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Plastid Targeting of Polyhydroxybutyrate Biosynthetic Pathway in Tobacco

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Abstract

Poly[(*R*)-(-)-3-hydroxyalkanoate] (PHA) synthase from *Aeromonas caviae* FA440 and β-ketothiolase and acetoacetyl-CoA reductase from *Ralstonia eutropha* H16 were targeted into tobacco plastid by utilizing a plastid-targeting signal peptide derived from the tomato ribulose-bisphosphate carboxylase (Rubisco) small subunit. The resulting transgenic tobacco plant expressed all of the introduced genes, and GC-MS analysis of chloroform extract of tobacco leaves demonstrated that the transgenic plant produced poly[(*R*)-(-)-3-hydroxybutyrate] (PHB). The productivity of PHB in plastids was about 100-fold greater than that in cytoplasm without any apparent deleterious effects on growth and seed production. Intracellular localization of PHB in the leaf of the transgenic plant was examined under epifluorescence microscopy after Nile blue A staining, and it was proven that PHB is formed in chloroplasts around the inside of cell walls.

Abbreviations

PHA, Poly[(*R*)-(-)-3-hydroxyalkanoate]; PHB, Poly[(*R*)-(-)-3-hydroxybutyrate]; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectroscopy; Rubisco, ribulose-bisphosphate carboxylase.

Poly[(*R*)-(-)-3-hydroxyalkanoates] (PHAs) are produced by various species of bacteria as storage material in response to nutrient limitation (Doi, 1990). PHAs are polymers of (*R*)-3-hydroxyalkanoates formed by PHA synthase and have the properties of biodegradability, thermoplasticity and elasticity. The production pathway of PHAs in plants may be a useful new carbon cycle using the solar energy, and the production system can be cost effective with a less adverse impact on the environment (Poirier, 1999).

In *Ralstonia eutropha* H16, poly[(*R*)-(-)-3-hydroxybutyrate] (PHB) is synthesized by the action of three enzymes (Peoples *et al.*, 1989a, 1989b); β-ketothiolase condenses two acetyl-CoA molecules to give acetoacetyl-CoA, and acetoacetyl-CoA reductase subsequently reduces acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA, which is then polymerized to PHB by the action of PHB synthase. By introducing these enzymes, PHB has been successfully produced in the cytoplasm and plastids of transgenic *Arabidopsis thaliana* (Poirier *et al.*, 1992; Nawrath *et al.*, 1994). However, *Arabidopsis* has no agronomic value, although it has the advantage of being a model organism for the synthesis of PHA in the oil crops. Recently, a number of transgenic *Arabidopsis* plants were obtained which accumulate a high content of PHB in plastids, however, the high levels of the polymer produced had severe effects on both *Arabidopsis* plant development and metabolism (Bohmert *et al.*, 2000). Thus, the absolute amount of PHB produced per transgenic *Arabidopsis* plant is still low. By contrast, tobacco has the advantage of being an agronomic model plant. We have previously demonstrated the PHB synthesis in the cytoplasm of transgenic tobacco (Nakashita *et al.*, 1999). However, the productivity of PHB was not high, possibly due to the low level of acetyl-CoA in the tobacco cytoplasm. In plants, the plastid has a high flux of carbon through acetyl-CoA because it is the site of fatty acid biosynthesis and storage. To investigate whether this flux could provide a significant increase in PHA synthesis without inhibiting the development and metabolism of tobacco plants, we targeted β-ketothiolase, acetoacetyl-CoA reduc-

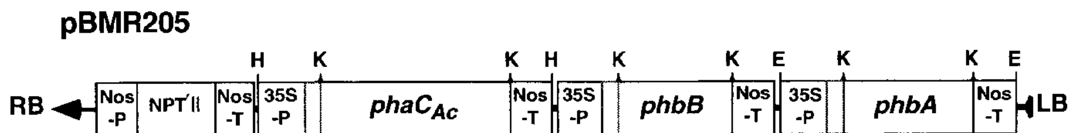

Fig. 1 Plasmid construct for the plant expression vector pBMR205. Gray boxes encode for the transit peptide and 24 amino acids of Rubisco small subunit protein. The *phaC*$_{Ac}$ gene encoding PHA synthase, the *phbA* gene encoding β-ketothiolase and the *phbB* gene encoding acetoacetyl-CoA reductase were conjugated in one vector derived from pBI121. NTPII, neomycin phosphotransferase; 35S-P, cauliflower mosaic virus 35S promoter; Nos-T, *Agrobacterium* nopaline synthase terminator; LB, left border of T-DNA; RB, right border of T-DNA; K, *Kpn*I; H, *Hin*dIII; E, *Eco*RI.

