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Polyhydroxybutyrate, a Biodegradable Thermoplastic, Produced in Transgenic Plants

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Polyhydroxybutyrate (PHB), a high molecular weight polyester, is accumulated as a storage carbon in many species of bacteria and is a biodegradable thermoplastic. To produce PHB by genetic engineering in plants, genes from the bacterium *Alcaligenes eutrophus* that encoded the two enzymes required to convert acetoacetyl–coenzyme A to PHB were placed under transcriptional control of the cauliflower mosaic virus 35S promoter and introduced into *Arabidopsis thaliana*. Transgenic plant lines that contained both genes accumulated PHB as electron-lucent granules in the cytoplasm, nucleus, and vacuole; the size and appearance of these granules were similar to the PHB granules that accumulate in bacteria.

Poly-D-(−)-3-hydroxybutyrate (PHB) is an aliphatic polyester that is accumulated by many species of bacteria as storage material (*1*). Both PHB and related polyhydroxyalkanoates (PHA) are renewable sources of biodegradable thermoplastic materials (*2*). The cost of PHB produced by bacterial fermentation is substantially higher than that of other biomaterials, such as starch or lipids, that accumulate in many species of higher plants. Therefore, we investigated the feasibility of transferring the capability for PHB synthesis from bacteria to higher plants.

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In the bacterium *Alcaligenes eutrophus*, PHB is derived from acetyl–coenzyme A (CoA) by a sequence of three enzymatic reactions (*3*). The first enzyme of the pathway, 3-ketothiolase (acetyl-CoA acetyl transferase; E.C. 2.3.1.9), catalyzes the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase; E.C. 1.1.1.36) subsequently reduces acetoacetyl-CoA to D-(−)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHB synthase to form PHB. The genes encoding the three enzymes involved in PHB synthesis in *A. eutrophus* have been cloned, and expression of the genes in *Escherichia coli* is sufficient for PHB production (*4–7*). Of the three enzymes involved in PHB synthesis in *A. eutrophus*, only the 3-ketothiolase is also found in the cytoplasm of higher plants, where it is involved in the synthesis of mevalonate, the precursor to isoprenoids.

Genes that encoded acetoacetyl-CoA reductase (*phbB*) and PHB synthase (*phbC*) were introduced into *Arabidopsis thaliana*

through Ti plasmid–mediated transformation. The coding sequences of the *phbB* and *phbC* genes were individually cloned into the binary Ti plasmid pBI121 so that the genes were under the transcriptional control of the constitutive cauliflower mosaic virus 35S promoter (*8*). Two series of transgenic *A. thaliana* plants, which contained either the *phbB* or *phbC* gene, were generated by transformation with *Agrobacterium tumefaciens* containing the Ti plasmid constructs (*9*).

Southern (DNA) blot and Northern (RNA) blot analysis of seven putative homozygous transgenic lines obtained by transformation with the *phbB* gene indicated the proper integration and transcription of the gene in the various lines (*10*). To assess whether the *phbB* mRNA was correctly translated and whether the polypeptide produced was functional, we assayed transgenic plants that had the *phbB* gene for acetoacetyl-CoA reductase activity (*11*). Enzyme activity was not detectable in leaf extracts of the untransformed wild-type plants. Leaf extracts of the seven transgenic lines exhibited specific activities ranging from 1.6 to 16.2 units per milligram of protein (*12*), compared to 1.4 units per milligram of protein for extracts of *Escherichia coli* DH5α that expressed the *phbB* gene present on the plasmid pTZ18U-PHB (*13*).

Southern blot and Northern blot analysis of three putative homozygous transgenic lines obtained by transformation with the *phbC* gene revealed proper integration and transcription of the gene in the various lines (*10*). However, transgenic plants that expressed *phbC* mRNA had no detectable PHB synthase activity. Similarly, expression of the *phbC* gene in *E. coli*, in the absence of *phbA* and *phbB* gene expression, did not result in significant PHB synthase activity (*7*). It has been postulated that in the absence of substrate synthesized by the *phbA* and *phbB* gene products, the PHB synthase may be unstable or inactive. Therefore, the absence of PHB synthase activity in transgenic plants that contained the *phbC* gene was not considered an accurate reflection of whether the enzyme would function in plants that contained the additional enzymes of the pathway.

