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REVIEW

Transgenic Plants Producing Polyhydroxyalkanoates

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Abstract. Currently, the polyhydroxybutyrate (PHB) copolymer, polyhydroxy-co-valerate (PHBV) is being produced commercially using a two-stage glucose / propionate fed batch fermentation process using Ralstonia eutropha. The economics of the manufacturing process are still a major barrier to the widespread use of polyhydroxyalkanoates (PHAs) and intensive efforts are being made to reduce the cost of production by means of bioprocess design and metabolic engineering of production strains. However, despite these improvements, the production costs are still high compared to petroleum-derived commodity plastics. An alternative strategy for lowering production costs that has been proposed is to develop transgenic plants that produce PHAs. This strategy is considerably cheaper because the PHAs production from plants does not require expensive fermentation equipment and processing facilities.

Keywords. Polyhydroxyalkanoates, Polyhydroxybutyrate, Polyhydroxy-co-valerate, Plastid, Transgenic plants

INTRODUCTION

Over the past 50 years, the development of synthetic petroleum-based polymers has considerably reduced mankind's dependence on the use of certain plant polymers, such as wood, cotton and rubber. Advances in plant genetic engineering, combined with the growing concerns about our environment and the decreasing petroleum reserves, have created new opportunities to use plants as bioreactor for production of novel renewable and environmentally friendly polymers such as polyhydroxyalkanoates (PHAs). The biotechnological use of this compound was first exploited in the 1980s by Imperial Chemical Industries (ICI) (Anderson and Dawes, 1990). ICI set up the bulk production of PHAs using an expensive bacterial fermentation approach using a polymer-accumulating bacterium, *Ralstonia eutropha*. The cost of production was high because the most useful polymer, polyhyroxybutyrate-co-valerate (PHBV), could only effectively synthesized by supplementing the fermentation medium with propionate. Potentially, plants offer an alternative large-scale factory for the production of complex products. In this review, a variety of PHAs having different physical properties have now been synthesized in a number of transgenic plants, including *Arabidopsis*, tobacco, rapeseed, cotton, alfalfa, flax and oil palm. Since the production of PHA in these plants were based on how the PHAs are synthesized in bacteria, a brief review of the types, properties, biosynthesis and commercialization of PHAs will first be elaborated.

Polyhydroxyalkanoates (PHAs). Polyesters like polyhydroxyalkanoates (PHAs) are a large group of polymers of 3-(R)-hydroxy fatty acids linked by an ester bond between the hydroxyl group and the carboxy group of an adjacent monomer (Sudesh *et al.*, 2000; Lenz and Marchessault, 2005). PHAs are osmotically inert compounds and they are optically active, biocompatible, biodegradable and hydrophobic. PHAs can be characterized by chain length, type of functional group and degree of unsaturated bonds. A higher degree of unsaturation increases the rubber qualities of a polymer, and different functional groups change the physical and chemical

Abbreviations. ACP, acyl carrier protein; bktB, gene coding for 3-ketothiolase; bar, gene coding for phosphinothricin acetyltransferase, CaMV35S, cauliflower mosaic virus 35S; CoA, coenzyme A; dwt, dry weight; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; ilvA, gene coding for threonine deaminase; PDC, pyruvate dehydrogenase complex; PHA, polyhydroxyalkanoate; phbA, gene coding for 3-ketothiolase; phbB, gene coding for acetoacetyl-CoA reductase; phbC, gene coding for PHA synthase; PHB, polyhydroxybutyrate; PHBV, polyhydroxy-co-valerate; RB7MAR, RB7 matrix attachment region; SCL, short-chain-length; tdcB, gene coding for threonine dehydratase; T-DNA, transferable-DNA; Tp, transit peptide.

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properties of a polymer (Madison and Huisman, 1999). PHAs are divided into two groups, i.e. short-chain-length PHAs (SCL-PHAs) that are comprised of Polyhydroxybutyrate (PHB) and the copolymer polyhydroxy-co-valerate (PHBV); and medium-chain-length PHAs (MCL-PHAs) that consists of 3-(R)-hydroxyhexanoate / 3-(R)-hydroxytetradecanoate monomers. The properties of PHAs vary with their composition. PHAs are mainly composed of R-(-)-3-hydroxyalkanoic acid monomers. Each type of PHA generally consists of 1,000-10,000 monomers, but most are synthesized by SCL monomers (Van der walle *et al.,* 2001).

