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ORIGINAL ARTICLE

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Expression of poly-3-(R)-hydroxyalkanoate (PHA) polymerase and acyl-CoA-transacylase in plastids of transgenic potato leads to the synthesis of a hydrophobic polymer, presumably medium-chain-length PHAs

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Abstract Medium-chain-length poly-3-(*R*)-hydroxyalkanoates (mcl-PHAs) belong to the group of microbial polyesters. The minimum gene-set for the accumulation of mcl-PHAs from de novo fatty acid biosynthesis has been identified in prokaryotes [B. Rehm et al. (1998) J. Biol Chem 273:24044–24051] as consisting of the Pha-C1 polymerase and the ACP-CoA-transacylase. In this paper, the synthesis of mcl-PHAs has been attempted in transgenic potato (*Solanum tuberosum* L.) using the same set of genes that were introduced into potato by particle bombardment. Polymer contents of transgenic lines were analysed by gas chromatography and by a new simple method employing a size-exclusion filter column. The expression of the Pha-C1 polymerase and the ACP-CoA-transacylase in the plastids of transgenic potato led to the synthesis of a hydrophobic polymer composed of mcl-hydroxy-fatty acids with carbon chain lengths ranging from C-6 to C-12 in leaves of the selected transgenic lines. We strongly suggest that the polymer observed consists of mcl-PHAs and that this report establishes for the first time a possible route for the production of mcl-PHAs from de novo fatty acid biosynthesis in plants.

Keywords ACP-CoA-3-(*R*)-hydroxyalkanoate transacylase · Co-transformation · Medium-chain-length poly-3-(*R*)-hydroxyalkanoates · Pha-C1 polymerase · *Solanum tuberosun*

Abbreviations ACP: Acyl carrier protein · CoA: Coenzyme A · FAB: Fatty acid biosynthesis · mcl-PHAs: Medium-chain-length poly-3-(*R*)-hydroxyalkanoates · 35-S: Cauliflower Mosaic Virus 35 S promoter · TPrbcS: Small subunit of ribulose bisphosphate carboxylase transit peptide

Introduction

Medium-chain-length poly-3-(*R*)-hydroxyalkanoates (mcl-PHAs) are polyesters of 3-(*R*)-hydroxy fatty acids with carbon chains ranging from C-6 to C-14, which are produced by a large number of fluorescent pseudomonads (Huisman et al. 1989; Timm and Steinbüchel 1990; Madison and Huisman 1999). The key enzyme for mcl-PHA biosynthesis is PHA-polymerase, which catalyses the esterification of the hydroxy group at the third carbon with the carboxy group of a separate coenzyme A (CoA)-activated 3-(*R*)-hydroxy fatty acid.

In their natural host, mcl-PHAs can be accumulated using intermediates derived from β-oxidation or from

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fatty acid biosynthesis (FAB). In the former case, fatty acids enter *β*-oxidation and 3-(*S*)-hydroxyacyl-CoA intermediates are converted into the enantiomer 3-(*R*)-hydroxyacyl-CoA, which is the precursor for mcl-PHA synthesis, by the action of a hydratase, an epimerase, or a reductase (reviewed by Madison and Huisman 1999). Under imbalanced conditions (limiting ammonia and excess of carbon), *Pseudomonas putida* and *P. aeruginosa* may accumulate mcl-PHAs from non-related carbon sources (i.e. sucrose, gluconate, acetone; Timm and Steinbüchel 1990; Huijberts et al. 1992), and precursors for PHA biosynthesis are provided by FAB. FAB normally employs the -(*R*)-enantiomer of 3-hydroxyacyl moieties. However, in this case, thio-ester activation takes place via the ACP, but not via CoA. Mutant strains of *P. putida* that were not able to accumulate mcl-PHAs from non-related sources led to the identification and cloning of the *phaG* gene, coding for an ACP-CoA-hydroxyacyl transacylase (Rehm et al. 1998). The analogous gene was subsequently cloned in *P. aeruginosa* (Hoffmann et al. 2000b). This enzyme catalyses the transfer of the 3-hydroxyacyl moiety from ACP to CoA (Rehm et al. 1998). Expression of the *phaG* gene in a number of recombinant prokaryotic hosts led to the accumulation of mcl-PHAs from FAB (Fiedler et al. 2000; Hoffmann et al. 2000a).

In a previous study, the Pha-C1 polymerase from *P. oleovorans* was successfully expressed in the cytoplasm of transgenic potato lines (Romano et al. 2003b). Evidence of enzyme activity was obtained after cell suspension cultures derived from transgenic potato lines expressing the Pha-C1 polymerase were fed with 3-(*R*)-hydroxyoctanoate (Romano et al. 2003b). In the present work, the synthesis of mcl-PHAs was studied in transgenic potato (*Solanum tuberosum* L.) using the set of genes known from prokaryotes (Rehm et al. 1998). The *Pseudomonas oleovorans* Pha-C1 polymerase (*phaC1* gene product) in combination with the *P. putida* ACP-CoA-transacylase (*phaG* gene product) was introduced by particle bombardment-mediated co-transformation into potato, and transgenic lines expressing the candidate genes were analysed.

Materials and methods

Plant material and transformation

In vitro-grown *Solanum tuberosum* L., genotype 1024-2 (*amf*, diploid; Jacobsen et al. 1989—Laboratory of Plant Breeding, Wageningen University & Research Centre, Wageningen) was used as starting material for particle bombardment-mediated co-transformation as described elsewhere (Romano et al. 2001, 2003a, 2003c). Untransformed and transgenic plants were cultivated in pots at 20°C, and a 16-h photoperiod with light supplied at an intensity of 150 mol photons m^{-2} s^{-1}.

