PRODUCTION OF POLYHYDROXYALKANOATES (PHAs) IN TRANSGENIC POTATO

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Proefschrift

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Contents

List of abbreviations

2-4-D	2,4-dichlorophenoxyacetic acid
35-S	cauliflower mosaic virus 35 S
ACP	acyl carrier protein
BAP	6 benzyl-amino-purine
bar	phosphinothricin acetyl transferase gene
CIM	callus inducing medium
CoA	coenzyme A
E-35-S	enhanced 35-S promoter
FAB	fatty acid biosynthesis
GA_3	gibberellic-acid
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
GUS	β-glucuronidase
HFG	Helium Flow Gun
IAA	indole-3-acetic-acid
luc	luciferase gene
Mcl	medium-chain-length
NAA	1-naphthalene acetic acid
nos	nopaline synthase
Pat	phosphinothricin acetyl transferase.
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
RT-PCR	reverse transcriptase PCR
Scl	short-chain-length
STS	silver-thio-sulphate
*uid*A	β-glucuronidase gene

CHAPTER 1
General Introduction[1]

[1] partly based on: **"**Romano, A., Vincken, J.-P., Raemakers, K., Mooibroek, H. and Visser, R. 2002. Potato genetic transformation and its application in polymer modification. In: R. Singh and P. Jaiwal (Ed.), Plant Genetic Engineering, Sci-Tech Publishing Company, Houston, USA, 3, *in press*.

The production of polymers as alternative renewable resources has gained increasing interest and is rapidly expanding. It is generally accepted that mineral oil, and thus oil-derived plastics, is a finite source (Gerngross & Slater, 2000). Furthermore, the accumulation of synthetic non-degradable plastics in the environment and their combustion products are a real pollution problem. Organisms are able to accumulate large amounts of carbon and energy in the form of osmotically inert polymers. These polymers include polysaccharides such as glycogen in bacteria and animals, and starch in algae and higher plants. Beside polysaccharides, other highly interesting natural storage compounds like polyesters can be formed by bacteria (Anderson & Dawes, 1990; Steinbüchel & Schlegel, 1991; Steinbüchel et al., 1992). Polyesters like 3-(*R*)-hydroxyalkanoates (PHAs; for a recent review, see Madison & Huisman, 1999) are a large group of polymers of 3-(*R*)-hydroxy fatty acids linked by an ester bond between the hydroxy group and the carboxy group of an adjacent monomer (Figure 1). PHAs are divided into two groups: short-chain-length PHA (scl-PHA) comprises poly-3-(*R*)-hydroxybutyrate (PHB) and the co-polymer poly-[3-(*R*)-hydroxybutyrate-co-3-(*R*)-hydroxyvalerate] (PHB / HV); medium-chain-length PHA (mcl-PHA) consists of 3-(*R*)-hydroxyhexanoate / 3-(*R*)-hydroxytetradecanoate monomers. PHAs are osmotically inert compounds and they are optically active, biocompatible, biodegradable and hydrophobic and possess properties which vary with their composition (Table 1).

The bacterium *Ralstonia eutropha* accumulates scl-PHA, which can be processed and applied in a way similar to commodity plastics. The

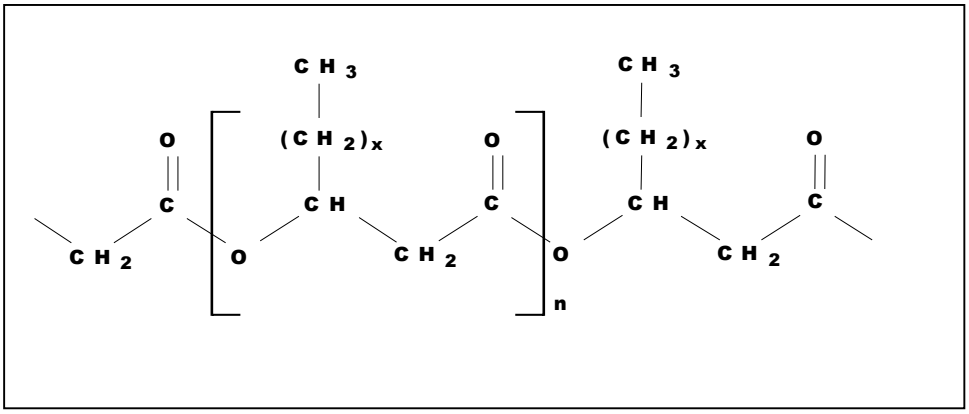

Figure 1. Structural formula of PHA. x = 1-11. Number of residues n= 1,000 – 3,000.

Table 1. Properties of scl- and mcl-PHA compared to polypropylene (Madison & Huisman, 1999). [a]: PHB: poly-3-(*R*)-hydroxybutyrate. PHB / HV: poly-[3-(*R*)-hydroxybutyrate-3-(*R*)-hydroxyvalerate], 80 - 20 %. PHB / (4)HV: poly-[3-(*R*)-hydroxybutyrate-4-(*R*)-hydroxyvalerate], 84 – 16 % PHO / HH: poly-[3-(*R*)-hydroxyoctanoate-3-(*R*)-hydroxyhexanoate], 89 – 11 %. PP: polypropylene.

[a]	PHB	PHB / HV	PHB / (4)HV	PHO / HH	PP
T_m (˚C)[b]	177	145	150	61	176
T_g (˚C)[c]	2	-1	-7	-36	-10
Crystallinity (%)	70	56	45	30	60
Extension to break (%)	5	50	444	300	400

heteropolymer PHB / HV is commercially known as Biopol®. Mcl-PHA is accumulated by fluorescent pseudomonads belonging to the rRNA group I (Timm & Steinbüchel, 1990; Huisman et al., 1991). Mcl-PHA are very versatile compounds, and can be used in a number of applications: e.g. packaging materials, elastomeric (rubber-like) materials, cheese coating, adhesives, coatings and paint binders (van der Walle et al., 1999 and 2001 and references therein). Several medical applications have been also reported (Williams et al., 1999) exploiting PHAs for tissue engineering (Sodian et al., 2000) or as stocks for the stereoscopic-drug industry (van der Walle et al., 2001 and references therein).

Synthesis of scl-PHA and mcl-PHA in natural hosts

The formation of PHB, which is the best known scl-PHA, is mediated by three enzymes (Figure 2), i.e. 3-ketothiolase (the *phb*A gene product), NADPH-dependent acetoacetyl-CoA reductase (the *phb*B gene product), and PHB-polymerase (the *phb*C gene product). The PHB-polymerase is classified as type-I polymerase. The *phb* genes, in *R. eutropha* and most of the other PHB-accumulating bacteria, are located in one operon (reviewed by Madison & Huisman, 1999). Fatty acids can be used as a substrate for PHB synthesis, which are metabolised *via* the β-oxidation cycle and generate acetyl-CoA. Acetyl-CoA can also be derived from various carbon sources because of its central role in bacterial metabolism. Therefore, PHB can be synthesised from a

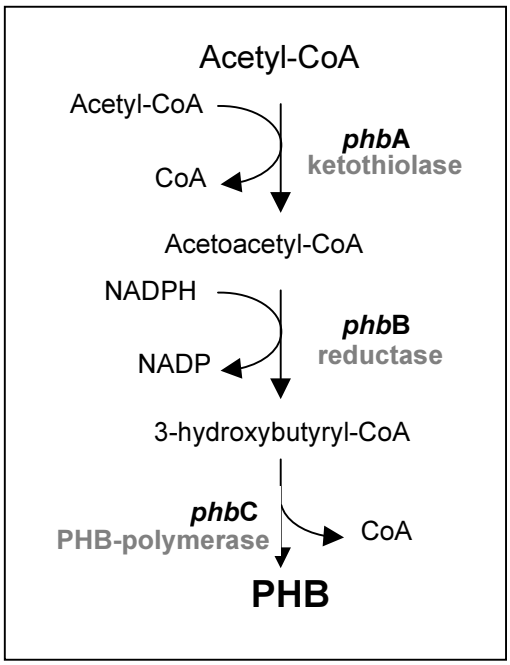

Figure 2. Metabolic pathway for the production of scl-PHA (PHB) in *R. eutropha*.

wide variety of substrates, whereby the intracellular acetyl-CoA pool (and free CoA) regulates PHB synthesis. The PHA-polymerase specific for mcl-PHA is classified as type-II. In both *P. oleovorans* (Huisman et al., 1991) and *P. aeruginosa* (Timm & Steinbüchel, 1992) the *pha* locus consists of two genes, namely *pha*C1 and *pha*C2, which code for two forms of mcl-PHA-polymerase, Pha-C1 and Pha-C2, respectively. In between the *pha*C1 and *pha*C2 genes, is located *pha*Z, which codes for the depolymerase. Downstream this cluster of three genes, several extra genes involved in the mcl-PHA metabolism are present and are currently being studied and characterised (Steinbüchel & Hein, 2001).

The substrate, 3-(*R*)-hydroxyacyl-CoA, for the mcl-PHA-polymerase can be furnished *in vivo* mainly through two pathways.

1. *From the β-oxidation.* During growth on mcl-fatty acids, *P. putida* (De Waard et al., 1993) and *P. oleovorans* (Gross et al., 1989) accumulate mcl-PHA consisting of monomers which are directly derived from the substrates or shortened by one or more 2-carbons units. β-oxidation proceeds through *S*-enantiomers of 3-hydroxyacyl groups. Different intermediates of the β-oxidation pathway can be converted into 3-(*R*)-hydroxyacyl-CoA (Figure 3). The trans-2-enoyl-CoA can be converted to 3-(*R*)-hydroxyacyl-CoA *via* the action of a hydratase. Genes coding for (*R*)-specific enoyl-CoA hydratases have been cloned from *Aeromonas caviae* (Fukui, 1998) and *P. aeruginosa* (Tsuge et al., 1999) and their role as precursor supplier for mcl-PHA biosynthesis from β-oxidation has been shown in recombinant *E. coli* (Tsuge et al., 1999). The involvement of the endogenous 2-enoyl-CoA-hydratase-II in transgenic *A. thaliana* for mcl-PHA precursors supply has been also

postulated (Mittendorf et al., 1998). In the second possible path, 3-ketoacyl-CoA can be converted into 3-(*R*)-hydroxyacyl-CoA by a reductase. A NADPH-dependent 3-ketoacyl reductase has been cloned from *P. aeruginosa* (Campos-Garcia et al., 1998) and it provided precursors to mcl-PHA biosynthesis from β-oxidation in recombinant *E. coli* (Ren et al., 2000b). The third possible path postulates that 3-(*R*)-hydroxyacyl-CoA is produced by an epimerase from the 3-(*S*)-hydroxyacyl-CoA. It has been postulated that an endogenous epimerase activity provided precursors to mcl-PHA biosynthesis in transgenic *A. thaliana* (in combination with the hydratase path; Mittendorf et al., 1998 and 1999; Allenbach & Poirier, 2000). It seems that all pathways contribute to mcl-PHA metabolism (Ren, 1997).

2. *From the fatty acids biosynthesis (FAB)*: under unbalanced conditions (limiting ammonia and excess of carbon) *P. putida* accumulates 25% of the cell dry weight of mcl-PHA from non-related sources (Huijberts et al., 1992).

FAB proceeds through 3-(*R*)-hydroxyacyl-ACP intermediates which can be transferred to a CoA molecule by the 3-(*R*)-hydroxyacyl-ACP-CoA-transacylase, coded by the *pha*G gene (Rehm, 1998), and eventually polymerised by the PHA-polymerase (Figure 3).

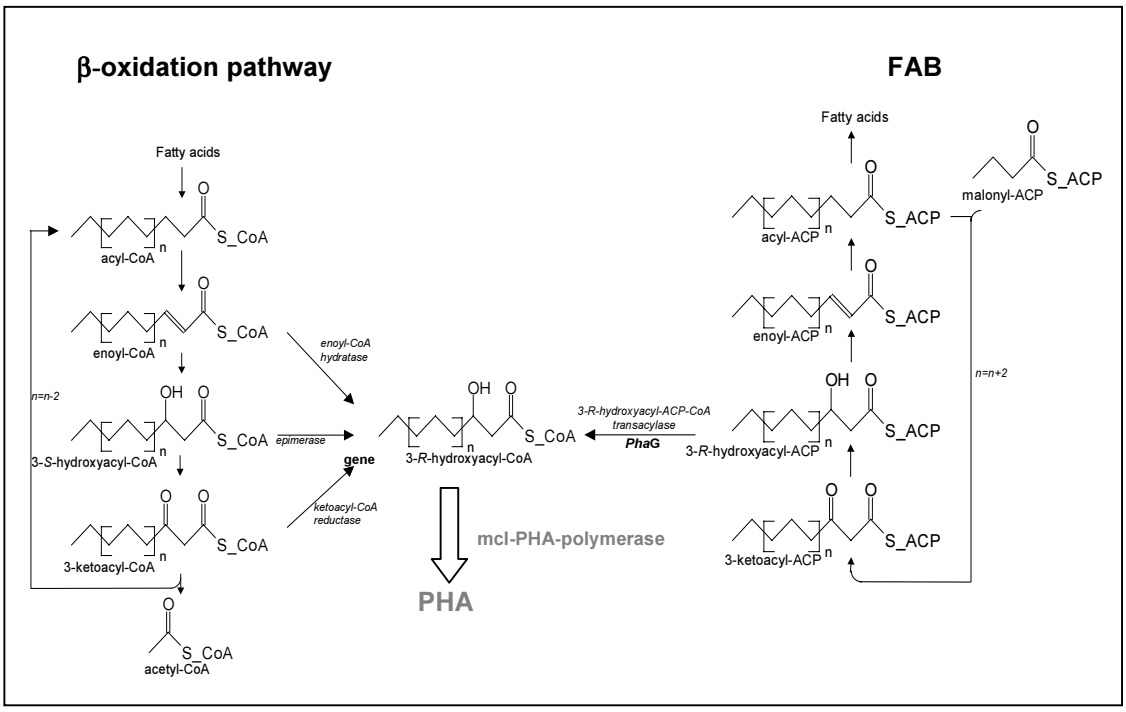

Figure 3. Metabolic pathway for the production of mcl-PHA in pseudomonads.

Production of PHAs in transgenic bacteria

Many publications describing the accumulation of PHAs in recombinant prokaryotic hosts are available now, and they largely contributed to clarify the PHA metabolism.

The expression of *phb*A, *phb*B and *phb*C genes from *R. eutropha* (Madison & Huisman, 1999) in *E. coli* leads to the accumulation of PHB starting from the available pool of acetyl-CoA. Also mcl-PHA accumulation has been achieved in recombinant hosts re-directing intermediates from both β-oxidation or from FAB upon transfer, in this latter case, of the acyl moieties from ACP to a CoA molecule by the action of the *pha*G gene (Fiedler et al., 2000; Hoffmann et al., 2000a). Klinke and co-workers (Klinke et al., 1999) showed that also the expression of the *pha*C1 gene from *P. oleovorans* in combination with a truncated, cytoplasmic form of the *E. coli tes*A gene, coding for the thioesterase-I (Cho & Cronan, 1993 and 1995) in recombinant *E. coli* deficient in one step of the β-oxidation, led to accumulation of mcl-PHA from FAB.

Production of PHA in transgenic plants.

A recent study indicates that PHB and mcl-PHA can be produced by optimised bacterial fermentation using cheap substrates (Choi & Lee, 2000; see also Chapter 7, general discussion). However, at the moment fermentative production of the polymer is still very expensive (Poirier et al., 1995; Choi & Lee, 2000; Lee & Choi, 2001) and the disadvantage of most biotechnological approaches employing prokaryotic, i.e. microbial systems is the need of relatively complicated bioreactor facilities to ensure high-grade end products.

From 1992 on, when Poirier and coworkers (1992) published the first evidence of PHB synthesis in *Arabidopsis thaliana*, the use of transgenic plants, for low-cost and bulk PHA production, using as substrates intermediates of the fatty-acid metabolism, is considered an alternative and attractive strategy. Table 2 summarises reports describing the accumulation of PHAs in transgenic plants. Nawrath et al. (1994) expressed the *phb*A, *phb*B and *phb*C genes from *R. eutropha* in the chloroplasts of *A. thaliana*, resulting in the accumulation of up to 14 % of the cell dry weight of PHB in the leaves.

Subsequently, PHB was synthesised in *Brassica napus* (Nawrath et al., 1995), and in *Gossypium hirsutum* (John & Keller, 1996), transformed with the *R. eutropha phb*B and *phb*C genes (Table 2).

The production of the heteropolymer PHB / HV, much more flexible and useful than the stiff and brittle homopolymer PHB for possible commercial applications, has been reported by Slater and coworkers (1999) in *A. thaliana* and in *B. napus*. In that work, the threonine deaminase, coded by the *E. coli ilv*A gene, was expressed in the chloroplast of *A. thaliana* and *B. napus* in combination with the *R. eutropha* broad-substrate-specific ketothiolase (coded by the *bkt*B gene), acetoacetyl-CoA reductase and PHB-polymerase. Threonine deaminase and the endogenously expressed pyruvate-dehydrogenase-complex provided propionyl-CoA, from which, *via* condensation with a molecule of acetyl-CoA, 3-hydroxyvaleryl-CoA was synthesised. The latter was subsequently incorporated into the growing chain together with 3-hydroxybutyryl-CoA by the action of the PHB-polymerase, thus yielding the heteropolymer PHB / HV. PHB has been produced in transgenic seeds of *B. napus* (Houmiel et al., 1999) by co-expressing alternatively two *R. eutropha* ketotiolases (coded by *phb*A and *bkt*B genes), acetoacetyl-CoA reductase and PHB-polymerase in leucoplasts. The accumulation of PHB has been also obtained by Hahn and coworkers (1999) in peroxisomes of transgenic maize, by Nakashita and coworkers (1999) in transgenic tobacco and by Bohmert and coworkers (2002) in transgenic tobacco and potato plants.

Also mcl-PHAs have been produced in *A. thaliana*: Mittendorf et al. (1998) achieved the accumulation of mcl-PHA by the sole expression of the *P. aeruginosa* Pha-C1 or Pha-C2 polymerases in peroxisomes. In this compartment, substrates for the mcl-PHA-polymerases were derived from β-oxidation. The physical properties of the mcl-PHA produced in these transgenic plants were still suboptimal, resulting in a very sticky material due to large amounts of unsaturated fatty acids and to the low molar percentage of C-6, C-8 monomers. The properties of the polymer have been improved by exogenously providing novel fatty acids in feeding experiments, or by co-expressing the Pha-C1 polymerase in peroxisomes and as acyl-ACP

thioesterase in plastids from *Cuphea lanceolata* (Mittendorf et al., 1999), thus resulting in a new pool of different monomers available for polymerisation.

In general, it is envisaged that the use of starch-storing plants will be more promising for PHA production, due to the generally larger productivity (oil-*versus* starch-content of starch crops compared to oil crops (van der Leij & Witholt, 1995). Moreover, the accumulation of PHA in starch-storing organs, like potato tubers, is expected to have less unwanted side effects on the normal development and fertility of the plant (Pool, 1989). Also the exploitation of FAB as source of substrates for PHA biosynthesis, compared to β-oxidation which is active only during a limited period of the life cycle, is considered more suitable for the accumulation of PHAs in large scale.

Nevertheless, only a few reports describe the use of starch-storing plants for the accumulation of PHB (Hahn et al., 1999; Bohmert et al., 2002).

Table 2. List of transgenic plants accumulating scl-PHA and mcl-PHA. *: indicates from which tissue PHAs have been extracted and quantified. [1]:*Ilv*A gene codes for the wild type threonine deaminase. [2]: *bkt*B gene codes for a ketothiolase form with broad substrate specifity. [3]: *pha*C gene from *Nocardia corallina* was used in parallel with the analogous gene from *R. eutropha*. [4]: *Fat*B3 gene from *Cuphea lanceolata* codes for a medium-chain acyl-ACP thioesterase. [5]: the value reported in the cited references (which is indicated between brackets) is expressed in percentage of polymer per fresh weight, and the yield per cell dry weight has been estimated considering that about 90 % of the fresh weight consisted of water. [6]: the percentage of polymer reported in the cited reference (indicated between brackets) is given per seed fresh weight. For the estimation of the corresponding percentage of polymer per dry weight, it has been considered that 15% of the seed weight still consisted of water.

Plant	Gene	Origin	Targeting	PHA	% polymer per cell dry weight	Reference
A. thaliana	*phb*B *phb*C	*R. eutropha* *R. eutropha*	cytoplasm	PHB	0.1 **(leaves)*	Poirier et al. 1992
A. thaliana	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	14 *(leaves)*	Nawrath et al. 1994
A. thaliana	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	40 *(leaves)*	Bohmert et al. 2000
A. thaliana	*bkt*B[2] *phb*B *phb*C *ilv*A[1]	*R. eutropha* *R. eutropha* *R. eutropha*[3] *E. coli*	plastid	PHB / HV	0.1 – 1.6 *(leaves)*	Slater et al. 1999; Valentin et al. 1999
G. hirsutum	*phb*B *phb*C	*R. eutropha* *R. eutropha*	cytoplasm	PHB	0.3 *(fibers)*	John & Keller 1996
B. napus	*phb*B *phb*C	*R. eutropha* *R. eutropha*	cytoplasm	PHB	0.02 –0.1 *(leaves)*	Nawrath et al. 1995; Poirier 2002a
B. napus	*bkt*B[2] *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	leucoplast	PHB	0.02 - 7.5 [6] (0.02 – 6.39) *(seeds)*	Houmiel et al. 1999
B. napus	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	leucoplast	PHB	0.02 – 9 [6] (0.02 – 7.7) *(seeds)*	Houmiel et al. 1999; Valentin et al. 1999
B. napus	*bkt*B[2] *phb*B *phb*C *ilv*A[1]	*R. eutropha* *R. eutropha* *R. eutropha*[3] *E. coli*	leucoplast	PHB / HV	0.7 – 2.3 *(seeds)*	Slater et al. 1999 Vlentin et al 1999
N. tabacum	*pha*C *phb*B	*A. caviae* *R. eutropha*	cytoplasm	PHB	0.01 [5] (0.001) *(leaves)*	Nakashita et al. 1999
N. tabacum	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	0.008– 0.32 *(leaves)*	Bohmert et al. 2002
Z. mays	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	peroxisome	PHB	2.0 [5] (0.2) *(cell suspensions)*	Hahn et al. 1999
Z. mays	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	5.7 *(leaves and stalks)*	Poirier 2002a
S. tuberosum	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	0.002 – 0.009 *(leaves)*	Bohmert et al. 2002
A. thaliana	*pha*C1 *pha*C2	*P. aeruginosa* *P. aeruginosa*	peroxisome	mcl-PHA	0.01 – 0.4 *(leaves)*	Mittendorf et al. 1998
A. thaliana	*pha*C1 *Fat*B3[4]	*P. aeruginosa* *C. lanceolata*	peroxisome plastid	mcl-PHA	0.08 – 0.6 *(leaves)*	Mittendorf et al. 1999

The following part of this chapter will describe the models designed to produce PHB and mcl-PHA in transgenic potato. Backgrounds on potato transformation are also outlined and the motivations for the development of a particle bombardment-mediated co-transformation system are discussed.