Polyhydroxybutyrate (PHB) and Polyhydroxy-co-valerate (PHBV). Most of the knowledge on bacterial production of PHB have been generated from studies with *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) because it naturally produces PHB and can produce up to 85% of its dry weight (dwt) when grown in media containing excess glucose. In this condition, PHB acts as a carbon reserve and an electron sink. Its production is limited by the amount of nitrogen and phosphate present in the growth medium (De Koning, 1995). When growth limiting conditions are applied, PHB is depolymerized by the action of an intracellular PHB depolymerase to give acetoacetate, which is then catabolized further to acetyl-coenzyme A (CoA) (Steinbuchel and Valentin, 1995). It was shown that the precursor for PHB in bacteria is acetyl-CoA. PHB decomposes into 3-hydroxybutyrate (3HB) monomers that bacteria and fungi can use as a carbon source. PHB also produces a metabolite normally present in the blood (D-3-hydroxybutric acid), making it an ideal production system for use in medical applications such as implants, suture filaments and drug carriers (Koosha *et al.*, 1989; Zinn *et al.*, 2001; Chen and Wu, 2005). Besides being "environmentally-friendly", PHB has good UV resistance, oxygen impermeability and resistance to hydrolytic degradation in moist air, making it ideal for use in disposable plastic products such as household containers, bags and wrapping films. However, PHB has some negative qualities, such as poor resistance to acids, bases and organic solvents. PHB is also "stiff and relatively brittle", so it has to be improved (modified) in order to be useful for making any commercial products.

The negative qualities of PHB for commercial uses have led to an increased interest to produce SCL-PHAs copolymers with improved qualities. Modification of PHB was carried out via incorporation of 3-hydroxyvalerate (3HV) monomers (3- or 5-carbon monomers). This decreases the crystalinity, melting point and stiffness, creating PHBV and related co-polymers that are more ideal for commercial applications (De Koning, 1995). More than 300 bacteria species producing PHB have been used to produce PHBV with different C3 to C5 monomers depending on the type and quantity of the carbon sources supplied in the growth media (Steinbuchel and Valentine, 1995). In the bacterium *Ralstonia eutropha*, the addition of propionic acid to the growth media containing glucose leads to the production of a different type of PHBV (Steinbuchel *et al.*, 1995). The bacterium *Rhodococcus rubber* also produces a different PHBV with high 3HV content when succinic acid was used as the carbon source (Williams *et al.*, 1994).

Biosynthesis of Polyhydroxybutyrate (PHB) and Polyhydroxy-co-valerate (PHBV). The existence of a PHB cycle was established in 1973 for *Ralstonia eutropha* by Gottingen and Hull (Senior and Dawes, 1973). There are three biosynthetic enzymes leading from the precursor acetyl-CoA, namely 3-ketothiolase, acetoacetyl-CoA and PHA synthase. The genes for these enzymes have been cloned and expressed in *Escherichia coli* independently by Schubert *et al.* (1988) and Slater *et al.* (1988), and have been sequenced by People and Sinskey (1989a; 1989b). The first enzyme, 3-ketothiolase (encoded by *phbA* gene), catalyzes the reversible condensation of two acetyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (encoded by *phbB* gene) subsequently reduces acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of a PHA synthase (encoded by *phbC* gene) to form PHB.

Ralstonia eutropha is a natural producer of PHB, and when provided with glucose and propionic acid in varying ratios, produces different types of PHBV (Senior and Dawes, 1973). Due to this, Haywood *et al.* (1988) revealed that additional condensation of propionyl-CoA with acetyl-CoA to form 3-ketovaleryl-CoA is required to produce PHBV. Subsequently, Slater *et al.* (1999) reported another type of 3-ketothiolase, designated as *bktB*, which has a higher specificity for propionyl-CoA and acetyl-CoA to 3-ketovaleryl-CoA. Reduction of 3-ketovaleryl-CoA to R-3-hydroxyvaleryl-CoA and the subsequent polymerization to form PHBV are catalyzed by the same enzymes involved in PHB synthesis. However, this pathway has not been completely elucidated. The conversion of succinate to propionyl-CoA is through the action of methylmalonyl-CoA mutase and either methyl malonyl-CoA decarboxylase or methylmalonyl-CoA: oxaloacetate transcarboxylase has been proposed to synthesize HV units from glucose (Madison and Huisman, 1999).

