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Synthesis of polyhydroxyalkanoate in the peroxisome of *Pichia pastoris*

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters naturally produced by bacteria that have properties of biodegradable plastics and elastomers. A PHA synthase from *Pseudomonas aeruginosa* modified at the carboxy-end for peroxisomal targeting was transformed in *Pichia pastoris*. The PHA synthase was expressed under the control of the promoter of the *P. pastoris* acyl-CoA oxidase gene. Synthesis of up to 1% medium-chain-length PHA per g dry weight was dependent on both the expression of the PHA synthase and the presence of oleic acid in the medium. PHA accumulated as inclusions within the peroxisomes. *P. pastoris* could be used as a model system to study how peroxisomal metabolism needs to be modified to increase PHA production in other eukaryotes, such as plants.

Keywords: β-Oxidation; Peroxisome; Polyhydroxyalkanoate; *Pichia pastoris*

1. Introduction

Polyhydroxyalkanoates (PHAs) are polymers of *R*-3-hydroxyalkanoic acids synthesized naturally by a wide variety of bacteria [1–3]. PHAs typically form intracellular inclusions of 0.2–1 μm and can accumulate to levels reaching nearly 80% of the bacterial dry weight (dwt). These polyesters have attracted industrial interest because of their properties as thermoplastics and elastomers, making them an attractive source of biodegradable and renewable polymers [1,2].

PHAs have been broadly divided into two classes, namely short-chain-length PHA (SCL-PHA) for polymers containing 3-hydroxyalkanoic acid monomers of three to five carbons, and medium-chain-length PHA (MCL-PHA) for polymers containing 3-hydroxyalkanoic acid monomers of six to 14 carbons [1,3]. SCL-PHAs have properties close to conventional plastics while MCL-PHAs are generally regarded as elastomers and rubbers [4].

MCL-PHAs are typically synthesized by Pseudomonads, such as *Pseudomonas oleovorans* and *Pseudomonas aeruginosa*. One major pathway for the production of MCL-PHAs is through the polymerization of 3-hydroxyacyl-coenzyme A (CoA) intermediates generated by the degradation of alkanoic or fatty acids via the β-oxidation cycle [1,3]. The nature of the PHA produced is thus directly related to the substrate used for growth and is typically composed of monomers that are $2n$ ($n \geq 0$) carbons shorter than the substrate [5].

One of the main limitations for the use of PHAs as biodegradable plastics used in high-volume low-value commodity products is their high production cost through bacterial fermentation relative to petroleum-derived plastics [2,6]. Synthesis of PHAs in crop plants has been seen as an attractive alternative for the commercial production of large amounts of PHAs at low cost [2,6,7]. Synthesis of PHB up to 40% of shoot dwt has been demonstrated in *Arabidopsis thaliana* cells [8–10]. Synthesis of the SCL-PHA co-polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) has also been demonstrated in the seed leukoplasts of *A. thaliana* and *Brassica napus*, with a maximum amount of 7–8% dwt [11]. MCL-PHA has been synthesized in *A. thaliana* using intermediates of the β-oxidation of fatty acids through the expression of a PHA synthase from *P. aeruginosa* in the peroxisome [12]. Only low amounts of polymer were produced ($< 0.6\%$ dwt) indicating the need for further genetic engineering before com-

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mercial production of MCL-PHAs in plants can be considered.

Synthesis of PHB has been achieved in insect cells [13] and *Saccharomyces cerevisiae* [14]. More recently, a low amount of MCL-PHA has also been demonstrated in *S. cerevisiae* expressing the PHA synthase of *P. aeruginosa* in the peroxisome [15]. These organisms are not directly regarded as vectors for the commercial production of PHAs but rather as model systems useful to learn how enzymes and metabolism can be modified to control the quantity and quality of PHAs produced in eukaryotes, and particularly in plants.

Pichia pastoris has been developed as a major model system for the study of various aspects of peroxisome biology, including peroxisome biogenesis and autophagy [16,17], protein targeting [18,19] and biochemical pathways of fatty acid degradation [19,20]. In contrast to *S. cerevisiae*, which grows relatively poorly on fatty acids, *P. pastoris* grows vigorously on fatty acids or methanol as the sole carbon source [16,21]. In this study, we examine whether *P. pastoris* could be used as a model organism for the study of MCL-PHA synthesis in the peroxisome.