Model to produce PHB in transgenic potato

As outlined above, the production of PHB in heterologous hosts, including plants, is well-documented in literature. Three genes from *R. eutropha*, namely the *phb*A, *phb*B and *phb*C genes, coding for a ketothiolase, an NADP-dependent acetoacetyl-CoA reductase and for the PHB-polymerase, respectively, are required for the accumulation of PHB. The corresponding proteins have been expressed in chloroplasts or in peroxisomes of plants (Nawrath et al., 1994; Hahn et al., 1999). In these heterologous hosts, the endogenous pool of acetyl-CoA was used as a precursor for PHB biosynthesis. A ketothiolase activity, required for the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, the first committed step in the PHB biosynthetic pathway, is endogenously present in the plant cytoplasm and it is involved in the synthesis of isoprenoids. Therefore, a combination of *phb*B and *phb*C represents the minimum donor gene set for the accumulation of PHB when relevant enzymes are expressed in plant cytoplasm (Poirier et al., 1992; John & Keller, 1996; Nakashita et al., 1999).

The synthesis of PHB is based on the consumption of acetyl-CoA as initial substrate. This molecule, being an essential intermediate in a number of metabolic pathways, is available in several cell compartments. This makes a metabolic engineering approach to produce PHB in transgenic potato plants straightforward. For these reasons, the possible synthesis of PHB in transgenic potato was employed as a model (Chapter 4) for subsequent attempts to produce mcl-PHA in transgenic potato (Chapters 5 and 6). The accumulation of PHB in potato was achieved after the expression of the *phb*B and *phb*C gene products in the cytoplasm of transgenic lines.

Models to produce mcl-PHA in transgenic potato.

After obtaining evidence for the synthesis of minimal amounts of PHB, new models were designed to produce mcl-PHA in transgenic potato. Three approaches were chosen and they are shown in figure 4. Given that the minimum gene set for mcl-PHA accumulation was not known at the beginning of this project, the first model was based on the sole expression of the Pha-C1 polymerase in the cytoplasm. The cytoplasm does not contain 3-(*R*)-hydroxyacyl-CoA, which is the monomeric substrate of the PHA-polymerase. Therefore, this substrate, in its non-CoA activated form, was added in the medium of cell suspension cultures in a feeding experiment (Figure 4, path 1. Chapter 5). Successful synthesis of mcl-PHA showed that the Pha-C1 polymerase was active in the potato cytoplasm. The next step to define the minimum gene-set required for mcl-PHA accumulation was the introduction of a pathway that would supply the 3-(*R*)-hydroxyacyl-CoA monomers *in vivo*. In the other two models represented in figure 4, FAB is employed to supply monomeric precursors. Rehm et al. (1998) reported the isolation and characterisation of the key gene for the accumulation of mcl-PHA from FAB in *P. putida*, *pha*G, which codes for a 3-(*R*)-hydroxyacyl-ACP-CoA transacylase. Later, Klinke et al. (1999) showed the accumulation of mcl-PHA from non-related sources after the expression of the mcl-PHA-polymerase and a truncated, cytoplasmic form, of the *E. coli* thioesterase-I (*'tes*A gene. Cho & Cronan, 1993 and 1995), in the cytoplasm of recombinant *E. coli*. The exploitation of FAB for the accumulation of mcl-PHA in plants would require that the expression of the candidate genes (*pha*G and *'tes*A) would be directed to the cellular compartment where FAB occurs, e.g. in the plastids. Free 3-(*R*)-hydroxyacyl-CoA and 3-(*R*)-hydroxyacyl-groups would originate from the action of the transacylase and the thioesterase-I on the FAB acyl-ACP intermediates. Subsequently, they would be incorporated in the polymer, directly, or after CoA activation, in case '*tes*A gene was used. In path 2 (Figure 4), the Pha-C1 polymerase is expressed in the cytoplasm. In this case, the 3-(*R*)-hydroxyacyl moieties derived from FAB, activated with a CoA molecule or not, would have to be exported to the cytoplasm, and, subsequently, polymerised. In path 3 (Figure 4 and Chapter 6) the Pha-C1

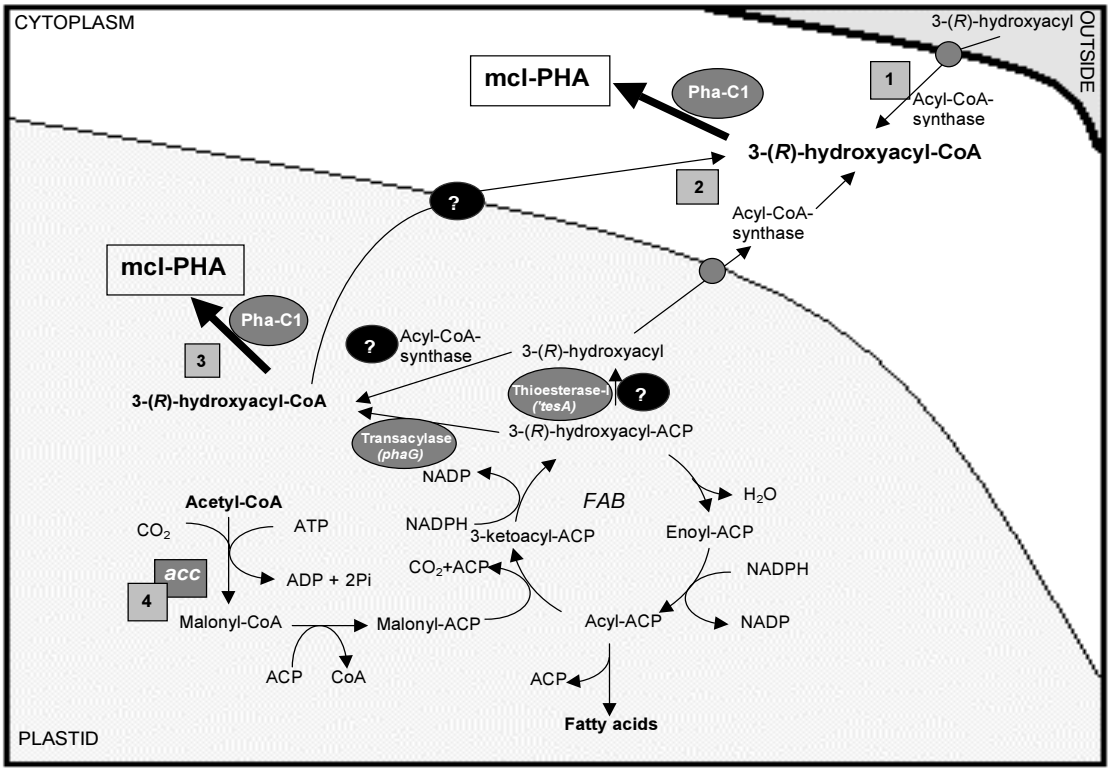

Figure 4. Models for the production of mcl-PHA in transgenic potato.
1 2 3 represent the three different possible pathways leading to mcl-PHA accumulation described in the text. 4 represents the key step controlling FAB.

polymerase is expressed in the plastids. Thus, the 3-(*R*)-hydroxyacyl-groups, released by the action of *pha*G or *'tes*A gene products, would be directly available for polymerisation in the same compartment. This is considered the most suitable system for mcl-PHA accumulation *in planta*. During the current project, path 2 was not addressed due to a number of doubtful steps that would be required in the metabolic engineering path. Also in path 3 some doubtful steps are present. These are indicated by question marks in figure 4. If the *pha*G gene would have been used in path 2, it is not certain whether CoA-activated and hydroxylated monomer would be efficiently transported through the double layer plastid membrane. The approach based on the thioesterase-I is questionable in both path 2 and path 3, because it is not known whether thioesterase-I would be active on hydroxy fatty acids formed during FAB. If thioesterase-I is only active on straight chain fatty acids, a few steps of the β-oxidation pathway, which is localised in the peroxisomes, would

be required. Finally, in path 3, it is not known whether the plastid acyl-CoA synthases are active on mcl-hydroxy fatty acids released by thioesterase-I.

A starch storing crop as site for PHA biosynthesis

PHA metabolism is closely related to fatty acid metabolism which would make oil crops logical hosts for the accumulation of PHAs. Indeed, the first species reported to accumulate PHAs have been oil accumulating plants (*Arabidopsis* and *Brassica*; Nawrath et al., 1994 and Nawrath et al., 1995). However, in terms of biomass starch crops are more productive (see Chapter 8) and thus, more profitable for accumulation of bulk PHAs. The predominance of carbohydrate metabolism in these plants could however yield various problems. It is expected that the re-direction of the carbon flux from carbohydrate metabolism to fatty acid metabolism in at least some starch crops would be required for the accumulation of PHAs at levels which will be suitable for industrial applications. It has been shown in transgenic potato, in which the antisense-ADP-glucose-phosphorylase (AGPase) was expressed, that starch synthesis was impaired and sucrose was accumulated as free sugar in the tubers (Müller-Röber et al., 1992). Thus, eventual over-expression, in these "low-starch" potato lines, of the genes involved in controlling and enhancing the glycolysis (phospho-fructokinase, cloned from *Solanum tuberosum* by Smith et al., 1993) to convert the sucrose into acetyl-CoA and overexpression of the genes involved in controlling fatty acid biosynthesis could enhance total fatty acid metabolism and provide precursors for PHA synthesis in large amounts. The enzyme which exerts most of the control over fatty acid biosynthesis is acetyl-CoA carboxylase (ACCase; Page et al., 1994), which catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Figure 4, path 4).

In conclusion, it is clear that a number of genes, involved on the one hand in the synthesis of PHAs, on the other hand in carbon flux re-direction, are needed for the accumulation of PHAs in transgenic potato. This demand had evident consequences in the choice of the transformation methodology and approach.

Transformation approach

Potato is the fourth most important food crop in the world following wheat, rice and maize. Several different methods exist to transfer foreign traits into potato, which are generally based on *Agrobacterium tumefaciens*-mediated transformation and, in a limited number of cases, on direct gene transfer (Romano et al., 2002). A large number of agronomically useful genes have already been transferred to potato. Most of these genes play a role in virus resistance (Douches et al., 1998), starch composition (Visser et al., 1997a; Visser et al., 1997b), oral vaccination (Mason & Arntzen, 1995) and antibodies production (Artsaenko et al., 1998). All are based on the transfer and the expression of one transgene. However, approaches involving the co-ordinated expression of more than one transgene gained considerable importance for novel agronomically relevant traits. One of these is the production of PHAs in transgenic plants.

The production of PHAs in potato requires that a whole new pathway, consisting of several transgenes, is introduced. There are a number of approaches to integrate several genes in one plant: *Agrobacterium*-mediated co-infection, re-transformation, crossing, and transformation with large plant vectors containing more than one gene besides the selectable marker. However, a few attempts aiming at simultaneous co-transformation using *Agrobacterium*, attempted during this project, were not successful. The scarcity of selectable markers (Romano et al., 2002) does not allow a re-transformation approach and the high heterozygosity of potato, results in the loss of the original genotype, and also makes crossing approaches not recommendable. The construction of large plant vectors is time consuming and the number of genes which can be delivered into one plasmid is limited. Furthermore, given that the minimum gene set for PHA accumulation was not completely elucidated at the time this work started, co-transformation with separate plasmids, allowing a certain flexibility in combining different genes, appeared much more amenable than the use of large plant vectors for each combination of genes to be tested. Thus, the use of particle bombardment mediated transformation (Klein et al., 1988) to simultaneously introduce all relevant genes was considered. Particle bombardment is based on the

acceleration of metal micro projectiles coated with DNA into living (plant) tissues. From the end of the 80s, particle bombardment has been employed successfully in transformation of many crops representing a substantial part of food crops (Christou, 1994; Christou et al., 1995a; Christou, 1995b; Christou, 1997) and nowadays the particle bombardment technique is used on a routine scale to engineer agronomically important cultivars. However, no protocol for particle bombardment-mediated transformation of potato was available. The initial part of this project was thus dedicated to the development of protocols for stable particle bombardment-mediated.

Scope and content of the thesis

The work described in this thesis is focussed on the development of methodologies to produce PHAs in transgenic potato. PHA production in plants requires the expression of multiple genes. Thus, as a prerequisite to achieve PHA accumulation in potato, procedures to simultaneously co-transform potato with several genes in separate DNA molecules were developed and were based on particle bombardment-mediated transformation. These protocols are described in Chapters 2 and 3. Chapter 3 also describes a procedure for particle bombardment-mediated transformation of potato using gene-cassettes (i.e. a DNA fragment comprising promoter – gene – terminator). These protocols were subsequently used to introduce the genes responsible for PHA biosynthesis in potato. Four methods were considered in total and are described in Chapters 4, 5 and 6. 1) The synthesis of PHB was attempted after the expression of *R. eutropha* acetoacetyl-CoA reductase and PHB-polymerase. This approach is described in Chapter 4. In this model, the cytoplasmic pool of acetyl-CoA would contribute to the synthesis of PHB. 2) Chapter 5 describes the expression of the *P. oleovorans* Pha-C1 polymerase in the cytoplasm of transgenic potato. The transcription and the translation of the *pha*C1 gene were confirmed. The polymerase was able to direct mcl-PHA synthesis using exogenously provided substrates.

Two possible routes were finally considered to direct intermediates from FAB to mcl-PHA biosynthesis and are described in Chapter 6. 3) A truncated form

of the *E. coli* thioesterase-I and the *P. oleovorans* Pha-C1 polymerase were expressed in the plastids of transgenic potato. The action of the thioesterase on 3-hydroxy intermediates of FAB, in combination with a plastidial acyl-CoA synthase, was expected to act as precursor supply for mcl-PHA. 4) The *P. putida* ACP-CoA-transacylase and the *P. oleovorans* Pha-C1 polymerase were expressed in the plastids of transgenic potato. The precursor supply route consisted in this case of the transfer of hydroxyacyl moieties from ACP (intermediates of FAB) to CoA. In Chapter 7, the results described in the previous chapters are discussed and future prospects of PHAs are outlined.

CHAPTER 2

Transformation of potato (*Solanum tuberosum*) using particle bombardment[1]

[1] Based on: Andrea Romano, Krit Raemakers, Richard Visser, Hans Mooibroek. 2001. Transformation of potato (*Solanum tuberosum*) using particle bombardment. Plant Cell Reports, 20:198-204

Abstract

Internodes, leaves and tuber slices from potato (Solanum tuberosum), genotype 1024-2, were subjected to particle bombardment. Transient expression was optimized using the uidA and the luc reporter genes coding for β-glucuronidase (GUS) and luciferase respectively. Stable transformation was achieved using the neomycin-phosphotransferase (nptII) gene, conferring resistance to the antibiotic kanamycin. The influence of biological parameters (tissue type, growth period before bombardment, pre- and post-bombardment osmoticum treatment) and physical parameters (Helium pressure, tissue distance) possibly known to affect stable transformation were investigated. Putative transgenic plants, which rooted in kanamycin-containing media, were obtained from all tissues tested with large differences in the efficiency: internodes (0.77 plants per bombarded explant), microtuber slices (0.10 plants per bombarded explant) and leaves (0.02 plants per bombarded explant). Southern blot analysis of putative transgenic plants confirmed the integration of the transgenes into plant DNA. The results indicate that an efficient particle bombardment protocol is now available for both transient and stable transformation of potato internodal segments, thus contributing to an enhanced flexibility in delivery of transgenes to this important food crop.

Key words: *Solanum tuberosum*, internodes, particle bombardment, regeneration, stable transformation.

Introduction

A range of different transformation procedures have been developed for potato which are either based on direct gene transfer into protoplasts or based on the use of *Agrobacterium*. *Agrobacterium* has been used successfully for transformation of leaves (De Block, 1988), internodes (Newell et al., 1991), and tubers (Ishida et al., 1989). Until now, no protocol was available for potato transformation using the biolistic technique. Particle bombardment is the most used method of transformation for those plants that are not susceptible to *Agrobacterium* transformation, like most of the monocots and some dicots (for a review, see Christou, 1994). Application of bombardment to plants that can be efficiently transformed by *Agrobacterium* has been limited only to model species such as tobacco (Klein et al., 1988; Tomes et al., 1990; Hunold et al., 1994), and *Arabidopsis thaliana* (Seki et al., 1991).

Nevertheless, the development of particle bombardment-mediated protocols in addition to *Agrobacterium* methodologies, enables the choice of the most suitable technology in relation to the experimental objectives. In the case of potato transformation, as long as one single gene trait is required, *Agrobacterium* could be the preferred technology. On the other hand, when the new traits require co-ordinated integration and expression of several genes, particle bombardment could lighten and speed up the necessary work. In fact, the currently used plasmids for *Agrobacterium*-mediated plant transformation can carry only 2 or 3 genes, at least one of which should be a selectable marker. Efficient cotransformation of two T-DNAs using two *Agrobacterium* strains has already been reported in *Brassica napus* (39% to 85% of cotransformation efficiency; De Block & Debrouwer, 1991), tobacco and *Arabidopsis thaliana* (De Neve et al., 1997; De Buck et al., 1998). However, to our knowledge, no report exists about the simultaneous integration of more than two T-DNAs into the plant genome, carrying 4-6 different genes with at least one selectable marker. This means that modification of traits based on multiple genes with *Agrobacterium* is time-consuming and laborious as it either requires re-transformation or crossing of single transgenic plants.

The efficiency of cointegration of plasmids using particle bombardment has

been well documented in literature. Usually, the cotransformation frequency for two plasmids is up to 85% (Bower et al., 1996; Wakita et al., 1998; Hilliou et al., 1999). It has been shown that more than 10 different plasmids can be co-delivered into the plant genome by particle-bombardment (soybean, Hadi et al., 1996 and rice, Chen et al., 1998).

Here we report the development of protocols for transient and stable potato transformation using the particle bombardment technique. The physical parameters for transient transformation were optimized using the *uid*A and *luc* reporter genes, encoding β-glucuronidase (GUS) and luciferase respectively, and for stable transformation using the neomycin-phosphotransferase (*npt*II) gene as selectable marker. Transgenic plants were obtained after bombardment of leaves, internodes and microtubers as starting tissue.

Materials and Methods

Plant Material.

Internodes, leaves and microtubers of in vitro grown *Solanum tuberosum*, genotype 1024-2 (*amf*, diploid; Jacobsen et al. 1989) were used in the particle bombardment experiments. Whole plants were regenerated from each tissue as follows: internodes were split longitudinally and cultured horizontally with the cut side up on CIM I Murashige and Skoog (1962) salts plus vitamins (MS), 2% sucrose, 2 mg / L 2-4 D, 0.5 mg / L zeatine, 0.8% Daishin agar (Duchefa), pH 5.8 before autoclaving. Whole leaves were cultured with their abaxial side on CIM II (MS, 3% sucrose, 2 mg / L BAP, 5 mg / L GA_3, 0.2 mg / L NAA, 0.8 % Daishin agar, pH 5.8 before autoclaving).

Microtubers were first induced from nodal segments of in vitro grown plants on MS, 8% sucrose, 1 mg / L BAP, 2.5 mg / L kinetine, 200 mg / L cefotaxime, pH 5.8 before autoclaving and solidified with 0.2 % gelrite (Duchefa). Microtubers with a diameter of 0.5 cm were cut in slices and cultured on CIM III (MS, 3% sucrose, 1 mg / L thiamine, 0.5 mg / L nicotinic acid, 0.5 mg / L pyridoxine, 1 mg / L zeatine, 0.5 mg / L IAA, 0.8% Daishin agar, pH 5.8 before autoclaving).

Internodal segments were transferred to shooting medium I (MS, 2% sucrose,

1 mg / L zeatine, 0.1 mg / L GA_3, 0.6 mg / L STS, 0.8% Daishin agar, pH 5.8 before autoclaving) 9 days later. Leaves were grown for 8 weeks on callus inducing medium II and then transferred to shooting medium II (MS, 3% sucrose, 2 mg / L BAP, 5 mg / L GA_3, 0.8 % Daishin agar, pH 5.8 before autoclaving). Tubers were cultured continuously on CIM III.

All explants were transferred to fresh medium every three weeks. Regenerating shoots were rooted on MS with 3% sucrose.

DNA constructs.