Commercialization of PHBV by Bacterial Fermentation. In response to the world oil crisis, Imperial Chemical Industries (ICI), and later Zeneca and Monsanto established an industrial scale (200,000 liter) fermentation based production of PHBV using *Ralstonia eutropha* which has been commercialized with the trademark Biopol. However, this approach was based on the use of expensive carbon sources, glucose and propionic acid, which makes the final production cost non-competitive, especially for bulk production. The cost of glucose and propionic acid to produce 1kg of PHBV was USD1.9, and the fermentation equipments, processing facilities including the energy costs was estimated as USD14.1 (Kessler *et al.*, 2001). Thus, the cost of PHBV using the natural producer *Ralstonia eutropha* is USD16/kg. Despite the

cost has been reduced using recombinant *Escherichia coli*, Choi and Lee (2000) evaluated that the cost of PHBV at a scale of 100,000 t/yr would only be USD3.95/kg. This price still cannot compete with the price of oil-derived plastics which is USD0.6~USD1.0/kg polypropylene and USD0.8~USD1.8/kg polyethylene. In addition, a lot of energy is required and this will impact the increasing of environment pollution. At least 19 times of electric, 22% of heat and 7 times of water is needed for the production of PHBV using the fermentation approach compared to the chemical approach for the production of polypropylene (Kessler *et al.*, 2001; Steinbüchel, 2005).

PRODUCTION OF POLYHYDROXYALKANOATES (PHAs) IN PLANTS

As the production of PHAs in bacteria is expensive, its production in plants may be an attractive alternative. Plants are ideal candidates for synthesizing PHAs because they have been shown to be efficient producers of biomass compared to bacteria. For example, *Ralstonia eutropha* and potato tuber accumulate up to 85% dwt of their commodities, PHB and starch, respectively. One hacter of potatoes can produce about 20,000 kg of starch at about USD0.2/kg compared to bacterially-produced PHB at USD15/kg (Somerville *et al.*, 1994; Pool, 1989). In addition to being highly productive, genetically engineered plants have demonstrated the ability to produce foreign proteins that are biologically active such as antibodies (Ma *et al.*, 2005). Plants are also the supplier of carbon sources through photosynthesis, so it is much more efficient to use plants as a direct carbon source for PHA production by eliminating the carbon input cost. In fact, the cost of producing PHAs in plants may even become comparable to petroleum-based plastics because of no cost of feedstock and fermentation settings. It was estimated that PHAs could potentially be produced at a cost of USD0.2 ~0.5/kg if plants could produced to a level of 20~40% dwt (Poirier, 2002; Scheller and Conrad, 2005). Another advantage to using plants is the presence in plant cells, acetyl-CoA, the precursor to the PHAs biosynthetic pathway. In general, the production of PHB from plant's acetyl-CoA required genetic engineering of *phbA*, *phbB* and *phbC* genes of *Ralstonia eutropha*, whereas, the *bktB*, *phbB*, *phbC* genes of *Ralstonia eutropha* and *ilvA gene* of *Escherichia coli* were required for PHBV production from plant's acetyl-CoA and propionyl-CoA. In order to locate the expression of these genes in plant cells, at least two important considerations has to be given to the most appropriate subcellular compartment, the presence of acetyl-CoA and the available space for storage of PHB and PHBV. In plant cells, acetyl-CoA is present in the cytoplasm, plastid, mitochondria and peroxisome. Thus, the synthesis of PHAs could be achieved in any of these compartments. However, considering the high storage space available, the cytoplasm and plastids seem to be the most appropriate subcellular compartments. Cytoplasm is normally occupied by oils bodies and plastids which act as storage tissues with a large storage capacity to store a high amount of starch and lipids.

Arabidopsis thaliana. The use of *Arabidopsis* as a model plant plays a crucial role in the understanding of other plant genes and their biological functions. The characteristics that make *Arabidopsis* an ideal model plant are its small size, sturdiness, fast growth, self-pollination, large number of progeny, small number of genes (5 chromosomes and 25,500 genes) and the entire genome has been sequenced (Sommerville *et al.*, 1994). More importantly, simplified transformation protocols facilitate positional cloning, insertional mutagenesis, and other transformation procedures, reducing the effort required to test any given DNA construct within plants. Due to this, *A. thaliana* was the first plant that was used as the experimental model for the production of PHAs in plants. The cytoplasm was initially targeted for PHB synthesis in *Arabidopsis* because the PHB biosynthetic enzymes could be directly expressed in this compartment without any modification of the proteins. Additionally, it was unnecessary to introduce the 3-ketothiolase enzyme, because it was already present in the cytoplasm as part of the mevalonate pathway (Poirier *et al.*, 1992). Another two enzymes, acetoacetyl-CoA reductase and PHA synthase, encoded by *phbB* and *phbC* genes of *Ralstonia eutropha*, respectively, driven by the *CaMV35S* promoter were cloned in two binary vectors. The two vectors were transformed into *Arabidopsis* plants, and transgenic *Arabidopsis* plants producing PHB were obtained by cross-pollinating transgenic plants expressing acetoacetyl-CoA reductase and PHA synthase. Approximately 0.1% dwt of PHB was produced in the leaf tissues. The low PHB yield of transgenic *Arabidopsis* plants revealed that the flux of acetyl-CoA in the cytoplasm is not sufficient to produce PHB. In addition, all transgenic *Arabidopsis* plants producing PHB exhibited a stunted phenotype. The diversion of acetyl-CoA from the plant's natural pathway for mevalonate formation may be a possible cause of the stunted phenotype. The mevalonate formation, such as synthesizing of flavanoid and sterols, involves in synthesis of plant growth hormones and cell membranes. Thus, small amount of acetyl-CoA in cytoplasm that been diverted to PHB was likely to change the amount of these components which severely affected plant growth. Although PHB synthesis was targeted for production in the cytoplasm, PHB granules were found in various compartments including the vacuole and nucleus but not in the plastids and mitochondria. This indicated that the double membrane of plastids and mitochondria may not allow the PHB to pass through into these compartments.

