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Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 22 May 2014.

To cite this article: Hideo NAKASHITA, Yuko ARAI, Toshiharu SHIKANAI, Yoshiharu DOI & Isamu YAMAGUCHI (2001) Introduction of Bacterial Metabolism into Higher Plants by Polycistronic Transgene Expression, Bioscience, Biotechnology, and Biochemistry, 65:7, 1688-1691, DOI: 10.1271/bbb.65.1688

To link to this article: http://dx.doi.org/10.1271/bbb.65.1688

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Biosci. Biotechnol. Biochem., **65** (7), 1688–1691, 2001

Preliminary Communication

Introduction of Bacterial Metabolism into Higher Plants by Polycistronic Transgene Expression

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Received February 5, 2001; Accepted April 24, 2001

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Multiple-gene transformation is required to improve or change plant metabolisms effectively; but this many-step procedure is time-consuming and costing. We succeeded in the metabolic engineering of tobacco plants by introducing multiple genes as a bacteria-type operon into a plastid genome. The tobacco plastid was transformed with a polycistron consisting of three bacterial genes for the biosynthesis of a biodegradable polyester, polyhydroxybutyrate (PHB). Accumulation of PHB in the leaves of the transgenic tobacco indicated that the introduced genes were polycistronically expressed. This "phyto-fermentation" system can be used in plant production of various chemical commodities and pharmaceuticals.

Key words: plastid transformation; operon; metabolic engineering; phyto-fermentation; polyhydroxybutyrate

The plastid, one of the major organelles in plant cells, provides the plant kingdom with a characteristic ability of photosynthesis. In another aspect of the role of plastids in plant cells, they acquired specialized functions as containers of synthesis and storage, which are usually localized in specific organs or tissues. They participate in the biosynthesis and storage of starch (amyloplast), carotenoids (chromoplasts) and lipids (elaioplast),[1] enabling plants to reserve nutrition to be used for growth and the next generation seedlings. The outstanding capabilities of production and storage have been utilized in agriculture for thousands of years and today come to lead the possibility of exploiting plastids as a factory of new chemical commodities by biotechnology techniques.[2,3]

This organelle, possessing its own genome, is likely derived from a free-living oxygen-evolving photosynthetic prokaryote[4] and the structure of the genome and the mechanism for decoding the genetic information within plastids are similar to those of prokaryotes.[5,6] Transformation of the plastid genomes of higher plants by homologous recombination has been established[7–10] and chimeric bacterial antibiotic resistance genes were introduced into plastid genomes to allow selection of the transformants. This method helped in the molecular study of function and regulation of plastid genes, such as the introduction of a mutation in a particular position in a plastid genome and the gene replacement with a heterogous gene.[11,12]

Considering that the structure and regulation of plastid genomes are similar to those of prokaryotes,[6,13] plastids have a wider possibility for production of various substances. In other words, if the bacterial polycistron can function in this organelle, the plastid can become a fermentation organism of secondary metabolites. Therefore we investigated the feasibility of giving a new character to plastids by introducing a bacterial operon into its genome. We chose PHB biosynthesis[14] as a marker to test the polycistronic expression of transgenes in plastids and the result obtained is reported in this paper.

Polyhydroxyalkanoates (PHAs) are a class of aliphatic microbial polyesters and renewable sources of biodegradable thermoplastic.[14] The most common PHA, poly-3-hydroxybutyrate (PHB), is produced by various bacteria and its biosynthesis from acetyl-CoA requires only three enzymes, which are usually encoded in an operon.[15–17] The *phb* operon of *Ralstonia eutropha*, encoding PHB biosynthetic enzymes, consits of *phbC*, *phbA*, and *phbB*. Acetoacetyl-CoA produced from acetyl-CoA by β-ketothiolase (*phbA*), is converted to 3-hydroxybutyrate by acetoacetyl-CoA reductase (*phbB*), and then poly-

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Abbreviations: PHB, polyhydroxybutyrate; PHA, polyhydroxyalkanoate; PCR, polymerase chain reaction

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merazed by PHB polymerase encoded by *phbC* (Fig. 1).

