Production of Polyhydroxybutyrate by Polycistronic Expression of Bacterial Genes in Tobacco Plastid

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Transgenic techniques are used to enhance and improve crop production, and their application to the production of chemical resources in plants has been under investigation. To achieve this latter goal, multiple-gene transformation is required to improve or change plant metabolic pathways; when accomplished by plant nuclear transformation, however, this procedure is costly and time consuming. We succeeded in the metabolic engineering of the tobacco plant by introducing multiple genes within a bacteria-like operon into a plastid genome. A tobacco plastid was transformed with a polycistron consisting of the spectinomycin resistance gene and three bacterial genes for the biosynthesis of the biodegradable polyester, poly[(R)-3-hydroxybutyrate] (PHB), after modification of their ribosome binding sites. DNA and RNA analysis confirmed the insertion of the introduced genes into the plastid genome and their polycistronic expression. As the result, the transplastomic tobacco accumulated PHB in its leaves. The introduced genes and the PHB productivity were maternally inherited, avoiding genetic spread by pollen diffusion, and were maintained stably in the seed progeny. Despite the low PHB productivity, this report demonstrates the feasibility of transplastomic technology for metabolic engineering. This “phyto-fermentation” system can be applied to plant production of various chemical commodities and pharmaceuticals.

Keywords: Biodegradable polyester — Metabolic engineering — Plastid transformation — Polycistron — Polyhydroxybutyrate.

Abbreviations: GC-MS, Gas chromatography-mass spectrometry; PCR, Polymerase chain reaction; PHB, poly-(R)-3-hydroxybutyrate; RBS, ribosome binding site.

Introduction

Genetic engineering of crop plants, involving the introduction and control of foreign genes, has been used not only for production of useful products but also as a medium for experimental modification of plant metabolism. One of the most promising results of this process has been the production in plants of poly[(R)-3-hydroxyalkanoate] (PHA), a class of biodegradable polyester (Poirier 2001). PHAs, produced by various species of bacteria as storage material, are polymers of (R)-3-hydroxyalkanoates formed by PHA synthase and have the useful properties of biodegradability, thermoplasticity and elasticity (Doi 1990). Production of PHAs in plants is expected to establish a new carbon cycle for commercial use using only light energy for production, a system which would have far less adverse effects on the environment than traditional polymer manufacturing.

Various bacteria produce the most common PHA, poly[(R)-3-hydroxybutyrate] (PHB), whose biosynthesis from acetyl-CoA requires only three enzymes, usually encoded in an operon (Huisman et al. 1991, Fukui and Doi 1997, Peoples and Sinskey 1989a, Peoples and Sinskey 1989b). The phb operon of Ralstonia eutropha, encoding the PHB biosynthetic enzymes, consists of phbC, phbA and phbB. Acetoacetyl-CoA, produced from acetyl-CoA by β-ketothiolase (phbA), is converted to 3-hydroxybutyrate by acetooacetyl-CoA reductase (phbB). This product is then polymerized by PHB polymerase, which is encoded by phbC (Fig. 1). The PHB biosynthetic genes are introduced into Arabidopsis (Poirier et al. 1992, Nawrath et al. 1994, Mittendorf et al. 1998), cotton (John and Keller 1996), corn suspension cell cultures (Hahn et al. 1997), Brassica napus (Houmiel et al. 1999), and tobacco (Nakashita et al. 2001, Nakashita et al. 1999, Arai et al. 2001) and the resultant plants accumulated PHAs in cytosol, plastids, or peroxisomes. These studies exhibited that, in both Arabidopsis and tobacco, the localization of PHB synthesis in plastid gave higher PHB yield rather than in cytoplasm or peroxisome. This
may be due to a high flux of carbon through acetyl-CoA in plastid, the site of fatty acid biosynthesis and storage.