For transient expression studies, 35-S-GUS, pAPP34, pJIT64 and pJIT100 were employed. Plasmid 35-S-GUS and pAPP34 contained the *uid*A gene controlled by the 35-S promoter and 35-S terminator, and by the E-35-S promoter and the *nos* terminator, respectively. Plasmid pJIT100 contained the *luc* gene and the *bar* gene (encoding the phosphinothricin acetyl transferase), both under control of the 35-S promoter and terminated by the 35-S polyadenylation region. Plasmid pJIT64 contained the *luc* gene driven by the E-35-S promoter and terminated by the 35-S polyadenylation region. Plasmids 35-S-GUS, pJIT64 and pJIT100 were kindly provided by the John Innes Research Institute, Norwich, United Kingdom. The E-35-S and *nos* terminator fragment for pAPP34 was obtained from pBIN35Snos (Rouwendal et al., 1997) digested with *Hin*dIII / *Eco*RI. This fragment was subcloned into pUC18 (Pharmacia) giving rise to pAPP23. The *uid*A open reading frame was amplified by PCR using primers designed to introduce *Nco*I and *Bam*HI sites at the 5'- and 3'-ends, respectively, of the gene. Plasmid pAPP33 was subsequently constructed by introducing the modified *uid*A gene into the *Nco*I site of pAMV-1 (Rouwendal et al., 1997). The *Bgl*II / *Bam*HI fragment from pAPP33 containing the AMV- *uid*A cassette was finally cloned into *Bam*HI site of pAPP23, giving rise to pAPP34. Plasmid 35-S-KAN (John Innes Center, Norwich, UK) and pDC2 (Plant Genetic Systems, Aventis N.V., Gent), were used for stable expression experiments. Vector 35-S-KAN contained the *npt*II gene driven by the 35-S promoter and terminator, whereas pDC2 contained the *npt*II gene driven by the *'tr*1 promoter (Velten et al., 1984), together with the *uid*A and the *bar* genes controlled by the *'tr*2 (Velten et al., 1984) and the 35-S promoters, respectively.

Particle bombardment.

DNA was coated onto gold particles (1.5-3.0 μm diameter, Aldrich) as described by McCabe et al. (McCabe et al., 1988). In short: 10 mg of gold particles were suspended in 150 mM NaCl, 60 mM Tris. Samples (20 μg) of plasmid DNA were added, followed by the addition of 100 μl spermidine (100 mM), 100 μl of poly-ethylene-glycol (100 mM) and 100 μl of $CaCl_2$ (2.5 M). After 10 minutes of incubation at room temperature, particles were washed twice with ethanol 100% and resuspended in 200 μl of absolute ethanol.

A home-made device, HFG, based on the model of the Particle Inflow Gun (Finer et al., 1992) was used. Ten μl of gold suspension were delivered per shot in a partial vacuum set at 50 mbar.

Transient expression in potato was optimized in a series of experiments where different parameters were tested: the effect of using a 0.2 mm mesh size screen between the tissue and the syringe filter, the number of shots per Petri-dish (1 or 2), different distances (3 - 15 cm) of the tissue from the syringe filter, different Helium pressures (1 - 10 bar), and 24 hours of osmoticum pre- and post-treatment (0.1 M sorbitol plus 0.1 M mannitol)

Between 8 - 10 leaves, 15 - 20 split internodes and 8 - 10 tuber slices were cultured to cover the whole surface of a 3.5 cm Petri-dish containing MS, 3% sucrose medium. GUS and *luc* assays were performed 24 hours after bombardment. Standard deviations (SD) were calculated from 3 or 4 Petri-dishes.

In the stable transformation experiments, the three types of tissues were cultured on 3.5 cm Petri-dishes containing the appropriate callus-inducing medium. In three different experiments, the effect of the Helium pressure (6, 8, 10 bar), of the period of growth before transformation (between 1 to 9 weeks) and the effect of different osmoticum treatments (0.0, 0.2, 0.3, 0.4, 0.5 M sorbitol, or 0.0, 0.2, 0.3, 0.4, 0.5 M sorbitol plus mannitol, each) were investigated. The plant material was covered with a 2 mm mesh-size screen and placed 9 cm below the syringe filter, bombarded twice with gold particles coated with DNA of 35-S-KAN. One day after bombardment, the explants were transferred to the appropriate callus-inducing medium (without osmoticum). Kanamycin (100 mg / L) was added to the medium during the

whole process including rooting of the regenerated shoots. In all experiments, 3 negative controls were included: a) non-bombarded explants cultivated on kanamycin-containing medium. b) explants bombarded with DNA-free gold suspension cultivated on kanamycin-containing medium. c) explants bombarded with pUC18 coated gold suspension and cultivated on kanamycin-containing medium.

GUS and luc detection.

Tissues were assayed for GUS activity by dipping the explants in 0.05 M NaH_2PO_4 / Na_2HPO_4, 0.1% Triton X-100, 1mM x-Gluc, and incubation for 24 hours at 37°C (Jefferson et al., 1987).

Tissues were assayed for luciferase activity by spraying with 0.15 mg / mL luciferin (Promega E 160). Luciferase activity was measured with a VIM intensified CD camera and an Argus-50 photon counting image processor (Hamamatsu Photonic System; Raemakers et al., 1996).

Southern blot analysis.

Genomic DNA was isolated from leaves of plants rooting on kanamycin-containing medium, and equal amounts of DNA were *Bam*HI restricted and blotted on a positively charged nylon membrane (Hybond^{TM}-N+, Amersham) using standard techniques (Sambrook et al., 1989). The filter was hybridized with a digoxigenin-labeled probe amplified by PCR from 35-S-KAN (*npt*II gene) as described by the manufacturer (Boehringer, Mannheim, Germany), or with a ^{32}P radioactively labeled probe using standard molecular biology techniques (Sambrook et al., 1989).

Results

Transient expression.

After the general physical parameters of the HFG were optimized, the tissue was placed 9 cm below the syringe filter, and a screen (0.2 mm mesh size) was placed 6 cm above the tissue. This resulted in a more even distribution of the particles and less damage in the central area, as previously observed with this kind of apparatus (Finer et al., 1992).

An extra 1.5 mm mesh size grid was placed in contact with the explants to maintain their position during shooting.

Transient expression (Figure 1a) was studied using plasmid 35-S-GUS, pAPP34 (E-35-S-GUS), pJIT100 (35-S-*luc*) and pJIT64 (E-35-S-*luc*).

In most cases, the use of the E-35-S promoter was associated with a more than 10 fold higher level of transient expression (Table 1). Only for leaves the difference between the two promoters associated with the *luc* gene was less pronounced.

In stable transformation experiments, the *'tr*2 promoter (pDC2) combined with the *uid*A gene was used. The number of blue spots observed with pDC2 was comparable with the results obtained with the *uid*A gene driven by the 35-S promoter of 35-S-GUS (results not shown).

Plasmid pAPP34 (E-35-S promoter) was chosen to optimize other parameters in transient expression experiments.

The minimum Helium pressure necessary to obtain transient expression was studied using internodes and leaves. At 1 bar less then 20 blue spots per Petri-dish were observed. The number of spots increased to more than 600 for leaves and around 150 for internodes when 7 bar Helium pressure was used (Figure 2). In another set of experiments, it was found that the transient activity increased up to a pressure of 8 bar. Above this pressure, no further

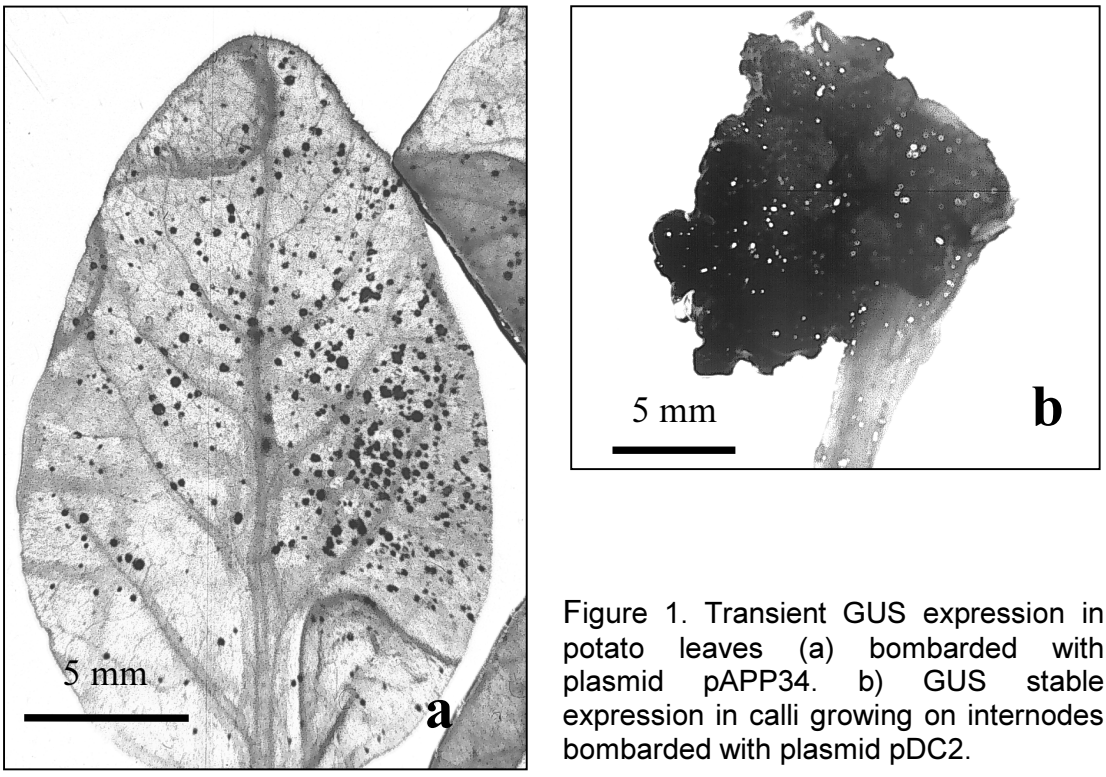

Figure 1. Transient GUS expression in potato leaves (a) bombarded with plasmid pAPP34. b) GUS stable expression in calli growing on internodes bombarded with plasmid pDC2.

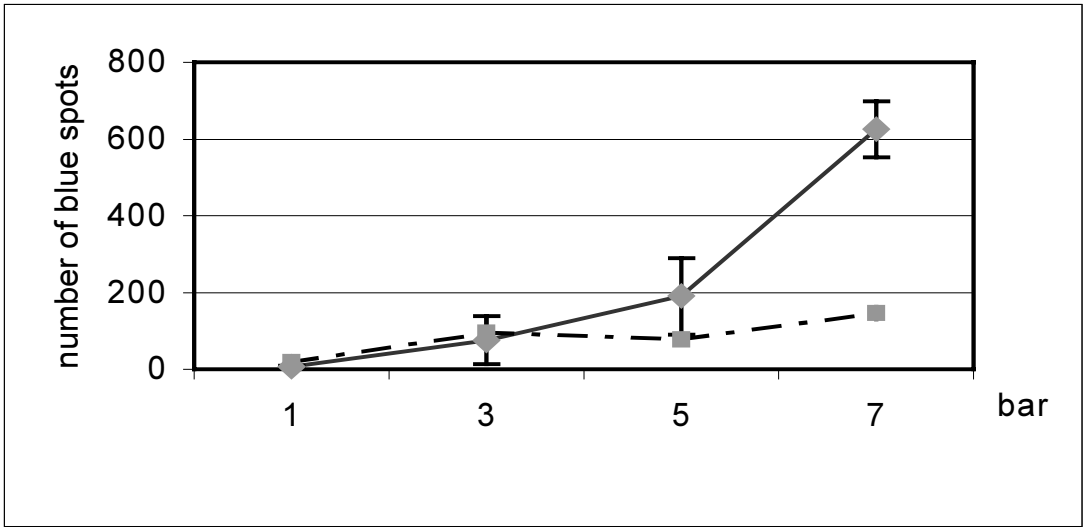

Figure 2: Number of blue spots detected in stems (■) and leaves (♦) at different Helium pressures. Bars indicate the standard deviation (from 3 to 4 Petri-dishes. Each Petri-dish contained 8 - 10 leaves or 15 - 20 split internodes.

rise of the transient GUS expression was detected. A pressure of 7 or 8 bar was chosen as standard in the following experiments on transient expression. Leaves treated with an osmoticum (0.1 M sorbitol plus 0.1 M mannitol) showed a significant increase in transient activity (1058 ± 105 blue spots per bombarded Petri-dish), compared to a treatment without osmoticum (626 ± 73 spots per bombarded Petri-dish). No differences were observed in internodes treated with or without osmoticum.

Several other parameters tested such as amount of DNA delivered per shot (1 μg versus 5 μg), volume of gold suspension delivered per shot (5 versus 10 μl), and gold resuspension (water versus ethanol) did not result in significant

Table 1. Transient expression of the *uid*A and the *luc* reporter genes driven by the 35-S and the E-35-S promoters. SD from 3 Petri-dishes

Promoter	Assay	Explant type		
		Stems	Leaves	Tubers
35-S-GUS	Number of blue spots per Petri-dish	13 ± 4	40 ± 23	ND
E-35-S-GUS		146 ± 17	626 ± 73	ND
35S-LUC	Number of photons per Petri-dish per minute	107 ± 31	167 ± 48	200 ± 101
E-35-S-LUC		1343 ± 631	224 ± 59	2195 ± 865

differences. However, the conditions that gave the best results were chosen for stable transformation experiments [2 bombardments per sample, 0.5 mg of DNA-coated gold particles (10 μl of gold suspension), resuspended in ethanol and carrying 1 μg of DNA].

Stable transformation.

The protocols used to regenerate plants from leaves, internodes and tubers were first tested on control explants for their efficiency. After 4 weeks of cultivation on CIM I-II and III, strong callus growth was observed on all three tissue types. Explants started to form shoots 3-4 weeks later. Leaves and internodes regenerated 10-30 shoots per explant, whereas 5-20 shoots were obtained from tuber slices. All shoots (100%) rooted after transfer to rooting medium.

In order to test any possible negative effect of bombardment on regeneration ability, explants were bombarded prior to cultivation on CIM. No visible injury was detected when 7 bar Helium pressure was used for bombardment of internodes or tubers slices. However, in leaves, brown spots were observed following bombardment. Nevertheless, regeneration efficiency was not affected.

In total three experiments were designed to investigate the effect of different parameters (Helium pressure, tissue age and osmoticum treatment) and different types of tissues (internodes, leaves and microtubers) used as starting material on stable expression. From 4 to 5 Petri-dishes were bombarded per treatment. Each Petri-dish contained 8 - 10 leaves, 15 - 20 split internodes or 8-10 tuber slices.

The use of different Helium pressures (6, 8 and 10 bar tested on leaves and internodes), the use of explants from donor plants grown for different periods of time (1, 4, 9 and 12 weeks, tested on leaves and internodes), did not result in significant differences. The use of different pre- and post-bombardment osmoticum treatments (tested on internodes and microtuber slices) had no effect on internodes. However, in the case when tuber slices were treated with media containing more than 0.4 M mannitol and 0.4 M sorbitol, most of the explants died within a few weeks.

Table 2. Total number of transgenic plants obtained after bombardment of leaves, internodes and microtuber slices. [a] Total number of plants rooted on kanamycin divided by the number of bombarded explants X100.

Tissue	Number of explants bombarded	Number of plants rooting on kanamycin
Tubers	413	17 (4%)[a]
Internodes	1462	455 (31%)[a]
Leaves	157	3 (2%)[a]

The kind of explant used as starting material proved to be an important parameter, resulting in large differences in transformation efficiency. In the three different experiments, in total 157 leaf explants were bombarded. Seven weeks after bombardment, a total of 7 calli were observed. In total 3 plants were harvested 15 weeks after bombardment (Table 2). When internodes and microtuber slices were used as starting material, green and healthy calli were observed 4 and 6 weeks after cultivation on kanamycin-containing media, respectively. In the case of internodes, the number of calli per initial explant varied between 0.20 and 0.86. The first shoots appeared 8 weeks after bombardment. The number of shoots per bombarded internode varied between 0.18 and 0.77. In total 775 independent calli were obtained, of which 470 regenerated a shoot. Ninety-six per cent of the shoots rooted on kanamycin-containing media.

In total 455 plants rooting on kanamycin-containing medium were obtained from 1462 bombarded internodes (Table 2). The best results were obtained when internodes isolated from 9 weeks grown donor plants, were bombarded at 8 bar Helium pressure and were pre-/-post-bombardment treated with 0.1 M mannitol. In this treatment 76% of the calli formed a shoot.

When microtuber slices were used as starting explants, the number of calli per bombarded microtuber slice varied from 0 (when media with 0.4 M mannitol plus 0.4 M sorbitol or more were used) to 0.44 (8 bar Helium pressure, 0.1 M mannitol plus 0.1 M sorbitol). The first shoots were collected 12 weeks after bombardment. Only 22 % of the calli regenerated a shoot. In the best treatment tested (8 bar Helium pressure, 0.1 M mannitol plus 0.1 M sorbitol), the number of shoots per bombarded tuber slice was not higher than 0.10.

In none of the experiments callus growth or formation of shoots was observed on the three negative controls.

In a fourth experiment pDC2 instead of 35-S-KAN was used to bombard internodes. This construct contained the *npt*II and the *uid*A genes, thus allowing rapid screening of the transgenic nature of calli (Figure 1b) and shoots. An average of 0.59 calli per bombarded internode was observed. Ten weeks after bombardment, all the 180 bombarded internodes were analyzed for GUS activity using the hystochemical assay. This showed that 88 % of the 107 tested calli expressed the gene.

Twelve out of the 13 plants tested were GUS positive.

Southern blot analysis.

Genomic DNA from 23 randomly chosen individual plants, which rooted on kanamycin-containing medium (results shown only for 9 of these plants in Figure 3) was extracted and digested with restriction enzymes. Fragments were separated by gel electrophoresis, and blotted onto nylon membranes for Southern blot analysis using a DIG- or radioactive-labeled *npt*II probe. All 23 plants were positive. According to the number of visible bands, the transgene number of inserts ranged from at least 1 to 5.

Genomic DNA from another set of 9 individual plants, was also digested with restriction enzymes in order to cut out the transgene expression cassette. All tested plants showed integration of the complete gene cassette (results not shown).

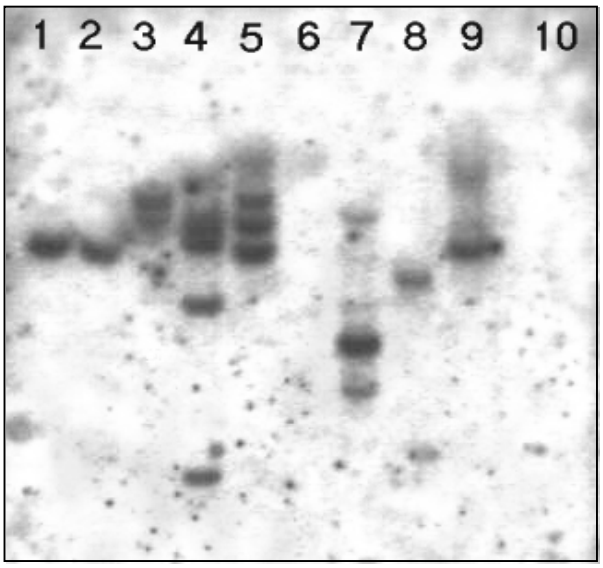

Figure 3: Southern blot analysis of genomic plant DNA digested with a restriction enzyme not cutting in the integrated plasmid (*Bam*HI). Lane 1-9: 9 independent plants rooted on kanamycin containing medium. Lane 10: negative control (genomic DNA from wild type plant).

Discussion

Agrobacterium transformation is the most commonly used gene transfer technique for potato. However, not all species can be efficiently transformed by *Agrobacterium*. Even in those species in which *Agrobacterium* is successfully employed, certain cultivars may be recalcitrant to transformation by *Agrobacterium*, as was shown for potato by Figueira Filho et al. (1994).

The use of protocols based on direct gene transfer of protoplasts is limited due to problems with regeneration and occurrence of somaclonal variation (Karp et al., 1982; Kumur, 1994). The development of protocols based on particle bombardment-mediated transformation of whole tissues or cell suspensions might overcome problems related to regeneration (Jahne et al., 1995).

Transformation protocols based on particle bombardment have already been developed for at least 20 species (Christou, 1992; Sanford et al., 1993; Christou et al., 1995a).

In this paper a protocol to transform potato by particle bombardment is presented. Transgenic plants were regenerated from leaves and microtuber slices with rather low efficiency (1.9 % and 4 % respectively; Table 2) when compared with the reported efficiencies of transformation by *Agrobacterium* [from 2.3 % (Visser et al., 1989) to 23 % (Yang & Zhou, 1997) and 31 % (Sheerman & Bevan, 1988), respectively]. The high level of GUS transient expression in leaves and microtubers suggests that the low efficiency of stable transformation is related to the regeneration protocol in the presence of the antibiotic regime.

The efficiency achieved in the experiments of internode transformation (Table 2) is higher than that previously reported for internode transformation by *Agrobacterium* (14 %; Newell et al., 1991). Nevertheless, it must be stressed that the internode transformation by *Agrobacterium* of the genotype 1024-2 can be as high as 200 % (2 independent shoots per internodal segment; unpublished results in our laboratories).

The time necessary to regenerate and collect the transgenic shoots is comparable with *Agrobacterium* transformation. Many parameters tested aimed at the reduction of tissue and cell damage (osmoticum treatment, and

varying the Helium pressure in order to find the right compromise between particle penetration and stable transformation) had no influence on stable transformation of internodes, indicating that this tissue is tolerant to mechanical injuries. This is, to our knowledge, the first report on potato transformation via particle bombardment. The protocol described was optimized for the 1024-2 genotype. The use of internodes as starting material gives a high efficiency of transformation in a relatively short period of time. It must to be confirmed whether the described protocol is applicable to other genotypes with the same efficiency.

Also for potato and those species or genotypes in which efficient transformation protocols based on *Agrobacterium* infection are available, particle bombardment can be advantageous in relation with the kind of transformation required. For example, multiple genes can be transferred in one transformation procedure by particle bombardment (Chapter 3).

An example of a multiple gene trait is the production of poly-3-(-hydroxybutyrate). In *Gossypium hirsutum*, poly-3-(-hydroxybutyrate) producing plants have been obtained after bombardment with one construct containing the *uid*A gene (used for selection) together with the *pha*B bacterial genes (acetoacetyl-CoA reductase) along with a second construct bearing *pha*C (poly-hydroxyalkanoate synthase). A high percentage of GUS selected plants also contained the *pha*C gene (John & Keller, 1996). This high frequency of codelivery of two plasmids has also been observed in other crops, and seems to be a general phenomenon. This high frequency of codelivery might also be used to produce marker-free plants via outcrossing of the marker gene. However, this is only feasible if the two plasmids integrate at different chromosomal sites.

