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Improved polyhydroxybutyrate (PHB) production in transgenic tobacco by enhancing translation efficiency of bacterial PHB biosynthetic genes

Ken'ichiro Matsumoto,[1,*] Kenjiro Morimoto,[1] Aoi Gohda,[1] Hiroaki Shimada,[2,3] and Seiichi Taguchi[1]

Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8 Kita-ku, Sapporo 060-8628, Japan,[1] Department of Biological Science and Technology, Tokyo University of Science, Yamazaki 2641, Noda 278-8510, Japan,[2] and Research Institute for Science and Technology, Tokyo University of Science, Yamazaki 2641, Noda 278-8510, Japan[3]

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Polyhydroxybutyrate [P(3HB)] was produced in the transgenic tobacco harboring the genes encoding acetoacetyl-CoA reductase (PhaB) and polyhydroxyalkanoate synthase (PhaC) from *Ralstonia eutropha* (*Cupriavidus necator*) with optimized codon usage for expression in tobacco. P(3HB) contents in the transformants (0.2 mg/g dry cell weight in average) harboring the codon-optimized *phaB* gene was twofold higher than the control transformants harboring the wild-type *phaB* gene. The immunodetection revealed an increased production of PhaB in leaves, indicating that the enhanced expression of PhaB was effective to increase P(3HB) production in tobacco. In contrast, codon-optimization of the *phaC* gene exhibited no apparent effect on P(3HB) production. This result suggests that the efficiency of PhaB-catalyzed reaction contributed to the flux toward P(3HB) biosynthesis in tobacco leaves.

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[**Key words:** Transgenic plant; *Nicotiana tabacum*; PHA synthase; Real-time PCR]

Bacterial polyhydroxyalkanoates (PHAs) are representative bio-based polyesters that is applicable for commodity plastics and thus considered as a potential alternative to petroleum-based plastics (1,2). PHAs are produced by numerous gram-negative (3) and positive bacteria (4–6) from inexpensive feed stocks, such as plant oils (7) and glycerol (8,9). For further reducing the cost of production, PHA productions in transgenic plants harboring bacterial PHA biosynthetic genes have been investigated because the plant system does not need bioreactors and feedstock for fermentation, which contribute to large portion of the entire cost. To date, PHA production in several plants, such as *Arabidopsis thaliana* (10–12), tobacco (13), sugar cane (14), and potato (15), has been reported. However, the low productivity of PHA has been a central obstacle to the commercial PHA production in plants.

We have succeeded in producing PHAs in *A. thaliana* using the engineered PHA synthases (PhaC) (16,17) and monomer supplying enzyme [3-ketoacyl-acyl carrier protein synthase III (FabH) (18)] genes, which allowed to synthesize PHA copolymers composed of short-chain-length and medium-chain-length monomers (12,19) During the course of this project, we found that the expression of the engineered enzymes (PhaC and FabH) increased the yields of PHA in the transgenic *A. thaliana*. These results suggested that increasing activity of PHA biosynthetic enzymes could achieve the higher yield of PHA in the transgenic plants. However, it has been reported that enrichment of the transcript from transgene driven by strong promoter and/or insertion of the multiple genes into genome often cause an unexpected gene silencing (20). Therefore, in this study, we altered codon usage of the PHA biosynthetic genes for improving the translation efficiency of their mRNAs in plants in order to increase the amount of the enzymes.

For this purpose, P(3-hydroxybutyrate) [P(3HB), or PHB]-producing transgenic tobacco was used as a model system. P(3HB) is a representative PHA that is produced from acetyl-CoA as the starting material by successive reactions composed of the following three enzymes: β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). Tobacco is a common model plant, of which the efficient and quick transformation method has been developed, and has an intrinsic pathway supplying acetoacetyl-CoA. Therefore, expressions of PhaB and PhaC were needed for P(3HB) production in tobacco. Hence, we created genetically modified *phaB* and *phaC* genes of *Ralstonia eutropha* (*C. necator*) (21) and investigated their effect on P(3HB) production.

* Corresponding author. Tel./fax: +81 11 706 6612.
E-mail address: mken@eng.hokudai.ac.jp (K. Matsumoto).

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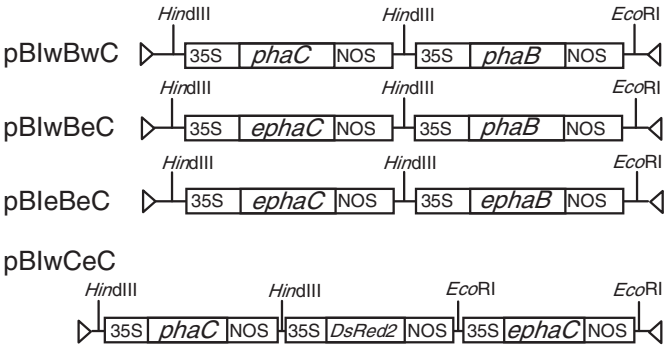

FIG. 1. Vectors used in this study. *phaC* indicates PHA synthase gene; *phaB*, acetoacetyl-CoA reductase gene; *ephaBC*, codon-optimized genes; 35S, 35S cauliflower mosaic virus promoter; NOS, nopaline synthase terminator. Triangles indicate the left and right boarder of the T-region.