DNA constructs

Plasmid 35-S-Kan containing the *nptII* selectable marker under the control of the Cauliflower Mosaic Virus 35 S promoter (35-S) and terminator was kindly made available by the John Innes Centre (Norwich, UK). The *phaG* gene was cloned by PCR [*Pyrococcus woesei* (*Pwo*) DNA polymerase] from genomic DNA of *P. putida* KT2442. Primers 5′-CCATGGGGCCAGAAATCG-CTGTACTTG-3′ and 5′-CGGGATCCTCAGATGG-CAAATGCATGC-3′ were used, which introduced, by primer extension, *Nco*I and *Bam*HI restriction sites at the 5′ and 3′ ends of the gene, respectively. The PCR product was cloned into the pGEM-T-easy vector (Promega), sequenced and subcloned into the *Nco*I and *Bam*HI sites of pAMV-1 (Rouwendal et al. 1997). TPrbsS was subsequently cloned upstream of the *phaG* gene and the *P. oleovorans phaC1* gene. The *Nco*I fragment containing the TPrbsS was amplified from genomic DNA of *S. tuberosum* cv. Saturna by G. Rouwendal et al. (Plant Research International Wageningen, personal communication) using primers 5′-GCGGA-CCATGGCTTCCTCAATTGTC-3′ and 5′-CTCCGC-CATGGGCCACACCTGCAT-3′ and was cloned into the *Nco*I sites of pAMV-1-*phaG* and pAPP62. The latter plasmid was constructed by cloning the *phaC1* gene into plasmid pAMV-1 (described in Romano et al. 2003b). *Bgl*II-*Bam*HI fragments from plasmids containing *amv*-TPrbcS-*phaC1* and *amv*-TPrbcS-*phaG* were cloned into pAPP23 (Romano et al. 2001) containing the enhanced 35-S (E-35-S), nopaline synthase (*nos*) terminator, and giving rise to the plant expression vectors pAPP100 and pAPP101, respectively.

PCR and Southern blot analyses

The DNA used as template for PCR analyses was extracted from plant leaf material using the Sigma GeneElute KIT according to the manufacturer's recommendations. PCR was performed with RedTaq (Sigma) as described by the manufacturer.

Genomic DNA for Southern blotting was isolated from leaf material using the CTAB protocol (Rogers and Benich 1994), digested and blotted on a positively charged nylon membrane using the Turboblotter system (Schleicher and Schuell) and standard molecular-biology techniques (Sambrook et al. 1989). Filters were hybridised with digoxigenin-labelled probes as described by the manufacturer (Boehringer, Mannheim, Germany).

Reverse transcription (RT)–PCR and Northern blot analyses

RNA was extracted as described elsewhere (Romano et al. 2003a). C-DNA was synthesised using the Superscript system (GibcoBRL) according to the manufacturer's recommendations, and 6 µl of cDNA solution was used for PCR amplification.

For northern blot analysis, 15 µg of total RNA was separated in a 2% agarose gel and blotted onto a positively charged nylon membrane using the Turboblotter system (Schleicher & Schuell). Filters were hybridised with digoxigenin-labelled probes as described by the manufacturer (Boehringer) and using standard techniques (Sambrook et al. 1989).

Polymer isolation and analyses

Leaf material, from plants grown in vitro or in pots, was ground in liquid nitrogen and freeze-dried. The lyophilised powder was washed with ethanol at 55°C for 48 h to remove fatty acids and chlorophylls, and the hydrophobic polymer was eventually solubilised from the defatted powder with chloroform at 55°C for 48 h. The washing and solubilisation steps were performed by shaking powder and solvents (500 ml) in glass flasks. During each step, the solvent was refreshed 3 times. The volume of the chloroform fraction containing hydrophobic compounds was reduced with a rotavapor, and the concentrated solution was analysed by gas chromatography (GC) after methanolysis (Lageveen et al. 1988). Methylesters were analysed by GC using a Carlo-Erba GC6000 apparatus equipped with a 25 m CP-Sil5CB capillary column (Chrompack).

Peaks corresponding to the monomeric units of the extracted polymer were identified by comparing their retention times with those of the monomers present in pure mcl-PHAs isolated from *P. putida* KT2442 (monomer composition shown in Table 1). Polymer quantities were determined from monomer peak areas in GC analysis. Separation of the "polymer-containing" fraction from other lipid and monomeric components was achieved using an Ultrafree CL filter column with Ultracel-PL membrane, nominal molecular weight limit (NMWL) 5,000 (Millipore), following the manufacturer's recommendations, and adapted to the purpose of polymer separation. In short, clarified plant extracts containing the polymer were resuspended in 2 ml of acetone, applied to the column and centrifuged at 5,000 *g* until the entire solution passed through the filter (approximately 10 h). Filters were washed 4 times with 2 ml of acetone and the "polymer-containing" phase (i.e. the fraction retained by the filter) and the "monomer-containing" phase (i.e. the fraction that passed through the filter) were finally resuspended in chloroform, methanolysed and analysed by GC. A series of controls were used to optimise the performance of the filter columns: pure mcl-PHA control consisting of polymer isolated from *P. putida* KT2442; pure monomer consisting of 3-(*R*)-hydroxyoctanoate obtained as described elsewhere (de Roo et al. 2002) and kindly provided by Guy de Roo; a mix of polymer and monomer.