The plastid, one of the major organelles in the plant cell, provides the plant kingdom with the characteristic ability of photosynthesis. In addition, plastids have acquired specialized functions as the sites of synthesis and storage of various compounds, usually localized in specific organs or tissues. They participate in the biosynthesis and storage of starch (amyloplast), carotenoids (chromoplast) and lipids (elaioplast) (Possingham 1980), enabling plants to reserve nutrition to be utilized for growth and the next generation of seedlings. The outstanding production and storage capabilities of plastids may allow their exploitation as factories for new chemical commodities. To manipulate plastids properly, targeting techniques using transit peptides has been exploited (Nawrath et al. 1994, Gallardo et al. 1995, Denis 1999, McFadden 1999). This procedure, however, especially when used to introduce multiple genes, requires the troublesome addition of a promoter, terminator, and transit peptide sequence into every gene. The plastid, which possesses its own genome, is derived from a free-living oxygen-evolving photosynthetic prokaryote (Gray and Doolittle 1982); both the structure of the genome and the mechanisms for decoding genetic information within plastids are similar to those of modern prokaryotes (Shinozaki et al. 1986, Mullet 1993). Thus, the introduction of a polycistron into the plastid genome can simplify the transformation procedure, requiring only one promoter and one terminator to control multiple genes, and eliminating the need for transit peptide sequences. This technique has been employed in the production of biologically active proteins (Kota et al. 1999, Sidorov et al. 1999, Staub et al. 2000, Cosa et al. 2001). The plastid is also suitable for the introduction of foreign metabolic pathways into plants, as mentioned above. Although the metabolic engineering in the plastid was achieved previously by direct targeting, plastid transformation using a complete bacterial operon has an advantage in that the expression of such a transgene is intrinsically regulated, producing the appropriate assortment and quantity of enzymes necessary for the complete metabolic pathway. Thus, the plastid transformation technique is expected to improve the existing metabolisms of plastid and even confer new functions upon this organelle.

In an earlier report, we preliminarily demonstrated that the introduction of a bacterial phb operon into the tobacco plastid genome resulted in the accumulation of PHB in the leaves (Nakashita et al. 2001). However, probably because of the poor matching of gene regulation mechanisms between the plastid and R. eutropha, the PHB productivity was, unexpectedly, less than 10 ppm, similar to that in the cytoplasm (Nakashita et al. 1999) and the evidence for polycistronic expression could not be detected. To facilitate the production of chemicals by polycistronic expression of transgenes in plastid, the regulatory sequences of the operon most likely require modification. In this paper, we describe the molecular evidence for the metabolic engineering by polycistronic expression and the higher PHB production in tobacco plastid by modification of regulatory sequences.

**Results**

**Tobacco plastid transformation for polycistronic expression**

The plastid rbcL and accD genes are located next to each other but are regulated in different operons, and the spacer region between them is suitable for the insertion of the foreign genes. We constructed a plastid transformation vector to introduce the phb genes in this region by two homologous recombination events. In the plastid transformation vector pPT06,
artificial operon, consisting of the spectinomycin resistance gene (aadA) and the phb genes, is under control of the constitutive promoter of the plastid rRNA operon (Prm) and the plastid psbA terminator sequence (psbA 3′), all of which is located between gene fragments of the rbcL and accD genes (Fig. 2). As previously reported, the utilization of the operon derived directly from the R. eutropha genome resulted in low PHB productivity, ranging from 2 to 8 ppm dry weight. Therefore, to facilitate translation in this study, all introduced genes were preceded by a synthetic ribosome binding site (RBS) designed after the rbcL gene. Tobacco plastid transformation was performed by the biolistic process. Following a round of selection on spectinomycin-containing medium, three lines of pPT06 transformants (PT1, PT2 and PT3) were obtained. Polymerase chain reaction (PCR) assays, using specific primers for aadA, phbC, phbA and phbB, confirmed the introduction of these genes in the selected lines (data not shown). It is reported that further regeneration cycles from the leaf sections gave homoplasmic plants (Svab and Maliga 1993, Shikanai et al. 1998), so we performed this operation. After the selection thorough regeneration, the 2nd regeneration plants PT1-1 to PT1-6, PT2-1 to PT2-6, and PT3-1 to PT3-8 were obtained. Several of these sublines were used for further cycles of selection and the 3rd and 4th regeneration sublines were established.