Because potato is a highly heterozygous, vegetatively propagated crop, such outcrossing includes a long-term breeding procedure.

Another advantage is the fact that in particle bombardment-mediated transformation no specific DNA regions are required for the DNA integration, like in the case of *Agrobacterium*, thus allowing the introduction into the plant genome of only the target genes without any extra redundant plasmid DNA (Fu et al. 2000).

Acknowledgement. This study has been funded by a Marie Curie Fellowship (contract number FAIRCT98-5036 granted to Andrea Romano). We would like to thank Linus van der Plas, Dick Vreugdenhil and Jan Springer, Marjan Bergervoet, Elly Janssen, Irma Straaman, Diego Orzaez and our colleagues at ATO for their technical assistance and advises.

CHAPTER 3

Transgene organisation in potato after particle bombardment-mediated (co)-transformation using plasmids and gene cassettes[1]

[1]Based on: Andrea Romano, Krit Raemakers, Jamila Bernardi, Richard Visser, Hans Mooibroek. Transgenes organisation in potato after particle bombardment-mediated (co-)transformation using plasmids and gene cassettes. *Submitted to "Transgenic Research"*

Abstract

Protocols for efficient co-transformation of potato internodes with genes contained in separate plasmids or gene cassettes (i.e. linear PCR fragments comprising a promoter-gene-terminator) using particle bombardment were established. Twenty-eight out of 62 (45 %) and 11 out of 65 (17 %) plants transformed with a plasmid containing the selectable marker contained one and two additional non-selected genes, respectively. When gene cassettes were used in transformation, 6 out of 8 plants were co-transformed. Expression analysis showed that 75 % to 80 % of the plants transformed with two transgenes expressed both of them, irrespective of the use of plasmids or gene cassettes.

Thirty-eight plants containing the *uid*A reporter-gene and the *npt*II selectable-marker have been characterised with respect to the molecular organisation of the donor DNAs. Seventeen out of 49 (35 %) *uid*A sites of integration contained one copy of the gene. Only 11 *uid*A sites (22 %) were linked to the site of integration of the selectable marker. When one site of integration contained several copies of the transgene, a predominance of 3' - 3' inverted re-arrangement repeats was observed.

Key words particle bombardment, co-transformation, *Solanum tuberosum,* gene cassette, site(s) of integration, copy number

Introduction

Several protocols for transformation of potato are available today (Romano et al., 2002), and genetic engineering of this crop has become routine in many laboratories. Potato has been improved with genes conferring resistance to viruses and pests (Hoekema et al., 1989; Douches et al., 1998) and improving e.g. starch quality (Visser et al., 1997a; Visser et al., 1997b; Schwall et al., 2000). In the above cases, *Agrobacterium tumefaciens* has been used as gene-transfer technique and the traits were controlled by one gene. However, many of the important traits are controlled by multiple genes, such as metabolic pathways, like the production of polyhydroxyalkanoates in transgenic plants (Nawrath, 1994; Slater et al., 1999)

In principle, transformation with multiple genes can be achieved in a number of ways (Romano et al., 2002): by crossing, re-transformation, usage of multiple gene constructs and simultaneous co-transformation using multiple plasmids. Due to the heterozyogous nature of potato, crossing results in the loss of the original genotype. Re-transformation is limited because of the scarcity of selectable markers (Romano et al., 2002) and is, furthermore, time consuming. The construction of multiple-gene transformation vectors is time consuming and difficult. Furthermore the number of genes transferred by one plasmid is limited. *A. tumefaciens*-mediated co-transformation with two bacterial strains, although successfully reported for other crops (De Block & Debrouwer, 1991; De Neve et al., 1997), has never been reported for potato. Also in our laboratory, attempts to achieve co-transformation with *A. tumefaciens* have failed (Romano et al., *unpublished*). Simultaneous co-transformation with separate plasmids would be less laborious, faster and more flexible.

In the first part of this work, a protocol recently published for transformation of potato *via* particle bombardment (Chapter 2) has been improved and an efficient protocol for particle bombardment-mediated co-transformation with two and three separate plasmids is presented. Also a protocol for gene cassette (i.e. a DNA sequence comprised of promoter-gene-terminator) transformation using particle bombardment is described.

One of the problems of all transformation methodologies, and in particular particle bombardment, is the fact that integration of the transgenes into the plant genome does not implicate expression of transgenes. Epigenetic and position effects, gene silencing, suppression and co-suppression phenomena often result in transgene inactivation.

It is thought that transgene expression is dependent on the pattern(s) of integration of alien DNA. However, detailed information on the relation between integration events and transgene expression is scarce and inconclusive (De Block & Debrouwer, 1991; Cooley et al., 1995; De Neve et al., 1997; Chen et al., 1998; De Buck et al., 1998; Kohli et al., 1998; Stoger et al., 1998; Kohli et al., 1999; De Buck et al., 2001). The second part of this work describes the analysis of integration events. The number of sites of integration for selected and non-selected genes, their copy-number and a possible correlation with the reporter gene activity, the occurrence of re-arrangements like inverted and tandem repeats and putative maps for the sites of integration, in transgenic potato plants obtained, are described.

Material and methods

Plant Material.

Internodes of *in vitro* grown *Solanum tuberosum*, genotype 1024-2 [*amf*, diploid; (Jacobsen et al., 1989)] were used in the transformation experiments. Whole plants were regenerated from internodes as described in Chapter 2. Twenty-nine plants transformed with pDC2 and used for expression analyses in the present work were obtained in Chapter 2.

DNA plasmids.

Plasmids, 35-S-Bar, 35-S-Kan, pAPP34, pJIT64, pDC2, pAPP60, pAPP63, pAPP100 and pAPP102 were used for co-transformation. Vectors 35-S-Bar and 35-S-Kan contained the *bar* and the *npt*II genes, respectively, both driven by the 35-S promoter and terminator. Plasmid pAPP34 (Chapter 2) contained the *uid*A gene controlled by the Enhanced-35-S promoter (E-35-S) and the *nos* terminator. Plasmid pJIT64 contained the *luc* gene driven by the E-35-S promoter and terminated by the 35-S polyadenylation region.

Plasmids 35-S-Bar, 35-S-Kan (www.pgreen.co.uk) and pJIT64 were kindly provided by the John Innes Research Institute, Norwich, United Kingdom. Plasmid pDC2 (kindly provided Plant Genetic Systems, Aventis N.V., Gent) contained the *npt*II gene driven by the *'tr*1 promoter (Velten et al., 1984), together with the *uid*A and the *bar* genes controlled by the *'tr*2 (Velten et al., 1984) and the 35-S promoters, respectively. Plasmids pAPP60, pAPP63, pAPP100 and pAPP102 were pUC-18 based plant expression vectors containing the synthetic forms of the *pha*G gene from *P. putida* (pAPP60), the *pha*C1 gene from *Pseudomonas oleovorans* (pAPP63 and pAPP100) and a truncated form of the *E. coli tes*A gene (pAPP102). These genes are involved in the production of polyhydroxyalkanoate (PHA). These plasmids will be described with more details in Chapters 5 and 6.

Gene cassettes (PCR fragments).

Gene cassettes containing the *npt*II or the *uid*A genes used for transformation were produced by PCR using the *Pyrococcus woesei* polymerase (Eurogentec) according to the manufacturer. The *npt*II cassette was amplified from plasmid pBIN19 [(Bevan, 1984). NCBI accession: U12540] using primers 5' CCAGCATATGCAGGAGGCCC and 5' CCCGCCATATGTCCTGTCAAAC resulting in a fragment containing the nopaline sinthase promoter and terminator controlling the gene. The *uid*A cassette containing the 35-S promoter and *nos* terminator was amplified using primers 5' GAAAGGGGGATGTGCTG and 5' CGGCTCGTATGCTGTGTG and using plasmid pBI221 (Clontech inc.) as template.

Particle bombardment.

Particle bombardment was performed as described in Chapter 2. Separate plasmids and gene cassettes were co-precipitated at 1 : 2 or 1 : 2 : 2 molar ratios of the selected / non-selected or selected / 2 non-selected DNA molecules, respectively. The total amount of DNA per mg of gold particles was kept constant (0.5 μg DNA per mg of gold).

*Assays for uid*A*, luc and bar reporter genes.*

The GUS hystochemical assay was performed by dipping the explants in 0.05 M sodium phosphate buffer pH 7.0, 0.1 % Triton X-100, 1 mM x-GlcA

(cyclohexylammonium. Duchefa), and incubating for 24 hours at 37°C (Jefferson et al., 1987).

The GUS fluorimetric assay was performed as follows: 40 - 60 mg of leaves from 4 weeks *in vitro* grown plants were homogenized in extraction buffer (0.05 M sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1 % Triton-100, 0.1 % SDS, 10 mM DTT). Fifty µl of extract plus 200 µl of 1 mM MUG (4-methylumbelliferyl-β-D-glucuronide. Duchefa) were incubated at 37°C for 30 minutes - 48 hours, depending on the individual *uid*A gene activity of each transgenic plant. The assay was stopped by adding 9 volumes of 200 mM Na_2CO_3 and the fluorescence was quantified using the biolumin™ (Molecular Dynamics). A standard curve for the 4-MU (7-hydroxy-4-methylcoumarin, Sigma) was used to correlate the fluorescence signal with the amount of hydrolysed substrate. The BCA™ protein assay (Pierce) was used to quantify the amount of total protein present in the extracts and performed as described by the manufacturer.

Tissues were assayed for luciferase activity by spraying with 0.15 mg / mL luciferin (Promega E 160). Luciferase activity was measured with a VIM intensified CD camera and an Argus-50 photon counting image processor (Hamamatsu Photonic System; Raemakers et al., 1996).

Pat-chlorophenolred (-CR) assay for *bar* activities was performed as previously described (Kramer et al., 1993). Leaf explants were dipped into 500 µl of MS (Murashige & Skoog, 1962) salts plus vitamins (Duchefa) liquid medium, 3 % sucrose containing 10 mg / L BASTA® and 50 mg / L CR. After incubation for 1 – 5 days, the CR turned yellow in those plants expressing the *bar* gene. A perfect correlation between the pat-CR assay, the integration of the *bar* gene into the plant genome (Southern blot) and the ability of plants to root in medium containing 20 mg / L of BASTA® was observed.

PCR and Long Template-PCR.

The DNA used as template for PCR analyses was extracted from plant leaf material using the Sigma Gene-Elute KIT according to the manufacturer's recommendations.

Standard PCR was performed with the RedTaq (Sigma) system and Long Template-PCR was performed using the Expand Long Template-PCR system (Roche) as described by the manufacturers. All Long Template-PCR products were blotted on a positively charged nylon membrane (Schleicher & Schuell) and hybridised with probes specific for the *uid*A (GUS probe C, 1701 – 3342 bp *Nru*I fragment of the *uid*A expression cassette, was used to prevent background derived from the GUS primers which were used both for preparing probes A and B and for Long Template-PCR analysis; Figure 1), the *npt*II genes (NPT probe) and pUC18 backbone.

Southern blot analysis.

Genomic DNA was isolated from leaves of plants rooted on kanamycin-containing medium using the CTAB protocol (Rogers & Benich, 1994). Fifteen μg of DNA were digested overnight at the optimal temperature of the used restriction endonuclease and blotted on a positively charged nylon membrane using the Turboblotter™ system (Schleicher & Schuell) and standard molecular-biology techniques (Sambrook et al., 1989). Filters were hybridised with digoxigenin-labeled probes (Figure 1) as described by the manufacturer (Boehringer, Mannheim, Germany).

Analyses of the Southern blot and Long Template-PCR data.

The number of copies of the *uid*A gene was evaluated using single cutter restriction enzymes. Restriction enzymes *Hin*dIII and *Bam*HI, cutting at the ends of the expression cassette, and *Bcl*I cutting at nucleotide 1719 behind the 5' - end of the expression cassette (Figure 1) were used. Evaluation of the copies of the *uid*A gene was made by subsequent hybridisation with probes specific to different parts of the gene (Figure 1). The number of sites of integration for each of the transgenes was determined using restriction enzymes which did not cut within the donor DNA. The linkage between *npt*II and *uid*A sites of integration was evaluated by Southern blot (see results) and Long Template-PCR using different combination of primers (a: GUSD – NPTC; b: GUSC – NPTC; c: GUSD – NPTD; d: GUSC – NPTD). If the *npt*II and the *uid*A genes were spatially linked, a PCR product would have been formed as a visible band on an ethidium bromide stained gel or after hybridisation with a specific probe (GUS, NPT or pUC18). In these analyses,

separate sites of integration were defined based on the resolution capacity of agarose gels (for Southern blot analysis or Long Template PCR analysis), i.e. around 20 Kb. To test if the Long Template-PCR approach for linkage evaluation was efficient, transgenic line PB996.15 in which the linkage between the *uid*A and the *npt*II sites of integration was confirmed by Southern blot, was analysed. Using different combination of GUS primers, Long Template-PCR was used to check the re-arrangements of the *uid*A gene when it was integrated in several copies within one *uid*A site of integration. With regard to this, the formation of PCR product(s) using primer combinations GUSA – GUSB, or GUSD – GUSC was consistent with a tandem repeat(s) organisation of the *uid*A genes within one *uid*A site of integration, primer combinations GUSA – GUSA or GUSD – GUSD with a 3' – 3' repeat(s) and primer combinations GUSB – GUSB or GUSC – GUSC with a 5' – 5' repeat(s).

RT-PCR.

RNA was extracted from young leaves as follows: plant material was ground in liquid nitrogen and RNA was extracted in 1 volume of RNA-extraction buffer (0.2 M Na-Acetate pH 5.0, 10 mM EDTA, 1 % SDS) and 1 volume of equilibrated-phenol at 65°C. One volume of chloroform was added and centrifuged for 10 minutes at 4°C. The water phase was re-extracted with chloroform and RNA was precipitated overnight at 4°C by adding 1/3 volume of LiCl 8 M. RNA was washed with LiCl 2 M and with ethanol 80% and finally re-suspended in water. C-DNA was synthesised using the Superscript™ system (GibcoBRL®) according to the manufacturer's recommendations and 6 μl of cDNA solution were used for PCR amplification.

Results

Transformation with two DNA constructs.

Co-transformation was first tested using two plasmids. One plasmid contained the *npt*II gene and kanamycin was used for selection of transgenic tissue. In total, about 2000 internodes were bombarded in 4 different experiments.

In the first experiment, plasmids pDC2 (*npt*II, *uid*A, *bar*) and pJIT 64 (*luc*) were co-bombarded (Table 1, row A). In the other three experiments (Table 1, row

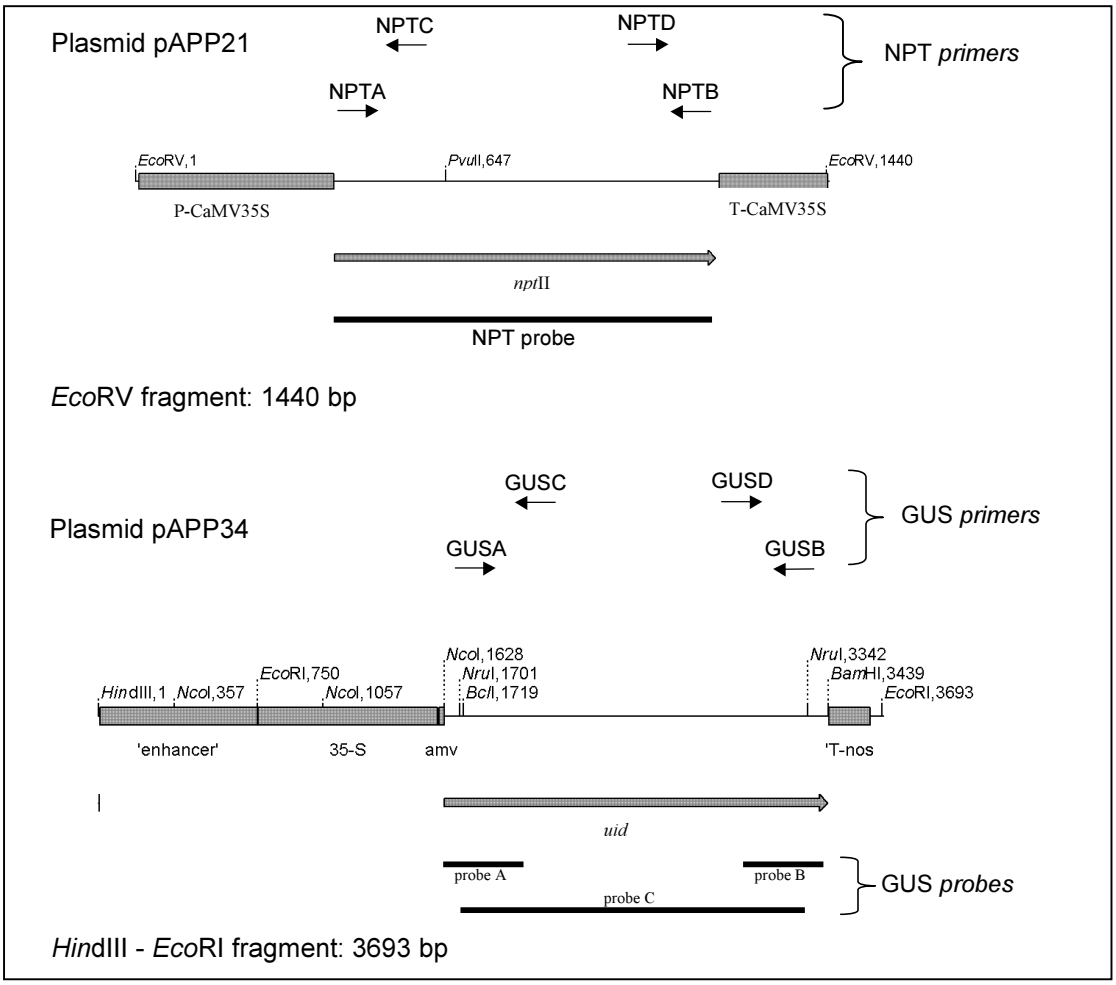

Figure 1. Schematic representation of the expression cassettes in plasmids 35-S-Kan and pAPP34 (not on scale). Restriction sites are indicated. The different probes and primers used for Long Template-PCR specific for the *uid*A and *npt*II genes are indicated. ➡) gene. ■) promoter or terminator. ■) probe.

Forward primers: $^{GUSA}\rightarrow$ / $^{GUSD}\rightarrow$ / $^{NPTA}\rightarrow$ / $^{NPTD}\rightarrow$. Reverse primers: $^{GUSB}\rightarrow$ / $^{GUSC}\rightarrow$ / $^{NPTB}\rightarrow$ / $^{NPTC}\rightarrow$).

B), 35-S-Kan (*npt*II) was co-bombarded with pAPP34 (*uid*A). In total, 271 plants resistant to kanamycin were obtained in the 4 experiments (Table 1, rows A and B), with an efficiency of transformation of 6 and 28 kanamycin resistant plants per 100 bombarded internodes using plasmid pDC2 and 35-S-Kan, respectively.

In total 182 of the 271 plants resistant to kanamycin were analysed for the expression of the reporter genes. In both construct combinations 68 plants (37 %) expressed the *uid*A or the *luc* gene.

Co-integration was studied on 62 plants bombarded with 35-S-Kan and pAPP34 (*uid*A gene) which were randomly chosen. Twenty-eight plants (45

Table 1. Transformation and gene expression results using different combination of plasmids. kan^R: plant resistant to kanamycin. [a]: number of transgenic plants obtained per 100 bombarded internodes. [b]: 3 plants did not express any of the non-selected genes, one plant expressed one of the two integrated non-selected genes.

	n. of genes & plasmids	N. of kan^R plants ([a])	Co-expression (2 plasmids)	Co-integration (2 plasmids)	Co-integration & co-expression (2 plasmids)	Co-integration (3 plasmids)	Co-integration & co-expression (3 plasmids)
A	1 *npt*II (pDC2) 2 *luc* (pJIT64)	45 (6 %)	16 / 43 (37 %)	--	--	--	--
B	1 *npt*II (35-S-Kan) 2 *uid*A (pAPP34)	226 (28 %)	52 / 139 (37 %)	28 / 62 (45 %)	22 / 28 (79 %)	--	--
C	1 *npt*II (35-S-Kan) 2 *uid*A (pAPP34) 3 *bar* (35-S-Bar)	35 (34 %)	--	10 / 20 (50 %)	9 / 10 (90 %)	11 / 65 (17 %)	6 / 10[b] (60 %)
D	1 *npt*II (35-S-Kan) 2, 3 PHA-genes	45 (5 %)	--	17 / 45 (38 %)	12 / 17 (71 %)		

%) gave a positive signal for the presence of the *uid*A gene in the genome using Southern blot or PCR analyses. Expression analysis showed that 6 of the 28 plants in which the integration of the *uid*A gene was observed, did not express the *uid*A gene and 22 plants (79 %) did show expression (Table 1).

Transformation with 3 donor DNA constructs.

Co-transformation with three separate plasmids, one containing the selectable marker (*npt*II gene) and the other two plasmids containing non-selected genes, was tested in three experiments. In the first experiment (Table 1, row C), 35 kanamycin resistant plants were obtained after bombardment of 102 internodes with plasmids 35-S-Kan (*npt*II), pAPP34 (*uid*A) and 35-S-Bar (*bar*). In the other two experiments (Table 1, row D), 45 kanamycin resistant plants were obtained after bombardment of more than 900 internodes with plasmid 35-S-Kan and two plasmids each containing a different gene involved in bacterial polyhydroxyalkanoate (PHA) biosynthesis. In the three experiments, in total 80 kanamycin resistant plants were obtained. Sixty-five of these plants were evaluated by PCR or Southern blot analyses for the integration into the plant genome of the genes from the non-selected plasmids (Table 1). Twenty-seven of the 65 (41 %) plants resistant to kanamycin had none of the non-selected genes. The integration of one of the two non-selected genes was observed in 27 out of 65 (41 %) plant. Eleven out of 65 plants (17 %) contained all non-selected genes (2 / 20 and 9 / 45 from the experiment with

the *uid*A / *bar* genes and with the PHA-genes, respectively). Subsequently, expression analyses were performed based on reporter-gene assays for the *bar* and *uid*A genes and on RT-PCR for the PHA-genes. In the case of integration of one non-selected gene, 6 out of 27 plants (22 %) did not express the gene and the remaining 21 (78 %) did show expression. In the case of integration of 2 non-selected genes, three out of 10 plants did not show expression, 1 expressed only one of the two genes and 6 plants expressed all transgenes.