Subsequently, the plastids were proposed by Nawrath *et al.* (1994) to be a more suitable compartment for the production of PHB. Plastids seemed to be a good alternative to the cytoplasm for several reasons. Since plastids are a site

of high flux of carbon, acetyl-CoA is used for fatty-acid biosynthesis, and plastids have a double membrane which keeps PHB isolated and prevents it from disrupting other compartments. Nawrath *et al.* (1994) successfully synthesized PHB in *Arabidopsis* plastids by fusing the N-terminal of *phbA*, *phbB* and *phbC* genes of *Ralstonia eutropha* with the transit peptide from the small subunit of Rubisco under the control of the *CaMV35S* promoter in three vectors. These vectors were introduced independently into *Arabidopsis* and the transgenic plants obtained were cross together to produce the transgenic plants expressing *phbA*, *phbB* and *phbC* genes which accumulating PHB up to 14% of the dwt. Growth stunted phenotypes were not observed in the transgenic plants but slight chlorosis were observed in the leaves of plants containing higher levels (>3% dwt) of PHB. The plastid targeted PHB enzymes was demonstrated when the size and structure of PHB granules which similar to granules produced in bacteria were only located in the plastids by electron microscopy. However, it proved difficult to obtain stable transgenic plants expressing plastid-targeted enzymes (3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase) by cross-pollination. This may due to the use of the same promoter and transit peptide in all three vectors which causes homology-dependent gene silencing among the genes in transgenic plants. To counteract this, Valentin *et al.* (1999) and Bohmert *et al.* (2000) created a multiple-gene transformation vector which contains the plastid-targeted PHB genes that allowed the entire pathway to be inserted into one single locus of the plant genome. Three expression cassettes derived from vectors constructed by Nawrath *et al.* (1994) were cloned in one vector and transformed into *Arabidopsis*. As results, transgenic *Arabidopsis* plants accumulating PHB up to 13% and 40% of the dwt were obtained by Valentin *et al.* (1999) and Bohmert *et al.* (2000), respectively. However, the high yield of PHB was accompanied by severe growth reduction and chlorosis. Analysis of the effect of PHB production on the levels of various plant metabolites indicated that PHB accumulation did not affect the fatty acid pool of the plant. The concentrations of many metabolites analyzed were affected, including organic acids of the tricarboxylic acid (TCA) cycle, whose concentrations may have been reduced upon diversion of acetyl-CoA to PHB production. In another study, the main reason for the growth reduction was identified to be the 3-ketothiolase (encoded by *phbA*) expression, which also led to a significant decrease in transformation efficiency (Bohmert et *al.*, 2002). Transformation of *phbA* gene in the chloroplast genome was able to produce normal transgenic *Arabidopsis* plants but 100% of these plants were sterile (Ruiz and Daniell, 2005). As an improvement for construction of multiple-gene transformation vectors, Kourtz *et al.* (2005) constructed a PHB transformation vector containing plastid-targeted *phbA* and *phbB* genes of *Ralstonia eutropha* with 26 a amino acid linker. Transgenic *Arabidopsis* plants accumulating PHB up to 7% of the leaf dwt were obtained when coexpressed with a plastid targeted *phbC* gene of *Ralstonia eutropha*. This work (Kourtz *et al.*, 2005) represented the first step towards simplifying the expression of PHB biosynthetic pathway in plants.