2. Materials and methods

2.1. Strains and culture conditions

Plasmids were maintained and propagated in *Escherichia coli* DH5α according to standard procedures. *P. pastoris* strain GS115 (*his4*) was obtained from Invitrogen (Groningen, The Netherlands). Transformation of *P. pastoris* was done by electroporation as previously described [22]. Transformants were grown on histidine-deficient medium (0.67% yeast nitrogen base without amino acids [Difco, USA], 0.5% ammonium sulfate, 2% glucose and 0.4 g l^{-1} of histidine drop-out supplement [Clontech, USA]). For PHA production, a stationary phase culture was harvested by centrifugation, cells were washed once in water and suspended at a 1:20 dilution in fresh histidine-deficient medium containing 0.1% glucose, 2% Pluronic-127 (Sigma, USA) and 0.1–0.8% (v/v) oleic acid. Cells were grown for an additional 3 days before being harvested for PHA analysis.

2.2. DNA constructs

The plasmid pTW70 is derived from vector pHILD2 (Invitrogen) and contains the promoter sequence of the *P. pastoris* Pox1 gene encoding an acyl-CoA oxidase [19]. The PHA synthase gene from *P. aeruginosa* modified at the 3′ end by the addition of a peroxisomal targeting signal derived from the *B. napus* isocitrate lyase gene was excised from the plasmid pART7-PhaC1-ICL [12] by a *Eco*RI-*Xba*I digestion and made blunt-ended by T4 DNA polymerase. The pTW70 vector was cut by *Bgl*II, made blunt-ended with Klenow DNA polymerase and ligated to the blunt fragment harboring the PHA synthase. The resulting plasmid pTW70-PHA was linearized by digestion with *Sal*I and transferred into *P. pastoris* by electroporation.

2.3. Western blot analysis

Analysis of the expression of the PHA synthase in *P. pastoris* transformants was done by Western analysis using anti-PHA synthase antibodies as previously described [15].

2.4. PHA analysis

Extraction and analysis of PHA from cells was done essentially as previously described [12,15] with minor modifications. Briefly, cells were harvested by centrifugation, washed twice in water and lyophilized. The dried material was then weighed (approx. 20–60 mg) and transferred to a glass tube. The material was extracted four or five times with warm (65°C) methanol to remove lipids, free fatty acids and acyl-CoA, including 3-hydroxyacyl-CoA, while PHA, which is insoluble in methanol, remains associated with the cells. After centrifugation and removal of the residual methanol, the material was suspended in 0.5 ml of chloroform to which 0.5 ml of methanol containing 3% sulfuric acid was added. The mixture was heated at 95°C for 4 h and cooled down on ice. One ml of 0.1% NaCl was added to each tube, the mixture was vortexed vigorously and centrifuged at $5000 \times g$ for 5 min. The chloroform phase was harvested and dried over anhydrous $MgCl_2$. The methyl esters of 3-hydroxyacids were analyzed by GC-MS using a Hewlett-Packard 5890 gas chromatograph (HP-5MS column) coupled to a Hewlett-Packard 5972 mass spectrometer. Monomers of 3-hydroxyalkanoic acids were identified by selecting for the ions with a mass-to-charge ratio of 103, which corresponds to the methyl-esterified 3-hydroxypropionic acid fragment common to all 3-hydroxyalkanoic acids. Identity of the 3-hydroxyalkanoic monomers detected in cell extracts was verified by comparison with commercial 3-hydroxyacid standards (Sigma, USA) [12].

2.5. Electron microscopy

Cells were fixed for 4 h at room temperature in 4–5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate pH 7.2 and 0.1% (w/v) Brij 35, followed by an overnight treatment in the same solution without Brij 35. The cells were then rinsed several times with 0.1 M sodium cacodylate pH 7.2, transferred to 2% osmium tetroxide for 4–8 h at room temperature, and then transferred to 2% uranyl acetate in 10% ethanol for 40 min. Cells were dehydrated through a graded series of ethanol with a final treatment in propylene oxide. Cells were embedded in Epon/Araldite

