SYNTHESIS OF MEDIUM-CHAIN-LENGTH POLYHYDROXYALKANOATES IN ARABIDOPSIS THALIANA USING INTERMEDIATES OF PEROXISOMAL FATTY ACID 𝛽-OXIDATION

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ABSTRACT Polyhydroxyalkanoate (PHA) is a family of polymers composed primarily of R-3-hydroxyalkanoic acids. These polymers have properties of biodegradable thermoplastics and elastomers. Medium-chain-length PHAs (MCL-PHAs) are synthesized in bacteria by using intermediates of the 𝛽-oxidation of alkanic acids. To assess the feasibility of producing MCL-PHAs in plants, Arabidopsis thaliana was transformed with the PhaC1 synthase from Pseudomonas aeruginosa modified for peroxisomal targeting by addition of the carboxyl 34 amino acids from the Brassica napus isocitrate lyase. Immunocytochemistry demonstrated that the modified PHA synthase was appropriately targeted to leaf-type peroxisomes in light-grown plants and glyoxysomes in dark-grown plants. Plants expressing the PHA synthase accumulated electron-lucent inclusions in the glyoxysomes and leaf-type peroxisomes, as well as in the vacuole. These inclusions were similar to bacterial PHA inclusions. Analysis of plant extracts by GC and mass spectrometry demonstrated the presence of MCL-PHA in transgenic plants to approximately 4 mg per g of dry weight. The plant PHA contained saturated and unsaturated 3-hydroxyalkanoic acids ranging from six to 16 carbons with 41% of the monomers being 3-hydroxyoctanoic acid and 3-hydroxyoctenoic acid. These results indicate that the 𝛽-oxidation of plant fatty acids can generate a broad range of R-3-hydroxacyl-CoA intermediates that can be used to synthesize MCL-PHAs.

Polyhydroxyalkanoate (PHA) is a family of polymers composed primarily of R-3-hydroxyalkanoic acids (1–3). Polyhydroxybutyrate (PHB) is the most well-characterized PHA (4). High molecular weight PHB is found as intracellular inclusions in a wide variety of bacteria (2). In Alcaligenes eutrophus, PHB accumulates up to 80% of the dry weight (dwt), with inclusions being typically 0.2–1 μm in diameter. Small quantities of PHB oligomers of approximately 150 monomer units also are found associated with membranes of bacteria and eukaryotes, where they form channels permeable to calcium (4). High molecular weight PHAs have the properties of thermoplastics and elastomers. Numerous bacteria and fungi can hydrolyze PHAs to monomers and oligomers, which are metabolized as a carbon source. PHAs thus have attracted attention as a potential source of renewable and biodegradable plastics and elastomers. PHB is a highly crystalline polymer with rather poor physical properties, being relatively stiff and brittle (5). In contrast, PHA copolymers containing monomer units ranging from three to five carbons for short-chain-length PHA (MCL-PHA) are less crystalline and more flexible polymers (6).

PHB has been produced in Arabidopsis thaliana and cotton expressing the A. eutrophus PHB biosynthetic enzymes (6–8). In Arabidopsis expressing the PHB pathway in the plastids, leaves accumulated up to 14% PHB per g of dwt (7). High-level synthesis of PHB in plants opened the possibility of using agricultural crops as suitable systems for the production of PHAs on a large scale and at low cost (3, 9, 10). PHB also was shown to be synthesized in insect cells expressing a mutant fatty acid synthase (11), and in yeast expressing the A. eutrophus PHB synthase (12).

