Synthesis of bioplastics in plants

During the past decade, much attention has been focused on the development of new biodegradable plastics. This interest has been fueled by a greater awareness of the detrimental effect of synthetic plastics on our environment and of problems associated with their disposal. Among the various biodegradable plastics available, there is a growing interest in the group of polyhydroxyalkanoates (PHAs), polyesters produced by bacteria (1–4). These polymers represent a valuable source of biodegradable and environmentally safe biomaterials which can be used in a wide range of products such as utensils and dishes for the food industry, films for packaging or hygienic products, medical implants and gauze, agricultural mulches, etc. In contrast to many biodegradable plastics that can be partially broken down to smaller nondegradable constituents, either photochemically or by nonenzymatic hydrolysis, PHAs can be fully degraded by microorganisms to water and carbon dioxide. Thus, consumer products made of PHAs can be degraded by including them in a compost (Fig. 1).

PHAs represent a large family of polymers, composed primarily of 3-hydroxyacids, which are produced as intracellular granules by a wide variety of bacteria (1–4) (Fig. 2). These polymers act as a carbon reserve and electron sink when bacteria are grown in media containing an excess of carbon while one or more essential nutrients are limiting (e.g., NO\textsubscript{3} or PO\textsubscript{4}\textsuperscript{3-}). When growth-limiting conditions are alleviated, the intracellular PHA is cleaved by a PHA depolymerase and then catabolized to acetyl-CoA. In addition to the intracellular PHA depolymerase, some bacteria and fungi possess extracellular PHA depolymerases which enable these organisms to utilize external PHAs as a carbon source.

Bacteria that synthesize PHAs can be subdivided into two general groups: the first one produces short chain-length PHAs (SCL-PHAs), having monomers three to five carbons in length (C\textsubscript{3}–C\textsubscript{5}), while the second group synthesizes medium chain-length PHAs (MCL-PHAs), with monomers ranging from C\textsubscript{6}–C\textsubscript{16} (1–4). SCL-PHAs generally are more rigid thermoplastics while MCL-PHAs behave as elastomers. Despite the large diversity of bacteria synthesizing SCL-PHAs, most of the knowledge has been obtained from \textit{Ralstonia eutropha} (formerly \textit{Alcaligenes eutrophus}). Synthesis of MCL-PHAs is mainly confined to the \textit{Pseudomonas} species, with most studies done on \textit{P. oleovorans}.

In \textit{R. eutropha}, polyhydroxybutyrate (PHB) is synthesized from acetyl-CoA by the sequential action of three enzymes: 3-ketothiolase (\textit{phaA}), acetoadecyl-CoA reductase (\textit{phaB}), and the PHA synthase (\textit{phaC}) (Fig. 3A). Copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (P[HB-HV]) can be produced by \textit{R. eutropha} using as substrates propionic or valeric acid mixed with glucose (Fig. 3A). P(HB-HV) is more flexible than PHB, which is relatively stiff and brittle, making P(HB-HV) a better polymer for a wide variety of applications. Until recently, P(HB-HV) was produced commercially under the trade name Biopol by Monsanto.

In \textit{Pseudomonas}, the pathway of MCL-PHAs synthesis is linked to the generation of 3-hydroxyacyl-CoA intermediates from the \textit{β}-oxidation cycle of fatty acid degradation (Fig. 3B). MCL-PHA is made exclusively of the \textit{R} isomer of 3-hydroxyacids. Since bacterial \textit{β}-oxidation generates S-3-hydroxyacyl-CoA intermediates,
additional enzymes are thought to be involved in generating R-3-hydroxyacyl-CoAs. When *P. oleovorans* is grown in media containing a fatty acid, the polymer is typically composed of monomers which are two carbons and multiples thereof shorter than the fatty acid used as the growth substrate. For example, growth of *P. oleovorans* on lauric acid (C12) generates PHA containing 31 mol% C12, 36 mol% C10, 31 mol% C8, and 2 mol% C6 monomers. The flexibility of the PHA synthase in accepting various substrates has been exploited with *Pseudomonas* for the synthesis of PHAs containing new functional groups such as double and triple bonds, hydroxy, epoxy, bromo, fluoro, cyano, and phenoxy groups. These functional groups enable the modification of the structure of the PHA after extraction of the PHA from bacteria, resulting in the generation of polymers with new interesting properties (5).