The data on expression of transgenes derived from separate plasmids were compared with the expression of the genes (one selected, *npt*II and two non-selected, *uid*A and *bar*) when they were delivered on one plasmid. Twenty-nine plants derived from particle bombardment with plasmid pDC2 were analysed. Twenty-six out of 29 (90 %) and 17 out of 19 (89 %) plants resistant to kanamycin expressed the *uid*A or *bar* genes, respectively. Both non-selected genes were expressed in 15 out of 18 (83 %) kanamycin resistant plants.

Gene cassette transformation.

In two experiments, one or two gene cassettes (PCR-fragments) were (co)-delivered into potato internodes. One gene cassette comprised the expression cassette (promoter-gene-terminator) for the *npt*II selectable marker and the second gene cassette contained the expression cassette for the non-selected *uid*A reporter gene.

When the *uid*A gene cassette was delivered, transient GUS expression was observed 48 hours after bombardment, although the level was very low as compared to the expression of the *uid*A gene delivered as part of a plasmid. Eight weeks after bombardment, calli expressing the *uid*A gene were observed on internodes. In the two experiments, 12 and 13 plants rooting on kanamycin containing medium were obtained from 200 and 251 bombarded internodes, respectively. For all plants, the integration of the *npt*II expression cassette was confirmed using Southern blot or PCR analyses.

When the *npt*II and *uid*A expression cassettes were co-delivered, 8 out of 11 kanamycin resistant plants showed integration of the *uid*A fragment.

Expression of the *uid*A gene was observed in 6 out of the 8 transgenic lines containing both delivered genes (selected and non-selected).

Internal variation of transgene expression.

Transgene activity was tested in a random sample of 20 transgenic plants resistant to kanamycin and expressing the *uid*A gene. From each independent transformant, one plant was subcultured and different multiplied shoots, originated from consecutive nodes, were tested for kanamycin resistance and *uid*A gene activity. All subcultures showed identical transgene activity (data not shown).

The activity of the transgenes was constant over more than 10 consecutive vegetative propagations (plants were propagated on fresh medium by sub-culturing apical nodes every 8 weeks). The presence or absence of the selection pressure during propagation did not affect the activity of the transgenes.

Molecular characterisation of co-transformed plants.

Twenty-four transgenic plants co-transformed with plasmids containing the *npt*II (35-S-Kan) and the *uid*A genes (pAPP34), 6 transgenic plants transformed with the *npt*II gene cassette, and 8 co-transformed with the *npt*II and *uid*A gene cassettes were further analysed with respect to the molecular organisation of the transgenes. Number of copy and sites of integration for the transgenes were evaluated by Southern blotting using a number of restriction endonucleases as described in materials and methods. Figure 2 shows the distribution of the sites of integration of the transgenes in 24 lines co-transformed using plasmid DNA. For the *uid*A gene, 11 plants showed one site of integration with 1 to 3 copies of the gene, 5 plants comprised 2 *uid*A sites with 2 to 4 copies of the gene. Three and 4 *uid*A sites were observed in the remaining 8 plants (4 each) with 3 to 6 copies of the gene. In total, most of the plants (16) contained 1 to 3 copies of the *uid*A gene and 8 plants, contained 4 to 6 copies. Like for the *uid*A, the *npt*II gene was inserted predominantly at one site (13 out of 24 plants) and the number of sites ranged from 1 to 5 (Figure 2). The same was observed in gene cassette

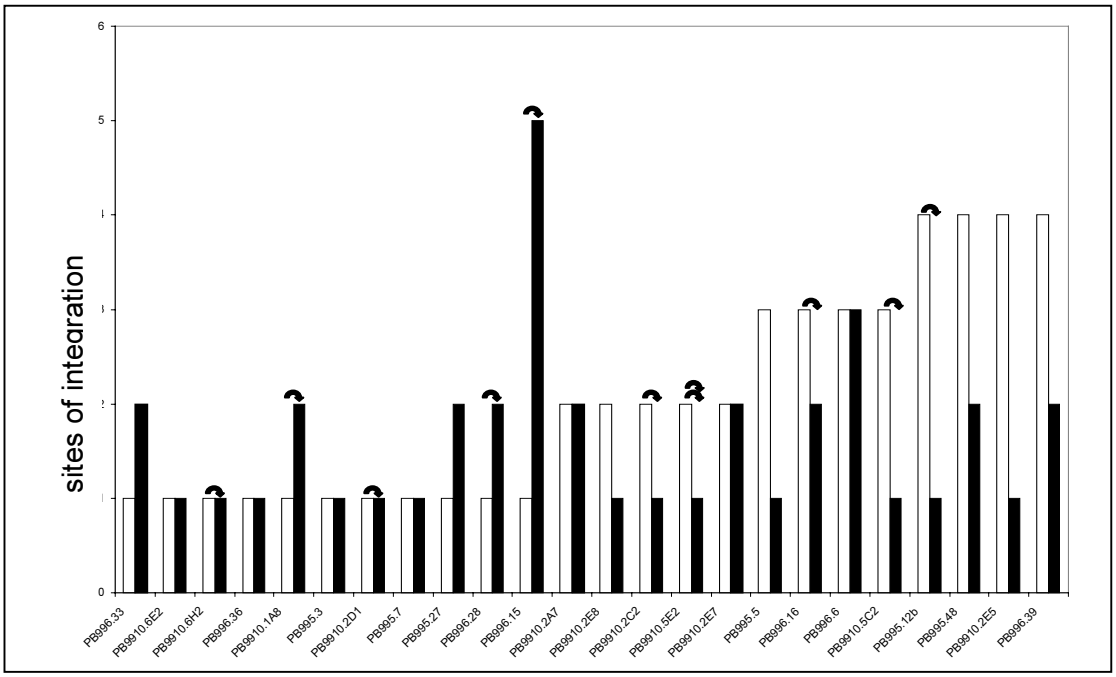

Figure 2. Distribution of transgenic lines according to their number of integration sites for the *uid*A (□) gene and *npt*II (■) gene among the set of 24 independent transgenic lines analysed and derived from plasmid transformation. The arrows indicate when a linkage between *uid*A – *npt*II sites of integration was observed in each plant.

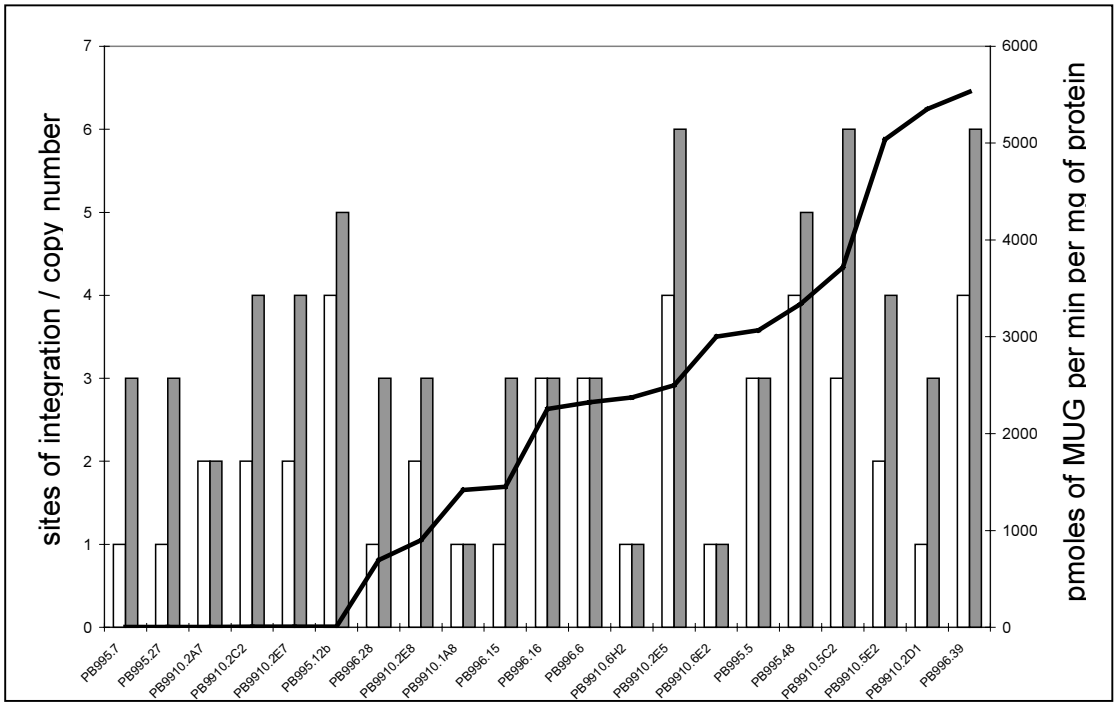

Figure 3. Correlation between number of sites of integration and copy numbers of the *uid*A gene with the corresponding GUS activities among 21 transgenic lines analysed (indicated on the X axis), belonging to the set of 24 lines, arranged according to increasing GUS expression. In this analysis, lines PB995.3, PB996.33 and PB993.36 were not included because no complete *uid*A expression cassette was found to be integrated. □) sites of integration. ▩) copy number.

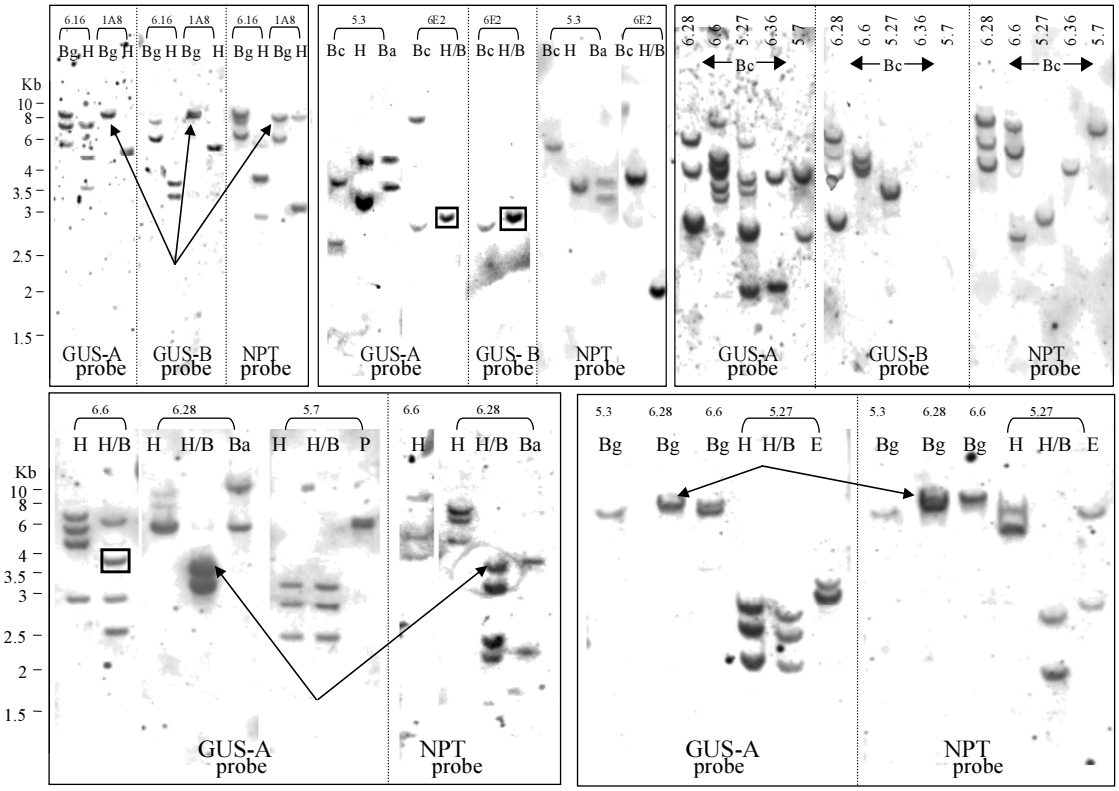

Figure 4. Examples of Southern blot analyses on transgenic lines PB9910.1A8 (lanes indicated as 1A8), PPB9910.6E2 (6E2), PB995.3 (5.3), PB996.6 (6.6), PB996.28 (6.28), PB996.36 (6.36), PB995.7 (5.7) and PB995.27 (5.27). The probes used for hybridisation are indicated and the portions of gene they are specific to are shown in figure 1. Control digestion of plasmid DNA together with genomic DNA was performed to exclude any possible inhibition of the DNA preparation on the restriction enzymes. Arrows indicate common hybridising bands. Bands corresponding to the complete *uid*A expression cassettes are squared. Bg: *Bgl*II. H: *Hin*dIII. B: *Bam*HI. Bc: *Bcl*I. P: *Pst*I. E: *Eco*RI. H/B: *Hin*dIII / *Bam*HI double digestion.

transformation with sites of integration and copy-number of the transgenes ranging between 1 to 3 and 1 to 5, respectively (results not shown).

The distribution of the sites of integration and copy number of the *uid*A gene, and the correlation with the *uid*A gene activity, in 21 plants belonging to the same set of 24 transgenic plants transformed with plasmid DNA is shown in Figure 3. No correlation between copy-number and number of integration sites with the GUS activity was found.

Linkage between the *npt*II and the *uid*A sites of integration was evaluated using Southern blot and Long Template-PCR analyses. Using Southern blot, bands which hybridised both with the *uid*A and the *npt*II probes indicated that transgenes were integrated at the same site. For example, lines PB9910.1A8

and PB9910.6E2 (Southern blot analysis is shown in Figure 4 lanes 1A8 and 6E2, respectively. A scheme of the site as deduced by molecular analyses is shown in Figure 5a and b) contained one single *uid*A site comprising one copy of the complete expression cassette, while in line PB9910.1A8 one of the two *npt*II sites is associated with the *uid*A site (the common hybridising band is indicated by the arrows in Figure 4), but in line PB9910.6E2 no linkage of the *uid*A to the *npt*II site of integration was observed. In this work (see below), the presence of non-cutter restriction sites within one transgenic site of integration was observed. Thus, those plants in which separate sites of integration for the *uid*A and the *npt*II genes were observed by Southern blot, were further evaluated for the presence of linkage by Long Template-PCR, using different combinations of GUS and NPT primers as described in materials and methods. After integration of Southern blot and Long Template-PCR data, it was deduced that in 10 out of 24 and 5 out of 11 (not shown) plants transformed using plasmids and gene cassettes, respectively, (about 45 %) *uid*A and *npt*II sites of integration were linked. Linkages for each individual transformant are shown by curved arrows in Figure 2. The presence in one plant of one *uid*A site of integration linked to a *npt*II site does not exclude that extra unlinked *uid*A sites may be present in the same plant. Table 2 summarises the results obtained from the analysis of the 49 sites of integration for the *uid*A gene which were distributed in the 24 transgenic lines analysed and derived from plasmid transformation. Eleven *uid*A sites resulted to be linked to an *npt*II site. This means that, apart from lines PB9910.1A8 and PB9910.6H2 where the unique *uid*A site was linked to a *npt*II site, in the remaining 8 plants in which *uid*A – *npt*II linkages were observed, extra unlinked sites of integration for the non-selected genes were also present. Among the 49 *uid*A sites of integration analysed, 17 comprised only one copy of the gene.

Long Template-PCR analysis, using different combinations of GUS primers (GUSA, GUSB, GUSC and GUSD), was also used to evaluate those *uid*A sites of integration, which seemed to be separated by Southern blot analysis.

*uid*A sites of integration analysed	Number (percentage) of *uid*A sites with only 1 copy of the gene	Number (percentage) of *uid*A sites unlinked to one *npt*II site	Copy organisation in multi-copy sites of integration[1]		
			→← 3'-3'	←→ 5'-5'	→→ Tandem
49	**17** (35 %)	**38** (78 %)	**19** (63 %)	**7** (23 %)	**4** (13 %)

Table 2. Summary of the molecular organisation of the sites of integration for the *uid*A gene in plants derived from plasmid transformation.
[1]: numbers (between brackets: percentages) of events in 30 events considered.

In 4 transgenic lines, it was found that, apparently unlinked *uid*A sites were instead linked and separated by non-plasmid sequences. In 2 lines (PB995.3 and PB996.28. Figure 4) non-cutting restriction sites were found within *npt*II sites of integration by Southern blot analysis, indicating, also in these cases, the intervening of non-plasmid sequences within one site of transgene integration (compare the number of *npt*II bands of lanes 5.3 and 6.28 using different non-cutter restriction enzymes, *Bcl*I, *Hin*dIII, *Bam*HI). In at least 3 lines (PB995.3, PB996.6 and PB996.16) one *uid*A expression cassette was disrupted (Figure 4 and 5c, d, e): in line PB995.3, although apparently there are two copies of the *uid*A gene (two *Hin*dIII and *Bam*HI bands) the integration process disrupted the gene presumably between primers GUSB and GUSD (PCR product was formed only when GUSA and GUSC primers were used. See scheme in Figure 5c). This was further confirmed by Southern blot analysis with *Hin*dIII / *Bam*HI digestion, resulting in the excision of the pUC18 backbone for all three lines (not shown).

A further analysis of one multi-copy *uid*A site of integration was performed using the Long Template-PCR as described in materials and methods. Using different combinations of GUS primers it was evaluated when two copies of the transgenes were positioned as an inverted repeat (5' - 5' or 3' - 3') or as a tandem repeat (3' - 5'). The presence of these re-arrangements was in each

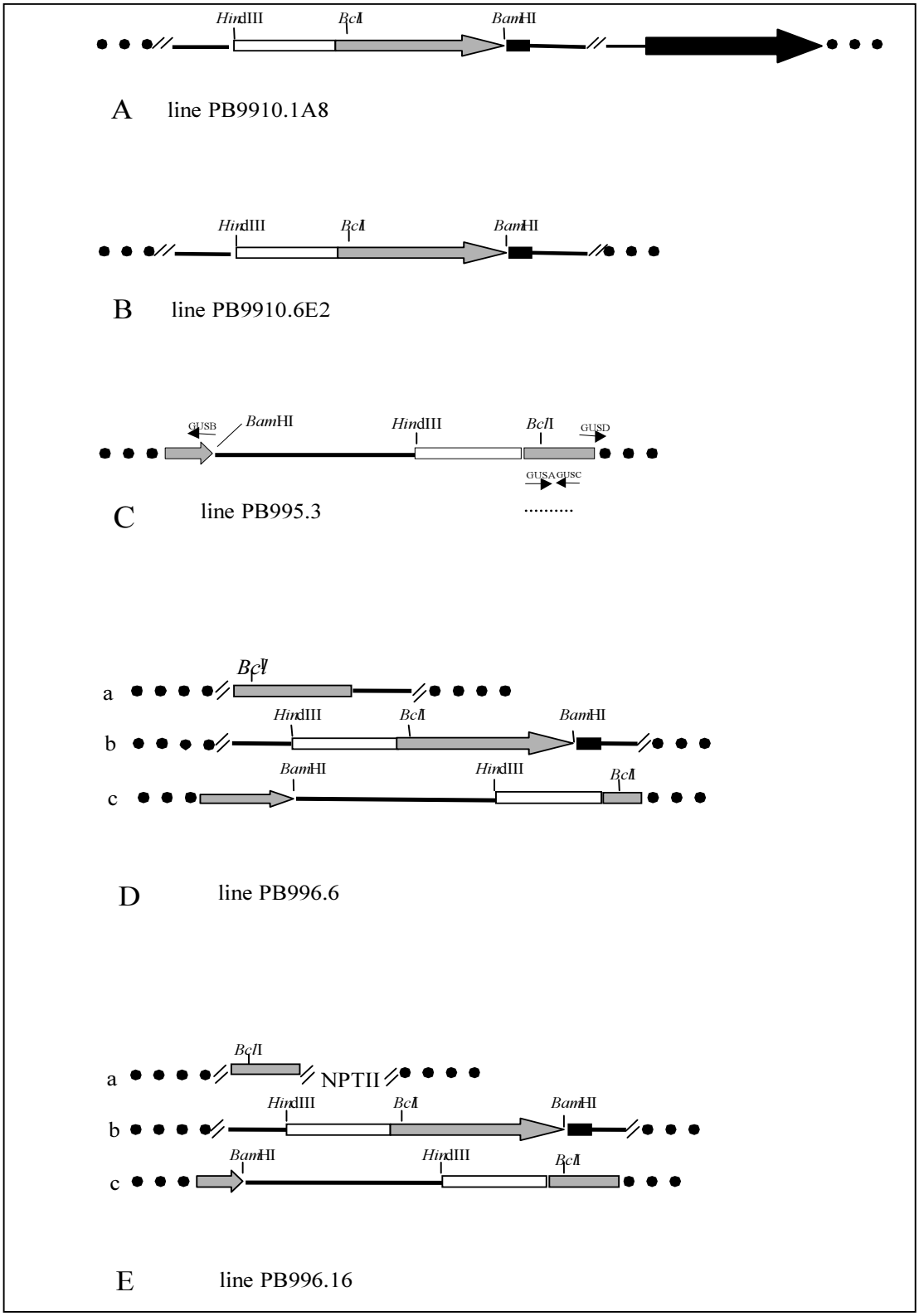

Figure 5. Schematic representation of sites of integration for the *uid*A gene in lines PB9910.1A8 (A), PB9910.6E2 (B) and PB995.3 (C) containing one *uid*A site of integration each; lines PB996.6 (D) and PB996.16 (E), containing 3 *uid*A sites of integration each. • • •) potato genomic DNA. ➡) *uid*A gene. ➡) *npt*II expression cassette from plasmid 35-S-Kan. □) E-35-S. ■) terminator. ‒) pUC18 backbone. $^{GUSA}\rightarrow$) primer. ·····) PCR product. //) gap.

case in agreement with the copy-number determination. In those plants in which multi-copy sites of integration were observed, 30 re-arrangement events were counted and analysed in total, and 19 consisted of 3' - 3' repeats re-arrangements, 7 events of 5' - 5' repeats and 4 of tandem repeats (Table 2).