A number of pseudomonads, including Pseudomonas putida and P. aeruginosa, accumulate MCL-PHAs when cells are grown on alkanic acids (1–3). The nature of the PHA produced is related to the substrate used for growth and typically is composed of monomers that are 2n (n ≥ 0) carbons shorter than the substrate. These studies indicate that MCL-PHAs are synthesized by the PHA synthase from 3-hydroxyacyl-CoA intermediates generated by the 𝛽-oxidation of alkanic acids (13, 14). To explore whether intermediates of 𝛽-oxidation of fatty acids could be used in MCL-PHA synthesis in plants, we have targeted the phaC1 synthase from P. aeruginosa to peroxisomes of transgenic Arabidopsis. We report here that plants expressing a P. aeruginosa PHA synthase modified for peroxisome targeting produce PHA containing saturated and unsaturated 3-hydroxyalkanoic acids ranging from six to 16 carbons. PHA inclusions are found within the glyoxysomes and leaf-type peroxisomes of dark- and light-grown plants, respectively, as well as in the vacuole.

MATERIALS AND METHODS

Plant Material. Arabidopsis thaliana, race Columbia, was transformed by the vacuum infiltration method (15) using the Agrobacterium tumefaciens strain GV3101. Transformants were selected on media containing Murashige and Skoog salts, 1% sucrose, 0.7% agar, and 50 μg/ml of kanamycin. Kanamycin-resistant plants subsequently were transferred to soil and grown under continuous fluorescent light at 19°C. In some experiments, plants were grown under constant agitation (100 rpm) for 1–2 weeks in liquid media containing Murashige and Skoog salts and 2% sucrose.

DNA Constructs. The phaC1 gene from P. aeruginosa was amplified by PCR from the plasmid pKS-::PP750EP using oligonucleotides modifying the 5’ and 3’ ends of the gene (16).

Abbreviations: PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; MCL-PHA, medium-chain-length PHA; ICL, isocitrate lyase; PTS, peroxisomal targeting sequence; MS, mass spectrometry; dwt, dry weight.

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At the 5’ end, the second codon AGT was modified to AGC to conform more closely with the general codon preferences of *A. thaliana* (17). At the 3’ end, the stop codon was deleted to fuse the PHA synthase with the last 34 amino acids of the *Brassica napus* isocitrate lyase (ICL) (18). The resulting modified *phaCl* gene was subcloned into the *EcoRI*-XbaI sites of pART7, followed by subcloning into the binary vector pART27 (19).

**Immunolocalization.** Whole plants were fixed for 2 hr at room temperature in 4% formaldehyde, 0.5% glutaraldehyde, and 50 mM sodium cacodylate, pH 7.3. The tissue samples were dehydrated in an ethanol series and embedded in LR White resin. Ultra-thin sections were mounted on formvar-coated gold grids and blocked with 0.8% BSA, 0.1% gelatine, 5% normal goat serum, and 2 mM sodium azide in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Grids were incubated for 1 hr at room temperature with antisera against PHA synthase (1:50), glycolate oxidase (1:2,000), or ICL (1:1,000) in the blocking solution followed by a 4-hr incubation at room temperature with a 1:50 dilution of gold-conjugated goat anti-rabbit antibodies (15 nm gold particles) in PBS. Immunolabeled sections were double-stained with uranyl acetate and lead citrate and viewed with a JEOL JEM transmission electron microscope.

**PHA Extraction and Analysis.** Fresh or dried frozen plant material was ground in a mortar and lyophilized. The powder was extracted with methanol in a Soxhlet apparatus for 24 hr followed by PHA extraction with chloroform for 24 hr. The PHA-containing chloroform was concentrated by using a Rotovap and extracted once with water to remove residual solid particles. PHA was precipitated by the addition of 10 vol of cold methanol and subsequently washed by two cycles of chloroform solubilization and methanol precipitation. The purified PHA dissolved in chloroform as well as the residual plant material remaining after Soxhlet extraction were transferred and lyophilized. The purified PHA was dissolved in chloroform and analyzed by GC as described.

