Production of Biodegradable Polyester by a Transgenic Tobacco

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The acetoacetyl-CoA reductase gene (phbB) of Ralstonia eutropha and the poly[(R)-(−)-3-hydroxyalkanoate] synthase gene (phaC4c) of Aeromonas caviae were introduced into tobacco plant by Agrobacterium mediated transformation method. The resulting transgenic tobacco expressed both introduced genes and the expression of these genes was confirmed by enzymatic analysis and western blotting. GC-MS analysis of the chloroform extract of tobacco leaves indicated that the transgenic plant produced biodegradable polyester, poly[(R)-(−)-3-hydroxybutyrate]. GPC analysis indicated that the number-average molecular weight (Mn) and polydispersity (Mw/Mn) were 32,000 and 1.90, respectively.

**Key words:** transgenic plant; polyhydroxybutyrate; PHA; Aeromonas caviae; Agrobacterium

Poly[(R)-(−)-3-hydroxyalkanoates] (PHA), consisted of (R)-(−)-3-hydroxyalkanoates such as (R)-(−)-3-hydroxybutyrate (3HB), (R)-(−)-3-hydroxypentanoate, and (R)-(−)-3-hydroxyhexanoate, are widely produced by microorganisms for energy storage (Fig. 1). These polyesters with desired qualities like thermoplasticity, biodegradability, and elasticity are useful for human society. This type of polymer has already been produced commercially using microorganisms. Production of this polymer in plants is expected to establish a new carbon cycle using the light energy, so that the production system will have a less adverse impact on the environment. With this aim, some plants were genetically engineered for polyester production. In these, only the genes that produce a homopolymer, poly[(R)-(−)-3-hydroxybutyrate](PHB) consisted of 3HB, were studied, and it is necessary to exploit other types of genes also for production of polyesters with useful characteristics, which has already been a focus in several laboratories.

The biosynthetic pathway of poly-3-hydroxybutyrate, the most common polyester in bacteria, has been studied in detail using Ralstonia eutropha (formerly Alcaligenes eutrophus) and the genes encoding the biosynthetic enzymes have already been cloned. β-Ketothiolase (phaA) synthesizes acetoacetyl-CoA from two molecules of acetyl-CoA produced by the TCA cycle, and acetoacetyl-CoA reductase (phbB) converts acetoacetyl-CoA into 3-hydroxybutyrate (3HB). PHA synthase (phbC) polymerizes 3HB to produce PHB, which is a type I PHA polymerase and uses only C3-C5 hydroxylactanoate units. Type II PHA synthases of Pseudomonas oleovorans and Pseudomonas putida were reported to use C3-C12 3-hydroxyalkanoates units.

Aeromonas caviae FA440 was known to produce a copolymer consisted of 3HB and 3-hydroxyhexanoate when cultured in a medium containing fatty acids. Recently, it was found that A. caviae FA440 had an enoyl-CoA hydratase, which enabled this strain to use fatty acid to produce PHA via β-oxidation pathway. To produce this copolymer, A. caviae has PHA synthase with a different substrate specificity that can polymerize C3 to C6-3-hydroxyalkanoate units in contrast to that of R. eutropha, which can use only C3 to C5 units.

Plant cells contain various organelles and the metabolisms are quite different from those in bacterial cells, and the C4 unit of 3-hydroxyalkanoate may be synthesized by addition of an acetyl-CoA unit to C4 unit during fatty acid synthesis. To test the feasibility of producing copolymer by changing the substrate specificity, the PHA synthase gene (phaC4c) from A. caviae and the acetoacetyl-CoA reductase gene (phbB) from R. eutropha were introduced into tobacco plants and the polyester production was investigated.