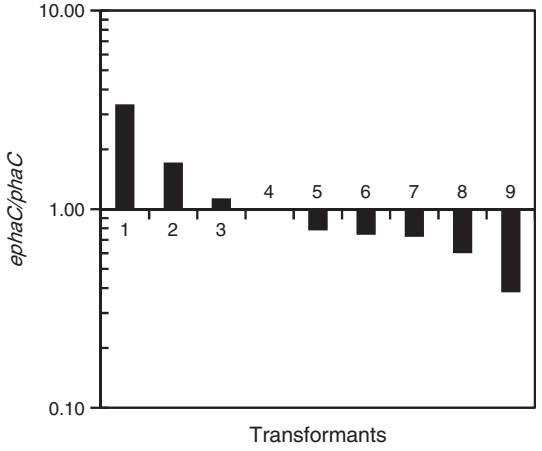

FIG. 2. Relative mRNA levels of *ephaC* versus wild-type *phaC* determined by qRT–PCR. Data were obtained from nine independent transformants of wCeC (1–9). The upward bars (1–3) indicate that the mRNA level of *ephaC* is higher than that of *phaC*, and the downward bars (4–9) indicate the opposite.

Chemiluminescence from the membrane was recorded on a ChemiDoc XRS imager (Bio-Rad).

Polymer analysis P(3HB) was extracted with chloroform from lyophilized leaves of transformants grown for five weeks after regeneration, as described previously (12). The extracted polymer was converted into ethyl 3HB by ethanolysis for quantification using gas chromatography/mass spectroscopy (GC/MS), as described previously (12).

RESULTS AND DISCUSSION

Expression of modified PHA biosynthetic genes in tobacco We constructed four vectors harboring the wild-type and codon-modified *phaC* and *phaB* genes (Fig. 1) for evaluating the effect of codon alteration on the transcription efficiency, translation efficiency, and P(3HB) production. We first compared the mRNA levels of the wild-type and codon-modified *phaC* genes. Nine wCeC transformants expressing both *phaC* and *ephaC* genes were generated for the comparative analysis of the expression levels of the genes without the position effect, which is known as a variation in transcription levels of transgenes depending on its integrated position in a chromosome of host plant (29). The qRT–PCR analysis of wCeC indicated that the relative amounts of mRNA of *ephaC* versus that of *phaC* were in the range of 0.38 to 3.3 (Fig. 2), and their geometric mean was 0.95. This result suggested that the alteration in codon usage of *phaC* did not influence its transcriptional efficiency.

Immunodetection of PhaB Next, nine transformants of each wBwC, wBeC, and eBeC were generated to evaluate the effect of

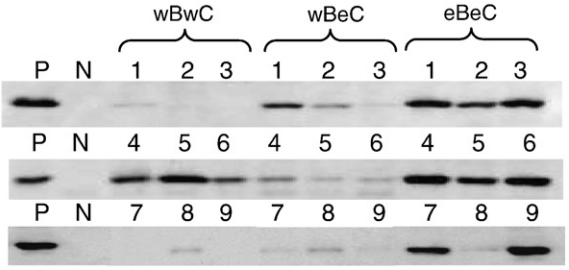

FIG. 3. Immunoblotting of PhaB using crude extract prepared from a leaf. P indicates crude extract of *Escherichia coli* expressing the *phaB* gene (positive control); N, wild-type tobacco (negative control). Numbers 1–9 indicate the independent transformants of each line.

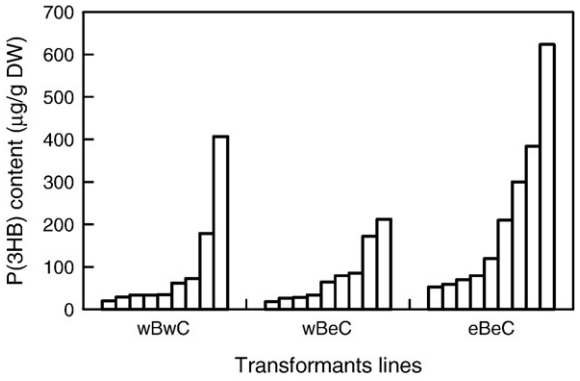

FIG. 4. P(3HB) content in transgenic tobacco. The polymer was extracted from lyophilized leaves and quantified using GC/MS. Data are sorted by P(3HB) content.