The production of the co-polymer PHBV in transgenic *Arabidopsis* plants was reported by Valentin *et al.* (1999) and Slater *et al.* (1999). In bacteria, 3-ketothiolase (encoded by *bktB* gene) catalyzes the condensation of acetyl-CoA and propionyl-CoA to form 3-ketovaleryl-CoA. Due to the small amount of propionyl-CoA present in the plastids, the plant's threonine was converted to propionyl-CoA by using a plastid-targeted threonine deaminase (*ilvA* gene from *Escherichia coli*), which converted threonine to 2-ketobutyrate, and then to propionyl-CoA by plant pyruvate dehydrogenase complex (PDC). As a result, transgenic *Arabidopsis* plants accumulating PHBV up to 1.5% of the dwt with 4~17 mol% 3HV were reported by Valentin *et al.* (1999), as compared to accumulation of up to 1.6% of the dwt containing 4% 3HV reported by Slater *et al.* (1999). The PHBV yields obtained by both studies are well below to the estimated 15% target required for commercialization proposed by Slater *et al.* (1999). An increased metabolic burden placed on the plant by the *ilvA* gene may be the reason for the low PHBV yields produced (Slater *et al.*, 1999). Additionally, Daae *et al.* (1999) found that changes in the expression levels of 3-ketothiolase (encoded by *bktB* gene) also influenced the flux to PHBV synthesis and the ratio of 3HV/3HB monomeric units. Increases in 3-ketothiolase were assumed to significantly increase the flux and reduce the 3HV/3HB ratio in the polymer. In contrast, increases in acetoacetyl-CoA reductase and PHB synthase activities increase both the flux to PHBV synthesis and the 3HV/3HB monomeric ratio in their model. Based on these assumptions, Daae *et al.* (1999) believed that high yields of PHBV can be achieved by increasing the expression of *phbB* and *phbC* genes.

Tobacco (Nicotiana tabacum). Increasing concerns about the dangers of cigarette smoking may limit the market for traditional tobacco products in the future. Hence, if tobacco could be used for other applications, such as the production of biodegradable plastics, tobacco farmers would potentially have a new market for their crop. As an attempt to produce PHB in tobacco cells, the *phbB* and *phbC* genes of *Ralstonia eutropha* driven by the *CaMV35S* promoter were transformed individually into tobacco plants and the resulting transgenic plants were cross-pollinated to produce PHB (Nawrath *et al.*, 1995). However, the resulting transgenic plants failed to produce any amount of PHB in the cytoplasm. Nawrath *et al.* (1995) concluded that the activity of *phbB* gene in the transgenic tobacco was approximately 100 times lower than in *Arabidopsis* plants. This may limit PHB production in tobacco. In another study, Nakashita *et al.* (1999) were able to produce trace amounts (0.001% dwt) of PHB in the cytoplasm of tobacco by expressing the *phbB* gene of *Ralstonia eutropha* and the *phbC* gene of *Aeromonas caviae,* each under the control of the *CaMV35S* promoter.

In another study, transgenic tobacco plants accumulating PHB up to 0.09% of the dwt were obtained when transformed with plastid-targeted *phbA* and *phbB* genes of *Ralstonia eutropha* and *phbC* gene of *Aeromonas caviae* (Arai *et al.*, 2001). The PHB produced in the plastids was higher than the production of PHB in the cytoplasm of tobacco. The low yields of PHB in tobacco compared to *Arabidopsis* were assumed to be due to lower expression of enzymes in tobacco especially *phbA* gene (Nawrath *et al.,* 1995). Due to these problems, Bohmert *et al.* (2002) transformed tobacco plants with the modified transformation vectors used earlier in *Arabidopsis* (Bohmert *et al.*, 2000). The first transformation vector carried the *phbA* gene driven by a pathogen-related protein (PRP) promoter of potato and the second transformation vector carried the *phbA* gene with an N-terminal fusion with transposable *Ac*-element (Ac) of maize. Up to 0.05% of the dwt of PHB was produced by transformation with the first transformation vector and 0.32% of the dwt was produced with the second transformation vector. The modification of *phbA* gene failed to yield high levels of PHB in tobacco. Another attempt to produce PHB by direct transformation of the plastid genome has also yielded minimal amounts of product. Lossl and co-workers (2003) reported the transformation of the plastid genome of tobacco with the *R. eutropha* PHB operon and obtained only 400 ppm dwt of PHB. In addition, growth inhibition and male sterility were observed in all transgenic tobacco. Due to these problems, Lossl *et al.* (2005) developed a trans-activation system to regulate transcription of PHB operon in plastid genome and obtained PHB up to 0.14% of the dwt. Lossl *et al.* (2005) concluded that the system could serve as an alternative to constitutive expression of PHB gene in the plastid genome. Nakashita *et al.* (2001) and Arai *et al.* (2004) also performed plastid transformation in tobacco using the same approach but obtained very low PHB content (<0.001% dwt). The failures may be due to the use of native *Ralstonia eutropha* operon, including the *Ralstonia eutropha* promoter, regulatory elements, and intergenic spacers that may not be ideal for transgene expression within the chloroplast of tobacco.