**RESULTS**

**Targeting the *P. aeruginosa* PHA Synthase to Plant Peroxisomes.** To target the PHA synthase from *P. aeruginosa* to peroxisomes of *Arabidopsis* cells, the carboxyl end of the protein was modified by adding the terminal 34 amino acids of ICL from *B. napus*. The terminal three amino acids of ICL are Ala-Arg-Met and represent a variant of the consensus peroxisomal targeting sequence (PTS) Ser-Lys-Leu (20). It has been shown previously that fusion of the carboxyl-terminal five amino acids of ICL to chloramphenicol acetyltransferase is sufficient to target the passenger protein to the peroxisomes in leaves and glyoxysomes in cotyledons of *Arabidopsis* (18). The fusion protein was shown to retain PHA synthase activity in bacteria by its ability to restore PHA synthesis when expressed in a PHA accumulation-deficient mutant of *P. putida* KT2440 NK2.3 (data not shown). The modified *phaCl* gene was subcloned into the binary vector pART27, putting the gene under the transcriptional control of the constitutive CaMV 35S promoter.

**Arabidopsis** primary transformants initially were screened by Western analysis for expression of the modified PHA synthase. In several *phaCl*-transformed plants, a band of 65 kDa was detected with anti-PHA synthase antibodies that was absent in plants transformed with the pART27 binary vector (data not shown). Transgenic line 3.3 was chosen for further studies as it proved to have a stable level of PHA synthase expression. Transgenic plants expressing the PHA synthase were vigorous and had a normal seed set.

Immunocytochemistry was performed to determine whether the modified PHA synthase expressed in transgenic plants was localized in peroxisomes in light-grown plants and glyoxysomes in dark-grown plants. Leaf-type peroxisomes from plants grown for 7 days in the light could be identified with anticytochrome oxidase antibodies in both vector-transformed line 21 and *phaCl*-transformed line 3.3 (Fig. 1A and B, respectively). Electron-lucent inclusions of 0.1–0.2 μm in diameter were present within peroxisomes from line 3.3 but not from the vector control. In plants grown for 1 day in the light followed by 6 days in the dark, the presence of glyoxysomes was revealed by the anti-ICL antibodies in both lines 21 and 3.3 (Fig. 1C and D, respectively). The presence of numerous electron-lucent inclusions filling the glyoxysomes of transgenic plants expressing the modified PHA synthase was also clearly evident (Fig. 1D). Anti-PHA synthase antibodies revealed that the PHA synthase was present in *phaCl*-transformed line 3.3 within the glyoxysomes of dark-grown plants and peroxisomes of light-grown plants, and localized with the inclusions (Fig. 1F and G, respectively). No reaction with any subcellular structures could be detected with the anti-PHA synthase antibodies in vector-transformed plants (Fig. 1E). In some cells, inclusions also could be detected in the vacuoles (Fig. 1H and I). These vacuolar inclusions were labeled with anti-PHA synthase antibodies but not with anti-ICL antibodies, indicating the presence of PHA synthase (Fig. 1I). The vacuolar inclusions tended to be more heterogeneous in size than those in glyoxysomes and peroxisomes, with some vacuolar inclusions reaching 1 μm. Taken together, these results show clearly that the modified PHA synthase is targeted to peroxisomes in light-grown plants and to glyoxysomes of dark-grown plants. Furthermore, expression of the PHA synthase in these organelles is associated with the presence of inclusions that are similar in size and appearance to PHA inclusions found in bacteria and PHB inclusions found in transgenic plants expressing the PHB biosynthetic enzymes from *A. eutrophus* (6–9). In the vacuole of *phaCl*-transformed plants, inclusions are found associated with the PHA synthase. This association parallels the situation found in bacteria where PHA synthase is found on the surface of inclusions (21).