The *aadA* gene, encoding aminoglycoside 3″-adenyltransferase, confers spectinomycin resistance on the tobacco nuclei and plastid.[8] The *aadA* gene, attached with a synthetic ribosome binding site designed after the abundant Rubisco large subunit, was modified by the polymerase chain reaction (PCR) to have *Bam*HI and *Bgl*II restriction enzyme sites at the 5′- and 3′-ends, respectively. The bacterial *phb* operon, consiting of *phbC*, *phbA*, and *phbB*, was cloned from *R. eutropha* genomic DNA by PCR using the following primers: 5′-ATGGATCCCGGGCAAGTACCTTGCCGACAT-3′ and 5′-TCCGGATCCTATGCCCAACAAGGCACTAAGA-3′. The *Bam*HI fragment obtained (4.98 kb) contained these genes including the 5′- and 3′-untranslated regions.[15] This *Bam*HI fragment was connected downstream of the *aadA* gene in the order specified above to generate an operon. This chimeric operon was inserted into a plastid transformation vector plasmid, which was constructed essentially as reported by Svab and Maliga,[8] to generate pPT12 (Fig. 2). In this plasmid, the inserted operon is under control of the constitutive promoter of the plastid rRNA operon (*P*rrn) and the plastid *psbA* terminator sequence, which was located between the *Sph*I-*Bam*HI *rbcL* fragment (0.8 kb) and the *Bam*HI- *Xho*I (1.2 kb) fragment of *accD*. These adjacent plastid genes, *rbcL* and *accD*, are used for homologous recombination during transformation, because they are regulated in different operons in the plastid genome and the region between them is suitable for the foreign gene insertion.

Tobacco plastid transformation was done by the biolistic process as previously described[11] using gold particles (1 μm) as a DNA carrier. Thirty leaves of aseptically grown *Nicotiana tabacum* cv Xanthi were used for bombardment. Following a round of selection on spectinomycin-containing medium, two lines of pPT12 transformants (lines 1 and 2) were obtained. PCR analysis, using specific primers for *aadA*, *phbC*, *phbA*, and *phbB*, confirmed the introduction of these genes into the selected lines (data not shown). Further regeneration cycles were performed from the leaf sections to obtain homoplasmic plants.[8,11] After two cycles of selection, the third generation plants were rooted and transferred to a greenhouse. These plants did not demonstrate any visible morphological changes.

All three *phb* genes are necessary to synthesize PHB in the plastid due to the lack of β-ketothiolase activity in this organelle (Fig. 1); the combination of *phbC* and *phbB* is sufficient to synthesize PHB in the cytosol.[2,18,19] The expression of these introduced genes in an operon in the plastid allowed the transformants to accumulate PHB. The leaves of pPT12 transformants, following chloroform extraction and ethanolysis, were analyzed by gas chromatography-

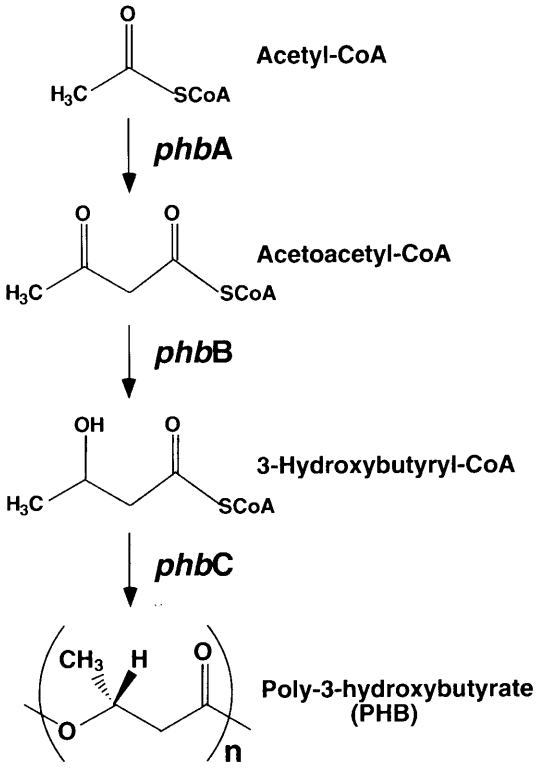

Fig. 1. Biosynthetic Pathway of Polyhydroxybutyrate.