Analysis of insertion of introduced operon

To confirm the insertion of the operon into the target site between the rbcL and accD genes we performed Southern blot analysis on total cellular DNA prepared from the two 1st regeneration lines of pPT06 transformants (PT2 and PT3). Using the 1.9-kb rbcL-accD fragment from the wild-type plastid genome as a probe (Fig. 3A), digestion with SphI or EcoRI revealed one (7.55 kb) or two (5.65 kb and 3.8 kb) bands, respectively (Fig. 3B). The same results were observed in the 2nd regeneration plants (sublines PT2-1 and PT3-2). These bands were not observed in samples of wild-type plants (Fig. 3B, lane W), although several other non-specific bands were detected. These results indicate that the introduced operon was inserted properly between rbcL and accD by homologous recombination (Fig. 3A). The intense signals of the non-inserted wild-type rbcL-accD fragments, a 2.45-kb fragment by SphI digestion and a 4.4-kb fragment after EcoRI digestion, indicated that the copy number of transformed plastid genomes was low compared to uninserted wild-type genomes. For further confirmation of transformation, we PCR-amplified the 5′- and 3′-end regions of the operon using primers specific for the adjacent plastid genome regions. Cloning and sequencing of these products corroborated earlier findings of successful insertion (data not shown).

Polycistronic transcription of introduced genes

The polycistronic expression of the introduced genes was analyzed by Northern blot analysis, but the detection of the polycistronically transcribed mRNA was not possible due to low amounts of the transcript present. We performed reverse transcription (RT)-PCR using primers designed to amplify the
two adjacent genes with their connecting region. The cDNA preparations of PT1 and PT3 were digested with EcoRI, located between phbA and phbB, to prevent the amplification of double-stranded DNA fragments derived from the plastid genome. To confirm the amplification of proper DNA fragments, not only the estimation of the size of the amplified fragment but also RT-PCR Southern blot analysis was performed. The results showed a gene fragment with the expected size (767-bp), which confirmed the presence of both phbA and phbB in the transcript (Fig. 4). Utilizing a HindIII site, the presence of phbC and phbA in the transcript was also confirmed by the amplification of an 816-bp fragment (Fig. 4). These results were also confirmed by sequence analysis of the amplified DNA fragments. These genes, located downstream of the aadA gene in the introduced operon, require Prm for their expression, indicating that the introduced operon was transcribed polycistronically into mRNA.

Polyester production in the transplastomic plant

Since the insertion and expression of the introduced operon in the plastid were confirmed, the transformants were expected to accumulate PHB. The leaves of pPT06 transformants, following chloroform extraction and ethanolysis, were analyzed by GC-MS. A peak, detected at 5.8 min, appeared only in the leaves of PT3-2 plants but not in the wild-type plants (Fig. 5A, B). The mass spectrum of this peak was identical to that of 3-hydroxybutyrate ethyl ester, which is derived from PHB (Fig. 5C). All three phb genes are necessary to syn-

![Fig. 4](https://academic.oup.com/pcp/article-abstract/45/9/1176/1857654/fig4)

**Fig. 4** Detection of polycistronic transcription of the introduced genes. Polycistronic expression of the introduced genes was detected by RT-PCR Southern blot analysis. EcoRI and HindIII indicate the restriction enzymes used to avoid contamination in cDNA samples from double-stranded DNA derived from plastid genomes. Results from PT1, PT3, and wild-type tobacco (lane W) are shown.