**Synthesis of PHA in Transgenic Plants.** To confirm the presence of PHA in transgenic plants expressing the peroxisome-targeted PHA synthase, plants homozygous for the *phaCl* genes (line 3.3) or the pART27 binary vector (line 21) were grown in liquid media. Typically, approximately 0.5 g of seeds was sterilized and grown for 13–21 days. Approximately 10–20 g of dry plant material were obtained and extracted for PHA. Analysis by GC-MS of the methanol trans-esterified chloroform extracts from *phaCl*-transformed plants revealed numerous peaks that were undetectable in extracts from vector-transformed plants (Fig. 2A and C). All novel peaks were found by mass spectra to be derivatives of 3-hydroxyacids based on the detection of the predominant ion with the mass-to-charge ratio value of 103 (Fig. 2B and D). This mass-to-charge ratio corresponds to the methyl-esterified 3-hydroxypropionic acid fragment common to all saturated 3-hydroxyalkanoic acids and unsaturated 3-hydroxyalkanoic acids with double bonds beyond carbon 3. Chloroform extracts from vector-transformed plants did not have any significant peaks detected.
possessing the mass-to-charge ratio value of 103 (Fig. 2D). Five peaks had mass spectra that were identical to standards of 3-hydroxyhexanoic acid (H6:0), 3-hydroxyoctanoic acid (H8:0), 3-hydroxydecanoic acid (H10:0), 3-hydroxydodecanoic acid (H12:0), and 3-hydroxytetradecanoic acid (H14:0). The identity of other peaks in plants extracts was based on the

Fig. 1. Immunolocalization of PHA synthase in the peroxisomes. Sections were prepared from cotyledons of phaC1-transformed line 3.3 (B, D, and F–I) and vector-transformed line 21 (A, C, and E) grown for 7 days in the light (A, B, and G) or 1 day in the light followed by 6 days in the dark (C–F, H, and I). Sections were incubated with antibodies against the glycolate oxidase (A and B), ICL (C and D), or PHA synthase (E–I). (H and I) Inclusions found in the vacuole. Bars indicate 500 nm and are white for light-grown cotyledons and black for dark-grown cotyledons. (Insets, B, D, and G) Magnification of inclusions and immuno-gold particles (×50,000).
comparison between their mass spectra and those of standard 3-hydroxyalkanoic acids as well as on the comparison with monomers found in PHA isolated from P. putida grown on hydrolyzed linseed oil and in which the monomer composition was determined by GC-MS as well as proton-detected multiple bond coherence NMR (22). These analyses revealed the presence of the following additional monomers in phaC1-transformed plants: 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxyhexadecenoic acid (H16:1), 3-hydroxyhexadecanoic acid (H16:0), 3-hydroxytetradecatrienoic acid (H14:3), 3-hydroxytetradecadienoic acid (H14:2), 3-hydroxytetradecenoic acid (H14:1), 3-hydroxydodecadienoic acid (H12:2), 3-hydroxydodecenoic acid (H12:1), and 3-hydroxyoctenocenoic acid (H8:1). Trace amounts of 3-hydroxynonanoic acid (H9:0), 3-hydroxydecanoic acid (H10:0), 3-hydroxyundecenoic acid (H11:0), and 3-hydroxystearic acid (H18:0) also were detected in phaC1-transformed plants. The position of double bonds within the unsaturated monomers could not be precisely determined by mass spectra and therefore was not assigned. Analysis of the residual plant powder remaining after Soxhlet extraction showed that approximately 60–75% of the PHA remained associated with the plant material (data not shown). The relative amount of monomers present in the chloroform-extractable PHA is presented in Table 1.

The accumulation of PHA was analyzed at different growth stages (Fig. 3). The highest amount of PHA was detected in 7-day-old seedlings, with approximately 4 mg/g of dwt. The amount of PHA in leaves of 30-day-old plant decreased to approximately 0.2 mg/g of dwt, whereas PHA increased to 0.9 mg/g of dwt in senesced leaves.

PHA purified from plants grown for 2 weeks in liquid media had a weight-average molecular weight (Mw) of 23,700, a number-average molecular weight (Mn) of 5,500 and a polydispersity (Mw/Mn) of 4.3. In contrast, PHA synthesized in P. putida grown on octanoic acid had a Mw of 151,500, Mn of 74,700, and polydispersity of 2.0. Thus, purified plant MCL-PHA had a significantly lower molecular weight and broader distribution compared with bacterial MCL-PHA.