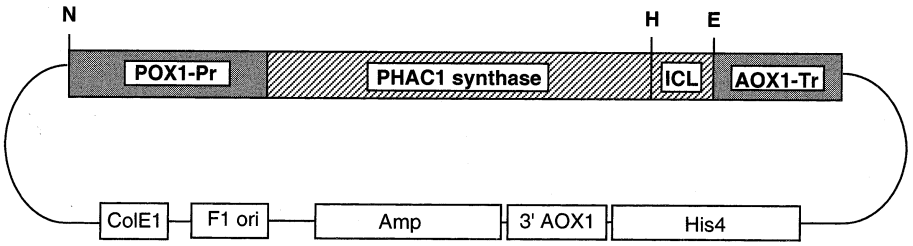

Fig. 1. DNA construct used to express the PHAC1 synthase of *P. aeruginosa* in *P. pastoris*. The PHAC1-ICL gene fusion is indicated by the striped boxes, while the promoter of the acyl-CoA oxidase gene (POX1-Pr) and the terminator of the alcohol oxidase 1 gene (AOX1-Tr) of *P. pastoris* are indicated by the shaded boxes. Open boxes denote the genes and regulatory elements found on the backbone of the vector pHIL-D2. E, *Eco*RI; H, *Hin*dIII; N, *Nde*I.

resin and polymerized for 3 days at 70°C. Fine sections of 50 nm were contrasted with a 2% aqueous solution of uranyl acetate for 6 min followed by lead citrate for 6 min. The grids were examined with a Philips Biotwin CM100 (Lab6 filament) transmission electron microscope.

3. Results and discussion

3.1. Expression of the PHA synthase from P. aeruginosa in recombinant P. pastoris

The majority of vectors designed for the expression of proteins in *P. pastoris* rely on the strong activity of the promoter of the alcohol oxidase 1 (AOX1) gene in cells grown in methanol. However, the AOX1 promoter is poorly active in cells grown in media containing fatty acids as the main carbon source [23,24]. The promoter of the *P. pastoris* Pox1 gene encoding the acyl-CoA oxidase was thus used in order to express a PHA synthase in *P. pastoris* growing on fatty acid-containing medium. A PHA synthase from *P. aeruginosa* modified at the carboxy-end by the addition of the peroxisomal targeting signal derived from the last 34 amino acids of the rapeseed isocitrate lyase [12] was cloned in front of the Pox1 promoter in the plasmid pTW70 [19] (Fig. 1). The rapeseed isocitrate lyase contains the carboxy-terminal tripeptide ARM, which has been shown to be an efficient peroxisomal targeting signal in both yeast and plants [25]. The resulting plasmid, named pTW70-PHA, was then integrated in the *P. pastoris* genome through recombination at the His4 locus.

Recombinant *P. pastoris* transformed with pTW70-PHA and growing in media containing either glucose or oleic acid as the main carbon source was tested for expression of the PHA synthase by Western blot analysis (Fig. 2). Cells growing in media containing 2% glucose and 2% Pluronic-127 do not express the PHA synthase in either the exponential phase or early stationary phase. However, PHA synthase was expressed in these cells in late stationary phase at which time the amount of glucose in the medium was below detection ($<0.002\%$). In contrast, cells growing in medium supplemented with 0.1% glucose, 2% Pluronic-127 and 0.1% oleic acid show strong expression of the PHA synthase in either the exponential phase or stationary phase. No protein cross-reacting with the PHA synthase antibody was found in cells growing in the same medium and transformed with the control vector pTW70. The detergent Pluronic-127 is a polyoxyethylene polymer containing no fatty acids and is used to ensure solubilization of the free oleic acid added to the medium. *P. pastoris* shows no significant growth in media containing only Pluronic-127 indicating that it cannot be used efficiently as a carbon source (data not shown).

The expression pattern of the Pox1 promoter is similar to other yeast promoters that are repressed by glucose but activated by either fatty acids, such as the *S. cerevisiae* catalase 1 (CTA1) promoter, or by methanol, such as the *P. pastoris* AOX1 promoter. For all these promoters, absence of glucose leads to a derepression of the promoter activity leading to low gene expression, while addition of fatty acids or methanol leads to full activity of the promoter and strong gene expression [26,27]. Together, these results show that the Pox1 promoter can be used to efficiently express a foreign protein in *P. pastoris* growing in media containing fatty acids.