![Fig. 5](https://academic.oup.com/pcp/article-abstract/45/9/1176/1857654/fig5)

**Fig. 5** Analysis of polyester production in plastid-transformed plants. (A) Gas chromatography analysis of the wild-type plant. (B) In the gas chromatography analysis, the transplastomic tobacco plant gave a new peak (arrow, 5.8 min). Data from subline PT3-2 grown on MS medium is shown. (C) Mass spectrum of the peak at 5.8 min of a sample prepared from a transplastomic plant shows a pattern specific to 3-hydroxybutyrate-ethyl ester. Its structure, with major fragmentation patterns, is also shown.
thesize PHB in the plastid due to the lack of β-ketothiolase activity in this organelle (Fig. 1); the combination of the phbC and phbB genes is sufficient to synthesize PHB in the cytosol (Poirier et al. 1992, Nawrath et al. 1994, Nakashita et al. 1999). Thus, accumulation of PHB indicated that the introduced phb operon was transcribed and translated properly in the transformed plastids. The PHB productivity of 1st and 2nd regeneration plants (PT1 to PT3 and their sublines) grown on MS medium was 50–160 ppm of the total dry weight. We previously reported that pPT12 plants introduced with the bacteria-derived operon in the plastids yielded 2–8 ppm PHB accumulation (Nakashita et al. 2001). Thus, the modification of RBS effectively increased the PHB productivity probably due to the enhanced translation of the PHB biosynthetic genes. However, the PHB productivity reduced to 10–60 ppm when these plants were cultured in a greenhouse. Compared to the culture on the soil, plants growing on MS medium containing sugars have lower photosynthetic activity, different metabolic activities in plastids, and different carbon flux into plastids for fatty acid biosynthesis and other secondary metabolisms. These could cause the reduced PHB productivity on, although the detailed mechanisms are unknown.

Analysis of regenerated sublines and seed progeny

Southern blot analysis (Fig. 3) indicated that the 2nd regeneration plants were not yet homoplasmic. The number of transformed genome copies was similar between the 1st and 2nd regenerations in both lines PT2 and PT3 as shown in Fig. 3. To obtain homoplasmic transformants, we repeated the selection of the regenerated plants; insertion of the introduced genes in the 3rd and 4th regenerations was confirmed by Southern hybridization (data not shown). Although some reports indicate that a second cycle of regeneration is enough to obtain homoplasmic plants (Svab and Maliga 1993, Maliga and Nixon 1998), all regenerated plants obtained in this study so far are, unexpectedly, not homoplasmic. The PHB productivity of 3rd and 4th regeneration plants is decreased to ca. 10 ppm due to unknown reasons.

These lines were self-pollinated and their progeny (T1) were analyzed. In the regenerated sublines derived from the two independently transformed lines PT1 and PT3, spectinomycin resistance was inherited in almost all seed progeny (97.5–100%), which is consistent with the maternal inheritance of plastids (Table 1). The few plants designated as “sensitive” exhibited retarded growth, but they were not bleached by spectinomycin, unlike wild-type plants. The next generation of seed progeny (T2) was also analyzed and showed similar results (Table 1). Although seed progeny are harvested by cultivation in a greenhouse without antibiotic selection pressure, Southern blot analysis indicates that the introduced genes were inherited and kept stably in the plastids of T1 plants (Fig. 6A). The ratios of the inserted genome copies to the wild-type genome copies were similar between T0 and T1 plants (PT1-2a, PT1-2, PT3-2, and PT3-2a), approximately 12–15%, as shown in Fig. 6A. The genome copies ratios of T2 plants (PT1-2b and PT3-2b, respectively) were also similar to T0 and T1 plants (data not shown). These results suggest that the foreign genes were not excluded by selection once they were incorporated into the plastid genome. Such stable integration was also confirmed by the fact that T1 plants accumulated PHB. The PHB productivity of T1 plants was 10–20 ppm, which was similar level to that of T0 plants, and was maintained also in T2 plants.

### Table 1 Inheritance of spectinomycin resistance to seed progenies

<table>
<thead>
<tr>
<th>Progeny</th>
<th>Lines a</th>
<th>Regenerations b</th>
<th>Total seeds c</th>
<th>Resistant d</th>
<th>Sensitive d</th>
<th>R/R+S (%) e</th>
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<tr>
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<td>166</td>
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<tr>
<td></td>
<td>PT1-2-1-1a</td>
<td>4th</td>
<td>220</td>
<td>156</td>
<td>3</td>
<td>98.1</td>
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<tr>
<td></td>
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<td>200</td>
<td>159</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
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<td>200</td>
<td>159</td>
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<tr>
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<td>4th</td>
<td>360</td>
<td>228</td>
<td>2</td>
<td>99.1</td>
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<tr>
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<td>92</td>
<td>91</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
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<td>374</td>
<td>364</td>
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<td></td>
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<td>0</td>
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</table>

a T₀ or T₁ progenies are indicated by addition of a letter a or b, respectively.