**Crops of biodegradable plastics**

The major drawback of (P(HB-HV)) copolymers and other bacterial PHAs is their high production cost ($5-15/kg), making them significantly more expensive than petrochemical plastics (<$1/kg). Plants produce a large amount of industrially useful compounds at low cost, such as starch and lipids, and are therefore good candidates for the production of bulk products at low price. With recent advances in plant biochemistry and molecular biology, there has been an increased interest in modifying plants for the production of novel commercially valuable compounds, including biopolymers (6). Thus, the large-scale production of polyhydroxyalkanoates (continued on page 771)
in crop plants became an attractive alternative. Production of PHAs on an agronomic scale could allow synthesis of biodegradable plastics in the range of 10^5–10^6 tons per year, quantities difficult to reach by bacterial fermentation alone. If PHA can be synthesized in plants to a level comparable to reserve lipids, i.e., approximately 10–40% dry weight, PHA potentially could be produced at a cost comparable to vegetable oil ($0.5/kg) and thus be competitive with petroleum-derived plastics (1,7).

**PHB production in plants**

Synthesis of PHA in plants was initially explored by expression of the PHB biosynthetic genes of *R. eutropha* in *Arabidopsis thaliana*, a plant related to rapeseed. The first experiments aimed at assessing the feasibility of synthesizing PHB in plants were done by expressing the PHB biosynthetic pathway in the cytoplasm (8). The *R. eutropha* genes encoding the acetoacetyl-CoA reductase (*phaB*) and PHA synthase (*phaC*) were expressed constitutively in transgenic *A. thaliana*. The highest amount of PHB obtained in these plants was approximately 0.1% of the shoot dry weight. Transmission electron microscopy revealed that PHB accumulated in the form of granules that were indistinguishable from bacterial PHA inclusions. Chemical analysis of the plant polymer indicated that it was of high molecular weight and had properties similar to bacterial PHB (9). Although PHB synthesis in plants could be demonstrated successfully, PHB accumulation was very low and the high level of expression of acetoacetyl-CoA reductase led to a reduction in growth of transgenic plants, with a corresponding decrease in seed yield. Both the low PHB production and the deleterious effect on plant growth were thought to be due mainly to the limited supply of cytoplasmic acetyl-CoA available for PHB, isoprenoid, and flavonoid biosynthesis (10). To solve this problem, it was necessary to find another cell compartment with a higher flux of carbon through acetyl-CoA.

In plants, fatty acid biosynthesis occurs in the plastid using acetyl-CoA as a precursor. The plastid is therefore a site of high flux of carbon through acetyl-CoA, particularly in developing seeds of oil-accumulating plants, such as *A. thaliana*, where up to 40% of the seed dry weight is triacylglycerols. Expression of the PHB biosynthetic pathway in plastics was demonstrated in transgenic *A. thaliana* (11). The *R. eutropha* phaA, phaB, and phaC genes were modified for targeting the proteins to the plastids, and the genes were constitutively expressed in *A. thaliana*. Transgenic plants accumulated PHB granules exclusively in the plastids (Figure 4). The maximal amount of PHB detected in presenescent leaves was 10–14% dry weight. In plants producing high levels of PHB, leaves showed chlorosis, indicating some defect in chloroplast function, although this phenotype was not accompanied by a reduction in fresh weight. Thus, redirecting the PHB biosynthetic pathway from the cytoplasm to the plastid resulted in a 100-fold increase in PHB production with limited deleterious effects on the plants (11).

Synthesis of PHB in crop plants was initiated in the early 1990s by Zeneca, which also produced PHB and P(HB-HV) by bacterial fermentation. Rapeseed was chosen as the initial target crop. Expression of the PHB pathway in the cytoplasm led to low levels of PHB accumulation in leaves, similar to the level detected in *Arabidopsis* (0.1% dry weight). In 1995, Zeneca sold its PHA business to Monsanto, which also took up the challenge to produce PHB in crops. Monsanto recently has shown that transgenic rape expressing the PHB pathway in the leucoplast of developing embryos accumulated PHB at levels up to 8% dry weight in mature seeds of heterozygous plants (12).