**Materials and Methods**

**Plasmid construction.** Polymerase chain reaction (PCR) was used to amplify the coding region of the phbB3 and phaC4c2 genes from the genomic DNA of R. eutropha and A. caviae, respectively. The primers were designed based on the sequence of the genes and contained convenient cloning sites. The primers used for the phbB gene were AGAGATCCCAAGGAGTG-TACATGACTCAG and ACTGAGCTCCGCGG-CTGCGGACTGGTGGA, and those for phaC4c gene were GAGATCCGGTGGTGAAGGATGATCAT-AGGCC and ACTGAGCTTCTACTCCAGGGATT-GTGG. Amplified phbB and phaC4c fragments were separately cloned into pCR2.1 (Invitrogen) and were se-
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Fig. 1. Structure of Poly[(R)-(−)-3-hydroxyalkanoate].

Quenced to confirm the primary structure. β-Glucronidase (GUS) gene of pBI121 (Clontech) was replaced with phbB gene fragment, generating a plasmid pBM202 by ligating with cauliflower mosaic virus 35S promoter (CaMV 35S) and Agrobacterium nopaline synthase terminater. phaC4c gene fragment was replaced with GUS gene of pBI221E, a modified pBI221 with an additional EcoRI site upstream of CaMV 35S, to generate pBM203E. The EcoRI fragment of pBM203E was inserted at the EcoRI site of pBM202 to generate a plant expression vector, pBM205E, containing the kanamycin resistance gene and CaMV 35S-regulated phbB and phaC4c genes.

Plant transformation. Nicotiana tabacum cv Samsun NN cultured on agarose solidified Murashige and Skoog (MS) basal medium9 (MS salts, pH 5.8, 3% sucrose, 0.7% agarose) for 3 weeks in a plant growth chamber was used for the transformation. The plant expression vector pBM205E was mobilized in Agrobacterium tumefaciens strain LBA4404 following the method described by Hufgen and Willmitzer.10 Tobacco transformation was done by the leaf disk method.11 About three weeks after infection with Agrobacterium, regenerated tobacco shoots on agarose solidified MS basal medium containing kanamycin (100 mg/l) and Clorafan (500 mg/l, Hoechst Japan) were picked up and transferred to the same medium for further selection. About two weeks after the second selection kanamycin resistant plants were obtained.

RNA analysis. Total RNA was extracted from young leaves (100 mg) of tobacco plants using RNAeasy (Qiagen) and 1.5 μg of each sample (12 μl) was denatured and used for CDNA synthesis. This was mixed with 0.5 μg of oligo-dT (Pharmacia), 20 μl of RNase inhibitor (Takara), 1 μl of 10 mM dNTP mix, and 200 μl of reverse transcriptase (Amersham) and the final volume was made up to 20 μl. The reaction mixture was incubated for 1 hour at 37°C. The reaction mixture (50 μl) for PCR contained 0.5 μl of CDNA preparation, 1 μl of 2.5 mM dNTP mix, a set of primers (10 pmol each), and 1.5 units of Taq polymerase (Boehringer-Manheim). PCR products were checked by 1.5% agarose gel electrophoresis and the expected size of amplified phbB and phaC4c gene fragments were 740 bp and 1,780 bp, respectively.

Western bloting. A protein preparation was made by homogenizing leaf tissue (1 × 1 cm) in 600 μl of 50 mM Tris-HCl buffer (pH 7.5, 4°C) followed by centrifugation for 20 min at 4°C and 15,000 rpm. Each sample (20 μg) was applied on a 8% SDS polyacrylamide gel (7 × 9 cm). After the electrophoresis proteins were electroblotted onto a nitrocellulose membrane. The membrane was then used for immunodetection using an antibody produced in a rabbit against a polypeptide of C-terminus 50 amino acids of PHA synthase. The horseradish peroxidase (HRP) linked anti-rabbit antibody was used as the secondary antibody and the HRP activity on the membrane was detected by ECL detection kit (Amersham).

Enzyme assay. Protein samples were prepared in the same manner as that for western blotting. The reaction mixture for acetoacetyl-CoA reductase assay contained 12 μM Mg2+, 0.5 mM dithiothreitol, 0.1 mM NADPH, 0.1 mM acetoacetyl-CoA, and 200 μg/ml protein sample in 60 mM potassium phosphate buffer (pH 7). The reaction was started by addition of the substrate. The reaction was performed at 30°C and A405 was recorded by spectrophotometer.

