

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

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Short-chain-length/medium-chain-length (SCL/MCL) polyhydroxyalkanoate (PHA) was produced in the plastids of *Arabidopsis thaliana*. Phe87Thr (F87T) mutated 3-ketoacyl-acyl carrier protein (ACP) synthase III (FabH) from *Escherichia coli*, and Ser325Thr/Gln481Lys (ST/QK) mutated polyhydroxyalkanoate (PHA) synthase (PhaC1) from *Pseudomonas* sp. 61–3, along with the β -ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) from *Ralstonia eutropha* (*Cupriavidus necator*) genes were introduced into *Arabidopsis*. The transgenic *Arabidopsis* produced PHA copolymers composed of monomers consisting of 4–14 carbons. The introduction of the engineered PHA synthase resulted in a 10-fold increase in PHA content compared to plants expressing the wild-type PHA synthase. In addition, expression of the engineered *fabH* gene in the plastid led to an increase in the amount of the SCL monomer, 3-hydroxybutyrate, incorporated into PHA, and contributed to supply of MCL monomers for PHA production.

Introduction

Polyhydroxyalkanoates (PHAs) are bacterial storage materials that can be processed into useful thermoplastic materials.¹ These polyesters are a biobased and biodegradable alternative to petrochemical plastics. Among biobased materials, an important feature of PHA is that the polymer is synthesized *in vivo* by biochemical reactions. Therefore, PHAs can be produced in various hosts by transferring and expressing the PHA biosynthetic genes. In planta, PHA production has a potential advantage over PHA production by fermentation in terms of cost because exogenous carbon feedstocks and fermentation facilities are not necessary for plant growth. Several PHA-producing transgenic plants have been made. For example, *Arabidopsis thaliana*,^{2,3} rapeseed,^{4–6} tobacco,⁷ potato,^{8,9} sugar cane,^{10,11} and switchgrass¹² have been used as recombinant hosts of the PHA synthetic suite of genes.

PHAs are typically synthesized by polymerization of (*R*)-3-hydroxyacyl-CoAs. PHA synthase (PhaC) is a key enzyme that catalyzes the polymerization. The specific activity and substrate

specificity of the PHA synthase greatly effects the *in vivo* accumulation, monomer composition, and molecular weight of the PHA polymers produced. PHAs are classified into two main classes: short-chain-length (SCL) PHA and medium-chain-length (MCL) PHA. SCL PHAs are highly crystalline thermoplastics and MCL PHAs are elastomeric in nature. Generally, these two classes of PHAs are produced by PHA synthases with narrow substrate specificity (for review see Nomura and Taguchi, 2007).¹³ However, PhaC1_{Ps} isolated from *Pseudomonas* sp. 61–3 has broad substrate specificity and is capable of synthesizing short-chain-length/medium-chain-length (SCL/MCL) PHA copolymers consisting of monomers composed of 4–12 carbons. Some of these SCL/MCL PHA copolymers have attracted industrial interest because these materials have superior properties as compared to SCL or MCL PHA and are easily processed.^{14,15}

Although the wild type PhaC1_{Ps} has broad substrate specificity, its overall activity toward SCL PHA monomers is relatively poor. In order to improve the functionality of this enzyme, we created highly active mutants of PhaC1_{Ps} that had enhanced activity toward SCL monomers and increased capacity to produce SCL/MCL PHA copolymers^{16,17} through *in vitro* evolutionary techniques.¹⁸ Although the highly active mutants were initially generated and selected in recombinant *E. coli*, the mutants were shown to exhibit enhanced function in plants.¹⁹ This result prompted us to investigate expression of a highly active mutant of PhaC1_{Ps} in *Arabidopsis* for SCL/MCL PHA production. We chose the S325T/Q481K (ST/QK) mutant¹⁷ with

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enhanced activity toward 3HB-CoA and shifted substrate specificity toward smaller monomers.