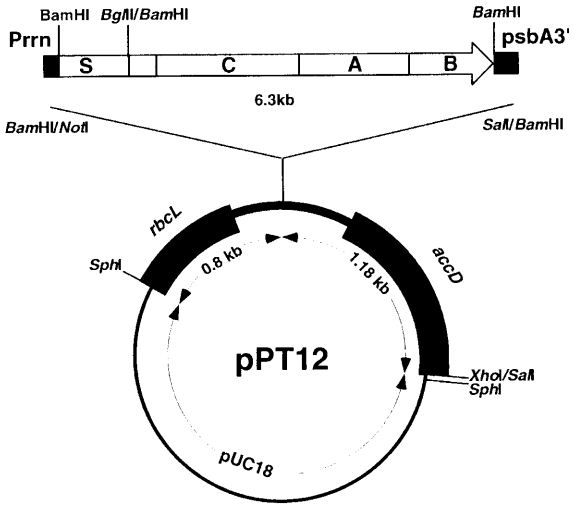

Fig. 2. Construct of Plastid Transformation Vector pPT12.
Tobacco plastid genes and bacterial genes are shown as closed and open boxes, respectively. Thick arrow indicates the orientation of the chimeric operon. Prrn, plastid rRNA operon promoter; S, *aadA*; C, *phbC*; A, *phbA*; B, *phbB*; psbA3′ plasid *psbA* terminator sequence.

mass spectrometry (GC-MS). A peak, detected at 6.5 minutes, appeared only in transformed plants (Fig. 3A). The mass spectrum of this peak was identical to that of 3-hydroxybutyrate ethyl ester (Fig. 3B), indicating unambiguously that the PHB biosynthetic enzymes expressed from the introduced *phb* operon functioned properly in the transformed plastids. The PHB productivities of line 1 and line 2 were 8 ppm

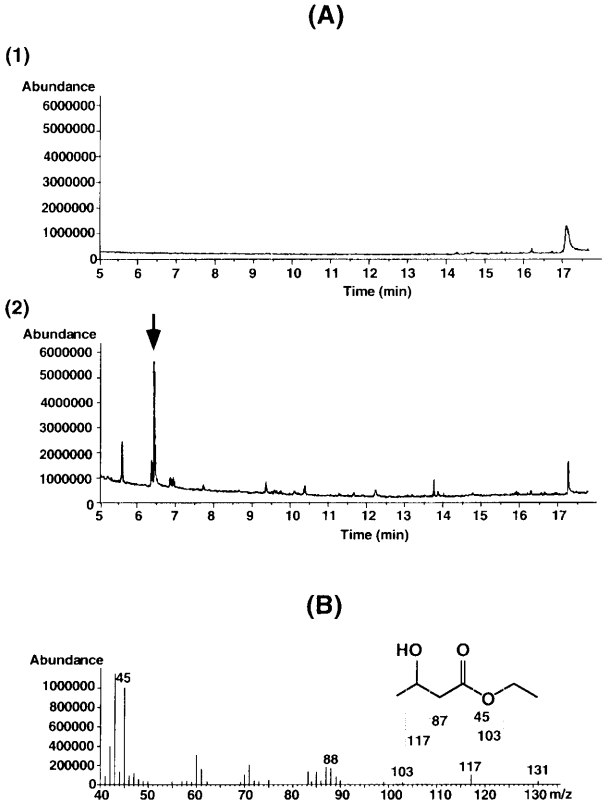

Fig. 3. GC-MS Analysis of Polyester Produced by Transgenic Tobacco Plant.

