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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.

[**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.

MATERIALS AND METHODS

Vector construction Modified *phaB* and *phaC* (*phaBC*) genes of *R. eutropha* were constructed as *Bam*HI/*Sac*I fragments by assembling oligonucleotides (purchased from Takara, Japan) based on the method previously described (22) and designated as *ephaB* and *ephaC*, respectively (accession numbers: AB591235 and AB591236). The GC content in *ephaB* (37%) and *ephaC* (45%) were lower than the wild-type *phaB* (63%) and *phaC* (67%) genes, respectively. The preferred codon usage in tobacco was chosen based on Codon Usage Database supplied by Kazusa DNA Research Institute, Japan (http://www.kazusa.or.jp/codon/). The modified genes possess the ACAATGG consensus sequence at the initial codon (23). The GGA

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

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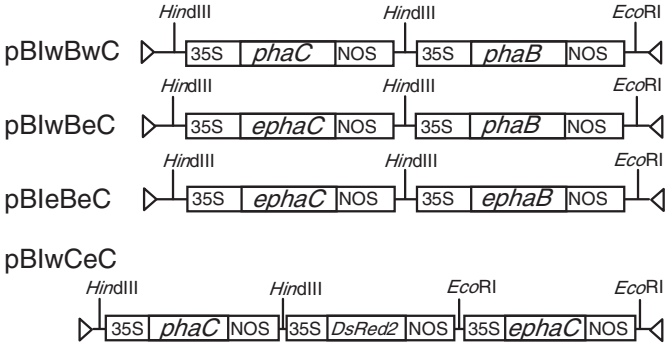

FIG. 1. Vectors used in this study. *phaC* indicates PHA synthase gene; *phaB*, acetoacetyl-CoA reductase gene; *ephaBC*, codon-optimized genes; 35S, 35S cauliflower mosaic virus promoter; NOS, nopaline synthase terminator. Triangles indicate the left and right boarder of the T-region.

codon was inserted next to the initial codon in *ephaB* gene for generating guanine at +4 position. The *Bam*HI/*Sac*I fragments of *phaB* and *ephaB* genes were inserted into pBI221 (Clontech, Japan), respectively, to connect the genes with the 35S cauliflower mosaic virus promoter and nopaline synthase terminator. The *Hin*dIII/*Eco*RI fragments of the yielded plasmids bearing *phaB* and *ephaB* genes were inserted into pBI121 (Clontech), respectively. Similarly, the *Bam*HI/*Sac*I fragments of *phaC* and *ephaC* genes were inserted into pBI221H (19), and the *Hin*dIII fragments of the yielded plasmids were inserted into pBI121 to construct three vectors shown in Fig. 1: pBIwBwC bearing wild-type *phaBC* genes, pBIwBeC bearing wild-type *phaB* and *ephaC* genes, and pBIeBeC bearing *ephaBC* genes. Additionally, the *Bam*HI/*Sac*I fragments of *ephaC* was inserted into pBI221E (19) and the *Eco*RI fragment of the yielded plasmid was inserted into pBI121DsRed2 together with the *Hin*dIII fragment of *phaC* to construct pBIwCeC (Fig. 1). The DsRed2 gene was not used for the selection of transformants in this study.

Transformation of tobacco The four vectors, pBIwBwC, pBIwBeC, pBIeBeC, and pBIwCeC, were introduced into tobacco (*Nicotiana tabacum* cv. Samsun NN) by the *Agrobacterium tumefaciens*-mediated method (24–26). The resultant transformants were designated as wBwC, wBeC, eBeC, and wCeC, respectively. Regenerated transformants were cultured on the agarose solidified Murashige–Skoog medium (27) (Wako Chemical, Japan) containing 3% sucrose and 100 mg/L kanamycin using a growth chamber equipped with fluorescent lamps under the environmental condition with 16 h light and 8 h dark at 25°C. The expression of the *phaC* and *ephaC* genes in the regenerated plants were confirmed by RT–PCR using the following primers: 5′-GTGCGCAACATGATGGAAGACC-3′ and 5′-GGCGCAAGAACGAGAAGGTA-3′ (*phaC*), 5′-GGTGCAGCAGCTTCTACACAA-3′ and 5′-CCATCATGTTCCTAACTCCT-3′ (*ephaC*) as described previously (12).