SCL/MCL PHA production in plants is more difficult than in bacteria, because MCL monomer supply is limited in plants. To date, two metabolic pathways are known to be involved in supplying MCL (*R*)-3-hydroxyacyl-CoAs (3HA-CoA). One monomer supplying pathway occurs via the fatty acid β -oxidation pathway, in which an *R*-specific enoyl-CoA hydratase (PhaJ) converts enoyl-CoA into 3HA-CoA.²⁰ In plants, β -oxidation occurs in the peroxisome. Targeting of PHA synthases into the peroxisome resulted in the accumulation of MCL²¹ and SCL/MCL⁷ PHA. However, the PHA content was very low in peroxisomes, probably because carbon flux and/or the volume of the peroxisome limited PHA accumulation. Another pathway for MCL PHA monomer supply is via the *de novo* fatty acid biosynthetic pathway. In *Pseudomonas* strains, 3-hydroxyacyl-acyl carrier protein (ACP)/CoA transacylase (PhaG) is thought to be a key enzyme for supplying 3HA-CoA monomers for PHA production.²² Overexpression of the *phaG* gene increased MCL PHA production in a recombinant *Pseudomonas* strain.²³ However, PhaG has little activity in heterologous hosts such as *Ralstonia eutropha* (*Cupriavidus necator*).²⁴ In fact, coexpression of *phaG* and a PHA synthase gene in *Arabidopsis* failed to accumulate PHA²⁵ and led to an accumulation of only trace amount of MCL PHA in potato.⁹

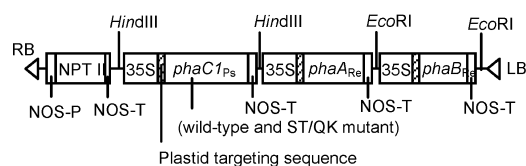
Previously, we reported an alternative MCL monomer-supplying pathway catalyzed by mutants of 3-ketoacyl-ACP synthase III (FabH).²⁶ The normal function of FabH is to catalyze the condensation of malonyl-ACP and acetyl-CoA to form acetoacetyl-ACP and CO₂ in the initial reaction of fatty acid biosynthesis. Overproduced FabH in recombinant *E. coli* has also been shown to supply acetoacetyl-CoA for P(3HB) production.²⁷ The variation of monomers produced for PHA production from unrelated carbon sources by FabH was expanded through the rational design of the enzyme. In previous studies, the amino acid at position 87 located in the substrate binding pocket based on crystal structure of the FabH protein, was substituted with other amino acids to expand the substrate specificity of the enzyme.^{28,29} The F87X mutants of FabH provide MCL 3-ketoacyl-CoAs, which are subsequently reduced into 3HA-CoA, through the *de novo* fatty acid biosynthesis pathway. Recombinant *E. coli* harboring F87T mutated *fabH* gene along with PHA synthase genes produced SCL/MCL PHA from unrelated carbon sources, such as glucose.²⁶ The result indicated that the FabH-mediated pathway could potentially be applied to various hosts, because the fatty acid biosynthesis pathway is conserved among many organisms, including plants. Plant fatty acid biosynthesis occurs in the plastid, thus targeting of engineered FabH into the plastid should enable the supply of MCL monomers for PHA biosynthesis.

In this study, we coexpressed the F87T mutated *fabH* gene in the plastid of *Arabidopsis* with the *phaC1_{Ps}* and β -ketothiolase and acetoacetyl-CoA reductase (*phaAB*) genes and compared PHA production in these recombinant plants with those expressing only *phaC1_{Ps}* and *phaAB_{Re}* genes. This report provides evidence for a new method to produce SCL/MCL PHA in plants facilitated by FabH and the PHA synthesis genes.