(A) Gas chromatography analysis of wild-type plant (1) and transgenic plant (2). Polyester was extracted from the leaves of the transgenic tobacco and analyzed after ethanolysis as previously described.[19] Arrow indicates the peak of 3-hydroxybutyrate-ethyl ester. (B) Mass spectrum of the peak at 6.5 min of a sample prepared from a transgenic plant shows the pattern specific to 3-hydroxybutyrate-ethyl ester. Structure with major fragmentation patterns is also shown.

and 2 ppm dry weight, respectively, which were close to that of the transgenic tobacco accumulating PHB in cytosol (*ca.* 10 ppm).[19] In *Arabidopsis*, however, it is reported that the PHB productivity by the targeted enzymes into plastid is 100 times higher than that in cytosol.[2,18] So we are continuing the attempt to produce the plant with higher PHB productivity and the detailed analysis of transformants obtained so far is now in progress.

To properly manipulate plastids, targeting techniques using transit peptides have been exploited;[20-22] this procedure, however, requires the troublesome addition of a promoter, a terminator, and a transit peptide sequence into every gene. The introduction of a polycistron into plastid genome can simplify these procedures, allowing one promoter and one terminator to control multiple genes without the necessity of transit peptide sequences.[23] It is reported that plastid transformation with polycistrons is effective for foreign protein production[24] We here demonstrate that this system is also applicable to introduce a foreign metabolism into plants.

Plastid transformation with polycistronic genes is both time and cost effective and gives ideally regulated expression. In addition, the transgene in the plastid genome is not affected by co-suppression caused by gene silencing,[25] often a serious problem during the production of transgenic plants, especially using genes homologous to host plant genes. These advantages can lead to the plant production of useful compounds, usually produced by bacterial fermentation, and also allow the improvement of conventional agricultural products. This system is supplied with materials and condition by the plant itself, based on solar energy, which could be called "phyto-fermentation" or "plasmic fermentation". Important for the application stage of genetically modified crops[20,21,26] is that transgenes contained within plastids are inherited maternally and will not spread outward into the environment by pollen diffusion,[27] enabling the safely programmed creation of new plants affording great benefits to human society with minimal ecological impact.

Acknowledgments

We thank Dr. K. Baba, Dr. T. Nakano, Dr. Y. Shimada, and Dr. K. Suzuki for useful discussions on this study. We thank Ms. Y. Kobayashi and Mr. T. Shimizu for assistance in culturing plants.

References

1) Possingham, J. V., Plastid replication and development in the life cycle of higher plants. *Annu. Rev. Plant Physiol.*, **31**, 113–130 (1980).
2) Nawrath, C., Poirier, Y., and Somerville, C., Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. USA*, **91**, 12760–12764 (1994).
3) Staub, J. M., Garcia, B., Graves, J., Hajdukiewicz P. T. J., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J. A., Spatola, L., Ward, D., Ye, G., and Russell, D. A., High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnol.*, **18**, 333–338 (2000).
4) Gray, M. W., and Doolittle, W. F., Has the endo symbiont hypothesis been proven? *Microbiol. Rev.*, **46**, 1–42 (1982).
5) Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., *et al.*, The complete sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.*, **5**, 2043–2050 (1986).
6) Mullet, J. E., Dynamic regulation of chloroplast transcription. *Plant Physiol.*, **103**, 309–313 (1993).
7) Svab, Z., Hajdukiewicz, P., and Maliga, P., Stable transformation of plastids in higher plants. *Proc. Natl. Acad. Sci. USA*, **87**, 8526–8530 (1990).
8) Svab, Z., and Maliga, P., High-frequency plastid