To produce plants containing both *phbB* and *phbC* genes, we cross-pollinated homozygous transgenic lines that had the *phbB* gene (lines RedB-2A, -2C, -2D, -2G, and RedD-3A) with homozygous transgenic lines that had the *phbC* gene (lines S8-1-2A and S8-1-2C). Leaf samples of 2- to 3-week-old hybrid plants (F1) were analyzed for the presence of PHB by gas chromatography of transesterified ethylated derivatives of chloroform-soluble material (Fig. 1). Extracts of F1 hybrids that expressed both the bacterial acetoacetyl-CoA reductase and the PHB synthase genes contained a novel compound that eluted with the same retention time as ethylhydroxybutyrate. The compound was not detected in transgenic plants that expressed only one of the two *phb* genes or in untransformed *A. thaliana* plants. Analysis of the compound by gas chromatography–mass spectrometry (GC-MS) confirmed that it had the same mass fragmentation pattern as a reference sample of ethylhydroxybutyrate (Fig. 2). Ethylhydroxybutyrate was not detected in chloroform extracts of plant tissues in

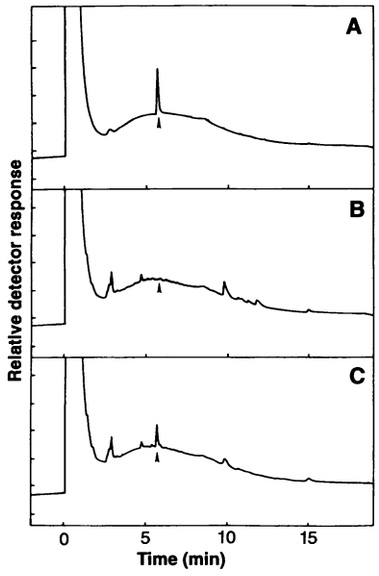

Fig. 1. Gas chromatographic evidence for PHB accumulation in transgenic plants. (**A**) Transesterified bacterial PHB (20 ng). (**B**) Transesterified chloroform extracts of leaves from wild-type *A. thaliana*. (**C**) Transesterified chloroform extracts of leaves from F1 hybrid between transgenic plants S8-1-2A (*phbC*$^+$) and RedB-2C (*phbB*$^+$). Arrowheads indicate elution time of ethylhydroxybutyrate. Between 5 to 50 mg of fresh or frozen plant shoot material was extracted in 1 to 2 ml of a chloroform and water mixture (1:1) for 16 hours at 65°C with constant agitation. This extract, which did not contain PHB, was discarded. The plant material was subsequently homogenized in water and reextracted in chloroform and water as described above. The chloroform phase was extracted twice with water. The products present in the organic phase were transesterified by acid ethanolysis, and one-hundredth of the final reaction mixture was analyzed by gas chromatography on a Hewlett-Packard 5890 series II GC (*18*). As a standard, bacterial PHB (Sigma) was used.

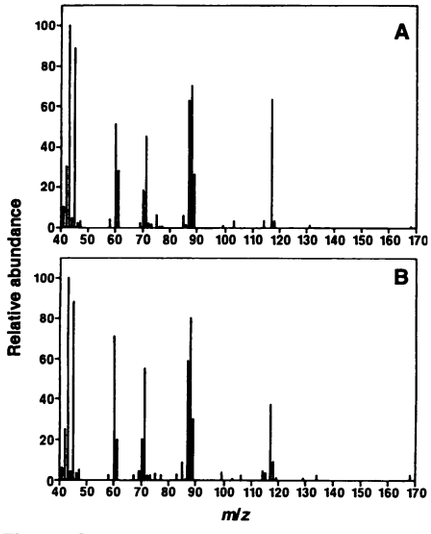

Fig. 2. Gas-chromatography–mass spectrometry analysis of PHB from bacteria and transgenic plants. (**A**) Mass spectrum of transesterified bacterial PHB. (**B**) Mass spectrum of the putative ethylhydroxybutyrate from F1 hybrid between S8-1-2A (*phbC*$^+$) and RedB-2C (*phbB*$^+$) (Fig. 1C). Electron impact mass spectral data was obtained on a JEOL JMS-AX505H mass spectrometer coupled with a Hewlett-Packard 5890 GC. The following parameters were used: source temperature, 200°C; ionization current, 100 μA; accelerating voltage, 3 keV (*19*); *m/z*, mass-to-charge ratio.