**PHA copolymers in plants**

PHA has relatively poor physical properties, being too stiff and brittle for most applications. There is thus a large interest in synthesizing PHA copolymers with better physical properties in plants. Two recent studies have shown that it is possible to synthesize both SCL-PHA and MCL-PHA copolymers in plants.

In one study, accumulation of MCL-PHA was obtained by expression of the PHA synthase from the bacterium *P. aeruginosa* in plant peroxisomes (13). The peroxisome was chosen because it is the site of fatty acid β-oxidation in plants, which can provide the 3-hydroxyacyl-CoA substrate to the PHA synthase for MCL-PHA synthesis. The *P. aeruginosa* PHA synthase was modified by the addition of a peroxisomal targeting signal from rapeseed isocitrate lyase at the carboxy end and expressed in *A. thaliana* under the control of a constitutive promoter (13). Analysis of transgenic plant material by gas chro-
matography and mass spectrometry demonstrated the synthesis of a complex PHA containing over 15 different monomers, ranging from C6 to C16 3-hydroxyacids, with some monomers containing up to three unsaturated bonds. Approximately 0.4% dry weight of PHA was produced in 7-day-old germinating seedlings. Analysis of transgenic tissues by electron microscopy revealed the presence of numerous PHA granules in the peroxisomes. These results indicated that, like bacteria, plants also have enzymes capable of generating the R-isomer of 3-hydroxyacyl-CoA from β-oxidation intermediates. It is hypothesized that a broad spectrum of R-3-hydroxyacyl-CoAs could be generated either by the epimerization of the S-3-hydroxyacyl-CoA, the hydration of 2-trans-enoyl-CoA by a enoyl-CoA hydratase II activity, or the hydration of 2-cis-enoyl-CoA generated from the β-oxidation of fatty acids having cis double bonds at the even position (13).

In order to assess the factors limiting the synthesis of MCL-PHAs in peroxisomes, transgenic plants were grown in liquid media supplemented with Tween detergents as a source of fatty acids. Addition of Tween-10, containing primarily lauric acid, resulted in a 10-fold increase in MCL-PHA accumulation. Addition of unusual fatty acids to the media resulted in the incorporation into the polyester of monomers derived from the β-oxidation of these fatty acids. For example, addition of tridecanoic acid, tridecenoic acid (C13:1, Δ12), or 8-methyl-nonanoic acid resulted in the production of PHA containing mainly saturated odd-chain, unsaturated odd-chain, or branched-chain 3-hydroxyacid monomers, respectively. These studies demonstrated that the plant β-oxidation pathway was capable of generating a large spectrum of monomers for PHA synthesis from unusual fatty acids and that the supply of fatty acids to the β-oxidation pathway was a factor limiting the accumulation of PHA in plants.

To overcome this limitation in vivo, the endogenous flux of fatty acids to β-oxidation was increased through genetic engineering by the expression of a medium-chain acyl-ACP (acyl carrier protein) thioesterase from Cuphea lanceolata. Previous studies done with transgenic rapeseed expressing the California bay lauroyl-ACP thioesterase had indicated that

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medium-chain fatty acids synthesized in plastids from leaves expressing the thioesterase were degraded by β-oxidation before any accumulation could be detected in vegetative tissues (14). It was thus hypothesized that expression of a medium-chain thioesterase in conjunction with a PHA synthase would create a higher flux of carbon toward fatty acid β-oxidation and PHA synthesis. Transgenic plants expressing the peroxisomal PHA synthase and the plastidial medium-chain acyl-ACP thioesterase from *C. lanceolata* produced 10-fold more PHA in mature shoots compared to plants expressing only the PHA synthase. PHA produced in double-transgenic plants contained mainly saturated monomers ranging from 6 to 10 carbons, consistent with the specificity of the *C. lanceolata* thioesterase for caprylyl-ACP, and in contrast to MCL-PHA produced from plants expressing only the PHA synthase, which was mainly composed of unsaturated long-chain monomers.