Results

Cloning of the *phaG* gene from *P. putida* KT2442

The *phaG* gene was cloned from *P. putida* strain KT2442 by PCR using primers designed on the *phaG* sequence of *P. putida* KT2440 (Rehm et al. 1998). *P. putida* KT2442 also accumulates mcl-PHAs from FAB (Huijberts et al. 1992). The cloned 888-bp DNA sequence codes for a

Table 1 Yields and monomer composition of the polymer accumulated by transgenic potato (*Solanum tuberosum*) lines, and mcl-PHAs accumulated by other recombinant and natural hosts using intermediates from FAB. Monomers derived from FAB are in *bold*. The presence of unsaturated monomers in *Pseudomonas oleovorans*, *P. fragi* and *P. putida* KT2442 results from the contribution of *β*-oxidation to PHA biosynthesis. *% PHAs*, mg of polymer/100 mg of dry mass; *H6*, 3-(*R*)-hydroxyhexanoic acid; *H8*, 3-(*R*)-hydroxyoctanoic acid; *H8:1*, 3-(*R*)-hydroxyoctenoic acid; *H10*, 3-(*R*)-hydroxydecanoic acid; *H12*, 3-(*R*)-hydroxydodecanoic acid; *H12:1*, 3-(*R*)-hydroxydodecenoic acid; *H12:2*, 3-(*R*)-hydroxydodecandienoic acid; *H14*, 3-(*R*)-hydroxytetradecanoic acid; *H14:1*, 3-(*R*)-hydroxytetradecenoic;*nd*, not detectable; –, not determined

Host	%PHA	**H6**	**H8**	H8:1	**H10**	**H12**	H12:1	H12:2	**H14**	H14:1
Potato line PB005.10	0.026	**2.5**	**29.7**	0	**46.8**	**20.9**	nd	nd	**nd**	–
Potato line PB005.11	0.0017	**1.3**	**42.1**	0	**36.7**	**20.2**	nd	nd	**nd**	–
P. aeruginosa KO1[a]	14.6	**1.8**	**47.2**	–	**45.9**	**5.1**	–	–	**–**	–
P. oleovorans[b]	46	**1**	**7.5**	–	**78**	**13.5**	–	–	**–**	–
P. putida PHAG$_N$-21[c]	50	**3.1**	**14.2**	–	**76.6**	**6.1**	–	–	**–**	–
P. fragi[d]	10	**1**	**16**	–	**69**	**10**	4	–	**–**	–
P. aeruginosa PAO1[e]	16.8	**3.3**	**23.7**	–	**63.5**	**9.6**	–	–	**–**	–
P. putida KT2440[f]	54	**3.1**	**24.2**	–	**66.4**	**6.3**	–	–	**–**	–
P. putida KT2442[g]	16–27	**1.7**	**21.4**	–	**63.6**	**3.8**	8.6	–	**0.1**	0.8

[a] Recombinant *phaG*-negative mutant harbouring an active *phaG* gene from the wild type strain (*P. aeruginosa* PAO1) and grown on gluconate (Hoffmann et al. 2000b)

[b] Strain not-accumulating mcl-PHAs from FAB, harbouring the *phaG* gene from *P. putida* KT2440 and grown on gluconate (Rehm et al. 1998)

[c] *P. putida phaG* negative mutant harbouring an active *phaG* gene from the wild-type strain (*P. putida* KT2440) and grown on gluconate (Rehm et al. 1998)

[d] Strain not accumulating mcl-PHAs from FAB, harbouring an active *phaG* from *P. putida* KT2440 and grown on gluconate (Fiedler et al. 2000)

[e] Untransformed strain grown on gluconate (Rehm et al. 1998)

[f] Untransformed strain grown on gluconate (Hoffmann et al. 2000a)

[g] Untransformed strain grown on glycerol (Huijberts et al. 1992)

295-amino-acid polypeptide with a mass of 33.8 kDa. The protein shares high homology with other cloned *phaG* genes. Compared to the *phaG* gene from *P. putida* KT2440 (Rehm et al. 1998), one nucleotide (C-17-A) and the corresponding amino acid (Ala-6-Asp) deviated. This residue is neither included in any conserved region nor in sequences known to be involved in catalysis. The consensus motif identified in all *phaG* genes, H X_4 D, is present in the *phaG* gene product from *P. putida* KT2442 as well. This motif has been proposed to be associated with enzymatic catalysis of the ACP-CoA transacylase (Rehm et al. 1998).

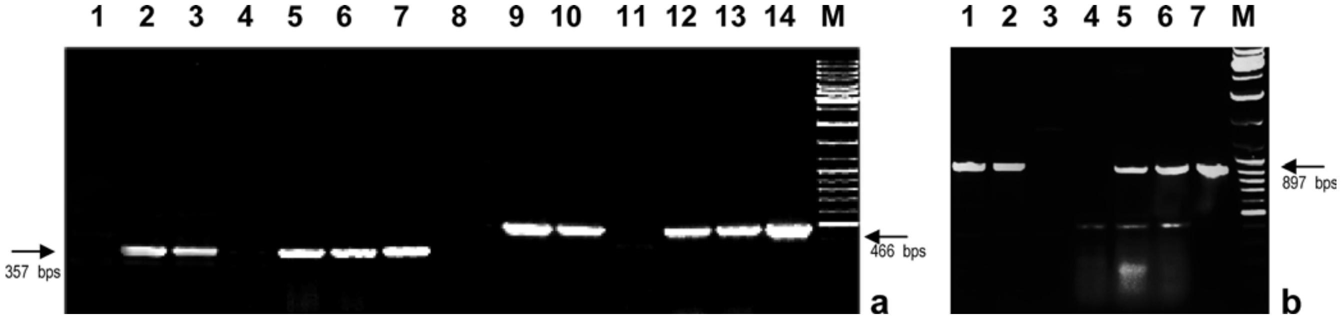
Transformation and selection of transgenic potato plants

More than 500 internodes were bombarded with plasmids pAPP100 (*phaC1*), pAPP101 (*phaG*) and plasmid 35-S-Kan containing the *nptII* gene needed for kanamycin selection of transgenic plants. Fourteen plants resistant to kanamycin were obtained, with an efficiency of transformation of 2.8 plants rooting on kanamycin per 100 bombarded internodes.