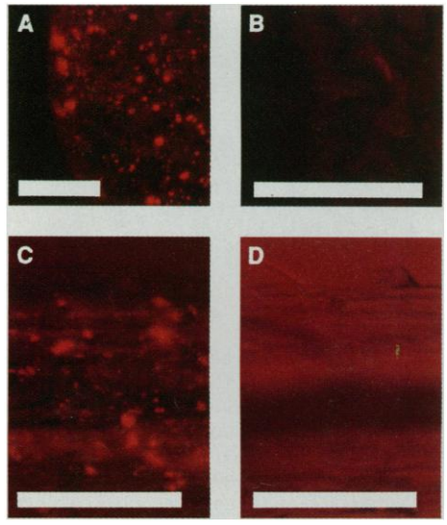

Fig. 3. Visualization of PHB granules by epifluorescence microscopy of tissues stained with Nile Blue A. Leaves (**A** and **B**) and roots (**C** and **D**) from PHB-producing F1 hybrids between RedB-2G (*phbB*$^+$) and S8-1-2C (*phbC*$^+$) (A and C) and from transgenic plants RedB-2G (*phbB*$^+$) that did not produce PHB (B and D) were fixed with glutaraldehyde, stained with Nile Blue A, and viewed by epifluorescence microscopy (Axiophot, Zeiss) under an excitation wavelength of 546 nm. Bars represent 50 μm. Plants were grown aseptically on Murashige and Skoog basal media (Sigma) that contained 1% sucrose and 0.8% agar. Roots and leaves of 2-week-old plants were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 8.0) for 3 hours. Tissues were rinsed in water and stained for 5 min in 1% Nile Blue A. Tissues were rinsed several times in water and soaked 1 min in 8% acetic acid followed by a final rinse in water.

which the cell wall had not been disrupted by homogenization. However, if the chloroform-extracted tissue was then homogenized and reextracted under identical conditions, ethylhydroxybutyrate was detected (Fig. 1). Because the cell wall is permeable to molecules with a molecular mass below ~60,000 daltons (*14*), these results indicate that the ethylhydroxybutyrate was derived from a large molecular size precursor. Thus, we conclude that transgenic plants that expressed both the bacterial acetoacetyl-CoA reductase and PHB synthase genes accumulated PHB.

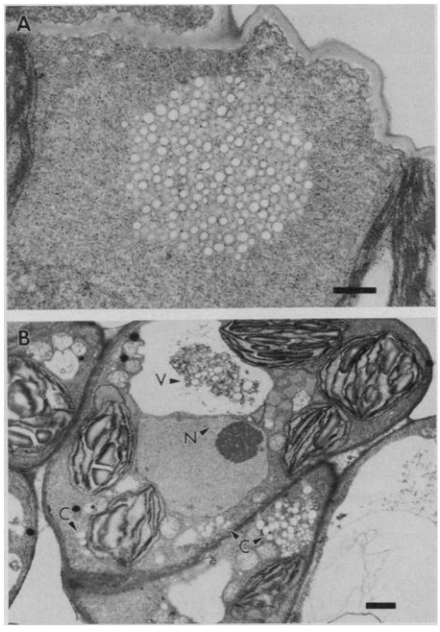

Fig. 4. Transmission electron micrographs of thin sections from PHB-positive transgenic *A. thaliana* plants. Transgenic line S8-1-2A (*phbC*$^+$) was pollinated with transgenic lines RedB-2D and RedB-2C (*phbB*$^+$). Tissue samples from 1- to 3-week-old F1 plants were analyzed by TEM. (**A**) Leaf mesophyll cells from a RedB-2D × S8-1-2A F1 hybrid with an agglomeration of granules in the nucleus. (**B**) Two adjacent mesophyll cells from a cotyledon of a RedB-2C × S8-1-2A F1 hybrid showing electron-lucent granules in the nucleus (N), vacuole (V), and cytoplasm (C). Arrows indicate agglomerations of electron-lucent granules. Bars represent 1 μm. Plant tissues were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1.5 to 2 hours at room temperature. The samples were washed four times in 0.1 M phosphate buffer (pH 7.2) and fixed with 1% OsO_4 in phosphate buffer for 2 hours at room temperature. The tissues were then dehydrated in a graded ethanol series and embedded in Spurrs epoxy resin. Sections of 80 to 90 nm were cut, placed on copper grids, and stained with 5% uranyl acetate for 30 to 45 min, followed by staining with Reynolds lead citrate for 3 to 4 min. Sections were viewed in a JEOL 100CX II transmission electron microscope operated at 80 kV.