Analysis of polyester. Leaves (50 g) were washed with 500 ml of 50% ethanol at 55°C four times followed by washing with 500 ml methanol at 55°C four times. Washed leaves were ground into powder and extracted with 500 ml chloroform for 12 hours at 60°C. The extract was filtered and concentrated, and polyesters were precipitated by addition of cold methanol. Precipitates were collected by filtration and washed with methanol, and then dissolved in chloroform. This green solution was recrystallized with n-hexane and the precipitate was collected by filtration and dissolved in chloroform. The PHA sample (0.5 ml) was mixed with 1.7 ml of ethanol and 0.2 ml of conc. HCl, incubated at 100°C for 4 hours, and analyzed by GC-MS. GC-MS analysis was done by a G1800 GCD system (Hewlett Packard) using a HP-5 column (Hewlett Packard). The crude PHA sample prepared by chloroform extraction of A. caviae cells was analyzed by GC-MS following ethanalysis.

Gel permeation chromatography (GPC) analysis was done at 40°C by a Shimazu 10A system using a Shimazu 6A refractive index detector and serial columns of Shodex K802 and Shodex K806M. Chloroform was used as an eluent at a flow rate of 0.8 ml/min and polystyrene standards was used to make a calibration curve.

Results

Transgenic tobacco that expresses the genes for phbB and phaC4c

The plant expression vector pBM205E (Fig. 2), containing both acetoacetyl-CoA reductase (phbB) and PHA synthase (phaC4c) genes, was introduced into tobacco plants by Agrobacterium mediated transformation following the leaf disk method, and 6 kanamycin resistant plants, BC1 to BC6, were obtained. There was no great difference in morphological phenotype or in growth rate between kanamycin-resistant and non-transgenic tobacco.

Expression of the introduced genes in the transgenic plants was analyzed by reverse transcription (RT)-PCR using specific primers for phaC4c and phbB. The result
Fig. 2. Construct of Plant Expression Vector pBM205E.
The phbB gene coding acetoacetyl-CoA reductase and the phaCAC gene coding PHA synthase were conjugated in one vector derived from pH121. NPTII, neomycin phosphotransferase; 35S-P, Cauliflower mosaic virus 35S promoter; Nos-P, Agrobacterium nopaline synthase promoter; Nos-T, Agrobacterium nopaline synthase terminator; LB, left border of T-DNA; RB, right border of T-DNA.

Fig. 3. Accumulation of PHA Synthase in Leaves of Transgenic Tobacco Plants.
Western blot analysis using anti-PHA synthase antibody confirmed the accumulation of 66 kDa protein of PHA synthase in a transgenic tobacco plant BC3. Bacterial PHA synthase was used as positive control (lane P) and lane N contained proteins prepared from a wild type tobacco plant.

Fig. 4. Acetoacetyl-CoA Reductase Activity in Transgenic Tobacco Plants.
The consumption of NADPH during the reduction of acetoacetyl-CoA (AA-CoA) to 3-hydroxybutyrate (3HB) catalyzed by acetoacetyl-CoA reductase was measured spectrophotometrically. The consumption of NADPH increased by addition of acetoacetyl-CoA in a sample prepared from transgenic plant, which was not observed in the wild-type plant.
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Fig. 5. GC-MS Analysis of Polyester Produced by Transgenic Tobacco Plants.
(A) In gas chromatography analysis, a transgenic tobacco plant gave a peak (4.4 min) which was observed in the sample prepared from bacterial PHB but not in a wild type tobacco plant sample. Arrows indicate the peaks of 3HB-ethyl ester. (B) Mass spectra of these peaks at 4.4 min showed the same pattern suggesting that these are identical compounds.

by chloroform extraction and GC-MS analysis after ethanolation of polyester. The sample prepared from the leaves of BC3 gave a peak at the same retention time as that of 3HB-ethyl ester prepared from bacterial PHB (Fig. 5A), which was not observed in the wild-type plant sample (data not shown). Identity of mass spectra of these peaks (Fig. 5B) indicated that 3HB-ethyl ester was generated by ethanolation from the transgenic tobacco extract and that a compound containing the 3HB unit accumulated in the leaves. Polyester productivity was very low (less than 10 µg per gram fresh weight) and did not affect the plant growth.