Quantitative real-time RT–PCR (qRT–PCR) Total RNA was extracted from the leaves of wCeC transformants, which were grown for one week after regeneration, using the TRIzol RNA extraction kit (Invitrogen, USA). cDNA was prepared with the ReverTra Ace reverse transcriptase (Toyobo, Japan). Primers and probes for the TaqMan assay of the *phaC* and *ephaC* genes (Table 1) were designed using Primer Express, version 3.0 (Applied Biosystems, USA). The amount of cDNA was determined using the average of three wells. The DNA fragments of *phaC* and *ephaC* ORFs were used for calibration.

Immunodetection The production of PhaB and PhaC proteins in transgenic tobacco was measured by immunoblot analysis using rabbit antisera of anti-PhaB, which was kindly provided by Dr. Kristi Snell of Metabolix Inc., and anti-PhaC (28). Crude extract was prepared by homogenization of a green leaf of the transformants, which had been grown for five weeks after regeneration, as described previously (12). In this study, a 2% protease inhibitor cocktail for plants (Sigma, USA) was added in the buffer to avoid proteolysis of the samples. The protein concentration of the crude extract was normalized by the Bradford method. After electrophoresis, the protein in the gel was electroblotted onto a PVDF membrane, which was subsequently subjected to immunodetection using the ECL Advance Western Detection Kit (GE Healthcare).

TABLE 1. Primers used for qRT–PCR assay.

Target		Sequence
phaC	Primers	5′-TGTTCTTGGCTTCTGTGTTGGT-3′
		5′-GGATGTTCACCTCTAGCTGCAA-3′
	Probe	5′-AACCATTGTTTCAACTGCA-3′
ephaC	Primers	5′-CGACGAGAGCGCGTTTG-3′
		5′-CAGCGGCTTGTACTGCAACA-3′
	Probe	5′-CGAGAACGAGTACTTCC-3′

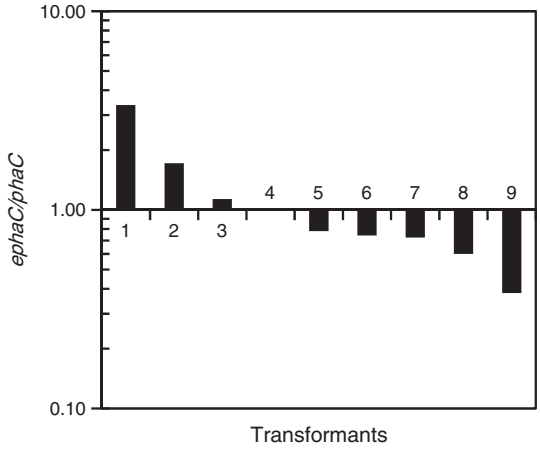

FIG. 2. Relative mRNA levels of *ephaC* versus wild-type *phaC* determined by qRT–PCR. Data were obtained from nine independent transformants of wCeC (1–9). The upward bars (1–3) indicate that the mRNA level of *ephaC* is higher than that of *phaC*, and the downward bars (4–9) indicate the opposite.

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

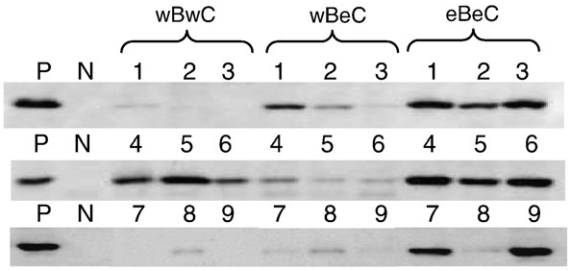

FIG. 3. Immunoblotting of PhaB using crude extract prepared from a leaf. P indicates crude extract of *Escherichia coli* expressing the *phaB* gene (positive control); N, wild-type tobacco (negative control). Numbers 1–9 indicate the independent transformants of each line.