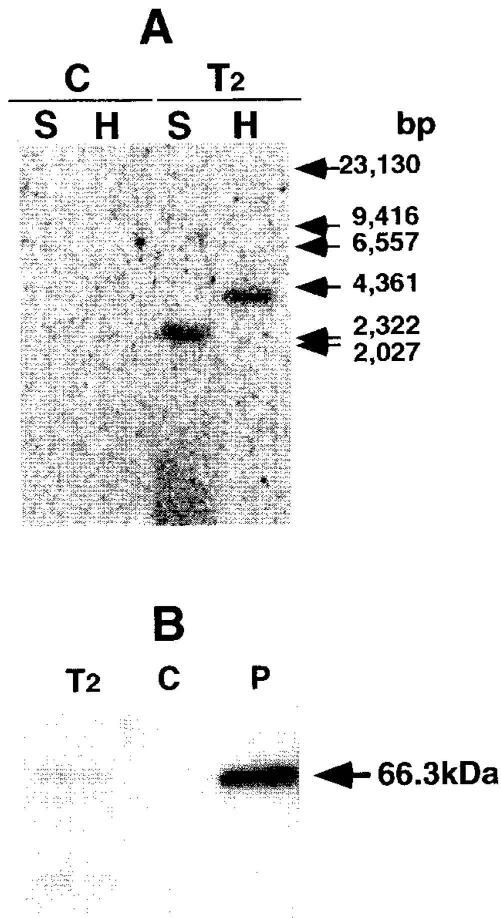

Fig. 2 Analysis of T_2 progeny of transgenic line rCBA10. (A) Southern blot analysis of the T_2 progeny of transgenic line rCBA10-15 (T_1). The total genomic DNA was digested with *Sph*I or *Hin*dIII, and hybridized with the *phbB* gene fragment as a probe. T_2, a self-pollinated progeny of rCBA10-15 (T_1); C, wild-type plant; S, *Sph*I; H, *Hin*dIII. (B) Immunoblot analysis of the T_2 progeny of transgenic line rCBA10-15 (T_1) using anti-PHA synthase antibody confirmed the accumulation of 66.3 kDa PHA synthase. T_2, a self-pollinated progeny of rCBA10-15 (T_1); C, wild-type plant; P, bacterial PHA synthase.

tase, and PHA synthase to the tobacco plastid.

The *phaC*$_{Ac}$ gene from *Aer. caviae* (Fukui *et al.*, 1997) and the genes *phbA* and *phbB* from *Ral. eutropha* (Peoples *et al.*, 1989a) were amplified by polymerase chain reaction (PCR) from the bacterial genomic DNAs. The primers used for the *phaC*$_{Ac}$ gene were TCAGGTACCGTTAGCCAACCATC-TTATGGCCCGCTGT and TCAGGTACCACTT-CCAGGGATTGTGCGCTCATGC, those for the *phbA* gene were CTTGGTACCGTTACTGACG-TTGTCATCGTATCCGCCG and ATAGGTA-CCCCCCGGAAAACCCCTTCCTCATTTGCGC, and those for the *phbB* gene were TAAGGTA-CCGTTACTCAGCGCATTGCGTATGACCG and TAGGGTACCAACCAGGCCGGCAGGTCAGCC-CATATGC. In order to target the PHA biosynthetic enzymes to the plastid, the corresponding genes were attached at the 5'-end with a sequence for the plastid-targeting signal peptide from the ribulose-bisphosphate carboxylase (Rubisco) small subunit of *Lycopersicon esculetum* (tomato).

The gene fragment encoding the 59-amino acid transit peptide with the 24-amino acid Rubisco small subunit was amplified by PCR from tomato cDNA (Pichersky *et al.*, 1986) using the primers GTAGGATCCCCGGGTCGATGGCTTCCTCTG-TCATTTCTTCAG and CTTGAGCTCTGATAT-CTGGTACCCAATTGCTCGTCAGACAAATCA-GG. Each gene was placed under the control of cauliflower mosaic virus 35S promoter and was attached to *Agrobacterium* nopaline synthase terminator to give the vector pBMR205 (**Fig. 1**). pBMR205 was transferred into *Agr. tumefaciens* strain LBA4404 following the method of Hofgen and Willmitzer (1988), then introduced into tobacco plants (*Nicotiana tabacum* cv. Samsun NN) by *Agrobacterium*-mediated transformation following the leaf disk method.