Genetic analysis of a transgenic tobacco
The transgenic tobacco BC3 (T₀) was self-pollinated and its progeny (T₁) was analyzed. Seventy-five among 97 BC3 T₁ plants showed kanamycin resistance, which fitted the segregation ratio of 3:1 (χ² = 0.278), and 32 out of 36 kanamycin-resistant plants were confirmed to accumulate PHA synthase in leaves. These results suggested that the genes were introduced at one site of a chromosome in this plant. And it was also indicated that the introduced genes were inherited following the Mendelian law. In young leaves of T₁ progeny of BC2, BC4, and BC5 lines also, the accumulation of PHA synthase was detected (data not shown), but the expression of the introduced genes was less compared with BC3.

Characterization of polyester
Polyester produced by the transgenic tobacco BC3 was purified and applied to GPC. The number-average molecular weights (Mn) and polydispersity (Mw/Mn) were 32,000 and 1.90, respectively. The existence of 3-hydroxypropionate and 3-hydroxyhexanoate units in the polyester produced by BC3 plant was not detected so far by GC-MS analysis.

Discussion
Various transgenic plants have been designed and produced by modification of protein structure, controlling the expression, and introduction of foreign genes to improve the quantity and the quality of products of crop plants, and to produce pharmaceutically important proteins and peptides. It is difficult to introduce new metabolisms into plant or alter the existing metabolism, but polyester production has been one of the most successful cases and has been reported in several plants. These polyester producing plants were introduced with PHB synthase from R. eutropha and were able to produce only a homopolymer, poly-3-hydroxybutyrate.
PHA synthase from A. caviae FA440 can use not only the C₄ monomer unit but also the C₂ and C₃ units as sub-
strates to produce copolymer. This study demonstrated that PHA synthase of \textit{A. caviae} can function in plant cells and produce at least a homopolymer consisted of 3-hydroxybutyrate. At present, it is not confirmed whether the transgenic tobacco BC3 can produce any copolymers because of its low productivity. This suggests that the change of substrate specificity is not enough to produce copolymer effectively in cytoplasm and engineering the metabolic pathway to supply the needed substrates such as the C₆ monomer unit may be necessary. The low polyester productivity in the transgenic tobacco indicates that the acetyl-CoA level in the cytoplasm of tobacco plants was low compared to that of \textit{Arabidopsis thaliana} which showed the reduction of growth due to the polyester production in cytoplasm.\(^\text{5}\)

\textit{A. caviae} FA440 uses C₄ to C₆ monomer units produced from fatty acids via the \(\beta\)-oxidation pathway.\(^\text{6}\) However, plants cannot be fed with fatty acids and the contribution of \(\beta\)-oxidation pathways existing in mitochondria and peroxisomes are not very high during plant growth except for the germination stage. Thus, to produce polyester exploiting \(\beta\)-oxidation by introducing \textit{A. caviae} enoyl-CoA hydratase, further modification of \(\beta\)-oxidation pathway will be necessary.

Another strategy to produce polyester effectively in plant cells is targeting the expressed proteins in plastids, where carbon flux through acetyl-CoA is high. This was tested in \textit{Arabidopsis} and the transgenic \textit{Arabidopsis} accumulated PHB in plastids effectively.\(^\text{15}\) In addition to the high carbon flux, fatty acids are also synthesized in plastids, which may enable the incorporation of several substrates such as C₄ and C₆ units in polyester. These studies are now in progress.

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**References**