**DISCUSSION**

Targeting of several proteins to the matrix of peroxisomes has been shown to rely on a tripeptide located at the extreme carboxyl terminus, referred to as PTS1 sequences (20). Addition of PTS1 sequences to carrier proteins has been shown in several cases to be sufficient for peroxisomal targeting of proteins in yeast, mammals, and plants (18, 20, 23). The B. napus ICL contains a PTS1 sequence, the terminal amino acids being Ala-Arg-Met. It previously has been shown that addition of the last five or 35 amino acids of ICL to chloramphenicol acetyltransferase (CAT) was sufficient for efficient targeting of the protein to peroxisomes in Arabidopsis (18). Furthermore, import of CAT was found in leaf-type peroxisomes containing the enzymes for photorespiration, as well as in glyoxysomes, which are the peroxisomes present in postgerminative seedlings and contain the enzymes of the glyoxylic cycle. Addition of the last 34 amino acids of the B. napus ICL to the P. aeruginosa PHA synthase was sufficient to direct the protein to the peroxisomes. In light-grown plants, the modified PHA synthase was localized in leaf-type peroxisomes expressing the glycolate oxidase. In dark-grown plants, the same enzyme was localized in glyoxysomes expressing the ICL. These results bring further support to the notion that leaf-type peroxisomes and glyoxysomes have similar import machinery and are able to import PTS1-containing proteins.

Transgenic plants expressing the PHA synthase in the peroxisomes were producing PHAs. This finding is demonstrated by both the appearance of PHA inclusions in cells as well as analysis of chloroform-soluble material by GC-MS. In both light- and dark-grown plants, the localization of the PHA synthase in the peroxisomes is associated with the presence of electron-lucent inclusions in the organelle. These inclusions were approximately 0.1–0.2 μm in diameter and were similar in size and appearance to PHA inclusions found in bacteria. Such inclusions were never found in control transgenic plants transformed with the binary vector only. Inclusions also were found in vacuoles of plants expressing the PHA synthase in the

**Table 1.** Monomer composition of chloroform-extractable PHA isolated from phaC1-transformed plant grown in liquid media

<table>
<thead>
<tr>
<th>Monomer</th>
<th>H6</th>
<th>H8</th>
<th>H8:1</th>
<th>H10</th>
<th>H12</th>
<th>H12:1</th>
<th>H12:2</th>
<th>H14</th>
<th>H14:1</th>
<th>H14:2</th>
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<td>%, w/w</td>
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<td>23</td>
<td>18</td>
<td>4.7</td>
<td>5.8</td>
<td>4.3</td>
<td>5.0</td>
<td>4.2</td>
<td>6.7</td>
<td>7.5</td>
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<td>2.0</td>
<td>2.0</td>
<td>5.6</td>
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<tr>
<td>SD</td>
<td>0.16</td>
<td>4.4</td>
<td>4.6</td>
<td>0.51</td>
<td>0.46</td>
<td>0.60</td>
<td>1.3</td>
<td>1.1</td>
<td>2.3</td>
<td>1.4</td>
<td>3.2</td>
<td>0.26</td>
<td>0.41</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Quantification of methyl esters was done on a GC with a flame ionization detector. Values were obtained from four separate PHA preparations from plants grown in liquid media for 13–21 days. Monomers present in trace amounts (H9:0, H10:1, H11:0, H13:0, and H16:1) were not quantified.
glyoxysomes. Immunocytochemistry demonstrated that the PHA synthase was associated with these vacuolar inclusions. In bacteria, the PHA synthase also is found associated with the inclusions (21). In previous studies it has been shown that transgenic Arabidopsis expressing the PHB biosynthetic pathway in the cytoplasm accumulated inclusions in the cytoplasm, vacuole, and nucleus (6, 9). The presence of PHA granules in several subcellular compartments within the same transgenic plant is thought to reflect the ability of the hydrophobic PHA granules to move through single membrane-bound organelles, such as the vacuole and peroxisome, instead of representing the simultaneous targeting of the PHA synthase to various organelles (6, 9).