codon alteration on translation efficiency of the PHA biosynthetic genes and P(3HB) production. The translation efficiencies of *phaB* and *ephaB* were determined by immunodetection of PhaB protein in the crude extracts prepared from leaves of wBwC, wBeC, and eBeC transformants. The PhaB proteins migrated as a single band by SDS–PAGE, size of which was consistent with that of the positive control (Fig. 3). The amounts of PhaB in the eBeC transformants tended to be enriched compared to those of wBwC and wBeC. Because the codon alteration unlikely affect the transcription efficiency as mentioned above, the increase in PhaB in eBeC should be due to the enhanced translation efficiency of the *ephaB* mRNA. On the other hand, PhaC protein in the same crude extract was not detected as a specific band on the membrane (data not shown). Nonspecific binding of anti-PhaC might hinder the detection of the PhaC protein.

P(3HB) analysis accumulated in the transgenic tobacco The effect of codon alteration on P(3HB) production was analyzed by determining the P(3HB) content in the transformants. The transformants of wBwC and wBeC accumulated 97 and 80 μg/g dry cell weight P(3HB) on average, respectively (Fig. 4), indicating that the introduction of *ephaC* had no positive impact on P(3HB) production. In contrast, the transformants of eBeC accumulated 211 μg/g cdw P(3HB) on average, a more than twofold increase over wBwC and wBeC. The increase in P(3HB) production was caused by the introduction of *ephaB* suggested that the efficiency of PhaB-catalyzed reaction may contribute to the flux toward P(3HB) biosynthetic pathway in transgenic tobacco. This hypothesis is consistent with the case of bacterial P(3HB) production, in which PhaB has been shown to be a rate-limiting factor (30). Thus, further enhancement of PhaB activity might increase the productivity of P(3HB). The contribution of the expression level of PhaC to P(3HB) content was not clear from the results. An improvement of immunodetection of PhaC will be necessary to clarify the problem.

In conclusion, we have demonstrated that the enhanced expression of PhaB effectively improved P(3HB) production in plants. The combined strategy of applying the codon-optimization to the highly active engineered enzymes involved in PHA biosynthesis (12,19) would be useful for further enhancement in PHA production in plants.