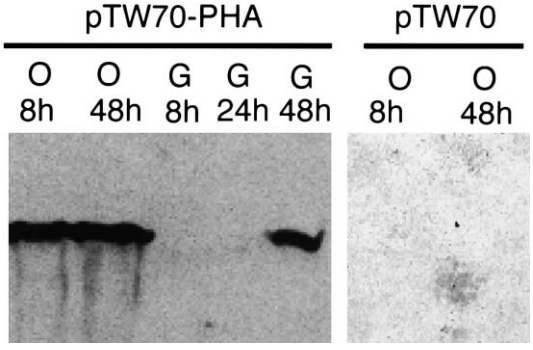

Fig. 2. Western blot analysis of PHA synthase expression in *P. pastoris*. Cells were transformed with the plasmid PTW70-PHA containing the PHA synthase or the control plasmid pTW70. Cells grown to stationary phase in histidine-deficient medium with 2% glucose were harvested, washed with water and inoculated at a 1:20 dilution in histidine-deficient medium containing either 2% glucose and 2% Pluronic-127 (G) or 0.1% glucose, 2% Pluronic-127 and 0.1% oleic acid (O). Cells were harvested either during the exponential phase (8 h), the early stationary phase (24 h) or late stationary phase (48 h) and processed for Western analysis with an anti-PHA synthase antibody.

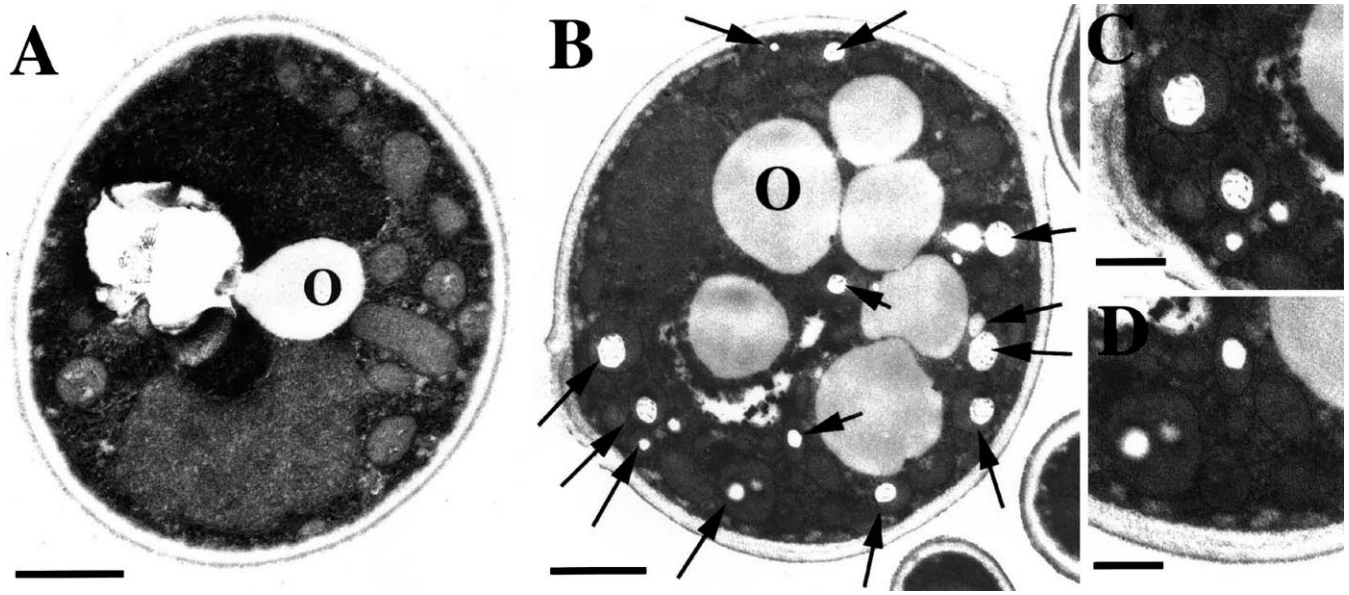

Fig. 3. Analysis of PHA inclusions in *P. pastoris*. Cells transformed with the control plasmid pTW70 (A) or the plasmid pTW70-PHA (B–D) were inoculated in medium containing 0.1% glucose, 2% Pluronic-127 and 0.1% oleic acid and grown for 3 days before being processed for TEM. C and D are close-up views of B. Arrows indicate the presence of PHA inclusions within membrane-bound organelles. Ob, oil body. Bars indicate 1 μm (A, B) and 0.5 μm (C, D).