In bacteria and plants, PHAs are composed of the R isomer of 3-hydroxyalkanoic acids because of the stereospecificity of the PHA synthase (1, 24, 25). Because β-oxidation of fatty acids in bacteria, mammals, and plants generates mainly the S isomer of 3-hydroxyacyl-CoAs from the hydration of enoyl-CoAs by the enoyl-CoA hydratase I (26, 27), synthesis of MCL-PHAs in these organisms implicates the presence of enzymes that can convert intermediates of β-oxidation to R-3-hydroxyacyl-CoAs. In bacteria synthesizing MCL-PHAs from alkanolic acids, R-3-hydroxyacyl-CoAs could be generated by a 3-hydroxyacyl-CoA epimerase, a R-specific enoyl-CoA hydratase II, or a R-specific ketoacyl-CoA reductase (2).

In yeast, β-oxidation normally occurs through R-3-hydroxyacyl-CoA intermediates, because this organism has only an enoyl-CoA hydratase II instead of an enoyl-CoA hydratase I (28). Our finding that plants expressing the peroxisomal PHA synthase produce MCL-PHAs with a whole range of saturated and unsaturated 3-hydroxyacids also suggests that plants have an enzyme capable of generating a broad range of R-3-hydroxyacyl-CoAs. Engeland and Kindl (29) have demonstrated the presence in cucumber seedlings of a homodimeric peroxisomal protein of 65 kDa having enoyl-CoA reductase activity (30). This protein is called the multifunctional protein of cucumber since it contains a domain having both enoyl-CoA hydratase II and R-3-hydroxyacyl-CoA epimerase activity (30). Both enoyl-CoA hydratase II and R-3-hydroxyacyl-CoA epimerase activities therefore could be implicated in the synthesis of a broad range of R-3-hydroxyacyl-CoAs in plants, which, in turn, could be used for synthesis of MCL-PHAs (Fig. 4).

β-Oxidation of unsaturated fatty acids with cis double bonds at the even-numbered carbon using the enoyl-CoA hydratase I generates R-3-hydroxyacyl-CoAs from 2-cis-enoyl-CoAs. This step represents a metabolic block because the R isomer of 3-hydroxyacyl-CoAs cannot be further metabolized by the S-3-hydroxyacyl-CoA dehydrogenase activity of the multifunctional protein. To circumvent this block, two pathways have been proposed (26). In one pathway, 4-cis-enoyl-CoA is metabolized by one cycle of the β-oxidation pathway to 2-cis-acyl-CoA, which then is hydrated to R-3-hydroxyacyl-CoA by the enoyl-CoA hydratase I. The conversion of R-3-hydroxyacyl-CoA to the S isomer is mediated either by the 3-hydroxyacyl-CoA epimerase or the combination of the reverse reaction of the enoyl-CoA hydratase II with the forward reaction of the enoyl-CoA hydratase I. In a second pathway, the 4-cis-enoyl-CoA first is oxidized by the acyl-CoA oxidase to 2-trans, 4-cis-dienoyl-CoA, converted to 2-trans-enoyl-CoA by a 2,4-dienoyl-CoA reductase, and further metabolized to 2-trans-enoyl-CoA by a Δ5,Δ2-enoyl-CoA isomerase. In contrast to the first pathway, the dienoyl-CoA reductase and isomerase pathway does not directly generate R-3-hydroxyacyl-CoAs, which could be used by the PHA synthase. Analysis of the monomer composition of MCL-PHAs in P. putida grown in oleic acids and linoleic acids showed that 3-hydroxyacyl-CoAs derived from the dienoyl-CoA reductase pathway were used in PHA synthesis (31). In plants, the activity of the dienoyl-CoA reductase is significantly lower than the enoyl-CoA hydratase II, indicating that the pathway through R-3-hydroxyacyl-CoAs may be prevalent (30).