Rapeseed (Brassica napus). Rapeseed is economically important as a source of edible oil, condiments, vegetables and cattle fodder. About 13% of world's edible oil output is produced from rapeseed. Since the flux of acetyl-CoA in the plastids is high in tissues producing oil, rapeseed is suitable plant for PHA production. Two groups have successfully produced PHB in *Brassica* seeds by expressing the PHB genes of *Ralstonia eutropha* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). Both groups constructed a multiple-gene transformation vector with each gene individually controlled by a seed specific hydroxylase promoter of *Lesquerella* and obtained a PHB content of up to 9% of the seed's dwt. Houmiel *et al.* (1999) demonstrated that the use of different 3-ketothiolases from *Ralstonia eutropha* had little effect on PHB production. *Brassica* plants transformed with a transformation vector expressing plastid targeted *phbA*, *phbB*, and *phbC* genes contained PHB up to 9% of the seed's dwt. Whereas *Brassica* plants transformed with a transformation vector expressing plastid targeted *bktB*, *phbB*, and *phbC* genes contained PHB up to 7.4% of the seed's dwt. Accordingly, Houmiel *et al.* (1999) suggested that PHB production could possibly be increased in *Brassica* seeds with the use of stronger promoters that were active during a longer period of seed development.

The production of PHBV in the plastid of *Brasicca* seeds (Valentine *et al.*, 1999) was carried out employing the strategies used to synthesize PHBV in *Arabidopsis* as was first reported by Slater *et al.* (1999). Seed-specific expression of the plastid-targeted *bktB*, *phbB*, *phbC* and *IlvA* genes with the *Lesquerella* hydroxylase promoter yielded plants containing 0.7~2.3% of the seed's dwt of PHBV with 2.3~6.4 mol% of HV. The yield observed in the seeds was slightly higher than the 0.2~0.8% of the leaf dwt observed in *Arabidopsis* by Valentin *et al.* (1999). However, the total HV monomer unit was lower than the 4~17% obtained in *Arabidopsis* (Valentin *et al.,* 1999). Slater *et al.* (1999) obtained similar results by transforming a similar transformation vector with 3-ketothiolase (*phbA* gene) from *R. eutropha*. Up to 1.6% of the seed's dwt of PHBV in *Brassica* seeds with 3 mol% 3HV was observed. Unfortunately, 25% of these plants had low seed recovery, low vigor and sterility, suggesting that some of the enzymes in the vector were not suitable for expression in *Brassica* plants. Interestingly, attempts to boost the incorporation of 3HV into the polymer by expressing a gene encoding *IlvA* gene failed since transformants containing this gene could not be obtained. The authors suggested that supplementary routes for the conversion of 2-ketobutyrate to propionyl-CoA, or alternative means for generating of propionyl-CoA, will most likely be needed to improve PHBV production in plants (Valentin *et al.,* 1999).

Cotton (Gossypium hirsutum). The quality of the cotton fiber for textile applications is dependent on many factors such as water absorption and thermal properties. The capability of the cotton fiber to absorb less water can be used as water barrier products such as diapers and carpets. Now days, most of winter clothing are produce from animal fiber, however, extinction of some animals such as Australia wolf made textile from animal is banned. Due to the naturally of PHB has good thermal adaptability and retain heat very well, the addition of PHB in the cotton fiber could conducted less heat, cooled down slower and took up more heat that conventional cotton fibers. Production of PHB in cotton fibers by transforming *phbB* and *phbC* genes from *Ralstonia eutropha* driven by fiber-specific E6 promoter of cotton and *CaMV35S* promoter, respectively was performed by John and Keller (1996). The PHB yield was 0.003~0.034% of the dwt. The accumulation was observed in the cytoplasm by electron microscopy. Unlike in *Arabidopsis* (Poirier *et al.,* 1992), transgenic cotton plants expressing *phbB* and *phbC* genes exhibited normal growth and the isolated fibers have

better insulating properties than the wild-type cotton. The fibers also exhibited higher heat capacities and lower thermal conductivities than control fibers (Chowdhury and John, 1998). The authors noted that the PHB production in cotton fibers needed to be increased several folds to be suitable for commercial applications.

Alfalfa (Medicago sativa). Most alfalfa plants are being used for forage and animal feed. Production of PHA in alfalfa plants could lead to a new commercial value since the plant also produces a high biomass for energy uses. Saruul *et al.* (2002) attempted to produce PHB in the leaves of alfalfa plants by using the multiple-gene transformation vectors constructed by Slater *et al.* (1999). Transgenic alfalfa plants expressing plastid-targeted PHB genes of *Ralstonia eutropha* driven by the *CaMV35S* promoter produced up to 1.8% of the dwt of PHB. Only 0.34% of the dwt of PHB was produced from transgenic alfalfa plants, which were transformed with a similar transformation vector using plastid-targeted *bktB* gene. Saruul *et al.* (2002) concluded that the expression of the two different 3-ketothiolase genes (*phbA* and *bktB*), had significant effect on the level of PHB produced in alfalfa leaves. Similar to the results with transgenic *Arabidopsis* plants producing PHB (Nawrath *et al.*, 1994), PHB granules were located in the chloroplasts of transgenic alfalfa plants demonstrating that PHB genes were targeted into the plastid. Growth stunting problems were not observed in the transgenic plants. Interestingly, F1 hybrid progeny obtained by crossing the transgenic alfalfa plants producing PHB with elite alfalfa germplasm also produced similar PHB content to the transgenic parental line. Due to this, Saruul *et al.* (2002) believed that PHB production in alfalfa was stable and dominantly inherited.