Materials and Methods

Plasmid Construction. pBIC1ABWT and pBIC1ABSTQK, have wild-type and ST/QK derivatives of the PHA synthase gene (*phaC1*), respectively, along with *phaAB* genes from *R. eutropha*. The plasmid pBIHC1AB harbors the F87T mutated *fabH*, *phaC1STQK*, and *phaAB* genes. All PHA biosynthetic genes were fusions with a plastid targeting

pBIC1ABWT, pBIC1ABSTQK



pBIHC1AB (ST/QK mutant)

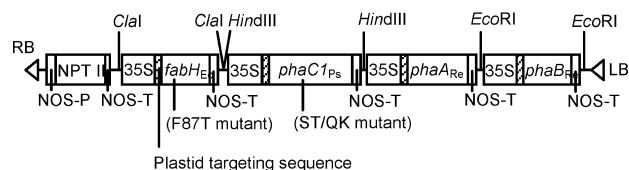


Figure 1. Plasmids map used in this study. 35S, 35S cauliflower mosaic virus promoter; NOS-P, nopaline synthase promoter; NOS-T, nopaline synthase terminator; LB, left border of T-DNA; RB, right border of T-DNA; NPT II, neomycin phosphotransferase gene; *phaB_{Re}*, NADPH-dependent acetoacetyl-CoA reductase gene from *R. eutropha*; *phaC1_{Ps}*, the wild-type and ST/QK mutated PHA synthase gene from *Pseudomonas* sp. 61–3; *fabH_{Ec}*, F87T mutated 3-ketoacyl-ACP synthase III gene from *E. coli*.

signal from *Lycopersicon esculentum* (tomato; Figure 1). All plasmids were constructed as described in the Supporting Information.

Plant Transformation. *Arabidopsis thaliana*, ecotype Columbia, was transformed by the floral dip method using *Agrobacterium tumefaciens* strain GV3101.³⁰ The transformants were selected on Murashige and Skoog (MS) agar medium (pH 5.8) containing 2.3 g/L MS salts (Wako Pure Chemicals, Japan), 1% sucrose, 0.7% agar, 25 mg/L kanamycin, and 50 mg/L Claforan (Hoechst, Germany). Kanamycin resistant plants were subsequently transferred to soil and grown in a greenhouse. The introduced genes in the transformants were detected by PCR using primers as described previously.¹⁹

Reverse Transcriptase-PCR (RT-PCR) Analysis. Total RNA was extracted from *Arabidopsis* using TRIZOL (Invitrogen, USA). Fresh leaves of *Arabidopsis* grown for 2 weeks were used for RNA extractions. Reverse transcription to produce cDNA was performed using MMLV reverse transcriptase following the protocol recommended by the manufacturer (Invitrogen). The *fabH* gene transcript was amplified from the cDNA using following primers; 5'-AGGTACCGTT-TATACGAAGATTATTGGTACT-3' and 5'-TGGTACCCTAGAAAC-GAACCAGCGCGGAGCC-3'. The actin gene transcript was amplified using the following primers; 5'-CTCAAGAAGTTCTCAGCAGTA-3' and 5'-TCACCTTCTTCATCCGCGAGTT-3'.

PHA Analysis. PHA analysis was performed by a GC/MS method, which was described previously⁷ but with the following modifications. After the seeds were harvested, the residual plants were lyophilized and ground into powder using a multi-bead shocker (Yasuikikai, Japan), and 10–100 mg of ground plants were transferred into 10 mL glass test tubes with screw caps. These samples were washed with 8 mL *n*-hexane at 50 °C three times for 4, 4, and 16 h, respectively. The test tubes were centrifuged at 1600 × *g* for 5 min and supernatant *n*-hexane was removed. Subsequently, the samples were further washed with 8 mL of 50% aq methanol at 50 °C three times for 4, 4, and 16 h, respectively. The washed plants were rinsed with water and lyophilized again. Next, the polyesters were extracted from plants in 6 mL of chloroform at 65 °C for 48 h. The chloroform extracts were filtered through glass wool to remove debris, and the chloroform was evaporated by nitrogen flow. Dried polyesters were washed with *n*-hexane and dried again. Polyesters were ethanolyzed and analyzed by GC/MS (Shimadzu, Japan) as described previously.⁷