b Regenerations indicate the times of regenerating operation.

c Number of tested seeds including germinated and not germinated seeds.

d Number of resistant or sensitive plants to 500 mg liter⁻¹ spectinomycin.

e Rate of spectinomycin resistant plants among total germinating plants.

f Transplastomic tobacco plant introduced with the aadA and gfp genes.
in the plastid genome is not affected by co-suppression caused by gene silencing (Dawson 1996), often a serious problem during the production of transgenic plants, especially using genes homologous to host plant genes. These advantages can lead to plant production of useful compounds usually produced by bacterial fermentation, and may also allow the improvement of conventional agricultural products. This system is supplied with materials and appropriate manufacturing conditions by the plant itself, based on solar energy, which could be called “phyto-fermentation” or “plasmic fermentation”. The application stage of genetically modified crops (Voelker et al. 1992, Gallardo et al. 1995, Shelton et al. 2000) is made much more feasible by the maternal inheritance pattern of transgenes contained within plastids, precluding their outward spread into the environment by pollen diffusion (Scott and Wilkinson 1999). This feature enables the safely programmed creation of new plants affording great benefits to human society with minimal ecological impact.

The artificial operon used in this study resulted in PHB productivity about 20-fold higher than the native bacteria-derived operon, due mainly to the use of a plastid-derived RBS to enhance translation. The PHB productivity reduced 5- to 10-times lower after the regeneration cycle or the culture in a greenhouse, which was, however, maintained in further regenerations and progenies. It has been reported that high levels of plastid accumulation of introduced protein can be achieved by the use of a suitable RBS sequence or by N-terminal addition of the final 14 amino acids of the GFP protein (Ye et al. 2001). These kinds of modifications, designed to stabilize or enhance the expression of the transgenes, will increase the efficiency of metabolic engineering. Much higher levels of PHB production (0.7% to up to 4% of fresh weight), with undesirable severe affection on plant growth, were reported in Arabidopsis plastids (Bohmert et al. 2000). This suggests that Arabidopsis, a member of the Brassica family, is more suitable for PHB production so far. For now, the plastid transformation technique is not easily applicable to plant species other than solanaceous plants (Sikdar et al. 1998, Sidorov et al. 1999, Ruf et al. 2001); however, in the near future, polycistronic metabolic engineering techniques will be developed for application to many other plant species.

Plant production of PHAs has been evaluated extensively with a focus on the economic potential of plastic production (Nawrath et al. 1994, Hahn et al. 1999, Nakashita et al. 1999).

**Discussion**

We have engineered tobacco plants to express enzymes constituting the PHB biosynthetic pathway by plastid transformation. The transplastomic plant accumulated the bacterial metabolite, PHB, in plastids as the result of the polycistronic expression of the introduced genes. To our knowledge, this is the first report presenting evidence at the molecular level for the establishment of metabolic engineering of higher plants by introduction of a bacteria-derived polycistron into a plastid genome. The plants obtained here are not homoplasmic, which may be evaluated as incomplete transplastomic plants. Nevertheless, the results obtained in this study showed the feasibility and difficulties of a new strategy for plant metabolic engineering. Plastid transformation with polycistronic genes is both time- and cost-effective, providing a source of well-regulated expression. In addition, the transgene in the plastid genome is not affected by co-suppression caused by gene silencing (Dawson 1996), often a serious problem during the production of transgenic plants, especially using genes homologous to host plant genes. These advantages can lead to plant production of useful compounds usually produced by bacterial fermentation, and may also allow the improvement of conventional agricultural products. This system is supplied with materials and appropriate manufacturing conditions by the plant itself, based on solar energy, which could be called “phyto-fermentation” or “plasmic fermentation”. The application stage of genetically modified crops (Voelker et al. 1992, Gallardo et al. 1995, Shelton et al. 2000) is made much more feasible by the maternal inheritance pattern of transgenes contained within plastids, precluding their outward spread into the environment by pollen diffusion (Scott and Wilkinson 1999). This feature enables the safely programmed creation of new plants affording great benefits to human society with minimal ecological impact.