3.2. Production of MCL-PHA

P. pastoris transformed with pTW70-PHA or the control plasmid pTW70 was grown for 3 days in media containing 0.1% glucose, 2% Pluronic-127 and various concentrations of oleic acid before being harvested for PHA analysis (Table 1). Cells transformed with control plasmid pTW70 and grown in media with or without 0.1% oleic acid did not produce any detectable PHA. Similarly, cells transformed with the PHA synthase and grown in media without oleic acid did not produce detectable PHA. In contrast, cells transformed with the peroxisomal PHA synthase synthesized PHA when grown in media containing oleic acid. The amount of PHA was found to increase progressively but non-linearly with the amount of oleic acid found in the medium. Whereas addition of 0.1% oleic acid resulted in the accumulation of 0.3% dwt PHA, addition of 0.8% oleic acid resulted in the production of 1.0% dwt PHA. Together, these data show that synthesis of PHA in *P. pastoris* is dependent on both the expression of the PHA synthase and the presence of oleic acid in the medium.

Table 1
Synthesis of MCL-PHA in recombinant *P. pastoris* grown in various media

Plasmid	Growth medium[a]	PHA % (w/dwt)
PTW70	GP	ND
	GPO 0.1%	ND
PTW70-PHA	GP	ND
	GPO 0.1%	0.3 ± 0.1
	GPO 0.2%	0.5 ± 0.1
	GPO 0.4%	0.7 ± 0.1
	GPO 0.8%	1.0 ± 0.1

[a]Cells were grown for 3 days in media containing either 0.1% glucose and 2% Pluronic-127 (GP) or 0.1% glucose, 2% Pluronic-127 and a variable amount of oleic acid (GPO). The concentration of oleic acid (v/v) is indicated by the percentage value. ND, not detected.

The monomer composition of the PHA produced in *P. pastoris* grown on oleic acid was determined by GC-MS to contain 7.2 mol% 3-hydroxyhexanoic acid, 50 mol% 3-hydroxyoctanoic acid, 24 mol% 3-hydroxydecanoic acid, 10 mol% 3-hydroxydodecanoic acid, 0.3 mol% 3-hydroxytetradecanoic acid and 8.5 mol% of 3-hydroxytetradecenoic acid. The monomers found in PHA synthesized in *P. pastoris* are between six and 14 carbons and correspond to the major 3-hydroxyacyl-CoA substrates that are accepted by the PHA synthase of *P. aeruginosa* and of other bacteria synthesizing MCL-PHA [1,3]. The monomer composition of *P. pastoris* PHA is also similar to PHA synthesized from intermediates of β-oxidation of oleic acid in *Pseudomonas putida* [28], as well as *A. thaliana* and *S. cerevisiae* expressing the PHA synthase from *P. aeruginosa* in the peroxisome [12,15]. In particular, the presence of both 3-hydroxytetradecanoic acid and 3-hydroxytetradecenoic acid agrees with the generation of the corresponding 3-hydroxyacyl-CoA by the β-oxidation of fatty acids having a *cis*-unsaturated bond at an odd-numbered carbon, such as oleic acid (18:1 Δ9*cis*) [29]. Together, these data show that external fatty acids are imported into cells, are degraded via the peroxisomal β-oxidation cycle, and that 3-hydroxyacyl-CoAs generated by the β-oxidation cycle are used for the synthesis of MCL-PHA in *P. pastoris*.

3.3. PHA inclusions detected by electron microscopy

P. pastoris transformed with either pTW70 control plasmid or pTW70-PHA were grown for 3 days in medium containing 0.1% oleic acid before being analyzed by transmission electron microscopy (TEM). Both types of cells showed the presence of oil bodies (Fig. 3A,B) as well as numerous peroxisomes. Growth of *P. pastoris* on either fatty acids or methanol has been previously shown to increase the number and size or peroxisomes [16,17]. However, only cells transformed with the PHA synthase and producing PHA showed the presence of small electronlucent inclusions within the peroxisomes (Fig. 3B–D). The apparent size of these inclusions is in the range of 0.1–0.2 μm in diameter. Both the size and general appearance of these inclusions, as seen by TEM, are very similar to PHA granules found in several bacteria [3] as well as in transgenic plants [8–10] and *S. cerevisiae* [15], indicating that, similar to PHA synthesized in these other hosts, PHA produced in *P. pastoris* accumulates in the form of inclusions.