Using Southern blot or PCR analyses, three lines were selected (PB005.10, PB005.11 and PB005.14) in which both non-selected transgenes were integrated. Northern blot or RT–PCR analyses were used to check which lines transcribed mRNAs of the expected size. Lines PB005.10 and PB005.11 were finally selected for further analyses. In these lines, molecular analyses showed integration and transcription of *phaC1* and *phaG* genes (Fig. 1). Line PB005.14 (the third line co-transformed with *phaC1* and *phaG*) grew very slowly and did not root on soil, but only in vitro, yielding too little material for further analyses.

All lines co-transformed with *phaC1* and *phaG* genes showed a dwarfed phenotype with curled and brittle leaves and strongly retarded growth. Transgenic plants transformed only with plasmid 35-S-Kan or with the *phaC1* gene or other reporter genes (Romano et al. 2001, 2003a) were never affected to this extent, indicating that the aberrant phenotypes observed in lines expressing the *phaC1* and *phaG* genes were not a result of the transformation procedure (Fig. 2).

Fig. 1 **a** PCR analysis on potato (*Solanum tuberosum*) genomic DNA and cDNA using primers specific for the 357-bp 5′ end of the *phaC1* gene (*lanes 1–7*) and primers specific for the 466-bp 3′ end of the *phaC1* gene (*lanes 8–14*). For each set of PCR reactions, a negative control lacking any template (only water) was included. PCR products derived from genomic DNA of the untransformed line (*lanes 1, 8*), PB005.10 (*lanes 2, 9*) and PB005.11 (*lanes 3, 10*) are shown. Expected sizes of the PCR products are indicated by arrows. The ability to amplify the 5′ (*lanes 2, 3*) and the 3′ (*lanes 9, 10*) ends of the *phaC1* gene suggests that the complete gene has been integrated into the plant genome. PCR products derived from cDNA of the untransformed line (*lanes 4, 11*), PB005.10 (*lanes 5, 12*) and PB005.11 (*lanes 6, 13*) are shown. The cDNA synthesis was primed with a poly-T oligo. Thus, the ability to amplify the 5′ (*lanes 5, 6*) and the 3′ (*lanes 12, 13*) ends of the *phaC1* cDNA indicates that the full transcript was synthesised in the transgenic lines. All RNA templates were treated with DNase prior to cDNA synthesis. As an extra control, cDNA synthesis reactions, lacking the reverse transcriptase, were processed in parallel till PCR analyses. This excludes the occurrence of false positives due to contaminating genomic DNA in the RNA used as a template for cDNA synthesis. *Lanes 7, 14*: plasmid positive control. **b** PCR analyses on potato genomic DNA and cDNA using primers specific for the amplification of the complete 897-bp *amv-phaG* cassette. *Lanes 1–3*, PCR on genomic DNA from PB005.10, PB005.11 and the untransformed lines, respectively; *lanes 4–6*, PCR on cDNA from the untransformed, PB005.10 and PB005.11 lines, respectively; *lane 7*, plasmid positive control. *Lane M*, molecular weight marker



Polymer analysis

A number of protocols used for PHA extraction from bacteria were adapted for use on plants and tested for their efficiency. At the end, the protocol illustrated in Fig. 3a was adopted because of its higher reproducibility compared to other protocols. Lyophilised powder derived from ground leaves was first washed with ethanol. This first step extracted fatty acids and chlorophylls, which dissolved in ethanol. Subsequently, hydrophobic compounds were extracted with chloroform. All harvested fractions (ethanol fraction, chloroform fraction, powder after ethanol and chloroform extractions) were analysed by GC after methanolysis of each sample. The compound eluted in each GC peak was identified by comparison with a standard of pure mcl-PHAs extracted from *P. putida*. The ethanol fractions of all samples contained fatty acids (Fig. 3b, chromatograms on the left). As expected, chromatograms of the chloroform fractions (Fig. 3b, centre) indicated the absence of mcl-PHAs in the untransformed line. However, in the two transgenic lines expressing the *phaC1* and the *phaG*