Flax (Linum usitatissimum). Flax is an annual plant cultivated in temperate climates and commonly used for fibre and linseed oil production. However, the use of flax fibres by the textile industry is limited because flax fibres are of lower quality than those of cotton origin. In order to improve at least the thermoplastic properties of the fibres, transgenic plants synthesizing PHB in their stem tissue were developed. Based on initial studies by Bohmert *et al.* (2000), Wrobel *et al.* (2004; 2007) successfully produced PHB in flax plastids by using two transformation vectors containing plastid-targeted PHB genes of *Ralstonia eutropha* driven by the *CaMV35S* promoter. The first transformation vector was constructed by Bohmert *et al.* (2000) in which the plastid-targeted *phbA* gene was placed under the control of a *CaMV35S* promoter and the second transformation vector contained a plastid-targeted *phbA* gene driven by a stem-specific promoter (14-3-3) of the 16R gene. The plastid-targeted *phbB* and *phbC* genes in both vectors were placed under the control of the *CaMV35S* promoter. The production of PHB in transgenic flax plants with the second transformation vector yielded up to 0.5% of the dwt which demonstrated a 40-fold higher PHB content than the transgenic flax plants expressing PHB genes transformed with the first transformation vector. Most of the transgenic flax plants transformed with the first transformation vector showed significant growth reduction and senescence but not so for transgenic flax plants producing PHB derived from transformation vector under a 14-3-3 promoter. Interestingly, the yield of seed production was two-fold higher than in wild type plants. The author concluded that the use of a stem-specific promoter for transgenes expression protected the transgenic plant from growth retardation and also provided higher PHB synthesis than transformation vectors driven by the *CaMV35S* promoter.

Oil palms (Elaeis guineensis and E. oleifera). *Elaeis guineensis*, commonly known as oil palm, is the most important species in the genus *Elaeis* belonging to the family Palmae. The second species is *E. oleifera* found in South and Central America and known as the American oil palm. Although significantly lower in oil-to-bunch content than its African counterpart, *E. oleifera* has a higher level of unsaturated fatty acids and has been used for the production of interspecfic hybrids with *E. guineensis*. *E. guineensis* is differentiated by its fruit pigmentation and characteristics. The most common types of fruit are *Dura*, *Tenera* and *Pisifera* that are classified according to endocarp or shell thickness and mesocarp content. *Dura* fruit has a 2-8mm thick endocarp and medium mesocarp content (35~55% of fruit weight), the *tenera* race has 0.5-3mm thick endocarp and high mesocarp content of 60~95% and the *pisifera* fruit has no endocarp and about 95% mesocarp (Rajainaidu *et al.*, 2000).

Traditionally, the breeding of oil palm has focused on yield improvement, oil content, slow height increment, oil quality and disease tolerance. Currently, the Malaysian Palm Oil Board (MPOB) has placed emphasis on the production of planting materials such as dwarf palms, PS1~PS4 types, which are small and have high iodine value, lauric and carotenoid content to meet industry and market needs (Rajanaidu *et al.*, 2000; Basri *et al.*, 2005; Basiron, 2007). As described earlier, the long generation time and the open pollinated behaviour of oil palm contribute to the slowness of breeding process besides requiring large amounts of planting material. Due to this, oil palm transformation was initiated since 1995 to develop transgenic oil palm with high oleic acid, high stearic acid, high ricinoleic acid, high palmitoleic acid and synthesizing biodegradable plastics (polyhydroxybutyrate). The production of PHAs in oil palm was initialed under a Malaysia-Massachusetts Institute of Technology (MIT) Biotechnology Partnership Programme (MMBPP) (Parveez, 2003). This project focused on the production of two types of PHAs; PHB and PHBV. In general, there were three long-term objectives of this project. The first objective was to initiate the production of PHAs in oil palm by genetic engineering the PHAs pathway in plastids, secondly, to study the oil palm biochemistry in which PHAs acts as a terminal carbon sink and lastly, to produce transgenic oil

palm synthesizing PHBV for commercialization. The project was divided into two phases.