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Plant production of PHAs has been evaluated extensively with a focus on the economic potential of plastic production (Nawrath et al. 1994, Hahn et al. 1999, Nakashita et al. 1999).
The value of plant PHA production as an alternative to plastics produced from petroleum sources is currently in dispute from both the practical and ecological points of view, including issues of production cost and CO₂ generation resulting from degradation (Gerngross 1999). Regardless of the result of this debate, however, this kind of technology will become necessary for society in the near future. It is important to evaluate the feasibility of PHAs as the source of a new type biomass like cellulose, starch and plant oil.

Homoplasmically transformed plants are important for the stable production of metabolites, but were not achieved during the course of this study. It has been reported that approximately half of all shoots grown during the second cycle of regeneration are homoplasmic (Svab and Maliga 1993, Maliga and Nixon 1998). Therefore, the plants obtained after regeneration cycles are expected to possess more than one copy of the transformed genome in each cell, and to be chimeric plants containing both transformed and non-transformed cells. This expectation is supported by the observation that T₁ progeny keep the introduced operon. It is reported that the plastid genome forms many types of structures; they are not only monomer to multimers with circular structure but also various sizes of linear, lasso-like fibers, and D-loop fibers structures (Lilly et al. 2001). Some unknown rearrangement should take part in conversion between these forms. Insertion of a large size of transgene used in this study might affect this rearrangement of plastid genomic DNA and resulted in the appearance of unknown fragments in Southern blot analysis (Fig. 3). Because the PHB productivity of the T₂ plans reduced after the cultivation on soil, some rearrangement mechanism occurred during the change of culture condition. Nevertheless, the productivity and the ratio of the inserted genome copies have been constant afterwards till in T₂ progenies. This suggests that the introduced genes exist stably in these lines, in some proportion depending on the size and the structure of the transgenes. On the other hand, it has been reported that PHB production in the cytosol or plastids of Arabidopsis reduced plant growth (Poirier et al. 1992, Bohmert et al. 2000). The accumulation of PHB in plastids may be lethal, disrupting the replication of plastids with high copies of the inserted genomes. This may be the reason for the small ratio of the inserted to non-inserted genome copies and also for the low PHB productivity. In addition, the accumulation of PHB seemed to influence embryo genesis and seed germination, resulting in a small number of plants. Some PHB-segregating progenies from segregating PHB-segregating populations have been reported (Poirier et al. 1992, Bohmert et al. 2000). The accumulation of polyester brought about by the introduced phb operon will be a useful tool to study not only the mechanism of fatty acid biosynthesis but also plastid genetics, including promoter function and RBS recognition. Polycistronic plastid expression will also be useful in the investigation of plastid biology, especially plastid gene regulation and function, differentiation into functionally specific types, and its evolution during endosymbiotic association with host cells. Also, it will be a potential technique to investigate secondary metabolism, including the biosynthesis of plant hormones.