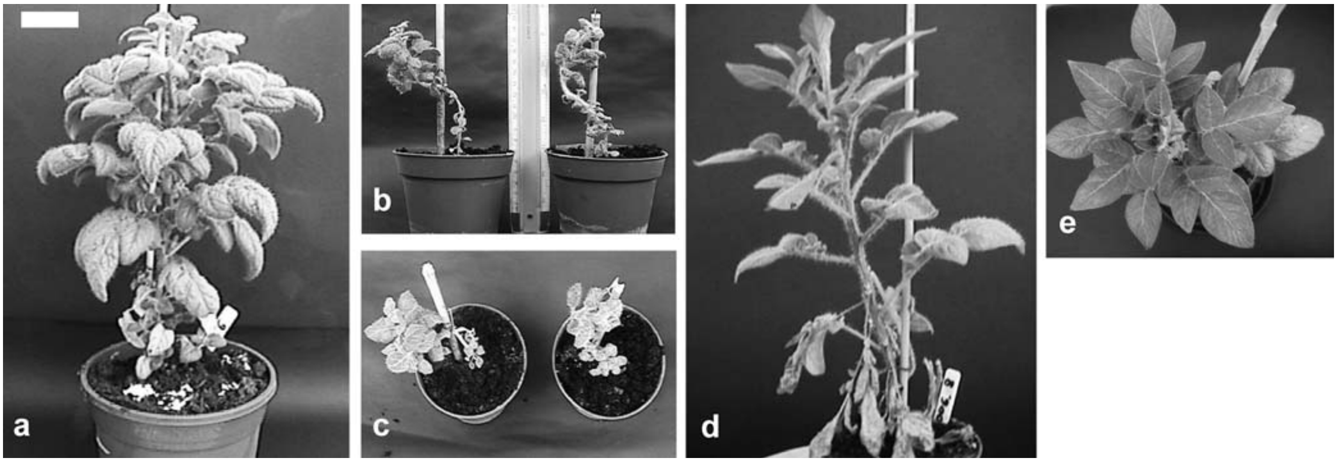


Fig. 2a–e Morphology of 6-week-old transgenic and untransformed lines of potato rooted on soil. **a** Untransformed line; **b,c** lines PB005.10 (*left*) and PB005.11 (*right*), double transformants *phaC1-phaG*; **d,e** example of a transgenic line transformed only with the *phaC1* gene. Bar = 5 cm

genes, peaks corresponding to the monomers expected to be present in mcl-PHAs (i.e. 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate) were observed. The powder remaining after the ethanol and the chloroform wash/extraction (Fig. 3b, chromatograms on the right) contained different components, including fatty acids, which were not further analysed, but did not contain any 3-hydroxy-monomer.

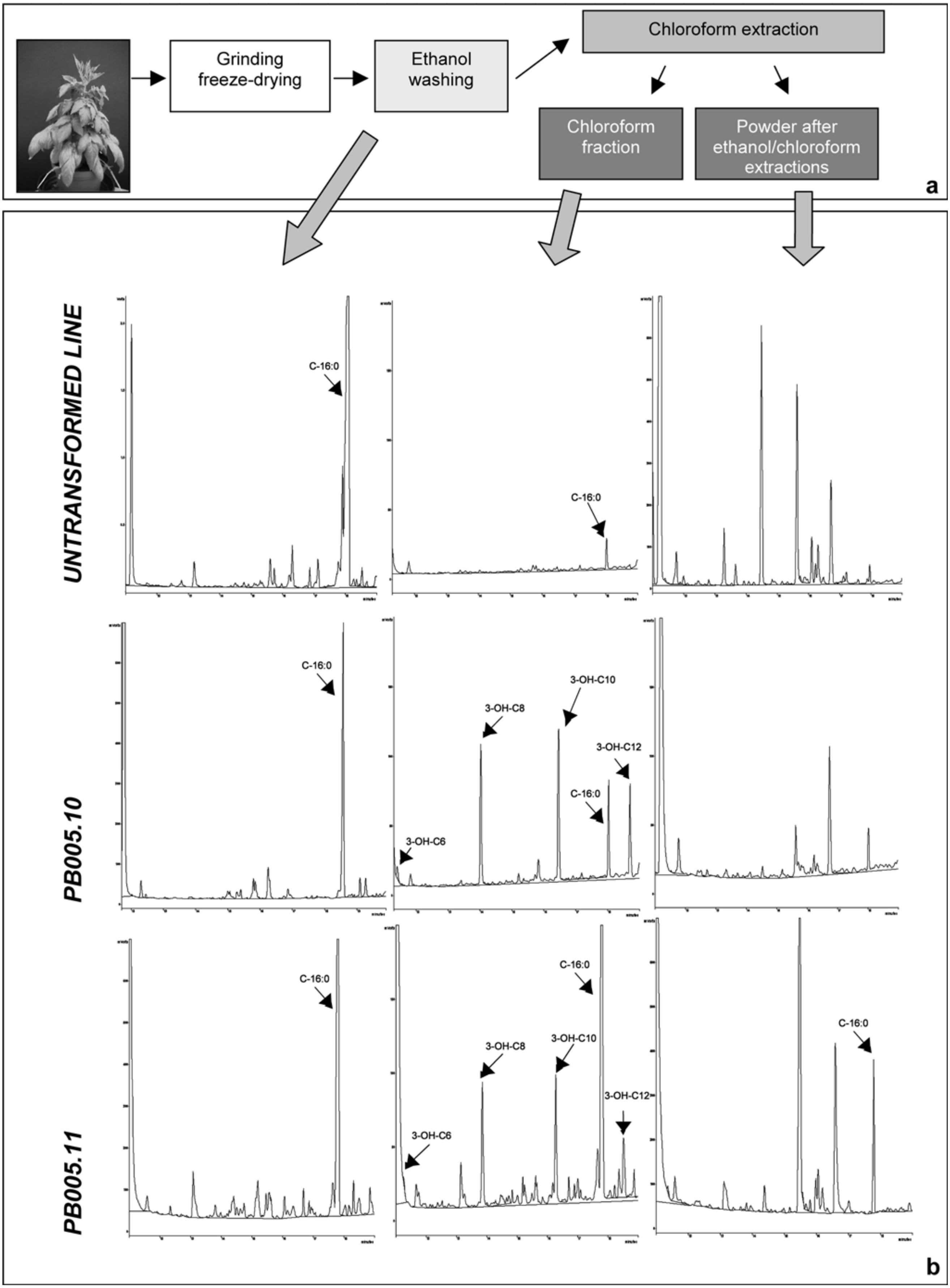
GC can only be used for the analysis of single monomeric units. To exclude the possibility that the peaks observed in transgenic lines PB005.10 and PB005.11, which we considered monomeric units of the polymer, were instead free 3-hydroxy fatty acids, a size-exclusion filter column was used to separate the putative polymer synthesised in the transgenic potato lines from other lipidic non-polymeric components present in the extract. A series of positive controls containing pure mcl-PHAs (from *P. putida*) or pure monomer [3-(*R*)-hydroxyoctanoate] or a mixture of mcl-PHAs and monomer were applied first to the column for optimisation of the procedure, and resulted in a perfect polymer/monomer separation (Fig. 4). Subsequently, plant extracts were separated using the size-exclusion columns, and after methanolysis the "polymer-containing" and "monomer-containing" phases were analysed by GC. 3-Hydroxyacyl units were observed only in the "polymer-containing" phase, indicating that they were indeed monomeric components of polymer separated from the total plant extract in the size-exclusion filter. Although these observations do not completely exclude other conclusions (see Discussion), they strongly suggest that the transgenic potato lines studied produced mcl-PHAs.