4. Conclusion

Expression in *P. pastoris* of a PHA synthase from *P. aeruginosa* modified for its targeting to the peroxisome was shown to lead to the synthesis of MCL-PHA in the peroxisome when cells are grown in media containing fatty acids. Both the monomer composition of the PHA synthesized in *P. pastoris* from oleic acid and the presence of inclusions within the peroxisomes indicate that MCL-PHA are synthesized using intermediates of the peroxisomal β-oxidation of fatty acid present in the medium. The maximal amount of PHA synthesized in *P. pastoris*, at 1% dwt, was slightly higher but comparable to MCL-PHA synthesized in either *A. thaliana* (0.6% dwt) [12] or *S. cerevisiae* (0.45% dwt) [15] but much lower than for bacteria, such as *P. putida*, that can accumulate up to 40% dwt PHA [28].

In contrast to *S. cerevisiae*, which grows relatively poorly in media containing only fatty acids as the carbon source, *P. pastoris* grows vigorously in similar media [16,21]. This indicates that *P. pastoris* has an efficient system to import and degrade fatty acids in peroxisomes and to utilize the carbon generated from β-oxidation for growth. Germinating seeds from plants storing triacylglycerides as the major carbon storage in their seeds, such as *A. thaliana* and *B. napus*, also efficiently degrade fatty acids in the peroxisome and use the released carbon for growth until the establishment of photosynthesis. From this work, it is proposed that *P. pastoris* could be used as a powerful model system to study how peroxisomal metabolism needs to be modified in order to increase MCL-PHA synthesis and apply this knowledge to plants.

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References

[1] Steinbüchel, A. and Hein, S. (2001) Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. Adv. Biochem. Eng. Biotechnol. 71, 81–123.
[2] Poirier, Y., Nawrath, C. and Somerville, C. (1995) Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Biotechnology 13, 142–150.
[3] Steinbüchel, A. (1991) Polyhydroxyalkanoic acids. In: Novel Biomaterials from Biological Sources (Byrom, D., Ed.), pp 123–216. MacMillan, New York.
[4] de Koning, G. (1995) Physical properties of bacterial poly((R)3-hydroxyalkanoates). Can. J. Microbiol. 41, 303–309.
[5] Lageveen, R.G., Huisman, G.W., Preusting, H., Ketelaar, P., Eggink, G. and Witholt, B. (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl. Environ. Microbiol. 54, 2924–2932.
[6] Poirier, Y. (1999) Production of new polymeric compounds in plants. Curr. Opin. Biotechnol. 10, 181–185.
[7] Poirier, Y. (2001) Production of polyesters in transgenic plants. Adv. Biochem. Eng. Biotechnol. 71, 209–240.
[8] Poirier, Y., Dennis, D.E., Klomparens, K. and Somerville, C. (1992) Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256, 520–523.
[9] Nawrath, C., Poirier, Y. and Somerville, C.R. (1994) Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91, 12760–12764.
[10] Bohmert, K., Balbo, I., Kopka, J., Mittendorf, V., Nawrath, C., Poirier, Y., Tischendorf, G., Trethewey, R.N. and Willmitzer, L. (2000) Transgenic *Arabidopsis* plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. Planta 211, 841–845.
[11] Slater, S., Mitsky, T.A., Houmiel, K.L., Hao, M., Reiser, S.E., Taylor, N.B., Tran, M., Valentin, H.E., Rodriguez, D.J., Stone, D.A., Padgette, S.R., Kishore, G. and Gruys, K.J. (1999) Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. Nature Biotechnol. 17, 1011–1016.
[12] Mittendorf, V., Bongcam, V., Allenbach, L., Coullerez, G., Martini, N. and Poirier, Y. (1999) Polyhydroxyalkanoate synthesis in transgenic plants as a new tool to study carbon flow through β-oxidation. Plant J. 20, 45–55.
[13] Williams, M.D., Rahn, J.A. and Sherman, D.H. (1996) Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eucaryotic fatty acid synthase. Appl. Environ. Microbiol. 62, 2540–2546.
[14] Leaf, T.A., Peterson, M.S., Stoup, S.K., Somers, D. and Srienc, F. (1996) *Saccharomyces cerevisiae* expressing bacterial PHB synthase produces poly-3-hydroxybutyrate. Microbiology 142, 1169–1180.
[15] Poirier, Y., Erard, N. and Macdonald-Comber Petétot, J. (2001) Synthesis of polyhydroxyalkanoate in the peroxisome of *Saccharomyces cerevisiae* by using intermediates of fatty acid β-oxidation. Appl. Environ. Microbiol. 67, 5254–5260.
[16] Gould, S.J., McCollum, D., Spong, A.P., Heyman, J.A. and Subramani, S. (1992) Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast 8, 613–628.

