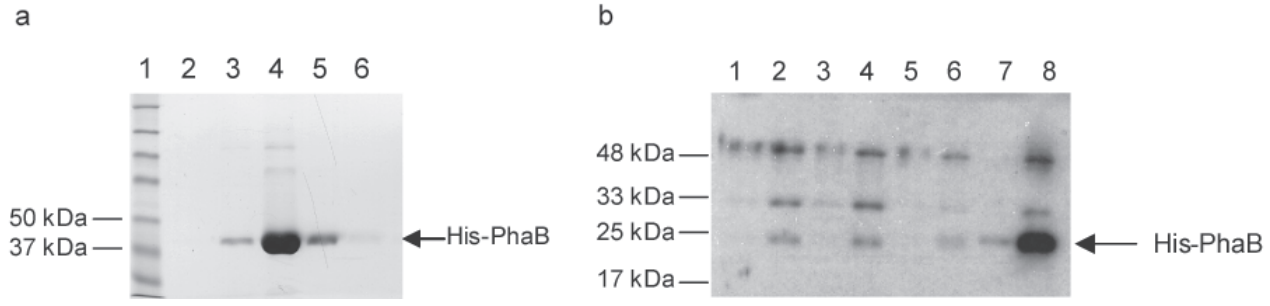

Figure 6. Purification and immunoblot analyses of His-tagged PhaB. a. SDS PAGE analysis of purified His-tagged PhaB. The marker in Lane 1 corresponds to the Prestained Broad Range Marker (New England Biolabs, Ipswich Massachusetts, USA. Lanes 2-6 correspond to elution fractions of 250 mM imidazole. b. Immunoblot analyses of anti-PhaB antibody. Antibody sample corresponds to rabbit HM3893 IgG elution 2 fraction, 1:2500 dilution. Lanes: 1, BL21(DE3) pLysS pET14b cells (0.16 μl), pre-induction; 2, BL21(DE3) pLysS pET14b cells (0.8 μl), pre-induction; 3, BL21(DE3) pLysS pET14b cells (0.16 μl), 2 hr post-induction; 4, BL21(DE3) pLysS pET14b cells (0.8 μl), 2 hr post-induction; 5, BL21(DE3) pLysS pET14b-PhaB cells (0.16 μl), pre-induction; 6, BL21(DE3) pLysS pET14b-PhaB cells (0.8 μl), pre-induction; 7, BL21(DE3) pLysS pET14b-PhaB cells (0.16 μl), 2 hr post-induction; 8, BL21(DE3) pLysS pET14b-PhaB cells (0.8 μl), 2 hr post-induction.

CONCLUSION

We have developed tools and reagents for the generation and analysis of bioplastic producing oil palm. We developed the modular vectors pME22 and pME26 which are suitable for engineering oil palm to produce homopolymers of hydroxybutyryl-CoA, and copolymers of hydroxybutyryl-CoA and hydroxyvaleryl-CoA, respectively. Each of the vectors contains bacterial open reading frames that have been engineered for expression of the genes in plants and transport of the heterologous proteins into the plastid. Plasmid pME22 directs the expression of BktB and PhaB, along with PHA synthase PhaC. Plasmid pME26 additionally directs the expression of the threonine dehydratase TdcB. TdcB catalyzes the conversion of threonine to 2-oxobutyrate, which can be converted to propionyl-CoA. BktB is a beta-ketothiolase that catalyzes the condensation of two molecules of acetyl CoA (or one molecule of acetyl CoA and one molecule of propionyl CoA) to acetoacetyl CoA (or 3-ketovaleryl CoA). PhaB catalyzes the reduction of acetoacetyl CoA (or 3-ketovaleryl CoA) to 3-hydroxybutyryl CoA (or 3-hydroxyvaleryl CoA). Thus, together, TdcB, BktB and PhaB can generate the substrate for production of the copolymer PHBV. We have generated antibodies against the N-terminal histidine-tagged (His-tag) versions of BktB and PhaB proteins of *Ralstonia eutropha*. The antibodies reported here can be applied as tools for the analysis of oil palm cells expressing the *bktB* and/or *phaB* genes. The expression vectors reported herein, and additional vectors with tissue-specific promoters and matrix attachment regions constructed at Universiti Kebangsaan Malaysia and the Malaysian Palm Oil Board's Advanced Biotechnology and Breeding Centre, were used for transformation of oil palm (Parveez *et al*., 2000; 2005; 2006; Jusoh, 2004; Abdullah, 2005; Joseph, 2005; Muad, 2005), the results of those studies. Production of bioplastics in plants is a long-term goal of the biodegradable and biorenewable plastics industry, and the vectors and antibodies reported here should be applicable in a wide range of plant hosts in addition to oil palm.

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