The observed monomer compositions and yields of putative mcl-PHA are indicated in Table 1. Line PB005.10 accumulated 0.26 mg putative mcl-PHAs per g of dry mass and line PB005.11 accumulated 0.017 mg putative mcl-PHAs per g of dry mass.

Discussion

In recent years, mcl-PHAs have gained worldwide interest because of their large number of possible applications (van der Walle et al. 1999, 2001). The use of transgenic plants for bulk PHA accumulation seems attractive because of the expected low cost of production compared to microbial fermentative PHA production (Nawrath et al. 1995). However, until now, most of the reports describing the production of PHAs in plants have focussed on polyhydroxybutyrate (PHB) biosynthesis (Poirier et al. 1992; Nawrath et al. 1994; John and Keller 1996; Hahn et al. 1999; Houmiel et al. 1999; Nakashita et al. 1999; Slater et al. 1999) whose range of applications is limited compared to that of mcl-PHAs. Until recently, knowledge of mcl-PHA biosynthesis was incomplete, which hampered the identification of the minimum gene-set required for its production in recombinant hosts. It has been shown that the PHA-polymerase alone was able to sustain mcl-PHA synthesis deriving precursors from β-oxidation in *Escherichia coli* (Langenbach et al. 1997), *Saccharomyces cerevisiae* (Poirier et al. 2001), *Pichia pastoris* (Poirier et al. 2002) and in the model plant *Arabidopsis thaliana* (Mittendorf et al. 1998). However, β-oxidation in plants is predominantly active during a limited part of the plant life-cycle (i.e., seed germination and senescence). Moreover, mcl-PHA accumulation, based on precursor supply from β-oxidation, would compete with the energy supply needed for the germination process.

FAB can also support mcl-PHA biosynthesis in many pseudomonads (Timm and Steinbüchel 1990; Huijberts et al. 1992). Mcl-PHA accumulation in transgenic plants from FAB would be more desirable because, being continuously active, FAB would be an excellent source of mcl-PHA precursors in large amounts. Rehm et al. (1998) showed that the metabolic link between FAB and mcl-PHA biosynthesis is accomplished by the action of ACP-CoA-transacylase, the *phaG* gene product. This


Grinding
freeze-drying
Ethanol
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Chloroform extraction
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a
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C-16:0
C-16:0
PB005.10
C-16:0
3-OH-C8
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3-OH-C12
C-16:0
3-OH-C6
PB005.11
C-16:0
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3-OH-C12
3-OH-C6
C-16:0
b

◄

Fig. 3a,b Polymer extraction and analysis of transgenic PB005.10, PB005.11 and untransformed lines of potato. **a** Polymer extraction procedure: plant material was first freeze-dried, subsequently washed with ethanol, and then mcl-PHAs were solubilised in chloroform. **b** Overview of the chromatographic analyses performed: residual ethanol from the washing steps containing fatty acids, chloroform containing the polymer (presumably mcl-PHAs), and the residual material were methanolysed and analysed by GC. *3-OH-C6*, 3-(R)-hydroxyhexanoate; *3-OH-C8*, 3-(R)-hydroxyoctanoate; *3-OH-C10*, 3-(R)-hydroxydecanoate; *3-OH-C12*, 3-(R)-hydroxydodecanoate; *C16:0*, palmitic acid. See text for explanation. Peaks corresponding to 3-(R)-hydroxytetradecanoate (3-OH-C14) are not visible because chromatograms were cut off to make the figure, only retention times between 11 and 19 min being shown

enzyme is able to direct precursors from FAB to mcl-PHAs in several prokaryotic hosts (Rehm et al. 1998; Fiedler et al. 2000; Hoffmann et al. 2000a, 2000b). In the present report, a eukaryote, i.e. potato, was used as host for the expression of the ACP-CoA-transacylase (*phaG* gene) and the Pha-C1 polymerase (*phaC1* gene). Proteins were targeted to the plastids of transgenic lines. Co-transformation with the *phaC1* and *phaG* genes resulted in a low efficiency of transformation compared with previous reports using other genes (Romano et al. 2001, 2003a, 2003c) and abnormal phenotypes were observed in all *phaC1*/*phaG* co-transformed plants. These transgenic plants could not be propagated for more than six to seven generations in vitro. When the three *phaC1*/*phaG* co-transformed plants were cultivated in pots, one did not survive (PB005.14) and two plants grew slowly, and neither tubers nor flowers were produced (Fig. 3). Most probably, the transacylase activity interferes with fatty acid metabolism yielding mcl-hydroxylated-fatty acids as a result of premature FAB termination, which may influence cell viability, regeneration and may result in a shortage of normal building blocks. Similarly, expression of the *phaG* gene in *A. thaliana* led to marked deleterious effects on plant growth (Poirier 2002). In contrast, the sole expression, in potato, of the Pha-C1 polymerase, both in the plastid and in the cytoplasm (Romano et al. 2003b), or the ACP-CoA-transacylase in the cytoplasm (data not shown), did not lead to such phenotypes. Unfortunately, no *phaG* single transfor-