In Arabidopsis, trienoic and dienoic fatty acids with an unsaturated bond at an even position (18:3, cis-9, 12, 15; 16:3, cis-7, 10, 13; 18:2, cis-9, 12; 16:2, cis-7, 10) represent approximately 50% and 80% of all fatty acids present in the seed and leaf, respectively (32). β-Oxidation of these fatty acids through the enoyl-CoA hydratase II/enoyl-CoA epimerase pathway therefore would generate a substantial amount of R-3-hydroxyoctenoyl-CoA (from 18:3 and 16:3) and R-3-hydroxyoctanoyl-CoA (from 18:2 and 16:2), which could be directly incorporated into MCL-PHAs without further modifications. In this perspective, it is striking that analysis of plant MCL-PHAs reveals that the major monomers present are 3-hydroxyoctenoic and 3-hydroxyoctanoic acids (Table 1), because expression of the P. aeruginosa PhaC1 synthase in its natural host or in Escherichia coli resulted in the synthesis of MCL-PHA with 3-hydroxydecanoic acid as the major monomer (33, 34). This result supports the hypothesis that, in Arabidopsis, β-oxidation of unsaturated fatty acids with cis double bonds at the even carbon involves the generation of R-3-hydroxyacyl-CoAs. It is also possible, how-
ever, that the specific activity of the plant enoyl-CoA hydratase I and S-3-hydroxyacyl-CoA dehydrogenase toward the β-oxidation intermediates may contribute, at least in part, to differential substrate availability to the PHA synthase and to the high H8:0 and H8:1 monomer content in the plant PHAs.

MCL-PHA synthesized in transgenic Arabidopsis has a lower molecular weight compared with bacterial PHAs. It is possible that a high level of PHA synthase relative to substrate availability in plant peroxisomes may result in a low molecular-weight polymer. A correlation between high PHA synthase activity and low PHB molecular weight has been described before in recombinant E. coli (35). Taking into consideration that the majority of the PHA remained associated with the plant material after Soxhlet extraction, it is possible that the “chloroform-extractable” PHA may be of lower molecular weight as compared with the PHA that remains associated with the plant material.

The highest amount of PHA was found in germinating seedlings (Fig. 3). The PHA content rose from 0.1 mg/g of dwt in seeds to 4 mg/g of dwt in 7-day-old germinating seedlings. During vegetative growth, PHA level in leaves decreased to 0.2 mg/g of dwt whereas it rose to 0.9 mg/g of dwt during leaf senescence. This pattern of PHA accumulation correlates with the activity of the β-oxidation cycle throughout plant development (27). The β-oxidation cycle is strongly induced upon seed germination, being involved in the mobilization of reserve lipids. It is, thus, at this stage that the highest amount of PHA is expected to be synthesized. After the establishment of photosynthesis and during vegetative growth, β-oxidation is present only at a very low level. Thus, while plant biomass increases considerably, PHA accumulation is expected to be minimal, resulting in a relative decrease of PHA amount when expressed per g of plant material. Upon senescence, β-oxidation is reactivated and thus more PHA is expected to be synthesized in these tissues. Together, these data indicate that the amount of MCL-PHA synthesized in transgenic plants expressing the PHA synthase in peroxisomes is modulated by the flux of fatty acids to the β-oxidation cycle.

The synthesis of MCL-PHAs in Arabidopsis demonstrates that a whole spectrum of useful polymers can be produced in plants, ranging from PHB, a stiff and brittle thermoplastic, to flexible and sticky elastomers such as MCL-PHAs. Furthermore, the incorporation of β-oxidation intermediates into plant PHA could be used as a tool to dissect the biochemical pathways involved in plant fatty acid degradation.

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