In addition, all 24 transgenic lines analysed, co-transformed with plasmid DNA, were checked for the presence and the site of integration of the plasmid backbone (pUC18) by Southern blot. In 22 lines, the bands hybridising with the plasmid backbone probe were common to the bands hybridising either with the *uid*A or the *npt*II probes, indicating that the integration of the DNA occurred as a unit (although sometimes not complete).

The analysis outlined above, extended to other lines, allowed to determine the structure of the *uid*A sites of integration in some transgenic plants (Figure 5).

Discussion

Co-transformation and expression analyses.

Co-transformation with separate plasmids using particle bombardment has been reported for several crops like gladiolus [more than 70 % co-transformation efficiency using two plasmids: (Kamo et al., 1995)], bean [40 – 50 %; (Aragao et al., 1996)], sugarcane [up to 90 %; (Bower et al., 1996)], rice [39 - 85 %; (Chen et al., 1998)] wheat [85 %; (Stoger et al., 1998)]. In this report, particle bombardment-mediated co-transformation, selecting only one DNA molecule, was studied in potato. Twenty-eight out of 62 (45 %) transgenic plants were co-transformed with one selected (*npt*II) and one non-selected (*uid*A) gene if separate plasmids were used for transformation. When gene cassettes (PCR fragments comprising promoter gene and terminator) were used, 8 out of 11 plants were co-transformed. Moreover, when 3 genes (only one of which was selected) were delivered in 3 separate plasmids, 11 out of 65 plants (17 %) were co-transformated with all genes. With *Agrobacterium* as well, co-transformation of two separate plasmids, contained in two bacterial strains and only one of which selected, was reported (De Block & Debrouwer, 1991; De Buck et al., 1998). However, *Agrobacterium*–mediated co-transformation with separate plasmids has never been reported for potato. In contrast with what Chen et al.

(1998) reported, no positive nor negative correlation was observed between efficiencies of transformation and co-transformation of genes in separate plasmids and molar ratios of the non-selected and selected plasmids, which was varied from 1 : 1 to 1 : 10 in one experiment (data not shown).

Co-expression of two integrated transgenes was 90 % in case the genes were delivered in one plasmid, and was 75 % - 80 % in case genes were delivered in separate plasmids or gene cassettes. In other crops, similar results have been obtained for DNA delivery, irrespective of the use of separate or co-integrate plasmids (Cooley et al., 1995; Stoger et al., 1998). The slight difference in the co-expression frequency observed using genes delivered in one plasmid *versus* genes delivered in separate plasmids, can be explained by the fact that, in the latter case, integration could have occurred into non-transcriptional active chromosomal sites.

Transgene activity was identical in plants derived by sub-culturing consecutive nodes from individual plantlets. This analysis, together with the histological observation that GUS positive calli growing on kanamycin containing medium appeared as discrete units with evenly distribution of the dye after the GUS assay, strongly indicated that regenerated transgenic plants were not chimeric. However, some form of chimerism could escape such analyses. Transgenes were stably maintained and expressed during at least 10 propagation cycles, irrespective of the use or not of selection pressure.

Molecular organisation.

The transgenes molecular organisation was studied in 38 plants, using Southern blot and Long template-PCR analyses. The donor DNAs integrated as units (complete or incomplete), like previously observed in rice (Cooley et al., 1995; Chen et al., 1998; Kohli et al., 1998; Kohli et al., 1999) and in wheat (Stoger et al., 1998).

The *uid*A gene was integrated predominantly in 1 - 3 copies. Similar results have been reported for particle bombardment mediated transformation of other crops (Chen et al., 1998; Kohli et al., 1998; Stoger et al., 1998). Correlation between transgene activity and copy number in previous reports are often conflicting. So also in this case.

In the present work, from 39 % to 55 % of the potato plants analysed showed at least two sites of integration for the *uid*A or the *npt*II genes. Moreover, 38 out of 49 (78 %) of the *uid*A sites analysed were positioned spatially separated from the *npt*II site (Table 2). Seventeen out of 49 (35 %) *uid*A sites analysed contained one single copy of the transgene. When re-arrangements of one *uid*A site were observed, a predominance of 3' – 3' inverted repeats (19 out of 30 events) was found. Future work will be dedicated to segregational analysis to check whether or not the sites of integration identified in this work were indeed segregating chromosomal loci. Although the integration patterns observed in this work were very heterogeneous, and sometimes very complex rearrangements of the transgenes were detected, a high percentage of the population of transgenic plants analysed showed separation between integration sites of selected and non-selected genes and a low copy number of the transgenes. This is an important difference compared to previous reports. In particle bombardment-mediated transformation of cereals (Chen et al., 1998; Kohli et al., 1998; Stoger et al., 1998; O'Kennedy et al., 2001), and in the case of two T-DNA *A. tumefaciens*-mediated co-transformation as well [Cheng et al. (1997) for wheat; De Block & Debrouwer (1991); De Neve et al (1997) for tobacco, *Brassica* and *Arabidopsis*], it was observed that all donor DNA molecules predominantly integrated at one site, with multi-copies of the transgene(s) rearranged. The same tendency has been also described in *Agrobacterium*-mediated transformation of potato (Kuipers et al., 1994; Wolters et al., 1998 and 2000). These differences can be related to the target tissue used in transformation, as postulated by Grevelding et al. (1993) and Tinland (1996). In internodal-somatic cells (used in these experiments) the homologous recombination machinery may be lowly active, thus resulting in low extrachromosomal multimerisation and in the subsequent low frequency of integration at one site. Interestingly, Fu et al., (2000) observed simple patterns of integration in rice plants transformed with gene cassettes as a result of the decreased amount of homologous sequences (pUC backbone) available for extrachromosomal recombination. In some plants, filler non-plasmid DNA sequences, of probably genomic origin, were observed between consecutive copies of a transgene. The same has been described by others (Cooley et al.,

1995; Kohli et al., 1998; Stoger et al., 1998; Wolters et al., 1998) and suggested that ligation of separate plasmids occurred during integration at the chromosome.

Conclusions.

Unique advantages can be received from the improvements on particle bombardment-mediated transformation described in this work. The presence of redundant bacterial vector DNA and antibiotic or herbicide resistance markers are under debate and subject to new government regulations. The recovery at high frequencies of single-copy sites of integration, in combination with plant breeding programmes could improve primary transformants and eliminate via out-crossing undesired sites (triggering to silencing or co-suppression, for example) but also to produce marker-free plants. The latter is however possible in different ways (Romano et al., 2002) including the use of none selection (Domínguez et al., 2002 and R. Visser, personal communication). The gene cassette co-transformation results in the elimination of redundant plasmid sequences.

Acknowledgements

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CHAPTER 4

Evidence of polyhydroxybutyrate synthesis in transgenic potato plants expressing the *Ralstonia eutropha* acetoacetyl-CoA reductase and the PHB-polymerase.

Abstract

Polyhydroxybutyrate (PHB) represents one member of a class of polyesters which are largely distributed in the prokaryote kingdom. Three enzymes (ketothiolase, acetoacetyl-CoA reductase and PHB-polymerase) are responsible for the synthesis of PHB in *Ralstonia eutropha*. The genes coding for the *R. eutropha* acetoacetyl-CoA reductase and PHB-polymerase (*phb*B and *phb*C, respectively) have been introduced into transgenic potato. In the cytoplasm of plants, a ketothiolase activity is endogenously present, being involved in isoprenoid biosynthesis. Low but detectable accumulations of PHB were observed in transgenic potato plants co-transformed with the *phb*B and *phb*C genes.

Key words: Particle bombardment, *Solanum tuberosum*, polyhydroxybutyrate, PHB-polymerase, NAPDH-dependent acetoacetyl-CoA reductase

Introduction

Short-chain-length polyhydroxyalkanoate (scl-PHA) comprises the homopolymer poly-[3-(*R*)-hydroxybutyrate] (PHB) and the heteropolymer poly-[3-(*R*)-hydroxybutyrate-co-3-(*R*)-hydroxyvalerate] (PHB / HV) consisting of hydroxybutyrate and hydroxyvalerate moieties linked *via* ester bonds. A wide range of microorganisms are capable of accumulating up to 80 % of their dry weight of PHB as storage of energy when grown under nutrient-limiting conditions (e.g. ammonia, oxygen, phosphate, etc. (Bebel et al., 2001). The best-known PHB-accumulating bacterium is *Ralstonia eutropha*. In this microorganism, the formation of PHB is mediated by three enzymes, i.e. 3-ketothiolase (*phb*A gene product), NADPH dependent acetoacetyl-CoA reductase (*phb*B gene product), and PHB-polymerase or PhbC (*phb*C gene product). Acetyl-CoA is the initial substrate for PHB biosynthesis and can be derived from various carbon sources because it plays a central role in bacterial metabolism. Alternative metabolic pathways, i.e. β-oxidation, FAB and TCA cycle *via* the methylmalonyl-CoA pathway, which can direct precursors to produce PHB in bacteria have been recently reviewed by Madison & Huisman (1999) and Bebel et al. (2001).

PHB and PHB / HV are useful materials for human society, because they can be used as commodity thermoplastics, they are biodegradable (van der Walle et al., 2001) and are derived from renewable resources. PHB / HV has been produced in bacteria at a scale of 200,000 Litres using a two step fermentation process (reviewed by Kessler et al., 2001b) and the polymer has been already commercialised with the trademark Biopol®. Although progress has been made in the past few years for the optimisation of fermentation processes (Choi & Lee, 2000), costs of fermentative PHB and PHB / HV production still remain high (Bebel et al., 2001; Lee & Choi, 2001) and cannot yet compete with the price of oil-derived plastics. The use of transgenic plants as a factory for the production of bulk PHAs, using atmospheric CO_2 and sun light as driving force, is considered an amenable approach for the production of PHAs at low price. PHB has been accumulated in a number of transgenic plants like *Arabidopsis* (Poirier et al., 1992; Nawrath et al., 1994; Bohmert et al., 2000), *Brassica* (Nawrath et al., 1995; Houmiel et al., 1999; Slater et al.,

1999; Poirier, 2002a), *Gossypium hirsutum* (John & Keller et al., 1996), *Nicotiana tabacum* (Nakashita et al., 1999) and *Zea mays* (Hahn et al., 1999) in various cell compartments, i.e. cytoplasm, peroxisomes, chloroplasts and leucoplasts (see Chapter 1, Table 2). In this work the production of PHB was attempted in potato after expression of the *R. eutropha* acetoacetyl reductase (*phb*B gene product) and the PHB-polymerase (*phb*C gene product) in the cytoplasm. The plant cytoplasm contains an endogenous ketothiolase activity similar to the ketothiolase activity involved in the PHB biosynthetic pathway which is coded by the *R. eutropha phb*A gene. The plant ketothiolase is involved in the biosynthesis of (poly)-isoprenoids. This chapter describes the accumulation of PHB in transgenic potato plants expressing the *phb*B and *phb*C genes. During the course of this work, also Bohmert and co-workers (2002) reported the production of PHB in transgenic potato and tobacco by the expression of the relevant enzymes in the plastids.

Material and methods

Plant Material and transformation.

In vitro grown *Solanum tuberosum*, genotype 1024-2 (*amf*, diploid; Jacobsen et al., 1989) was used as starting material for particle bombardment-mediated co-transformation as described in Chapters 3 and 4.

DNA constructs.

*R. eutropha phb*B and *phb*C were amplified by PCR from plasmid pSK2665 (kindly given by Alexander Steinbüchel, Munster University, Germany) and cloned into pGEM-T-easy plasmid (Promega) giving rise to plasmids pGEM-*phb*B, pGEM-*phb*C (Springer and coworkers; unpublished results). Restriction sites *Nco*I / *Bam*HI were introduced at the 5’ and the 3’ termini, respectively. Plasmid pAPP47, containing the *npt*II selectable marker driven by the *nos* promoter and terminator and containing the E-35-S and *nos* terminator was constructed as follows: the *nos*-promoter / *npt*II / *nos*-terminator cassette was amplified by PCR using primers 5’ CCCGCCATATGTCCTGTCAAAC and 5’ CCAGCATATGCAGGAGGCCC and plasmid pBIN19 (Bevan, 1984) as a template. These primers introduced the *Nde*I restriction site at the 5’ and 3’ termini of the *npt*II cassette, which was subcloned into the *Nde*I site of

pAPP23 (Chapter 2) containing the E-35-S and *nos* terminator. The *Pyrococcus woesei* (Pwo) DNA polymerase was used according to the manufacturer's recommendations (Eurogentec). The *phb*B and *phb*C genes were extracted by *Nco*I / *Bam*HI digestion from plasmids pGEM-*phb*B, pGEM-*phb*C, and cloned into *Nco*I / *Bam*HI of plasmid pAMV-1 containing the *amv* (5´-untranslated region of the alfalfa mosaic virus; Rouwendal et al., 1997) and giving rise to plasmids pAPP72 (*amv-phb*C) and pAPP73 (*amv-phb*B). *amv-phb*B and *amv-phb*C cassettes were isolated by *Bam*HI / *Bgl*II digestion from plasmids pAPP73 and pAPP72, respectively, and fragments were cloned into the *Bam*HI sites of plasmids pAPP23 (resulting in plasmid pAPP74) and pAPP47 (plasmid pAPP76), respectively. In this way, plasmid pAPP76 carried both the *npt*II selectable marker and the *phb*C gene.

PCR and Southern blot analyses.

All molecular techniques were performed as described in Chapters 2 and 3 and using standard protocols (Sambrook et al., 1989).

RT-PCR and northern blot.

RNA isolation from leaf material, cDNA synthesis and PCR were performed as described in Chapter 3. For northern blot analysis, 15 μg of total RNA were 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, Mannheim, Germany) and using standard techniques (Sambrook et al., 1989).

Cell suspension cultures.

Cell suspension cultures were induced from friable calli in liquid medium MS salts (Murashige & Skoog, 1962) plus vitamins (Duchefa), sucrose 3 %, 2-4 D, 2 mg / L. Ten mL of suspension were subcultured every 10 days in 40 mL of the fresh medium. Friable calli were induced on MS salts plus vitamins agar, sucrose 2.5 %, casein hydrolysed 0.1 %, myo-inositol 0.1 %, 2-4-D 3 mg / L, kinetine 0.2 mg / L, thiamine pyrophosphate 0.5 mg / L, pyridoxine 0.5 mg / L, nicotinic acid 0.5 mg / L, glycine 2 mg / L, folic acid 0.5 mg / L, biotine 0.05 mg / L, pH 5.7 from *in vitro* grown microtubers. Microtubers were induced from nodes of *in vitro* grown plantlets as described in Chapter 2.

Feeding experiment.

Sodium acetate (Sigma) and 3-(*R*-*S*)-hydroxybutyrate sodium salt (Sigma) were solubilised in 50 mM buffer phosphate pH 7 at a concentration of 300 mM. The substrates were added 5 days after the suspensions had been subcultured to a final concentration of 1 mM. The treatment was repeated every 2 days.

Sodiumdodecylsulfate polyacrylamide gel electophoresis (SDS-PAGE) and immunoblotting.

Antibodies specific for the PHB-polymerase were available and were used to check the expression of the *phb*C gene and the synthesis of the corresponding enzyme. Fresh plant material was homogenised in 100 mM Tris buffer, pH 7, containing the Complete^{TM} mini protease inhibitor cocktail (Roche) and proteins were separated by SDS-PAGE (Laemmli, 1970) in a Mini PROTEAN II system (Bio-Rad) under reducing denaturing conditions. Proteins were stained with Comassie Brilliant Blue (CBB R-350, Pharmacia). For western blot analysis, proteins were blotted electrophoretically onto a PVDF filter (Millipore) using a Mini Trans-Blot Cell (Bio-Rad) in CAPS (2.2 g / L) and 10 % ethanol. Filters were blocked overnight with 5 % skim milk powder in TBST (0.1 M Tris-HCl, pH 7.5; 1.5 M NaCl; 0.1 % Tween-20) at 4°C. Subsequently, filters were incubated for 2 hours with anti-PhbC-polymerase antibodies (1:1000), specific for the *R. eutropha* enzyme, and eventually with alkaline-phosphatase-conjugated anti-rabbit secondary antibodies (Sigma) in TBST containing 1 % skim milk powder. The antibodies were a gift from Prof. Y. Poirier, University of Lausanne, CH. The filters were washed in TBST and in detection buffer (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl) and antibodies-protein complexes were detected by incubating the filters in detection buffer containing 5-bromo-4-chloro-3-indoylphosphate 0.5 mM and nitro-blue-tetrazolium 0.1 mM (Amersham Pharmacia Biotech).

*NADP-dependent acetoacetyl-CoA reductase (phb*B *gene product) assay*.

Seventy mg of fresh leaf material were homogenised in 600 µl assay buffer (100 mM phosphate buffer, pH 7.0, 0.5 mM dithithreitol, 12 µM $MgCl_2$). The debris was pelleted, and the clear lysate was used for measuring the acetoacetyl-CoA reductase activity in 1 mL volume, in the presence of 1.5 mM

NADPH (Sigma). The consumption of NADPH was followed spectrophotometrically at 340 nM (molar extinction coefficient, $E^{mM}_{(340)}$ = 6.22) and activities were deduced by the oxidation rate of the NADPH in the presence of 0.3 mM acetoacetyl-CoA (Sigma) minus the oxidation rate of the same extract without the substrate (blanc). Reactions were started by the addition of the substrate (or blanc).

PHB isolation, Gas Chromatography (GC).

Leaf material from pot-grown plants was ground in liquid nitrogen and lyophilised. Cell suspension cultures (fed and not fed with the substrates) were washed three times with tap water, ground in liquid nitrogen and freeze dried. The lyophilised powder was washed with ethanol at 55 °C for 48 hours to remove fatty acids, and PHB was subsequently extracted from the de-fatted powder with chloroform at 55 °C for 48 hours. After the chloroform was evaporated, PHB was resuspended in 1 mL of chloroform, methanolysed and analysed by GC. Methanolysis was performed as described by Lageveen (Lageveen et al., 1988). Methylesters were analysed by CG on a Carlo-Erba GC6000 apparatus (Carlo Erba) equipped with a 25 m CP-Sil5CB capillary column (Chrompack). Alternatively methanolysis and GC analyses was performed directly on the de-fatted powder.

Results

Transformation and selection of transgenic lines.

Plasmid pAPP74 (*phb*B gene) and pAPP76 (*phb*C gene and *npt*II selectable marker) were precipitated onto gold particles at a 2 : 1 molar ratio and delivered by particle bombardment into potato internodes as described in Chapters 2 and 3. Kanamycin was used to select transgenic plants. About 330 internodes were bombarded and 25 plants resistant to kanamycin were obtained (lines PB0010). Genomic DNA was extracted from leaf material and screened for the integration of the *phb*B and *phb*C genes by Southern blot or PCR analyses (Table 1). Seven plants were found co-transformed with the *phb*B and *phb*C genes. Two plants transformed only with the *phb*C gene were also selected for further analyses (Table 1). Transcription of the *phb*B gene and expression of acetoacetyl-CoA reductase was assessed by RT-PCR

Table 1. List of potato transformants with respective molecular, enzymatic and PHB analysis data. [1]: genotypes B and C indicates the integration into the plant genome of the *phb*B and the *phb*C genes determined by Southern blot or PCR analyses.
-: absent. nd: experiment not performed.

Lines	Genotype ([1])	*phb*B mRNA	PhbB activity	PhbC western blot	Evidence of PHB
Wild type	--	-	-	-	-
PB0010.1	BC	-	-	-	-
PB0010.2	BC	+	+	-	0.7 μg / g
PB0010.4	BC	+	+	+	Traces
PB0010.5	BC	+	+	-	-
PB0010.7	BC	+	+	-	Traces
PB0010.10	BC	+	+	-	nd
PB0010.16	BC	nd	nd	-	nd
PB0010.8	C	nd	nd	+	nd
PB0010.13	C	nd	nd	-	-

(Figure 1a) and enzymatic assays (Figure 1b) in 6 out of 7 co-transformed plants (Table 1; one plant, PB0010.16 showed extremely delayed growth and was not further analysed). Only one line (PB0010.1) did not show *phb*B mRNA nor acetoacetyl-CoA reductase activity. Transcription of the *phb*C gene was also monitored in the transgenic lines by RT-PCR and northern blot analyses. However, most of the transgenic lines showing integration of the *phb*C gene, showed little or no *phb*C mRNA. Western blot analyses with PhbC-antibodies (Figure 2) was subsequently performed and a clear signal was associated only with 2 of the 9 transgenic lines analysed showing integration of the *phb*C gene (PB0010.4 and PB0010.8, see Table 1).