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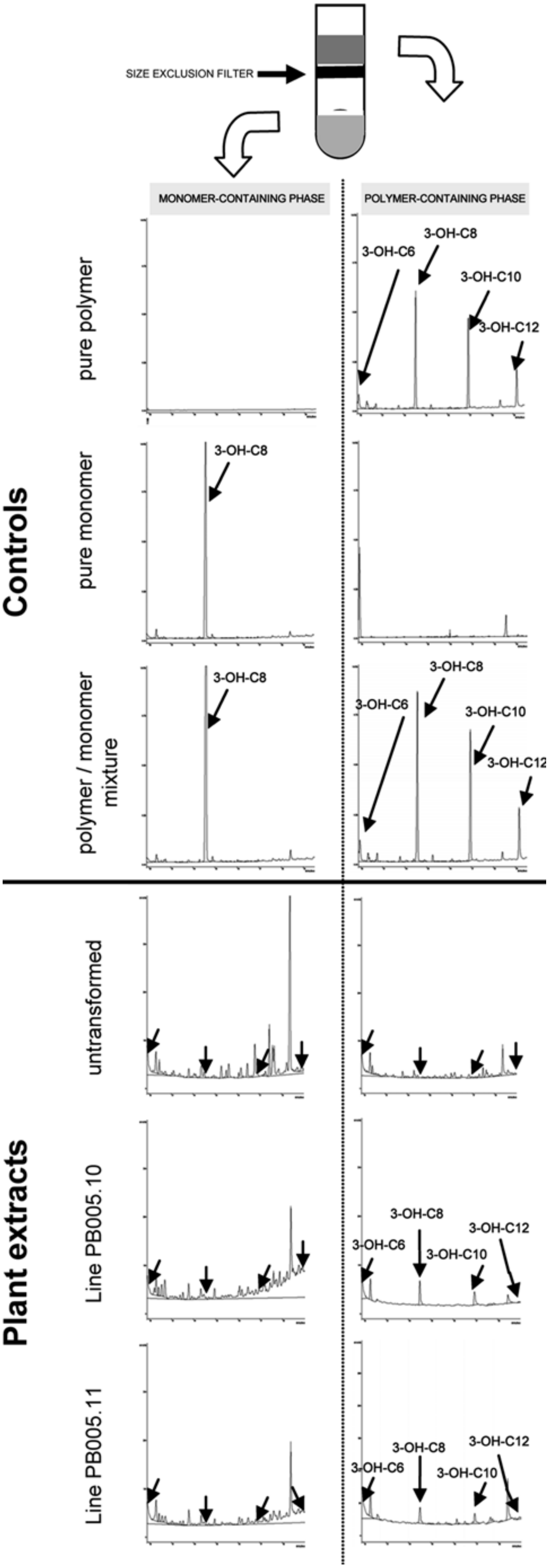

Fig. 4 Size-exclusion column filtration used to separate the polymer (in the *dark grey* fraction on top of the column) from other monomeric contaminants (in the *light grey* fraction, bottom of the column). Fractions were subsequently resuspended in chloroform, methanolysed and analysed by GC. The chromatograms at the top represent the separation of three different controls: pure polymer consisting of mcl-PHAs containing 3-hydroxy fatty acids with carbon chains ranging between C-6 and C-12; pure monomer consisting of 3-(*R*)-hydroxyoctanoate; blend of monomer and polymer. The chromatograms at the bottom represent the monomer (*left*) and polymer (*right*) fractions observed in the untransformed potato line and in lines PB005.10 and PB005.11. Arrows indicate the expected retention time of the monomeric units of mcl-PHAs. *3-OH-C6*, 3-(R)-hydroxyhexanoate; *3-OH-C8*, 3-(R)-hydroxyoctanoate; *3-OH-C10*, 3-(R)-hydroxydecanoate; *3-OH-C12*,3-(R)-hydroxydodecanoate

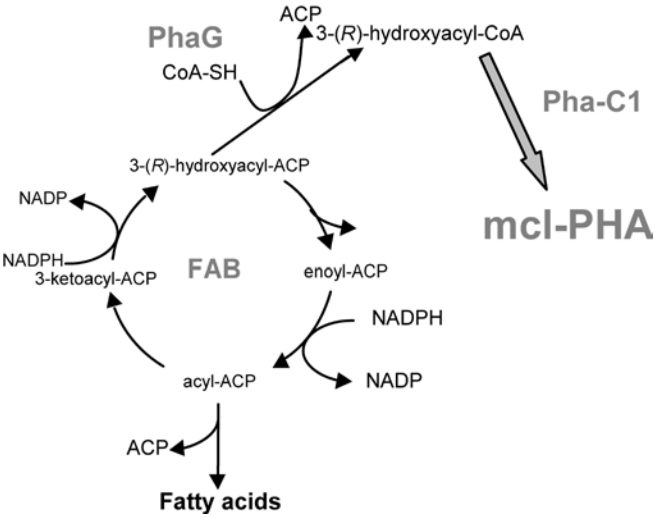

Fig. 5 Postulated metabolic pathway for the synthesis of mcl-PHAs in potato plastids using intermediates from FAB and based on the expression of the Pha-C1 polymerase (*phaC1* gene product) and the ACP-CoA-transacylase (*phaG* gene product). The ACP-CoA-transacylase transfers 3-(*R*)-acyl intermediates of the FAB from ACP to CoA. CoA activated moieties are subsequently polymerised by the Pha-C1 polymerase

mant expressing the protein in the plastids was obtained to further support this hypothesis.