Among 15 kanamycin resistant plants, rCBA4 and rCBA10 were confirmed to express the three introduced genes by the reverse transcription (RT)-PCR analysis. Further analysis of these two lines was done by immunoblotting and enzymatic analysis (Nakashita *et al.*, 1999). In immunoblot analysis, a

significant amount of PHA synthase was detected in the protein sample prepared from the leaves of a transgenic line rCBA10, however, it was not confirmed in rCBA4. The enzymatic activity of acetoacetyl-CoA reductase encoded by the *phbB* gene was determined by measuring the consumption of NADPH during the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyrate (3HB). The protein sample prepared from the leaves of rCBA10 also showed acetoacetyl-CoA reductase activity in the range of 2.6×10^{-2} units/mg of protein. Therefore, a transgenic line rCBA10 was used for further studies.

The transgenic tobacco rCBA10 (T_0) was self-pollinated to give its progeny. Among 277 rCBA10 (T_1) tested, 249 plants showed kanamycin resistance. Then the kanamaycin-resistant plants (T_1) were self-pollinated again to give their progenies. All of the T_2 plants derived from the rCBA10-15 T_1 line showed kanamycin resistance. Such stable integration of the introduced transgene was confirmed by Southern blot analysis. The total genomic DNA of T_2 progeny of rCBA10-15 (T_1) was digested with *Sph*I or *Hin*dIII, and hybridized with the ^{32}P-labeled *phbB* gene fragment as a probe. A single band was detected in both digested samples, and no bands were detected in the wild-type plant (**Fig. 2A**). Therefore, it is suggested that the T_2 progeny of transgenic line rCBA10-15 (T_1) contains a single copy of the transgene in its genome. The accumulation of PHA synthase in this line was also confirmed by immunoblot analysis (**Fig. 2B**).

Accumulation of polyester in plant tissue was examined by gas chromatography-mass spectroscopy (GC-MS) analysis. Lyophilized plant materials were ground into powder and extracted with methanol followed by PHB extraction with chloroform. The chloroform extracts were esterified by acid ethanolysis and analyzed by GC-MS using a G1800 GCD system with an HP-5 column (Hewlett Packard). The samples prepared from the leaves of rCBA10 gave a peak at the same retention time as that of authentic 3HB-ethyl ester, which was not observed in the sample from the wild-type plants (**Fig. 3A** and **3B**). The mass spectrum of this peak was identical to that of the authentic sample (**Fig. 3C**). The polyester productivity of whole plant leaves was 0.09 dry weight %, which is much higher than the productivity of PHB in tobacco cytoplasm (ca. 0.001 dry weight %) (Nakashita *et al.*, 1999). By changing the site of PHB production from cytoplasm to plastid, the level of PHB production increased in tobacco as reported in *Arabidopsis* (Nawrath *et al.*, 1994; Poirier, 1999). These findings suggest that the plastid metabolism is available for high-level PHB production. Next, PHB production at the various growth stages was examined by using T_2 progenies of the rCBA10-15 (T_1) line. While PHB production in young green leaves and fully expanded leaves was 0.006 and 0.079 dry weight %, respectively, PHB accumulation in the senescent leaves was 0.155 dry weight %. This suggests that PHB is continuously produced and gradually accumulates throughout plant growth.

Intracellular localization of PHB in the leaf of rCBA10 plant was examined under an epifluorescence microscopy after Nile blue A staining (Ostle and Holt, 1982). Leaves of transgenic tobacco plants were fixed with glutaraldehyde, and cut into thin sections. The thin sections were stained with Nile blue A. Nile blue A binding to PHB granule emits strong orange or red fluorescence by excitation at wavelengths of 460 nm or 546 nm, respectively. The leaf of rCBA10 showed foci of orange (460 nm) or red (546 nm) fluorescence in chloro-