[17] Sakai, Y. and Subramani, S. (2000) Environmental response of yeast peroxisomes. Aspects of organelle assembly and degradation. Cell Biochem. Biophys. 32, 51–61.
[18] Liu, H., Tan, X., Russell, K.A., Veenhuis, M. and Cregg, J.M. (1995) *PER3*, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. J. Biol. Chem. 270, 10940–10951.
[19] Koller, A., Spong, A.P., Lüers, G.H. and Subramani, S. (1999) Analysis of the peroxisomal acyl-CoA oxidase gene product from *Pichia pastoris* and determination of its targeting signal. Yeast 15, 1035–1044.
[20] Kalish, J.E., Chen, C.I., Gould, S.J. and Watkins, P.A. (1995) Peroxisomal activation of long- and very long-chain fatty acids in the yeast *Pichia pastoris*. Biochem. Biophys. Res. Commun. 206, 335–340.
[21] Veenhuis, M., Mateblowski, M., Kunau, W.-H. and Harder, W. (1987) Proliferation of microbodies in *Saccharomyces cerevisiae*. Yeast 3, 77–87.
[22] Scorer, C.A., Clare, J.J., McCombie, W.R., Romanos, M.A. and Sreekrishna, K. (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. Biotechnology 12, 181–184.
[23] Johnson, M.A., Waterham, H.R., Ksheminska, G.P., Fayura, L.R., Cereghino, J.L., Stasyk, O.V., Veenhuis, M., Kulachkovsky, A.R., Sibirny, A.A. and Cregg, J.M. (1999) Positive selection of novel peroxisome biogenesis-defective mutants of the yeast *Pichia pastoris*. Genetics 151, 1379–1391.
[24] Snyder, W.B., Koller, A., Choy, A.J., Johnson, M.A., Cregg, J.M., Rangell, L., Keller, G.A. and Subramani, S. (1999) Pex17p is required for import of both peroxisome membrane and lumenal proteins and interacts with Pex19p and the peroxisome targeting signal-receptor docking complex in *Pichia pastoris*. Mol. Biol. Cell 10, 4005–4019.
[25] Olsen, L.J. (1998) The surprising complexity of peroxisome biogenesis. Plant Mol. Biol. 38, 163–189.
[26] Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A. and Gingeras, T.R. (1987) Expression of the *LacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. Nucleic Acids Res. 15, 3859–3876.
[27] Horter, H., Ammerer, G., Hartter, E., Hamilton, B., Rytka, J., Bilinski, T. and Ruis, H. (1982) Regulation of synthesis of catalases and iso-cytochrome c in *Saccharomyces cerevisiae* by glucose, oxygen and heme. Eur. J. Biochem. 128, 179–184.
[28] de Waard, P., van der Wal, H., Huijberts, G.N.M. and Eggink, G. (1993) Heteronuclear NMR analysis of unsaturated fatty acids in poly(3-hydroxybutyrate). Study of β-oxidation in *Pseudomonas putida*. J. Biol. Chem. 268, 315–319.
[29] Gurvitz, A., Mursula, A.M., Yagi, A.I., Hartig, A., Ruis, H., Rottensteiner, H. and Hiltunen, J.K. (1999) Alternatives to the isomerase-dependent pathway for the β-oxidation of oleic acid are dispensable in *Saccharomyces cerevisiae*. J. Biol. Chem. 274, 24514–24521.