Previously, it was shown (Romano et al. 2003b) that proper substrates [3-(*R*)-hydroxyacyl-CoA] for PHA synthesis are absent in the plant cytoplasm, and that the expression of the Pha-C1 polymerase in this compartment does not result in the synthesis of mcl-PHAs unless the monomeric substrate is provided exogenously. Similarly, sole expression of Pha-C1 polymerase in the plastids did not result in the synthesis of any polymer, because of the absence of proper substrates. However, the coordinate expression of the Pha-C1 polymerase and the ACP-CoA-transacylase in the plastids of transgenic potato lines did lead to the synthesis of a hydrophobic polymeric compound, as shown by the size-exclusion experiment. The yields in the two transgenic lines analysed, were 0.017 mg and 0.26 mg of polymer/g of cell dry weight (Table 1). Transgenic *A. thaliana* expressing the mcl-PHA-polymerase in the peroxisomes accumulated similar amounts of mcl-PHAs in leaves (0.2 mg/g of cell dry weight) and 20 times more polymer (4 mg/g of cell dry weight) in germinating seedlings (Mittendorf et al. 1998). The monomer composition of the polymer accumulated in the two transgenic potato lines analysed in the present report is shown in Table 1 and consisted of 3-(*R*)-hydroxyalkanoates with carbon chains ranging from C-6 to C-12. No unsaturated monomers were formed. This proves that FAB had provided the precursors, since double bonds are introduced in the elongating fatty acid only after palmitic acid is formed (Ohlrogge and Browse 1995). The same was observed in mcl-PHAs accumulated in natural or recombinant prokaryotes (Table 1). In contrast, when β-oxidation intermediates were directed into mcl-PHA biosynthesis in natural hosts (De Waard et al. 1993), recombinant yeast (Poirier et al. 2001, 2002) or recombinant *A. thaliana* (Mittendorf et al. 1998, 1999; Allenbach and Poirier 2000), the monomer composition also included unsaturated monomers.

However, it should be noted that GC and size-exclusion analyses alone would not allow mcl-PHAs to be distinguished from other kinds of polymers. For instance, cutin is a polyester with an analogous structure to that of PHAs (Kunst and Samuels 2003) and with similar chemical properties. Thus, it might theoretically be possible that 3-hydroxyacyl monomers were incorporated into cutin-like structures rather than into PHAs. Yet such a biosynthetic pathway seems actually very unlikely for the following reasons. For the synthesis of mcl-PHAs, the respective substrates and all metabolic steps needed are clustered in one cell compartment (i.e. plastids). If mcl-hydroxy fatty acids were involved in the cutin polymer network, a series of biosynthetic and/or carrier-mediated steps would be necessary. As mcl-hydroxy fatty acids are synthesised inside the plastids, they would have to be transferred to the cytoplasm, either as CoA-derivatives or as hydroxy fatty acids. In the first case, a carrier in the plastid membrane for mcl-hydroxyacyl-CoA should exist which, to our knowledge, has not been described. In the second case the activity of a thioesterase, able to handle mcl-hydroxyacyl-CoAs and converting them into the corresponding mcl-hydroxy fatty acids, in combination with a carrier for these mcl-hydroxy fatty acids would be necessary. In the latter case, the hydroxy-fatty acids need again to be converted into the CoA-activated groups, again by a cytoplasmic acyl-CoA-synthase able to handle mcl-hydroxy fatty acids. In both cases, the mcl-hydroxyacyl-CoAs must be transported from the cytoplasm into the cell wall by a (largely unknown) carrier mechanism and would have to polymerise subsequently (together with the ‘normal’ C16 and C18 cutin monomers) forming the cutin network. Summarising, we have found no indications or evidence supporting such a “cutin-hypothesis”.

In conclusion, all these observations and the strong similarities of the polymer produced in transgenic potato with mcl-PHAs accumulated by natural and recombinant hosts using FAB as the precursor supply (Table 1) allow us to suggest that the observed polymer consisted of mcl-PHAs and that it was formed according to the scheme depicted in Fig. 5. Additional analyses (like NMR, or infrared-spectrometry, which were hampered by the small amount of polymer produced) will be required not only to unambiguously confirm the nature of the observed material, but also to check whether blends of PHAs and other polymers were formed. In contrast to bulk PHA production, the presence of blends would be very challenging for the future development of new materials and novel applications aimed at creating added-value compounds, like blends of endogenous plant materials and PHAs (John and Keller 1996), provided that higher productivities and simple extraction methods can be developed. Regarding this last issue, the evident relation between fatty acid and PHA metabolism, would make oil-crops logical candidates for any metabolic engineering approach aimed at PHA synthesis. Nevertheless, because of the larger productivity of starch crops than oil crops in terms of biomass (van der Leij and Witholt 1995) it remains to be determined which crop is the best PHA producer. In this context, the use of tuber-specific promoters in combination with metabolic engineering strategies to redirect the carbon flux from starch to FAB and mcl-PHA synthesis (i.e. using low-starch potato cultivars/transformants as hosts), may further improve the mcl-PHA production and may prevent the drawbacks observed because of the expression of candidate genes in leaf chloroplasts. Modified PHA polymerases with increased activity have been recently isolated (Amara et al. 2002). The use of these enzymes will certainly be of great value in future experiments aimed at PHA production in plants.

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