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

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Running title: Codon optimization for PHB production in tobacco

Keywords: transgenic plant; *Nicotiana tabacum*; PHA synthase; realtime PCR

## **Abstract**

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 the PhaB-catalyzed reaction to be a rate-determining step of P(3HB) biosynthesis in tobacco leaves.

## **INTRODUCTION**

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) have 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)]-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;  $\beta$ -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* (*Cupriavidus necator*) (21), and investigated their effect on PHA 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 number; AB591235 and AB591236). 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 *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 *Hind*III/*Eco*RI fragments of the yielded plasmids bearing *phaB*, and *ephaB* genes were inserted into pBI121 (Clontech, Japan), respectively. Similarly, the *Bam*HI/*Sac*I fragments of *phaC*, and *ephaC* genes were inserted into pBI221H (19), and the *Hind*III 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 *Hind*III 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 (23-25). The resultant transformants were designated as wBwC, wBeC, eBeC and wCeC, respectively. Regenerated transformants were cultured on the agarose solidified Murashige-Skoog medium (26) (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 realtime 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 ReverTraAce 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 (27). 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). 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 PHA 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 (28). 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 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 the 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  $\mu\text{g/g}$  cell dry 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  $\mu\text{g/g}$  cdw P(3HB) on average, a more than twofold increase over wBwC and wBeC. This result indicated that the introduction of *ephaB* led to the increase in P(3HB) production.

The increases in PhaB protein (FIG. 3) and P(3HB) accumulation (FIG. 4) in eBeC transformants revealed that enhanced PhaB expression was effective to increase the P(3HB) content. This result suggested that PhaB-catalyzed reaction may be a rate-determining step in the 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 (29). Thus, our result suggested that further enhancement of PhaB activity might increase the productivity of P(3HB). In contrast, introduction of *ephaC* exhibited little effect on P(3HB) production. This indication stands in contrast to our previous result of enhanced PHA production using highly active PHA synthases in *A. thaliana* (12,19). The difference may be due to the fact that the activity of PHA synthase from *R. eutropha* was stronger than other PHA synthases, as indicated by microbial studies (30).

In conclusion, we have demonstrated that the enhanced expression of PhaB effectively improves PHA production in plants. These results have also provided information on the rate-determining step in PHA production. The combined strategy of applying the codon-optimization to highly active engineered enzymes involved in PHA biosynthesis (12,19) would be useful for further enhancement in PHA production in

plants.

## ACKNOWLEDGMENTS

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## Figure captions

FIG. 1. Vectors used in this study. *phaC*, 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 was obtained from nine independent transformants of wCeC.

FIG. 3. Immunoblotting of PhaB using crude extract prepared from a leaf. P, crude extract of *Escherichia coli* expressing the *phaB* gene (positive control); N, wild-type tobacco (negative control). The 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.

Table 1. Primers used for qRT-PCR assay

target		Sequence
<i>phaC</i>	primers	5'-TGTTCTTGGCTTCTGTGTTGGT-3'
		5'-GGATG TTCACCTCTAGCTGCAA-3'
	probe	5'-AACCAT TGTTTCAACTGCA-3'
<i>ephaC</i>	primers	5'-CGACGAGAGCGCGTTTG-3'
		5'-CAGCGGCTTG TACTGCAACA-3'
	probe	5'-CGAGAACGAGTACTTCC-3'

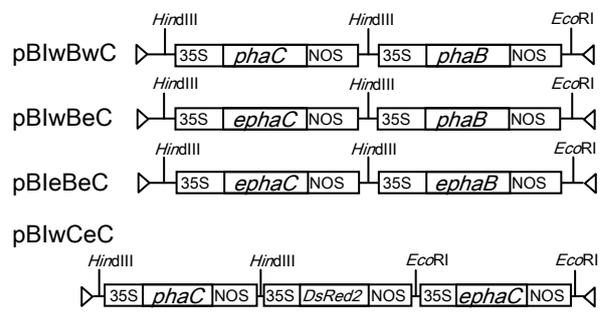


FIG. 1

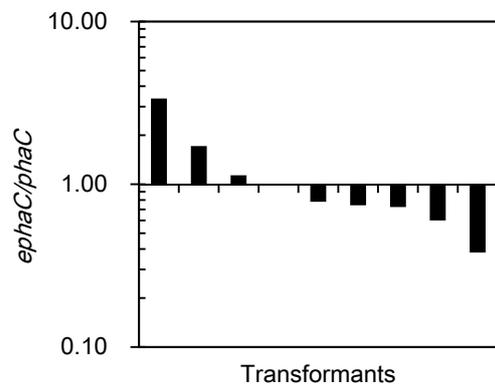


FIG. 2

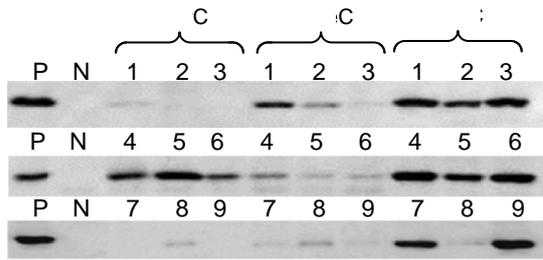


FIG. 3

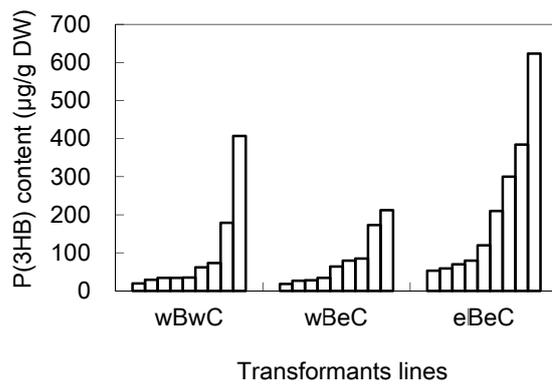


FIG. 4