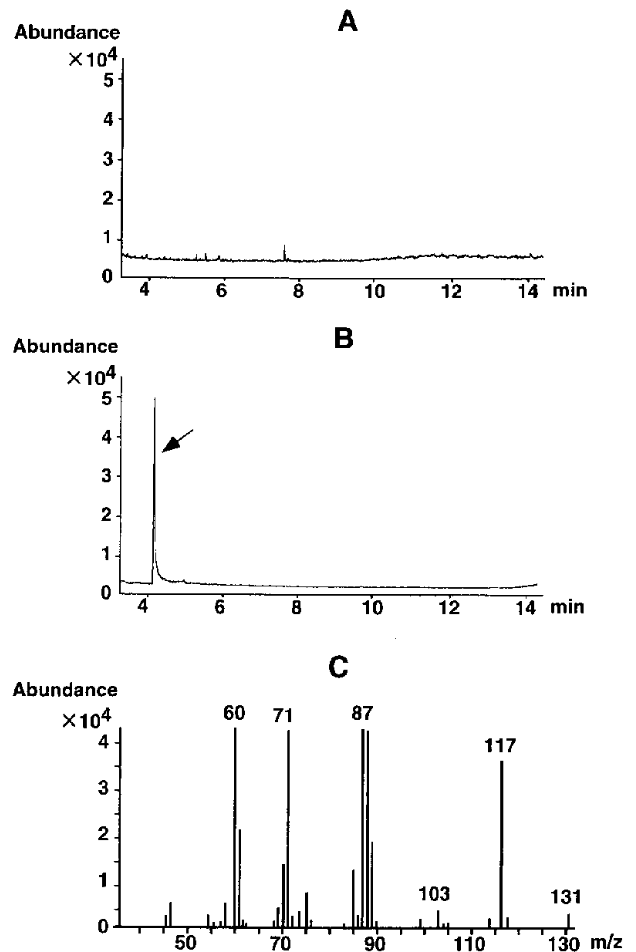

Fig. 3 GC-MS analysis of polyester produced by the transgenic tobacco plant. (A) Gas chromatography analysis of a wild-type plant. (B) Gas chromatography analysis of a transgenic tobacco plant. Arrow indicates the peak of 3HB-ethyl ester. (C) Mass spectrum of the peak at 4.2 min showed the same pattern as that of standard sample.

plasts around the inside of cell walls, which represents agglomerations of PHB granules (**Fig. 4**). Since these were not observed in any wild-type plants, the result indicates that PHB is produced and accumulates in plastids of transgenic plants.

In this study, we attempted to target the enzymes for polyester production to the tobacco plastids with a high flux of fatty acids from acetyl-CoA. As a result, PHB production increased about 100 times compared with the level expressed in the tobacco cytoplasm. In *Arabidopsis*, a strong negative correlation between PHB accumulation and plant growth was observed (Bohmert *et al.*, 2000). However, the transgenic tobacco plant reported in the present study accumulated PHB in plastids without any readily apparent deleterious effects on growth and seed production. In tobacco, although PHB productivity per weight was lower than in *Arabidopsis*, the total amount of PHB per plant was higher than in *Arabidopsis*, which indicates that tobacco is a better possible host crop for PHA production. However, the PHB production in tobacco cultivated in the field is currently estimated to be at most 1.5 g/kg dry weight, which is unfortunately not high enough for practical use.

PHA synthase encoded by the $phaC_{Ac}$ gene can use not only the C_4 monomer unit but also the C_5 and C_6 units as substrates for polyester synthesis. Although the production of copolymers was not observed in any transgenic lines tested in this study, efficient production of homopolymer PHB in the transgenic tobacco rCBA10 was confirmed as described above. This suggests that engineering the metabolic pathway to supply sufficient substrate (such as C_6 unit) would be necessary for the copolymer synthesis. Metabolic engineering of *Arabidopsis* for poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer production was reported (Slater *et al.*, 1999), however, redirecting the metabolic flow of intermediates from amino acid biosynthesis resulted in abnormal accumulation of ammonium ions in the cells to give an adverse effect on the plant, suggesting that homeostatic physiological conditions are also quite important for copolymer production.

For PHA synthesis in plastids, (*R*)-3-hydroxyacyl-ACP intermediates in fatty acid biosynthesis must be converted to the corresponding CoA derivatives. The *phaG* gene that encodes (*R*)-3-hydroxyacyl-ACP:CoA transacylase, which is supposed to directly link fatty acid biosynthesis and PHA biosynthesis, has been identified in *Pseudomonas putida* KT2440 (Rehm *et al.*, 1998) and *Pse. aeruginosa* (Hoffmann *et al.*, 2000). On the other hand, recently, for transformation of chloroplasts in higher plants, particle bombardment is used to introduce transgenes into leaf chloroplasts where integration of the foreign DNA is directed by homologous recombination (Svab *et al.*, 1990). Tobacco is the most stable plant for transformation of a plastid genome, but *Arabidopsis* is not yet established (Sikdar *et al.*, 1998). We have previously demonstrated that the tobacco plastid can be transformed with a polycistron consisting of three bacterial genes for the PHB biosynthesis, which resulted in, however, lower PHB content than reported here (Nakashita *et al.*, 2001). To improve the productivity and monomer content of polyester, introducing the *phaG* gene to the plastid genome of the rCBA10 progenies is now in progress.

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