For the large-scale preparation of PHA, lyophilized plants were washed with *n*-hexane and methanol for 48 h, respectively, using a Soxhlet extraction system. After the methanol was evaporated, the polyester was extracted in boiling chloroform for 48 h. The chloroform

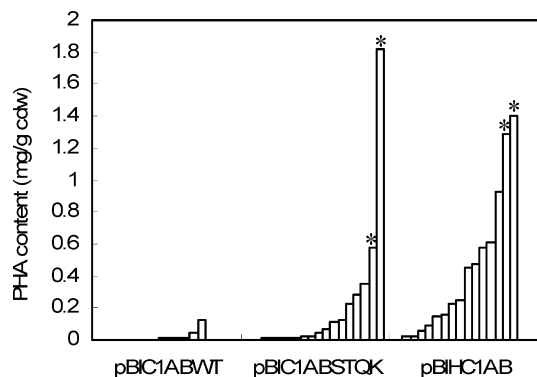


Figure 2. PHA contents in T1 lines of *Arabidopsis* transformants harboring pBIC1ABWT (14 lines), pBIC1ABSTQK (18 lines), and pBIHC1AB (16 lines). Asterisks indicate plant lines selected to generate T3 progeny.

extract was passed through a paper filter and PTFE membrane, and the solvent was removed by evaporation. Subsequently, the polyester was redissolved in 1 mL chloroform, and 10 mL of *n*-hexane was added to precipitate the polyesters. The supernatant was removed by centrifugation at $1600 \times g$ for 5 min. The precipitate was dried and again dissolved in 1 mL of chloroform, and the polyester was precipitated by the addition of 10 mL of methanol. The methanol precipitation step was repeated two times, after which, the purified polyester was subjected to GC/MS analysis.

Results

Arabidopsis was transformed using the plasmids pBIC1ABWT and pBIC1ABSTQK (Figure 1). A total of 14 and 18 transformants were obtained for pBIC1ABWT and pBIC1ABSTQK, respectively. The T1 transformants harboring the wild-type PHA synthase gene accumulated no or small amounts of PHA (up to 0.1 mg/g cell dry weight (cdw); Figure 2). However, introduction of the ST/QK mutated PHA synthase gene remarkably enhanced PHA content up to 1.8 mg/g cdw, indicating that ST/QK mutant was highly active in the plastid of *Arabidopsis*. Thus, we used the ST/QK mutant for further studies. Secondary monomer units larger than 3HB were not detected in the PHA isolated from T1 transformants.

Next, pBIHC1AB (Figure 1) was introduced into *Arabidopsis* and 16 transformants were obtained. The expression of *fabH* gene was confirmed by RT-PCR (data not shown). The introduction of engineered *fabH* gene led to a 2-fold increase in PHA content by average compared to pBIC1ABSTQK lines (Figure 2). Because the PHA produced consisted of mostly 3HB monomers, it can be concluded that the expression of the engineered FabH led to an increase in the supply of 3HB-CoA. Trace signals corresponding to MCL secondary monomer units were detected from some of the samples. However, the intensities of the peaks were too low to be quantified.