1. *Construction of PHB and PHBV transformation vectors.* The strategies for the construction of PHB and PHBV transformation vectors were largely based on previously published work on construction and analysis of plant transformation vectors, particularly transformation vectors for production of PHAs in plants. The *phbA*, *phbB* and *phbC* genes of *R. eutropa* (People and Sinkeys, 1989a; People and Sinkeys, 1989b; People and Sinkeys, 1989c) were identified and used as PHB genes for transformation, whereas the *bktB*, *phbB*, *phbC* of *R. eutropha* and *tdcB*, encoding the enzyme threonine dehydratase of *E. coli* (Goillouet *et al.*, 1999) were used as PHBV genes. Although researchers have previously demonstrated that the targeting of the 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. In contrast, *tdcB* gene functions more effectively in producing the precursor of PHBV as it is not subjected to produce inhibition (Goillouet *et al.*, 1999). These genes were targeted to be expressed in the plastids of oil palm cells by fusing the genes with a transit peptide oil palm acyl-carrier-protein (ACP) gene (Rasid *et al.*, 1999). These genes were linked together and adjacent to the selectable marker, *bar* gene. All the linked genes were flanked with RB7MAR to stabilize the transgene expression, to minimize gene silencing due to positional effects, and to increase the expression levels. The construction of the PHB and PHBV transformation vectors driven by three different constitutive promoters (CaMV35S, Ubiquitin, Actin) was first reported in 2001 (Parveez, 2003; Masani *et al.*, 2008) and was used as a model vector to produce PHAs in oil palm. The oil palm fruits are the main target for production of PHAs in transgenic oil palm because its mesocarp contains high flux of acetyl-CoA that acts as a substrate for fatty acid and PHAs. Accordingly, modifications were made for the mesocarp-specific promoter (MSP1) (Siti Nor Akmar and Zubaidah, 2003) driving the PHB and PHBV gene (Parveez, 2003). Further manipulations were performed by addition of antisense acetyl-CoA carboxylase (*ACCase*) gene of oil palm (Sambanthamurthi *et al.*, 2002) and substitution of bacterial 3-ketothiolase (*phbA*) genes with oil palm 3-ketothiolase gene with the hope that the activity of *ACCase* will be blocked and diverted towards the production of PHB and PHBV, and these minimizing the involvement of non-oil palm genes in transgenic oil palm as well as to aid in comparing the activity of both enzymes and for selection for the maximum synthesis of PHB and PHBV in oil palm cells, respectively (Masani, 2006).
2. *Transformation of PHB and PHBV trans-formation vectors into oil palm.* Biolistic-mediated transformation method was chosen to facilitate PHA production in transgenic oil palm. Current work involves transformation of the vectors into oil palm cultures and Basta-resistant embryogenic calli have been obtained. Regeneration of resistant calli is on going and few hundred plantlets have been obtained, and have been transferred onto soil in a contained nursery. PCR analysis revealed that more than 90% of transgenic lines were positive for the Basta-resistance gene and also for the PHB or PHBV genes (Parveez, 2003; Parveez *et al.*, 2008).

CONCLUSIONS AND FUTURE PROSPECTS

As reviewed above, the production of PHAs in transgenic plants is well-documented with different approaches. Based on these reports, for the successful production of PHAs in transgenic plants, it is important to follow these recommendations:

- All genes needed for the synthesis pathway of PHB and PHBV have to be introduced in plant tissues as a new pathway in a single locus.
- All the PHB and PHBV genes have to be targeted into a subcellular compartment of plant tissues.
- All the PHB and PHBV genes have to be expressed in specific tissues in order to minimize possible negative effects on the transgenic plants.

The problems arising from these studies such as altered plant phenotypes, low product yields, transgene stability, and difficulties associated with expressing multiple gene simultaneously in one plant should be used as guidelines to produce PHAs in transgenics plants. The metabolic profiling techniques described by Bohmert *et al.* (2000) can be used to study the changes in metabolism of transgenic plants created upon expression of PHAs transgenes. Improved methods for multiple transgene expression is also required to efficiently coordinate expression and prevent gene silencing (Matzke and Matzke *et al.,* 1994) due to multiple repeated genetic elements such as the use of different tissue-specific promoters. Further studies to generate complex metabolite PHAs pathway involving heterogenous and endogenous genes in plant cells could be studied for further modifications of plant metabolism without affecting the plant phenotype. New transformation technologies should be applied to produce PHAs in plants such as plastid transformation with polycistronic genes which may be more efficient as the transgenes in the plastid genome is not affected by gene silencing and inherited maternally (Nakashita *et al.*, 2001; Grevich and Daniell, 2005; Murphy, 2007).

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