Similar results were obtained with the F1 progeny of four different crosses involving four independent *phbB* transgenic lines and two independent *phbC* lines. The amount of PHB accumulated in leaves ranged from approximately 20 μg per gram of fresh weight for F1 hybrids between RedD-3A and S8-1-2C to approximately 100 μg per gram of fresh weight for F1 hybrids between RedB-2C and S8-1-2A.

Bacteria accumulate PHB as electron-lucent granules of 0.2 to 0.5 μm in diameter surrounded by a 2-nm-thick layer of electron-dense material (*15*). Bacterial PHB granules stained with Nile Blue A emit orange or red fluorescence at excitation wavelengths of 460 and 546 nm, respectively (*16*). To determine if similar granules could be detected in F1 hybrids shown to be positive for PHB production by GC-MS analysis, we examined plant tissues with epifluorescence microscopy and transmission electron microscopy (TEM). In all tissues of PHB-producing plants stained with Nile Blue A, bright foci of red fluorescence with an approximate maximum diameter of 10 μm were observed (Fig. 3). Similar granular red fluorescence was never observed in untransformed *A. thaliana*, in transgenic plants that expressed only one of the *phbB* or *phbC* transgenes, or in PHB-producing tissues not stained with Nile Blue A. Insoluble material partially purified from tissues of PHB-producing plants was shown to contain PHB granules by GC-MS and epifluorescence microscopy (*10, 17*).

Cells in the mature leaves, cotyledons, and roots of PHB-producing plants had agglomerations of electron-lucent granules (Fig. 4). These granules were detected in all analyzed F1 hybrids that expressed both *phbB* and *phbC* genes. Similar granules were never detected in the parental transgenic lines that expressed only one of the *phb* genes or in untransformed *A. thaliana*. The granules were detected in the nucleus, vacuole, and cytoplasm of the F1 hybrid tissues. No granules could be detected in the chloroplast. In the nucleus, individual granules were found to reach a maximum size of approximately 0.2 μm. In the vacuoles and cytoplasm, the granules were generally larger and reached a maximum diameter of approximately 0.5 μm. At higher magnification, the granules appeared to be surrounded by electron-dense material. Both the size and appearance of these granules were very similar to granules observed in bacteria accumulating PHB (*15*).

The polypeptide products of the *phbB* and *phbC* genes used here are expected to be located in the cytoplasm of *Arabidopsis* cells, as the genes lack sequences that encode organelle-specific targeting signals. The accumulation of PHB granules in the nucleus and vacuole of transgenic hybrid plants was unexpected. The nuclear localization of granules could result from the entrapment of existing cytoplasmic granules during reassembly of nuclear membranes at mitotic telophase. However, because the vacuolar membrane does not break down during any stage of the cell cycle, this result does not explain how the granules accumulated in this organelle. An alternate possibility is that PHB granules may be capable of crossing the membranes of the nucleus and vacuole.

Expression of large amounts of acetoacetyl-CoA reductase in transgenic plants caused a significant reduction in growth and seed production relative to wild-type plants. For example, for the transgenic lines RedB-2G and RedB-2C, which expressed approximately 5 and 9 units of acetoacetyl-CoA reductase activity per milligram of protein, respectively, the fresh weight of 22-day-old shoots was reduced to 45% and 19% of wild type, respectively. Seed production was reduced in approximately the same proportion. This phenotype could be the result of the diversion of a significant amount of acetyl-CoA or acetoacetyl-CoA away from an essential biochemical pathway such as isoprenoid biosynthesis. Expression of the PHB synthase, by itself, had no apparent effect on the growth or vigor of transgenic plants. However, the F1 hybrids that contained both genes were more severely stunted in growth than plants that contained only the acetoacetyl-CoA reductase gene, which could result from either a more severe depletion of substrate from the mevalonate pathway or a noxious effect of the PHB granules.

The present report of synthesis of PHB in plants represents a first step toward the production of novel biopolymers in plants through genetic engineering. Production of a large quantity of PHB or PHA in plants will require additional genetic manipulations to divert reduced carbon away from endogenous metabolic pathways and to regulate the tissue specificity, timing of expression, and cellular localization of the enzymes involved. It might be possible to divert carbon from synthesis of storage lipids toward PHB production in plastids, where accumulation of PHB granules may not have deleterious effects.