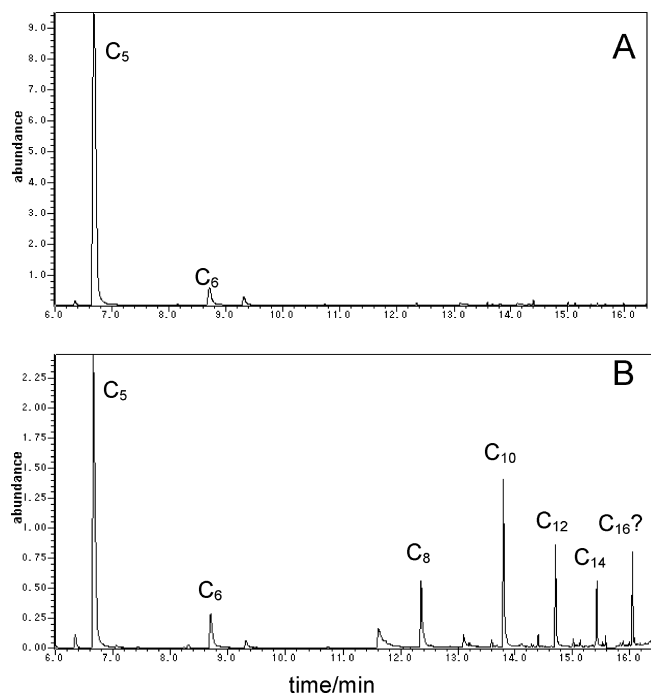


Figure 3. GC/MS analysis of purified PHA isolated from T3 line of the transgenic *Arabidopsis*. (A) Transformant harboring the *phaC1*_{PS} (ST/QK) and *phaAB*_{Re} genes, (B) transformant harboring the *fabH*_{Ec} gene (F87T) along with *phaCIAB* genes. The amount of ethyl-3-hydroxybutyrate, which was eluted at 4.6 min, was separately determined using diluted samples.

We chose T1 transformants with high PHA contents for both pBIC1ABSTQK and pBIHC1AB, as indicated in Figure 2, and established homozygous lines of their T3 progenies to analyze the monomer composition. A total of 30 transformants of each homozygous T3 line were combined and subjected to GC/MS analysis to detect the incorporation of various secondary monomer units (Figure 3 and Table 1). The gas chromatograph revealed a peak at 16 min that is likely 3-hydroxyhexadecanoate because the *m/z* ratio for the peak was typical for a 3-hydroxyalkanoate (data not shown). An unexpected result was that PHA produced by *Arabidopsis* harboring the *phaCIAB* genes (ST/QK mutant) was comprised of 3-hydroxyvalerate (3HV, 1.8 mol %) and 3-hydroxyhexanoate (3HHx, 0.2 mol %) units as well as 3HB monomers (Table 1), suggesting that there is an intrinsic MCL monomer supplying pathway in the plastid. The transformants harboring the *fabH* and *phaCIAB* genes produced PHA consisting of monomers with 4–14 carbons long, indicating that the engineered FabH contributed to an increased supply of MCL monomers for PHA production in *Arabidopsis*.

Discussion

SCL/MCL PHAs are potentially flexible thermoplastic polymers with a wide range of applications. This study has revealed

Table 1. Content and Monomer Composition of PHA Accumulated in the Transgenic *Arabidopsis* Harboring the PHA Biosynthetic Genes^a

vector	PHA content (T1; mg/g)			monomer composition ^b (T3; mol %)						
	number of plants	avg	max	3HB (C ₄)	3HV (C ₅)	3HHx (C ₆)	3HO (C ₈)	3HD (C ₁₀)	3HDD (C ₁₂)	3HTD (C ₁₄)
pBIC1ABWT	14	0.03	0.12							
pBIC1ABSTQK	18	0.21	1.8	97.8 ± 0.2	2.0 ± 0.3	0.2 ± 0.1				
pBIHC1AB	16	0.42	1.4	99.5 ± 0.2	0.41 ± 0.16	0.075 ± 0.041	0.006 ± 0.002	0.005 ± 0.004	0.003 ± 0.001	0.001 ± 0.001

^a PHA contents were determined using whole plant body samples but excluded the roots. ^b 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3HTD, 3-hydroxytetradecanoate. Monomer composition represents the averages ± standard deviations of two lines, which was determined using 30 transformants for each line.