PHB analysis

PHB was extracted from leaves of transgenic lines PB0010.1, PB0010.2, PB0010.4, PB0010.5, PB0010.7. The single *phb*C transformant (PB0010.13) and the wild type line were used as negative controls. First, lyophilised material was washed with warm ethanol to solubilise lipids and chlorophylls but not PHB, which is not soluble in ethanol. The de-fatted powder or the PHB

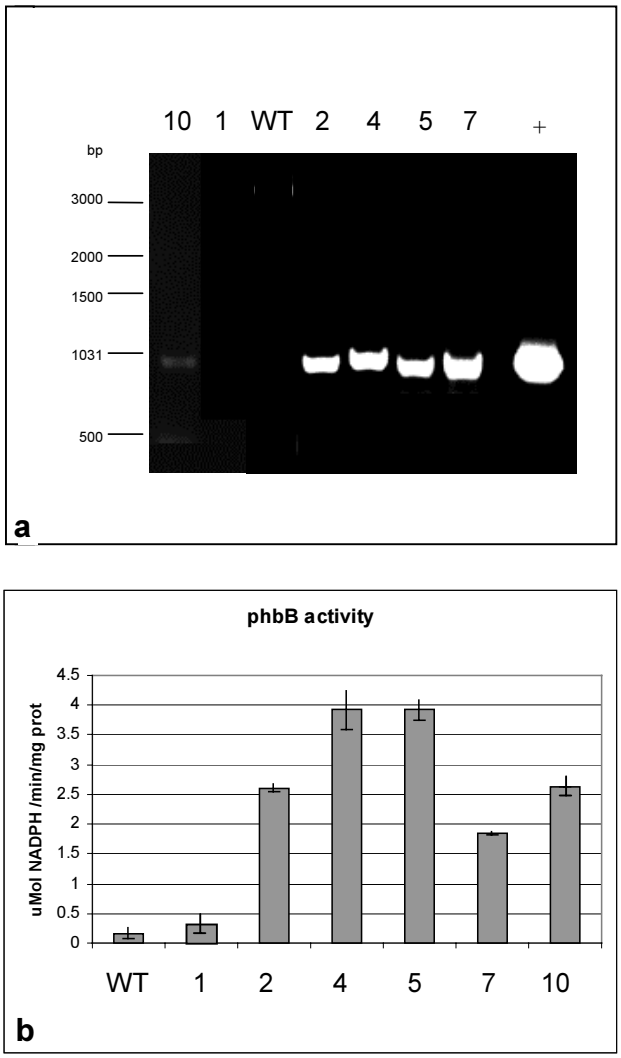


Figure 1. mRNA and enzymatic assay for the *phb*B gene and gene product. Codes 1, 2, 4, 5, 7 and 10: transgenic potato lines PB0010.1, PB0010.2, PB0010.4, PB0010.5, PB0010.7, PB0010. 10. Code WT: wild type potato line a) RT-PCR analyses on 5 transgenic lines and the wild type to check the transcription of the *phb*B gene. Lane +: plasmid positive control. b) results of the acetoacetyl-CoA reductase enzymatic assay on transgenic plants showing transcription of the *phb*B gene and the wild type. Bars indicate standard deviations, n= 3 – 5.

extracted with chloroform from the de-fatted powder was subsequently methanolysed and analysed by GC.

In 3 transgenic lines expressing both the acetoacetyl-CoA reductase and the PhbC polymerase (Table 1), the presence of small amounts (PB0010.2) or traces (PB0010.4 and PB0010.7) of PHB were observed (Figure 3). 'Traces' refer to amounts of polymer close to the limit of detection (around 0.5 ng), which were difficult to quantify. No evidence of PHB was obtained in the wild type line and in transgenic line PB0010.13, in which the integration and expression of the *phb*B gene had not been observed. The maximum amount of PHB detected in line PB0010.2 was 0.7 μg of PHB per g of dry weight.

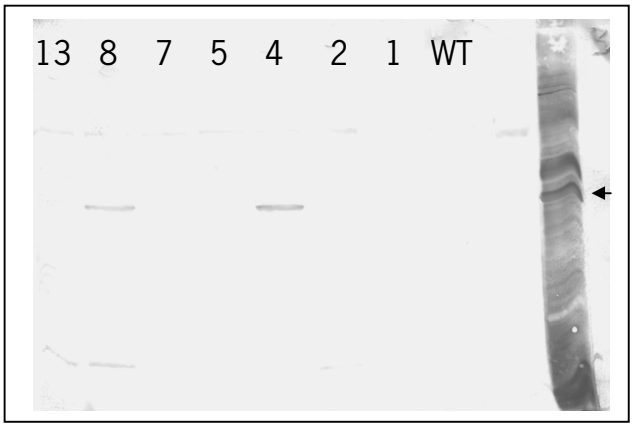
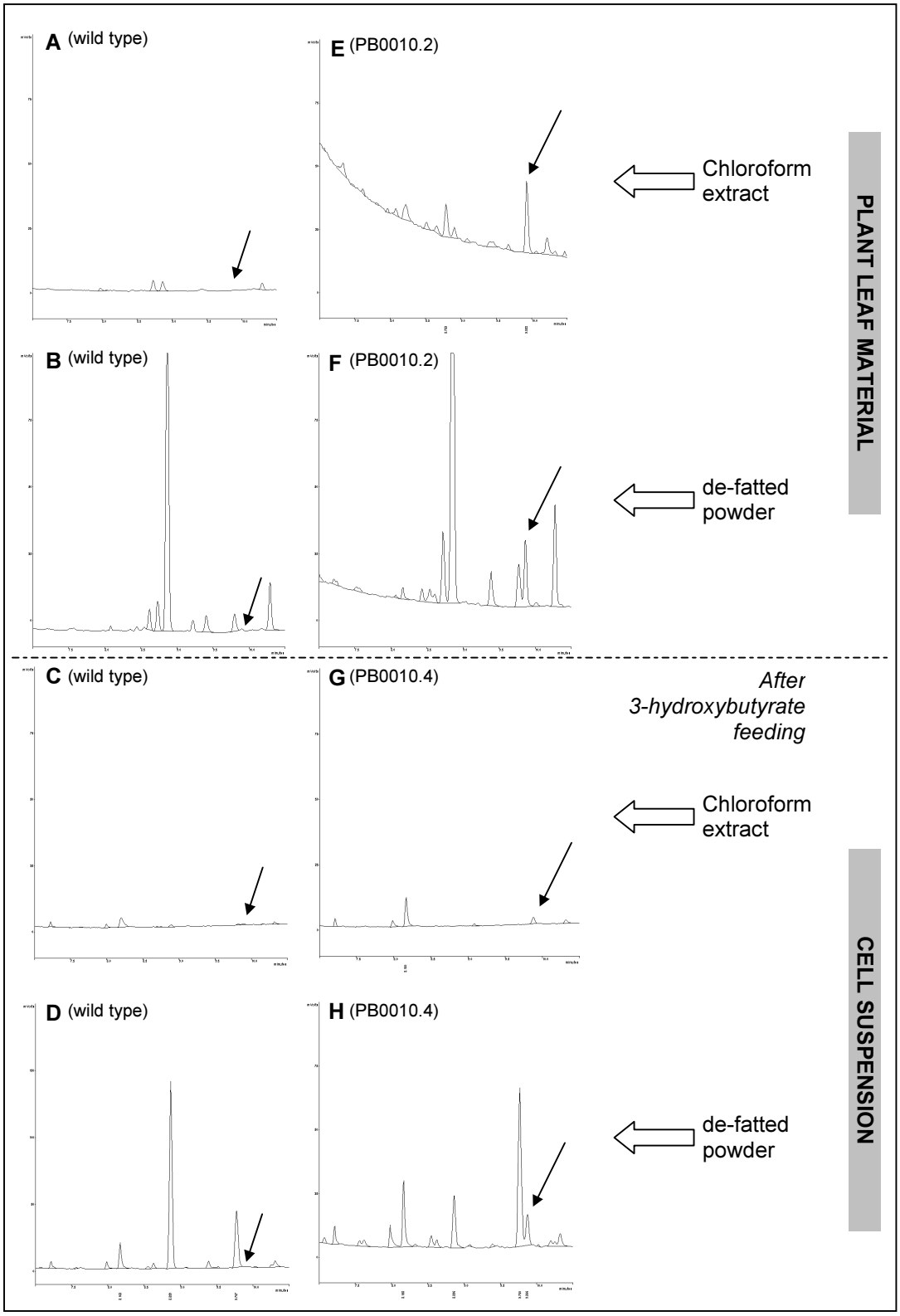


Figure 2. Western blot analyses using antibodies specific for the PHB-polymerase on transgenic potato lines PB0010.1 (lane 1), PB0010.2 (lane 2), PB0010.4 (lane 4), PB0010.5 (lane 5), PB0010.7 (lane 7), PB0010.8 (lane 8), PB0010.13 (lane 13), and wild type line (lane WT). The lane on the right contains a crude extract from *R. eutropha*. The arrow indicates the expected migration of the PhbC polymerase.

Feeding experiment

Given the low yield of PHB in transgenic lines expressing both the PhbC polymerase and the acetoacetyl-CoA reductase, a substrate feeding approach was chosen to investigate whether the bottleneck of the system resided in the substrate availability or somewhere else (gene expression, translation, protein folding etc.). Cell suspension lines were established from the wild type line and 2 transgenic lines (PB0010.2, PB0010.4) which showed minor amounts of PHB accumulation. It was not possible to establish cell suspension lines from the other transgenic lines, which were not analysed with the feeding approach. The accumulation of PHB was monitored after the suspension lines were fed with acetate (initial substrate of the PHB biosynthetic pathway) or 3-(*R*-*S*)-hydroxybutyrate (PHB monomer). The feeding treatment did not affect growth of the suspension lines (not shown), as reported also in Chapter 5. Both cell suspension cultures did not show any increase in PHB content, remaining at a level close to the detection limit (Figure 3, 3-hydroxybutyrate feeding).

Figure 3: GC analyses of transgenic and wild type potato lines. A, B, C, D: wild type. E, F: transgenic line PB0010.2. G, H: transgenic line PB0010.4. Chromatograms A, E (top row of chromatograms) represent the profiles of the chloroform extracts, containing PHB in the transgenic lines but not in the wild type. PHB content of the wild type (no PHB) and transgenic line PB0010.4 after they had been fed with 3-hydroxybutyrate are shown in chromatograms C and G, respectively. Chromatographic profiles of the de-fatted powder (i.e. after the first washing with ethanol) methanolysed and directly used for GC analyses for the same transgenic and wild type lines are represented in chromatograms B, F (wild type and PB0010.2) and D, H (wild type and PB0010.4, after feeding). The arrows indicate the expected retention times for 3-hydroxybutyrate monomers.


A (wild type)
E (PB0010.2)
Chloroform extract
PLANT LEAF MATERIAL
B (wild type)
F (PB0010.2)
de-fatted powder
C (wild type)
G (PB0010.4)
After 3-hydroxybutyrate feeding
Chloroform extract
CELL SUSPENSION
D (wild type)
H (PB0010.4)
de-fatted powder

Discussion

Several plant species have been engineered, receiving the bacterial PHB metabolic pathway, mainly from *R. eutropha* (see Chapter 1). In some cases, the initial substrate for the synthesis of PHB was derived from the cytoplasmic pool of acetyl-CoA (Poirier et al., 1992; Nawrath et al., 1995; John & Keller et al., 1996; Nakashita et al., 1999; Poirier, 2002a). However, the carbon flux through acetyl-CoA in the cytoplasm is limited, but it is abundant in those cell compartments where fatty acid metabolism occurs (i.e. in plastids, where FAB employs acetyl-CoA as initial substrate, and in peroxisomes, where acetyl-CoA is the final product of β-oxidation). In several reports, relevant proteins were targeted to the plastids (Nawrath et al., 1994; Houmiel et al., 1999; Slater et al., 1999; Bohmert et al., 2000; Bohmert et al., 2002) or peroxisomes (Hahn et al., 1999) and PHB was accumulated in transgenic plants.

In the present report, the ability of potato to produce PHB was tested after the coordinated expression of the *R. eutropha* acetoacetyl reductase (*phb*B gene product) and phbC polymerase (*phb*C gene product) in the cytoplasm of transgenic plants. Like Nakashita and coworkers observed (1999), not all the potato lines analysed in this report that showed integration of the *phb*C gave evidence of its expression (transcription and translation). However, no correlation was found between the presence of the PhbC polymerase at detectable levels (western blot), activity of the acetoactyl–CoA reductase and PHB content and most PHB was found in a line in which the expression of the PhbC polymerase was below the detection limit of western blotting. Slater et al. (1999) and Nawrath et al. (1994) made similar observations in *Arabidopsis*. Line PB0010.2 accumulated 0.7 ng / mg cell dry weight of PHB (7×10^{-5} %). After the relevant proteins were expressed in potato chloroplasts, Bohmert and colleagues (2002) observed the accumulation of PHB at amounts corresponding to 100 times the amount reported here ($2 - 9 \times 10^{-3}$ %). Interestingly, the similar discrepancies between cytoplasmic *versus* plastidial PHB accumulation have been reported for *Arabidopsis* (Poirier et al., 1992; Nawrath et al., 1994), *Brassica* and tobacco (Chapter 1, Table 2). This agrees with the idea that in the cytoplasm the flux of acetyl-CoA is limiting. However, important differences in the total flux of acetyl-CoA among plant species are

also evident. To check whether the substrate availability represented a bottleneck in the system used in the present report, acetate was provided to cell suspension lines derived from plants accumulating PHB. Hydroxybutyrate was used in a parallel feeding experiment to identify possible bottlenecks (acetoacetyl-CoA condensation, acetoacetyl reduction and presence of NADPH reducing power, or polymerisation step). However, no effect was observed after these treatments. This suggests that not only the substrate limitation may hamper the accumulation of PHB, but gene expression-related obstacles may be important as well, which agrees with the western blot data on PHB-polymerase expression. Similarities in observations among different groups suggests the occurrence of biological, rather than technical limitations. Moreover, the same set of genes used here for potato transformation have also been successfully expressed in the cytoplasm of the oleaginous yeast *Cryptococcus curvatus* leading to the accumulation of PHB up to 2.5 % of the cell dry weight (Springer, personal communication).

A decrease in the PHB content during consecutive vegetative propagation of the transgenic plants was observed and may have resulted from gene inactivation / silencing, as reported also by Poirier (2001b and 2002a). No additional morphological abnormalities were observed in the potato lines (apart form one line which was slightly growth-retarded, PB0010.10 and one which was aberrant due to somaclonal variations, PB0010.16).

In conclusion, this report and the work of Bohmert and colleagues (2002) show that potato can be a host for the expression of prokaryotic genes involved in PHB biosynthesis and for PHB accumulation. The yield of PHB produced in transgenic plants appears to be far too low for commercial production of polyesters in transgenic crops in the near future. Nevertheless, new applications, not based on bulk-polymer production but on added value to existing or novel plant compounds, can attract considerable interest in the future. One example of such an application is the modification of cotton fibres as shown by John & Keller (1996) through the incorporation of PHB into the fibres. Similar manipulation of other plant polymers could lead to the production of blends or novel products with novel properties.

Acknowledgements

The authors would like to thank Jan Springer and Cristina Turi for helping in constructing plant expression vectors. This study has been funded by a E. U. Marie Curie Fellowship (contract number FAIRCT98-5036) and by an ATO-IAC grant.

CHAPTER 5

Expression of the *Pseudomonas oleovorans* Pha-C1 polymerase and evidence of medium-chain-length polyhydroxyoctanoate synthesis in transgenic potato cell suspension cultures after precursor feeding[1]

[1] Based on: Andrea Romano, Dick Vreugdenhil, Diaan Jamar, Linus H. W. van der Plas, Guy de Roo, Bernard Witholt, Gerrit Eggink, Hans Mooibroek. Evidence of medium-chain-length polyhydroxyalkanoate accumulation in transgenic potato lines expressing the *Pseudomonas oleovorans* Pha-C1 polymerase in the cytoplasm. *Submitted to "Biochemical Engineering Journal"*

Abstract

The *pha*C1 gene from *Pseudomonas oleovorans*, coding for the Pha-C1 polymerase, was introduced into the potato genome. Transgenic callus and plant lines in which the transgene was transcribed and translated were selected. Cell suspension cultures from the wild type and transgenic lines were established and the substrate for the Pha-C1 polymerase, 3-(*R*)-hydroxyoctanoate, was provided to the growth medium. In the transgenic lines, but not in the wild type or in transgenic cell suspension cultures without Pha-C1 expression, evidence of medium-chain-length polyhydroxyalkanoate accumulation was observed after feeding with the substrate in the growth medium.

Key Words

Particle bombardment, *Solanum tuberosum*, medium-chain-length polyhydroxyalkanoate, Pha-C1 polymerase, cell suspension culture.

Introduction

Fluorescent pseudomonads belonging to the rRNA group I accumulate granules that contain medium-chain-length polyhydroxyalkanoate (mcl-PHA), consisting of 3-(*R*)-hydroxyalkanoic acids with carbon chains ranging from C-6 to C-12 (Huisman et al., 1989; Timm & Steinbüchel, 1990). The PHA-polymerase is the key enzyme for the synthesis of mcl-PHA using 3-(*R*)-hydroxyacyl-CoA derived from fatty acid metabolism. In these pseudomonads, the *pha*-operon (Huisman et al., 1991; Timm & Steinbüchel, 1992) comprises the *pha*C1 and *pha*C2 genes coding for the Pha-C1 and Pha-C2 polymerases, respectively. These PHA-polymerases are classified as type II, and are specifically active on mcl-alkanoic acids. The two PHA-polymerases share 54 – 50 % identity at the protein sequence level and have a molecular weight of 62 - 64 kDa. Several additional genes are present (Steinbüchel et al., 1992; Timm & Steinbüchel, 1992; Klinke et al., 1999; Prieto et al., 1999b) in the operon which are involved in PHA degradation or in structural aspects of the PHA granules (Steinbüchel & Hein, 2001; Kessler & Witholt, 2001a). A high degree of identity (69 – 80 %) at the amino acid level is observed between corresponding mcl-PHA-polymerases from different species and 40 % identity is observed with the *Ralstonia eutropha* polyhydroxybutyrate- (PHB)-polymerase, (Huisman et al., 1991; Timm & Steinbüchel, 1992), which is active on short-chain-length alkanoates (C4 and C5 carbon units). PHB-polymerase (type I) and type II polymerase contain a lipase box (Huisman et al., 1992) and it has been postulated that the catalytic mechanism involves residues Cys-296 and Cys-430 (*Pseudomonas oleovorans* numeration. Huisman et al., 1991).

Both Pha-C1 and Pha-C2 polymerases are functional proteins which are able to catalyse mcl-PHA formation independently from each other when expressed in heterologous hosts (Langenbach, 1997; Qi et al., 1997) including plants (Mittendorf et al., 1998). Overproduction of the mcl-PHA-polymerase in *E. coli* did not result in a corresponding increase of PHA yield, and most of the protein was recovered as inactive protein in inclusion bodies (Ren et al., 2000a). However, solubilisation of the trapped protein resulted in

active polymerase, and this enzyme was capable of *in vitro* synthesis of mcl-PHA without any additional component.

In this work, the Pha-C1 polymerase of *P. oleovorans* has been expressed in the cytoplasm of transgenic potato lines. Although plant cytoplasm does not contain any PHA precursors due to the compartimentation of fatty acid metabolisms in plants [ß-oxidation occurs mainly in peroxisomes and fatty acid biosynthesis (FAB) in the plastids], the ability of potato to express the Pha-C1 polymerase and to accumulate mcl-PHA was tested using a feeding approach, exogenously providing the substrate [3-(*R*)-hydroxyoctanoate] to cell suspensions of the selected transgenic lines. Evidence for mcl-PHA accumulation in the transgenic lines expressing the *pha*C1 gene, but not in the wild type line nor in transgenic lines not expressing the Pha-C1 polymerase, was obtained. This is the first report of accumulation of mcl-PHA in a starch-storing crop.

Material and methods

Plant Material and transformation.

In vitro grown *Solanum tuberosum*, genotype 1024-2 (*amf*, diploid; Jacobsen et al., 1989) was used as starting material for particle bombardment-mediated co-transformation as described in Chapters 2 and 3. Transgenic cell suspension lines were derived from both regenerated plants resistant to kanamycin and from calli resistant to kanamycin growing on bombarded internodes as described below.

DNA constructs.

Plasmid 35-S-Kan containing the *npt*II selectable marker under the control of the 35-S promoter and terminator, coding for the neomycin-phospho-transferase, conferring resistance to kanamycin was kindly provided by the John Innes Centre (Norwich, UK). Plasmid pAPP63 (Figure 1) contained the *pha*C1 gene from *P. oleovorans* under the control of the Enhanced 35-S promoter (E-35S) and *nos*-terminator and was constructed as follows (Figure 2): Pwo (*Pyrococcus woesei*) DNA polymerase (Eurogentec) was used, as described by the manufacturer, for PCR amplification using plasmid pET101 (*pha*C1 gene: Ren, 1997) as a template. Primer extension was used in order

to introduce the *Nco*I and *Bam*HI restriction sites at the 5' and 3' ends of the gene. In order to avoid the possible occurrence of PCR mistakes in the gene (1680 base pair long), upstream and downstream linkers were created by PCR using primers 5'-CGCGGATCCACCATGGGTAACAAGAACA-3' and 5'-TGACGAACTGGCCGCGGCTGATG, annealing at position 351 - 373 (which includes the restriction site *Sac*II at position 357) and at the 5' of the *pha*C1 gene and using primers 5'-CCCTGCCGGCCGCCTTCCACG-3' and 5'-TCCGGATCCTCAACGCTCGTGAACGTA-3', annealing at position 1209 - 1228 (including a site *Xma*III at position 1214) and at the 3' of the *pha*C1 gene. All PCR products were cloned into pGEM-T-easy vectors (Promega) and sequenced. The complete *pha*C1 gene was re-assembled in plasmid pAPP57 using the two linkers and the 857 base pairs *Sac*II / *Xma*III fragment from pET101 containing the central part of the wild-type gene as described in detail in Figure 2. The synthetic *pha*C1 gene was extracted by *Nco*I / *Bam*HI restriction from plasmid pAPP57 and cloned in pAMV-1 (Rouwendal et al., 1997) giving plasmid pAPP62. The *Bgl*II / *Bam*HI fragment from pAPP62 was finally cloned into the *Bam*HI site of pAPP23 (Chapter 2), giving rise to pAPP63 (Figure 1).

PCR and Southern blot analysis.

All molecular techniques were performed as described in Chapters 2 and 3 and using standard protocols (Sambrook et al., 1989). PCR analyses on genomic DNA were performed using DNA isolated with the Sigma Gene-Elute KIT according to the manufacturer's recommendations and using the upstream and downstream linker primers to monitor the integration into the plant genome of the *pha*C1 gene.