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X-ray Structure of T4 Endonuclease V: An Excision Repair Enzyme Specific for a Pyrimidine Dimer

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The x-ray structure of T4 endonuclease V, an enzyme responsible for the first step of a pyrimidine-dimer–specific excision-repair pathway, was determined at a 1.6-angstrom resolution. The enzyme consists of a single compact domain classified into an all-α structure. This single domain has two distinct catalytic activities: it functions as a pyrimidine dimer glycosylase and as an apurinic-apyrimidinic endonuclease. The amino-terminal segment penetrates between two major helices and prevents their direct contact. The refined structure suggests the residues involved in the substrate binding and the catalysis of the glycosylation reaction.

Ultraviolet (UV) irradiation causes formation of pyrimidine dimers within DNA that are lethal and mutagenic in vivo. The first step of the excision repair pathway of UV-damaged DNA is strand scission of the DNA in the vicinity of a pyrimidine dimer (*1*). The enzyme T4 endonuclease V, encoded by the *denV* gene of bacteriophage T4, is responsible for this step in bacteriophage-infected *Escherichia coli* (*2*, *3*). Although the enzyme is a rather small protein (138 amino acids), it has two distinct catalytic activities (*4*–*12*): it acts as a pyrimidine dimer glycosylase and as an apurinic-apyrimidinic endonuclease (Fig. 1). This latter reaction proceeds through the β-elimination of the 3′-phosphate of an abasic site rather than by the actual hydrolysis of the phosphodiester bond (*13*–*15*). Before binding to a pyrimidine dimer, the enzyme nonspecifically binds by electrostatic forces and scans the double-stranded DNA (*6*, *11*, *12*, *16*). Once the enzyme has specifically bound to a pyrimidine dimer, the DNA is incised at the 5′-glycosyl bond in the dimer, and, subsequently, scission of the phosphodiester bond occurs at the exposed backbone.

We report the three-dimensional (3-D) x-ray structure of the enzyme and discuss its functional implications. Combined with results from site-directed mutagenesis, the examination of the structural features allows the identification of residues participating in the substrate binding and the catalytic reaction.

Crystals of T4 endonuclease V (*17*) belonging to the space group $P2_1$, with unit cell parameters of a = 41.4 Å, b = 40.1 Å, c = 37.5 Å, and β = 90.01°, contain one molecule per asymmetric unit and diffract x-rays beyond 1.6 Å resolution.

An initial electron density map was calculated at 2.5 Å resolution with multiple isomorphous replacement (MIR) phases, which were obtained with five heavy-atom derivatives (Table 1). Consistent with the high value of the figure of merit, the electron densities were sufficiently well defined to allow the discernment of most residues even in a minimap, and thus an unambiguous chain tracing could be achieved. The $2F_o - F_c$ map after refinement with the restrained least-square program PROLSQ (Fig. 2) gave a final R value of 0.196 (*18*).

The enzyme T4 endonuclease V is composed of a single compact domain. The molecule has a roughly ellipsoidal shape with dimensions 50 by 42 by 40 Å. The enzyme consists of three α helices, five reverse turns, and extended chain segments and loops, but it contains no β structure (*19*) (Fig. 3). The enzyme should thus be classified into the all-α type of structure (*20*); 45% of its residues are located in α helices (Fig. 4). The first α helix (H1, residues 14 through 38) is centrally kinked at Pro^{25}, creating an inclination of 20°. All of the five reverse turns lie on the external surface of the molecule and in close proximity to NH_2- or COOH-terminal ends of the α helices, except for one reverse turn (Q98 to F100) (*21*).

The arrangement of α helices in this enzyme is unusual. The three helices, H1 (14 through 38), H2 (64 through 82), and H3 (108 through 124), stand side-by-side (Fig. 3), and their termini are covered by a caplike loop around the COOH-terminus.

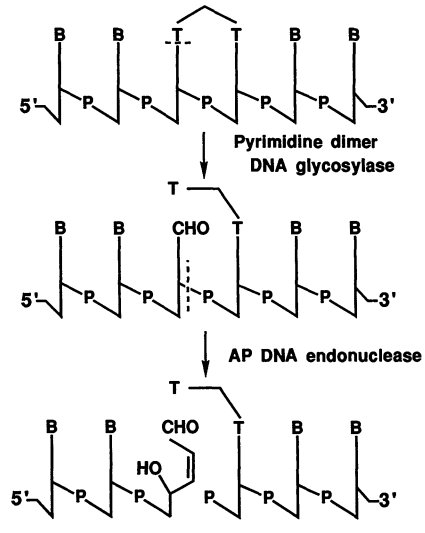

Fig. 1. Two distinct catalytic activities of T4 endonuclease V. AP, apurinic or apyrimidinic.

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