These results clearly showed that the quality and quantity of MCL-PHAs in plants can be modulated by the co-expression of enzymes responsible for the synthesis of unusual fatty acids together with a peroxisomal PHA synthase. Furthermore, these studies indicate that analysis of the nature and proportion of monomers found in peroxisomal MCL-PHA can be used in basic plant biochemistry to elucidate the pathways involved in the degradation of unsaturated and unusual fatty acids, while the quantity of PHAs can be used to study flux of fatty acids toward peroxisomal β-oxidation.

In an alternative approach aimed at the synthesis of P(HB-HV) copolymers in plants, scientists at Monsanto co-expressed in the plastids a threonine deaminase from *Escherichia coli* along with the PHB biosynthetic pathway (12) (Figure 3A). Threonine deaminase catalyzes the conversion of threonine to α-ketobutyrate, which itself can be converted, albeit at low efficiency, to propionyl-CoA by the action of the pyruvate dehydrogenase complex. The propionyl-CoA can be condensed with acetyl-CoA to form valeryl-CoA, which can then be used for P(HB-HV) synthesis. Expression of the threonine deaminase together with the PHB biosynthetic pathway in leucoplasts of rapeseed embryos resulted in P(HB-HV) accumulation to 2.3% dry weight in seeds, with a type of polymer from seedlings. Alternatively, one also can envisage producing PHA in the leaves or stems of a variety of crops, thus adding value to the plant biomass that is often regarded as a waste material. It has also been suggested that carbohydrate-accumulating crops, such as potato or sugar beet, could be used to produce PHA. In this last example, synthesis of PHA would be dependent on indirectly diverting carbon away from carbohydrate synthesis and toward PHA.

Important factors affecting the commercial viability of PHA production in crops are the extraction and the purification of the polymer.

**Bringing PHA synthesis to the field**

It has now been shown that it is possible for plants to synthesize a variety of PHAs having properties ranging from stiff plastic (PHB), softer plastic [P(HB-HV)], and elastomers (MCL-PHAs). The challenge for the future is to reach commercial levels of the right PHA in an appropriate crop. It is estimated that the break-even point for commercial viability of PHA production in plants would be approximately 15% dry weight PHA, assuming a relatively low decrease in biomass yield. From this perspective, the accumulation of PHB in rapeseed embryos of up to 8% dry weight in heterozygous plants appears promising.

Several strategies for synthesis of PHA in crops can be envisaged. Synthesis of PHAs in seeds of oil crops has several advantages. PHB and P(HB-HV) synthesis is dependent on an abundant source of acetyl-CoA, which can be provided in the leucoplast of developing seeds of oil crops. These PHAs could thus be harvested from dry mature seeds. For MCL-PHA synthesis, the large triacylglycerol reserve which is degraded through β-oxidation during germination could enable the harvest of this type of polymer from seedlings. Alternatively, one also can envisage producing PHA in the leaves or stems of a variety of crops, thus adding value to the plant biomass that is often regarded as a waste material. It has also been suggested that carbohydrate-accumulating crops, such as potato or sugar beet, could be used to produce PHA. In this last example, synthesis of PHA would be dependent on indirectly diverting carbon away from carbohydrate synthesis and toward PHA.

Important factors affecting the commercial viability of PHA production in crops are the extraction and the purification of the polymer. Although PHA synthesized in bacteria can account for up to 85% dry weight and can be relatively easily purified by protease and detergent treatment, extraction of 10–20% PHA from plant material is likely to be more difficult, adding to the production cost of the polymer. Finally, one must keep in mind that high-level synthesis of PHA in any crop is likely to require the coordinated and stable expression of numerous transgenes affecting several metabolic pathways. Synthesis of P(HB-HV) requires at least four transgenes affecting fatty acid and amino acid biosynthetic pathways. Thus, in comparison to the first-generation transgenic crops actually in the field, which typically express only one or two transgenes, the successful development of a PHA-producing crop represents a very complex and challenging genetic engineering project.

**References**

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