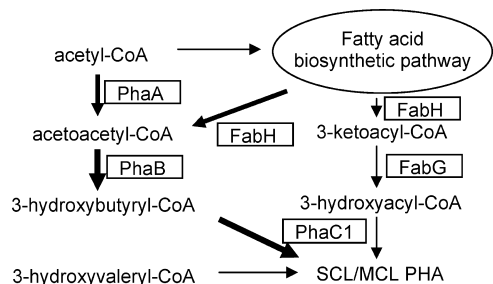


Figure 4. Proposed pathway for SCL/MCL PHA biosynthesis in plastids. PhaA, β -ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC1, PHA synthase; FabH, 3-ketoacyl-ACP synthase III (KASIII); FabG, 3-ketoacyl-ACP reductase.

new methods to produce SCL/MCL PHA in transgenic plants. Through the use of engineered, highly active PHA synthases, we have shown that an intrinsic MCL-supplying pathway is present in plastids. In addition, the expression of the engineered FabH (F87T) monomer supplying enzyme enabled incorporation of MCL monomers consisting of 8–14 carbons into PHA in *Arabidopsis*. This is the first report of SCL/MCL PHA being produced in the plastid of a plant and of functional expression of the engineered *fabH* gene in a eukaryotic host. Because the fatty acid biosynthetic pathway is conserved among plants, the FabH mediated pathway should be applicable to other plant species.

The proposed PHA biosynthetic pathway in *Arabidopsis* is illustrated in Figure 4. Acetyl-CoA is synthesized in the plastid for fatty acid biosynthesis but can be intercepted by the β -ketothiolase (PhaA) for SCL PHA monomer supply. β -Ketothiolase catalyzes condensation reaction of two molecules of acetyl-CoA that yields acetoacetyl-CoA, which is subsequently reduced to 3-hydroxybutyryl-CoA (3HB-CoA) by the PhaB enzyme. FabH also provides acetoacetyl-CoA from the initial reactions of fatty acid biosynthetic pathway. The results of this study indicate that the amount of 3HB monomer was increased by expression of FabH, suggesting that supply of acetoacetyl-CoA is a rate-determining step for polymer production in plastids. In addition, the F87T mutation in FabH has altered substrate specificity, so that the engineered enzyme can supply MCL 3-ketoacyl-CoA from the fatty acid biosynthetic cycle. The results of this study suggest that the engineered FabH proteins have a higher specificity toward SCL substrates as compared to MCL substrates, resulting in a high mol % of SCL monomers incorporated into PHA. Because the substrate specificity of FabH appears to be a limiting factor for MCL supply, further engineering of FabH is essential to produce SCL/MCL PHA with higher MCL monomer fractions.

In the plastid, the ST/QK highly active mutant was shown to accumulate larger amounts of PHA as compared to the wild-type PHA synthase gene (Table 1). This result was in contrast to our previous result where both the wild-type and ST/QK mutant produced nearly identical amounts of SCL/MCL PHA in the peroxisome,³¹ indicating that the rate-determining factor for PHA biosynthesis in the plastid is different than that in peroxisome. In the peroxisome, the supply of monomers and the size of the peroxisome itself are thought to be limiting factors for PHA accumulation. However, in the plastid, more monomers and space are available. Therefore, the activity of the PHA synthase has been shown to affect the productivity of PHA.

Conclusion

The F87T mutant of FabH and S325T/Q481K mutant of PHA synthase were functionally expressed in transgenic *Arabidopsis*.

These transformants produced SCL/MCL PHA consisting of monomers with 4–14 carbons long. The result suggested that in vitro engineered PHA synthase and fatty acid biosynthetic enzymes are functional in *Arabidopsis* and may be used to produce PHA via photosynthetic fixation of CO₂. We are continuing further engineering of FabH and PHA synthase. Productivity of SCL/MCL PHA should be improved by combining these beneficial engineered enzymes.

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Supporting Information Available. Experimental details for the construction of plasmids used in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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