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transformation in tobacco by selection for a chimeric aadA gene. *Proc. Natl. Acad. Sci. USA*, **90**, 913–917 (1993).
9) Carrer, H., Hockenberry, T. N., Svab, Z., and Maliga, P., Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Mol. Gen. Genet.*, **241**, 49–56 (1993).
10) Sidorov, V. A., Kasten, D., Pang S. Z., Hajdukiewicz, P. T J., Staub, J. M., and Nehra, N. S., Stable chloroplast transformation in potato: Use of green fluorescent protein as a plastid marker. *Plant J.*, **19**, 209–216 (1999).
11) Shikanai T., Endo T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A., Directed disruption of the tobacco ndhB gene impairs cyclic electron flow around photosystem I. *Proc. Natl. Acad. Sci. USA* **95**, 9705–9709 (1998).
12) Kofer, W., Koop, H. U., Wanner, G., and Steinmuller, K., Mutagenesis of the genes encoding subunits A, C, H, I, J and K of the plastid NAD(P)H-plastoquinone-oxidoreductase in tobacco by polyethylene glycol-mediated plastome transformation. *Mol. Gen. Genet.*, **258**, 166–173 (1998).
13) Jacob, F., and Monod, J., Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**, 318–356 (1961).
14) Doi, Y., *Microbial polyesters*, VCH publishers , New York, (1990).
15) Peoples, O. P., and Sinskey, A. J., Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. *J. Biol. Chem.*, **264**, 15298–15303 & 15298–15303 (1989).
16) Huisman, G. W., Wonink, E., Medima, R., Kazemier, B., Terpstr, P., and Witholt, B., Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*: Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. *J. Biol. Chem.*, **226**, 2191–2198 (1991).
17) Fukui, T. and Doi, Y., Cloning and analysis of poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. *J. Bacteriol.*, **179**, 4821–4830 (1997).
18) Poirier, Y., Dennis, D. E., Klomparens, K., and Somerville, C., Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. *Science*, **256**, 520–523 (1992).
19) Nakashita, H., Arai, Y., Yoshioka, K., Fukui, T., Doi, Y., Usami, R., Horikoshi, K., and Yamaguchi, I., Production of biodegradable polyester by a transgenic tobacco. *Biosci. Biotechnol. Biochem.*, **63**, 870–874 (1999).
20) Voelker, T. A., Worrell, A. C., Anderson, L., Vleibaum, J., Fan, C., Hawkins, D. J., Adke, S. E., and Davies, H. M., Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science*, **257**, 72–74 (1992).
21) Gallardo, F., Miginiac-Maslow, M., Sangwan, R. S., Decottignies, P., Keryer, E., Dubois, F., Bismuth, E., Galvez, S., Sangwan-Norreel, B., Gadal, P., and Cretin, C., Monocotyledonous C-4 NADP+-malate dehydrogenase is efficiently synthesized, targeted to chloroplasts and processed to an active form in transgenic plants of the C-3 dicotyledon tobacco. *Planta*, **197**, 324–332 (1995).
22) McFadden G. I., Plastids and protein targeting. *J. Eukaryot. Microbiol.*, **46**, 339–346 (1999).
23) Kota, M., Daniell, H., Varma, S., Garczynski, S. F., Gould, F., and Moar, W., Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistance insects. *Proc. Natl. Acad. Sci. USA* **96**, 1840–1845 (1999).
24) Cosa, B. D., Moar, W., Lee, S. B., Miller, M., and Daniell, H., Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nature Biotechnol.*, **19**, 71–74 (2001).
25) Dawson, W. O., Gene silencing and virus resistance: a common mechanism. *Trends Plant Sci.*, **1**, 107–108 (1996).
26) Shelton, A. M., Tang, J. D., Roush, R. T., Metz, T, D., and Earle, E. D., Field tests on managing resistance to Bt-engineered plants. *Nature Biotechnol.*, **18**, 339–342 (2000).
27) Scott, S. E., and Wilkinson, M. J., Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. *Nature Biotechnol.*, **17**, 390–392 (1999).

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