Materials and Methods

Construction of plastid transformation vectors

The adaA gene, encoding aminoglycoside 3′-adenylytransferase, confers spectinomycin resistance on the tobacco nuclei and plastid (Svab and Maliga 1993). The adaA gene, attached to a synthetic RBS designed after the rbcL gene, was modified by PCR to possess BamHI and BglII restriction enzyme sites at the 5′- and 3′-ends, respectively. The PHB biosynthetic genes, phbA, phbB and phbC, were cloned from R. eutropha genomic DNA. A synthetic RBS sequence, 5′-GGGAGGAGGGG-3′, designed after the adaA gene, was added to each phb gene by PCR to facilitate their translation. Restriction enzyme sites were added to the phb genes as shown in Fig. 2. The primers used were as follows: 5′-ATCGGATTCTAGGGAGGGAATCATGGCGACC-GGCAAAAGGCGCG-3′ and 5′-AGCAAGTTTCTACATGGAAGGGAGGGGACCTG-3′ to modify phbC, 5′-ATCAAGCTTAAGGGAGGAACAATGACTGACGTTGTCATCG-3′ and 5′-AGGAATATCC-CTTGAATGAGGGAGGGACCTGACGTTGTCATCG-3′ to modify phbA, and 5′-ATCGGATTCTAGGGAGGGAATCATGGCGACC-GGCAAAAGGCGCG-3′ and 5′-AGAGAGATCCAGGGGACAATGAGGGAGGGGACCTGACGTTGTCATCG-3′ to modify phbB. The chimeric adaA and phb genes were connected in the same order as seen in Fig. 2 to generate an operon. The synthetic operon was inserted into a plastid transformation vector plasmid (Nakashita et al. 2001) to generate pPT06 (Fig. 2). In this plasmid, the operon is under control of the constitutive promoter of the plastid RNA operon (Prn) and the plastid psbA terminator sequence. This cassette was located between the SphI-BamHI rbcL fragment (0.8 kb) and the BamHI-Xhol (1.18 kb) fragment of the SphI-BamHI rbcL fragment.

Plant transformation

Tobacco plants (Nicotiana tabacum cv. Xanthi nc) were grown aseptically on agar solidified Murashige-Skoog (MS) medium (Murashige and Skoog 1962). Leaves were used for bombardment, according to the method described by Svab and Maliga (1993), using gold particles (1 μm) as a DNA carrier. Transgenic callic and shoots were selected on RMOM medium (Svab and Maliga 1993) containing 500 mg liter⁻¹ spectinomycin. Resistant shoots were then rooted on
MS medium containing 500 mg liter⁻¹ spectinomycin. An additional regeneration cycle was performed from the leaf sections of these rooted plants in the same selective medium. Transformants, rooted on MS medium, were transferred to soil, and cultured in a greenhouse.

Analysis of plastid genomic DNA

Total cellular DNA was extracted from the leaves of plants, PT2, PT2-1, PT3 and PT3-2, and used for Southern blot analysis. DNA digested with SpI or EcoRI was separated by electrophoresis through a 0.8% agarose gel, transferred to a Hybond N+ membrane (Amer sham), and then probed with a 32P-labeled 1.9-kb KpnI-XhoI fragment of wild-type plastid genomic DNA (Fig. 3a). The detection and quantification were performed with BAS2500 image analyzer (Fujiﬁlm).

Analysis of transcript by RT-PCR analysis

Total RNA was extracted from the leaves of rooted plants using Trizol reagent (Lifetechnologies). First-strand cDNA was synthesized directly from 4 μg RNA using random hexamers. To eliminate contaminations from double-stranded genomic DNA, cDNA preparations were treated with EcoRI or HindIII, located in the connecting region between phbA and phbB or between phbC and phbA, respectively, to digest the genomic DNA. RT-PCR was performed by using the following primer sets: for EcoRI digestion, 5′-ACCCCGAGACCTGG-ACCTGAT-3′ and 5′-CCCCGTACCCAGGAGATGGATGA-3′, and for HindIII digestion, 5′-CGACCTGGTGTTGGAACACT-3′ and 5′-CCATGATAGCTTCGCTC-3′. PCR products were used for a Southern blot that was probed with a 32P-labeled phb operon fragment (4.9 kb).

Analysis of polyester

Polyester was extracted from tobacco leaves as previously described (Nakashita et al. 1999). The samples were treated by ethanolysis and then analyzed by gas chromatography-mass spectrometry (GC-MS), performed by a GCMS-QP5050A (Shimazu) using an HPLC (Hewlett Packard) and scanning m/z 40 to 400. The samples were treated by ethanolysis and then analyzed by gas chromatography-mass spectrometry (GC-MS), performed by a GCMS-QP5050A (Shimazu) using an HPLC (Hewlett Packard) and scanning m/z 40 to 400.

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References


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