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References

1. **Doi, Y.:** Microbial polyesters, VHC publishers, New York (1990).
2. **Sudesh, K. and Iwata, T.:** Sustainability of biobased and biodegradable plastics, Clean Soil Air Water, **36**, 433–442 (2008).
3. **Steinbüchel, A. and Valentin, H. E.:** Diversity of bacterial polyhydroxyalkanoic acids, FEMS Microbiol. Lett., **128**, 219–228 (1995).
4. **Jo, S. J., Maeda, M., Ooi, T., and Taguchi, S.:** Production system for biodegradable polyester polyhydroxybutyrate by *Corynebacterium glutamicum*, J. Biosci. Bioeng., **102**, 233–236 (2006).
5. **Mifune, J., Grage, K., and Rehm, B. H. A.:** Production of functionalized biopolyester granules by recombinant *Lactococcus lactis*, Appl. Environ. Microbiol., **75**, 4668–4675 (2009).
6. **Ramsay, B. A., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., and Ramsay, J. A.:** Production of poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acids, Appl. Environ. Microbiol., **56**, 2093–2098 (1990).
7. **Lee, W. H., Loo, C. Y., Nomura, C. T., and Sudesh, K.:** Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors, Bioresour. Technol., **99**, 6844–6851 (2008).
8. **Cavalheiro, J. M. B. T., de Almeida, M. C. M. D., Grandfils, C., and da Fonseca, M. M. R.:** Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol, Process Biochem., **44**, 509–515 (2009).
9. **Bormann, E. J. and Roth, M.:** The production of polyhydroxybutyrate by *Methylobacterium rhodesianum* and *Ralstonia eutropha* in media containing glycerol and casein hydrolysates, Biotechnol. Lett., **21**, 1059–1063 (1999).
10. **Poirier, Y., Dennis, D., Klomparens, K., and Somerville, C.:** Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants, Science, **256**, 520–523 (1992).
11. **Bohmert, K., Balbo, I., Kopka, J., Mittendorf, V., Nawrath, C., Poirier, Y., Tischendorf, G., Trethewey, R. N., and Willmitzer, L.:** Transgenic Arabidopsis plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight, Planta, **211**, 841–845 (2000).
12. **Matsumoto, K., Nagao, R., Murata, T., Arai, Y., Kichise, T., Nakashita, H., Taguchi, S., Shimada, H., and Doi, Y.:** Enhancement of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production in the transgenic *Arabidopsis thaliana* by the in vitro evolved highly active mutants of polyhydroxyalkanoate (PHA) synthase from *Aeromonas caviae*, Biomacromolecules, **6**, 2126–2130 (2005).
13. **Arai, Y., Shikanai, T., Doi, Y., Yoshida, S., Yamaguchi, I., and Nakashita, H.:** Production of polyhydroxybutyrate by polycistronic expression of bacterial genes in tobacco plastid, Plant Cell Physiol., **45**, 1176–1184 (2004).
14. **Purnell, M. P., Petrasovits, L. A., Nielsen, L. K., and Brumbley, S. M.:** Spatio-temporal characterization of polyhydroxybutyrate accumulation in sugarcane, Plant Biotechnol. J., **5**, 173–184 (2007).
15. **Romano, A., Vreugdenhil, D., Jamar, D., van der Plas, L. H. W., De Roo, G., Witholt, B., Eggink, G., and Mooibroek, H.:** Evidence of medium-chain-length polyhydroxyoctanoate accumulation in transgenic potato lines expressing the *Pseudomonas oleovorans* Pha-C1 polymerase in the cytoplasm, Biochem. Eng. J., **16**, 135–143 (2003).
16. **Kichise, T., Taguchi, S., and Doi, Y.:** Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase, Appl. Environ. Microbiol., **68**, 2411–2419 (2002).
17. **Takase, K., Taguchi, S., and Doi, Y.:** Enhanced synthesis of poly(3-hydroxybutyrate) in recombinant *Escherichia coli* by means of error-prone PCR mutagenesis, saturation mutagenesis, and in vitro recombination of the type II polyhydroxyalkanoate synthase gene, J. Biochem., **133**, 139–145 (2003).
18. **Nomura, C. T., Taguchi, K., Taguchi, S., and Doi, Y.:** Coexpression of genetically engineered 3-ketoacyl-ACP synthase III (*fabH*) and polyhydroxyalkanoate synthase (*phaC*) genes leads to short-chain-length/medium-chain-length polyhydroxyalkanoate copolymer production from glucose in *Escherichia coli* JM109, Appl. Environ. Microbiol., **70**, 999–1007 (2004).
19. **Matsumoto, K., Murata, T., Nagao, R., Nomura, C. T., Arai, S., Arai, Y., Takase, K., Nakashita, H., Taguchi, S., and Shimada, H.:** Production of short-chain-length/medium-chain-length polyhydroxyalkanoate (PHA) copolymer in the plastid of *Arabidopsis thaliana* using an engineered 3-ketoacyl-acyl carrier protein synthase III, Biomacromolecules, **10**, 686–690 (2009).
20. **Baulcombe, D. C.:** RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants, Plant Mol. Biol., **32**, 79–88 (1996).
21. **Schubert, P., Steinbüchel, A., and Schlegel, H. G.:** Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*, J. Bacteriol., **170**, 5837–5847 (1988).
22. **Xiong, A. S., Yao, Q. H., Peng, R. H., Duan, H., Li, X., Fan, H. Q., Cheng, Z. M., and Li, Y.:** PCR-based accurate synthesis of long DNA sequences, Nat. Protoc., **1**, 791–797 (2006).
23. **Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A.:** Selection of AUG initiation codons differs in plants and animals, EMBO J., **6**, 43–48 (1987).

24. **Hofgen, R. and Willmitzer, L.:** Storage of competent cells for Agrobacterium transformation, Nucleic Acids Res., **16**, 9877 (1988).
25. **Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T.:** A simple and general method for transferring genes into plants, Science, **227**, 1229–1231 (1985).
26. **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).
27. **Murashige, T. and Skoog, F.:** A revised medium for rapid growth and bio-assays with tobacco tissue cultures, Physiol. Plant., **15**, 473–497 (1962).
28. **Jo, S. J., Matsumoto, K., Leong, C. R., Ooi, T., and Taguchi, S.:** Improvement of poly (3-hydroxybutyrate) [P(3HB)] production in *Corynebacterium glutamicum* by codon optimization, point mutation and gene dosage of P(3HB) biosynthetic genes, J. Biosci. Bioeng., **104**, 457–463 (2007).
29. **Peach, C. and Velten, J.:** Transgene expression variability (position effect) of Cat and Gus reporter genes driven by linked divergent T-DNA promoters, Plant Mol. Biol., **17**, 49–60 (1991).
30. **Kichise, T., Fukui, T., Yoshida, Y., and Doi, Y.:** Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant *Ralstonia eutropha* and effects of PHA synthase activity on in vivo PHA biosynthesis, Int. J. Biol. Macromol., **25**, 69–77 (1999).