RT-PCR and northern blot.

RNA isolation from plant / cell suspension material, cDNA synthesis and PCR were performed as described in Chapters 3 and 4. For northern blot analysis, 15 μg of total RNA were separated on a 2 % agarose gel and blotted on a positively charged nylon membrane using the Turboblotter^{TM} system (Schleicher & Schuell). The filters were hybridised with ^{32}P labelled probe using standard techniques (Sambrook et al., 1989).

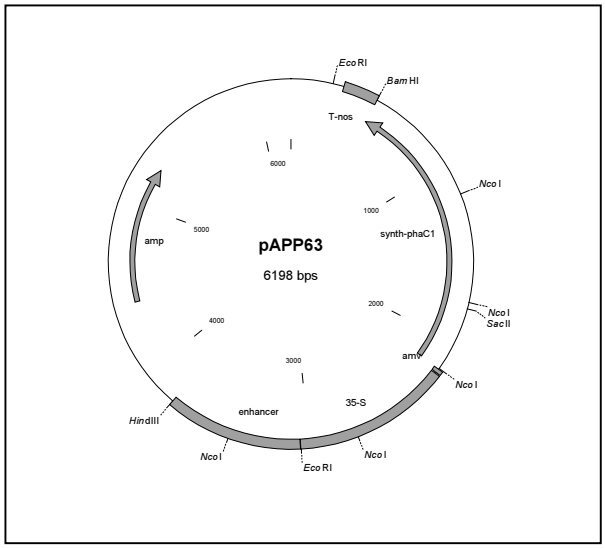
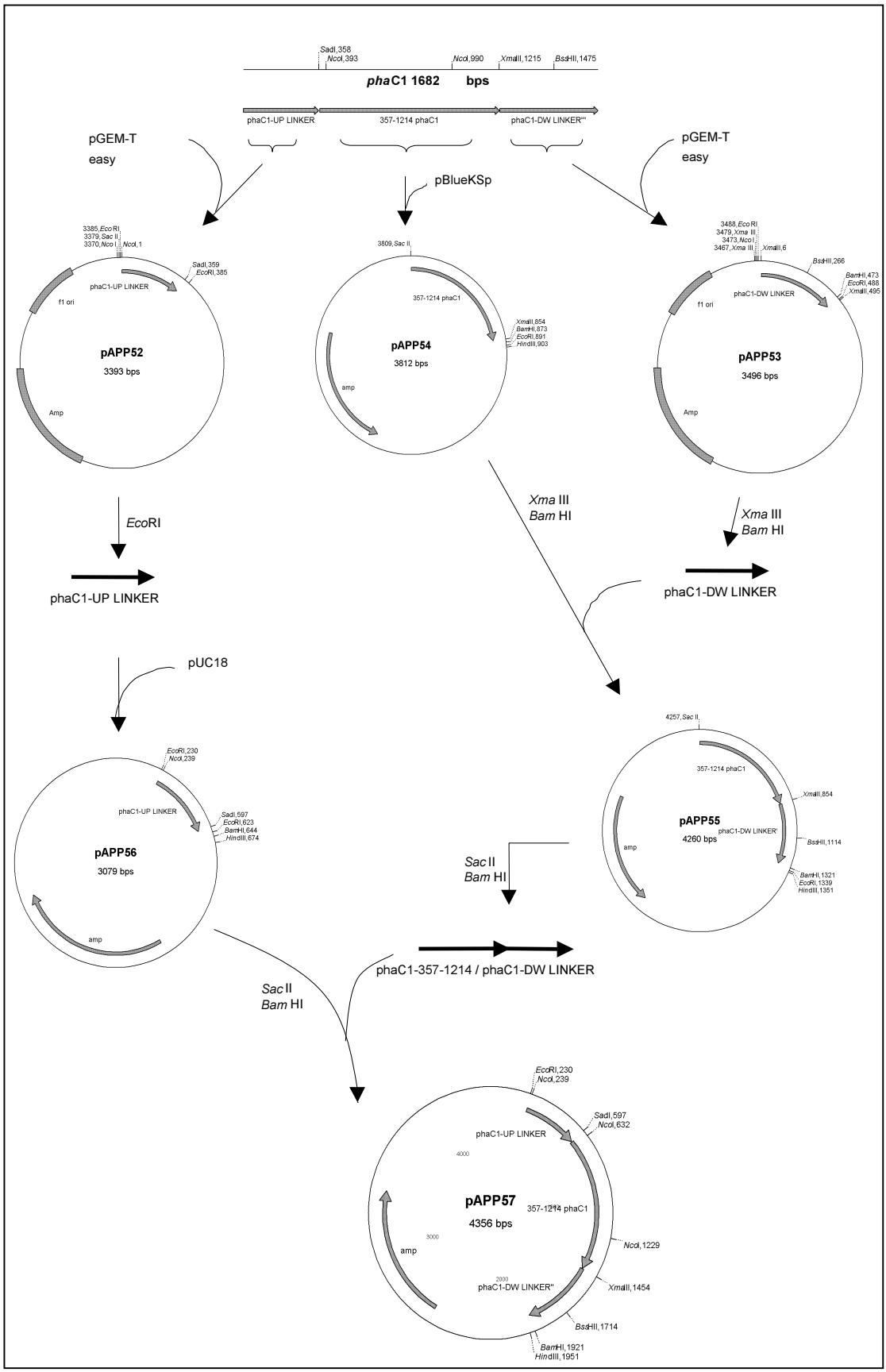


Figure 1. Schematic representation of plasmid pAPP63 containing the *pha*C1 gene from *P. oleovorans* under the control of the E-35S and *nos* terminator.

Sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Protein extraction, SDS-PAGE and protein blotting were performed as described in Chapter 4. After protein blotting, filters were blocked overnight with 5 % skim milk powder in TBST (0.1 M Tris-HCl, pH 7.5; 1.5 M NaCl; 0.1 % Tween-20) at 4°C. Subsequently, filters were incubated for 2 hours with anti-Pha-C1 antibodies (1:10000. Kraak et al., 1997) and with alkaline-phosphatase-conjugated anti-rabbit secondary antibodies (Sigma) in TBST containing 1 % skim milk powder. Antibodies-protein complexes were detected using 5-bromo-4-chloro-3-indoylphosphate and nitro-blue-tetrazolium (Amersham Pharmacia Biotech; Chapter 4).

Figure 2. Construction of plasmid pAPP63 (Figure 1). The *pha*C1 upstream linker (phaC1-UP-LINKER, 357 bp, including *Nco*I and *Sac*II restriction sites at the 5' and the 3' ends, respectively) and the *pha*C1 downstream linker (phaC1-DW-LINKER, 466 bp, including *Xma*III and *Bam*HI restriction sites at the 5' and the 3' ends, respectively) were amplified by PCR and cloned in pGEM-T-easy vectors giving rise to the pAPP52 (upstream linker) and pAPP53 (downstream linker). The *Sac*II / *Xma*III 857 bp wild-type fragment of the *pha*C1 was cut off from pET101 and cloned into *Sac*II / *Xma*III of pBlueKSp (Promega) giving rise to pAPP54. Plasmid pAPP55 was created by cloning the 466 bp *Xma*III / *Bam*HI fragment from pAPP53 into pAPP54 digested with *Xma*III / *Bam*HI restriction endonucleases.
The 393 bp *Eco*RI fragment from pAPP52, comprising the upstream linker, was cloned into the *Eco*RI site of pUC18 (Pharmacia) giving plasmid pAPP56. Eventually, the 1323 bp *Sac*II / *Bam*HI fragment from pAPP55 was cloned into *Sac*II / *Bam*HI sites of pAPP56, thus yielding pAPP57, containing the complete synthetic *pha*C1 gene which was subsequently cloned into plasmid pAPP23 (see Material and methods).


SadI,358
NcoI,393
NcoI,990
XmaIII,1215
BssHII,1475
phaC1 1682 bps
phaC1-UP LINKER
357-1214 phaC1
phaC1-DW LINKER''
pGEM-T easy
pBlueKSp
pGEM-T easy
3385,EcoRI
3379,Sac II
3370,NcoI
NcoI,1
SadI,359
EcoRI,385
phaC1-UP LINKER
f1 ori
pAPP52
3393 bps
Amp
3809,Sac II
357-1214 phaC1
XmaIII,854
BamHI,873
EcoRI,891
HindIII,903
pAPP54
3812 bps
amp
3488,EcoRI
3479,Xma III
3473,NcoI
3467,Xma III
XmaIII,6
BssHII,266
BamHI,473
EcoRI,488
XmaIII,495
phaC1-DW LINKER
f1 ori
pAPP53
3496 bps
Amp
EcoRI
phaC1-UP LINKER
Xma III
Bam HI
Xma III
Bam HI
phaC1-DW LINKER
pUC18
EcoRI,230
NcoI,239
phaC1-UP LINKER
SadI,597
EcoRI,623
BamHI,644
HindIII,674
pAPP56
3079 bps
amp
4257,Sac II
357-1214 phaC1
XmaIII,854
pAPP55
phaC1-DW LINKER'
4260 bps
BssHII,1114
BamHI,1321
EcoRI,1339
HindIII,1351
amp
Sac II
Bam HI
phaC1-357-1214 / phaC1-DW LINKER
Sac II
Bam HI
EcoRI,230
NcoI,239
SadI,597
NcoI,632
phaC1-UP LINKER
4000
pAPP57
4356 bps
357-1214 phaC1
3000
amp
NcoI,1229
3000
phaC1-DW LINKER''
XmaIII,1454
BssHII,1714
BamHI,1921
HindIII,1951

Cell suspension.

Friable calli were induced on MS (Murashige & Skoog, 1962) salts plus vitamins (Duchefa), 0.8 % agar, supplemented with sucrose 2.5 %, casein hydrolysed 0.1 %, myo-inositol 0.1 %, 2-4-D 3 mg / L, kinetine 0.2 mg / L, thiamine pyrophosphate 0.5 mg / L, pyridoxine 0.5 mg / L, nicotinic acid 0.5 mg / L, glycine 2 mg / L, folic acid 0.5 mg / L, and biotine 0.05 mg / L, pH 5.7 from *in vitro* grown microtubers or from calli resistant to kanamycin that emerged from bombarded internodes. Cell suspension cultures were started from friable calli on MS salts plus vitamins liquid medium supplemented with sucrose 3 %, and 2-4 D, 2 mg / L and maintained by subculturing 10 mL of suspension in 40 mL of the same medium every 10 days. Microtubers were induced from nodes of *in vitro* grown plantlets as described in Chapter 2.

Viability test using the fluorescein diacetate (FDA).

To check the percentage of living cells, the FDA staining method was performed as described by Hoeberichts et al. (2001).

Feeding experiment.

Pure 3-(*R*)-hydroxyoctanoate was produced as described by de Roo et al. (2002). After solubilisation in buffer phosphate 50 mM, pH 7, at a concentration of 100 mM, the substrate was added to the suspension, 5 days after subculturing, to a final concentration of 1 mM. The substrate feeding was repeated every 2 days.

PHA isolation, Gas Chromatography (GC) and GC-MS (Mass Spectrometry) analyses.

Cell suspensions (fed with the substrate or not fed) were washed three times with tap water, ground in liquid nitrogen and freeze dried. The resulting powder was washed with ethanol at 55 °C for 48 hours to remove fatty acids, and the content of the de-fatted powder was analysed by GC after methanolysis as described by Lageveen (1988). In short, 50 – 100 mg of powder was stirred at 110 °C for 5 hours in 2 mL of methanol supplemented with sulphuric acid 15 % and 2 mL of chloroform. Methanol / chloroform phases were separated by adding 1 mL of water, and the methylesters in the chloroform phase were analysed by GC on a Carlo-Erba GC6000 (Carlo Erba) equipped with a 25 m CP-Sil 5CB capillary column (Chrompack). GC-MS

spectra were obtained using a Carlo-Erba HRGC/MS gas chromatograph equipped with a CP-Sil 5CB column attached by a direct interface to a Carlo Erba QMD 1000 mass spectrometer.

Results

Transformation and selection of transgenic lines

Plasmids 35-S-Kan and pAPP63, containing the *npt*II selectable marker and the *P. oleovorans pha*C1 gene, respectively, were simultaneously introduced in potato cells by particle bombardment mediated co-transformation as described in Chapters 2 and 3. In short, plasmids 35-S-Kan and pAPP63 were co-precipitated on gold particles at a molar ratio of 1 : 2 and were delivered using the Helium Inflow Gun device to *in vitro* grown potato internodes. Transgenic plants were regenerated and 30 lines were rooted in the presence of kanamycin. Thirty-eight calli growing on kanamycin containing medium but which did not accomplish regeneration, were maintained and propagated on callus medium containing kanamycin (100 mg / L). Genomic DNA was extracted from plant or callus lines resistant to kanamycin and screened for the integration of the *pha*C1 gene by Southern blot or PCR analyses. Finally, 12 co-transformed lines (6 callus and 6 plant lines) containing the *pha*C1 gene were finally selected and 10 of these showed, by northern blot or RT-PCR analyses, that the transgene was transcribed. Western blot analysis confirmed that the Pha-C1 polymerase was expressed in 5 of these 10 lines (Figure 3).

Table 1. List of plant and callus lines selected and used in the feeding experiments. Results of western blot and GC analyses (after 3-hydroxyoctanoate feeding) are indicated. CDW: cell dry weight.

cell suspension lines	derived from	Pha-C1 Western blot	mg mcl-PHA / g CDW
Wild type	Plant	-	-
PB002.1A1	Callus line	-	-
PB002.1E1	Callus line	-	-
PB002.1F1	Callus line	+	0.022
PB002.1L1	Plant	+	9.7
PB002.2A1	Plant	+	2.0
PB002.3.4	Callus line	+	0.9
PB002.3.5	Plant	-	-

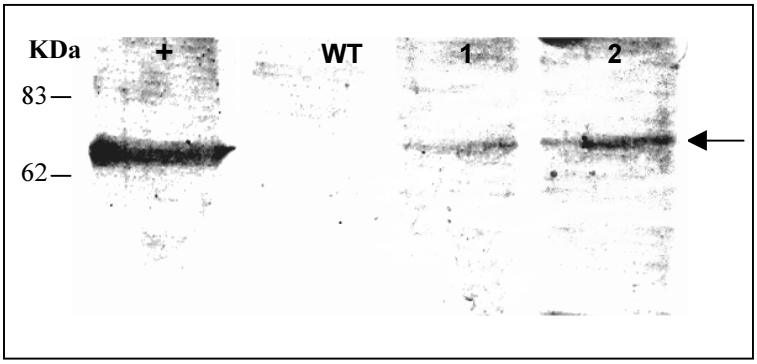
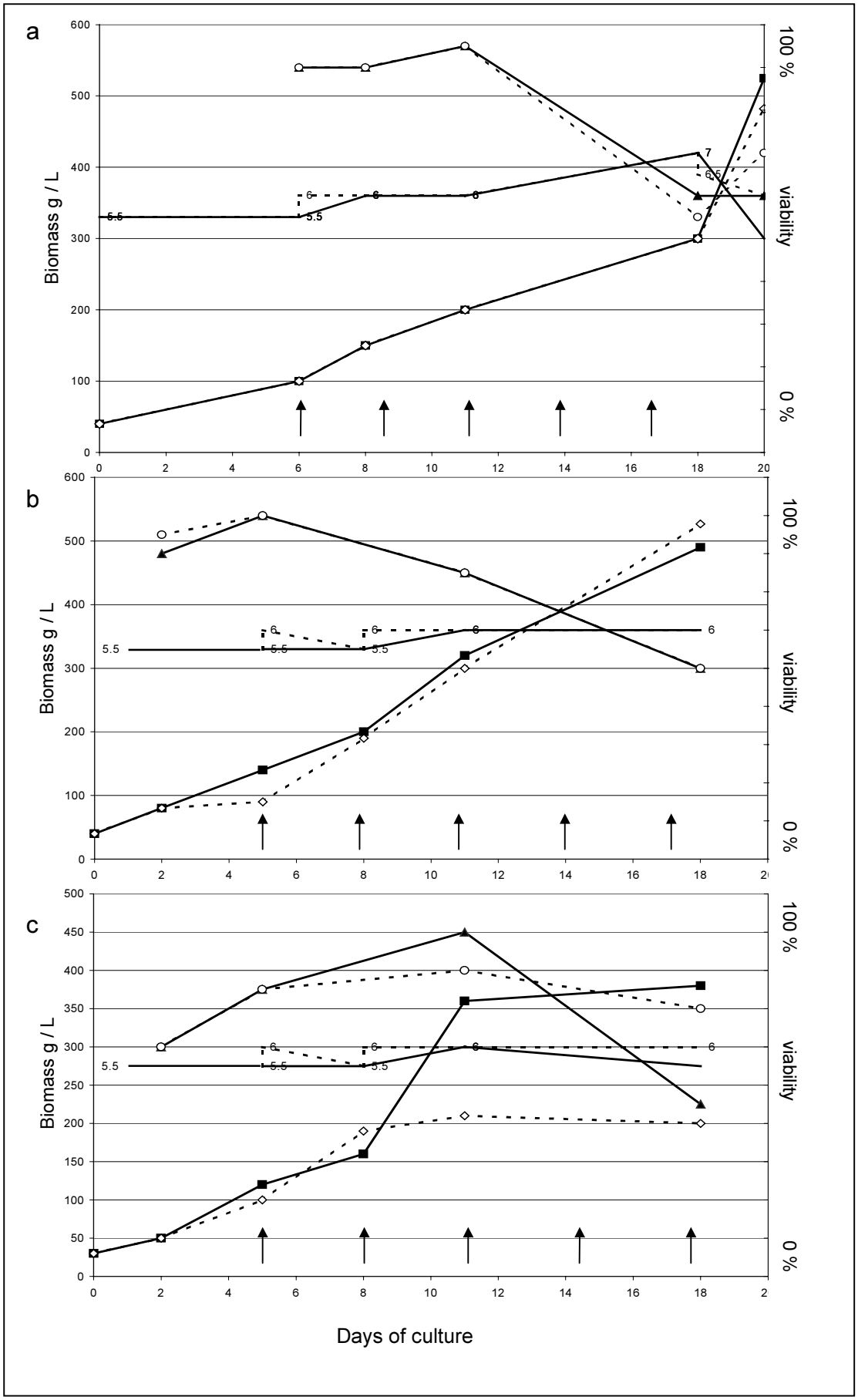


Figure 3. Western blot analyses using antibodies raised against the Pha-C1 polymerase of *P. oleovorans*. The Pha-C1 polymerase band is indicated by an arrow. Lane +: purified Pha-C1. Lane WT: wild type line. Lanes 1 and 2: two independent transgenic lines.

Feeding experiment

Cell suspensions were established from the wild type line and 7 transgenic lines, 4 lines which showed and 3 lines which did not show the expression of the Pha-C1 polymerase by western blot analyses (Table 1). Both transgenic plants and calli were used as starting material. Feeding with 3-(*R*)-hydroxyoctanoate was started after the initial early lag phase of the growth curve had finished and the substrate was supplemented to the growing medium at a final concentration of 1 mM every two days. Too early addition of the substrate resulted in the death of the cell suspension. Preliminarily, different parameters (biomass, pH, cell viability) were monitored during feeding, to check to which extent the treatment affected growth and survival of the suspensions (Figure 4). Furthermore, depletion of the substrate from the growing medium was monitored in the wild type and the transgenic lines. The substrate was detectable in the growing medium by GC analysis after its addition, and it was completely depleted after two days.

Figure 4. Preliminary optimization of the feeding experiment on cell suspension lines derived from the wild type (a) PB002.1F1 (b) and PB002.1A1 (c) lines. Biomass: -■-) without feeding; --◇ ---) after feeding with 3-hydroxyoctanoate. Cell viability: -▲-) without feeding: ---o---) after feeding with 3-hydroxyoctanoate. pH: ▬) without feeding; -----) after feeding with 3-hydroxyoctanoate. Arrows indicate the time of addition of 3-(*R*)-hydroxyoctanoate at a final concentration of 1 mM.


a
b
c
Biomass g / L
viability
100 %
0 %
Days of culture
5.5
6
6.5
7

The absence of substrate depletion in a negative control without cells (medium plus substrate) suggested that it was indeed taken up into the cell, as previously observed in bacteria (de Roo, personal communication) and for other forms of fatty acids (Allenbach & Poirier, 2000). No significant differences in growth related parameters were observed between cell suspensions that were or were not exposed to the substrate.

PHA analysis

The PHA content of the cell suspensions was analysed by GC. In the extraction procedure, lyophilised material was first washed with warm ethanol. Ethanol solubilised lipids and chlorophyll, while PHA, soluble in chlorinated solvent and not in ethanol, was retained in the powder. The remaining content of the powder (including PHA) was subsequently methanolysed and analysed by GC.

In the 4 transgenic lines expressing the Pha-C1 polymerase (Table 1), only after 3-(*R*)-hydroxyoctanoate feeding, a peak was observed at the expected retention time corresponding to the pure substrate used in the experiment, (Figure 5a), but no peak was evident when the same lines were not exposed to the substrate. Moreover, in the wild type and transgenic lines in which thepolymerase was not expressed, no peak, at the same expected retention time, was observed, irrespective of whether the substrate had been supplemented or not (Figure 5a). The amount of polyhydroxyoctanoate detected ranged between 0.022 – 9.7 mg of polyhydroxyoctanoate per g of dry weight. GC-MS analysis (Figure 5b) was subsequently performed. The spectra of the methyl-esters extracted from the lines were analysed and werecompared with the spectra of the substrate. This confirmed the GC data, i.e. the peak observed in the transgenic lines expressing the Pha-C1 corresponded to 3-(*R*)-hydroxyoctanoate. Considering the total amounts of polyhydroxyoctaonate produced in separate batch cultures and the amount of 3-(*R*)-hydroxyoctanoate fed during the experiment the ratio depleted-substrate / polymerised-substrate ranged between 150 and 1800. These results suggest that the substrate provided from outside the cell, upon entrance into the cytoplasm, was either degraded (wild type and Pha-C1 negative lines) or in

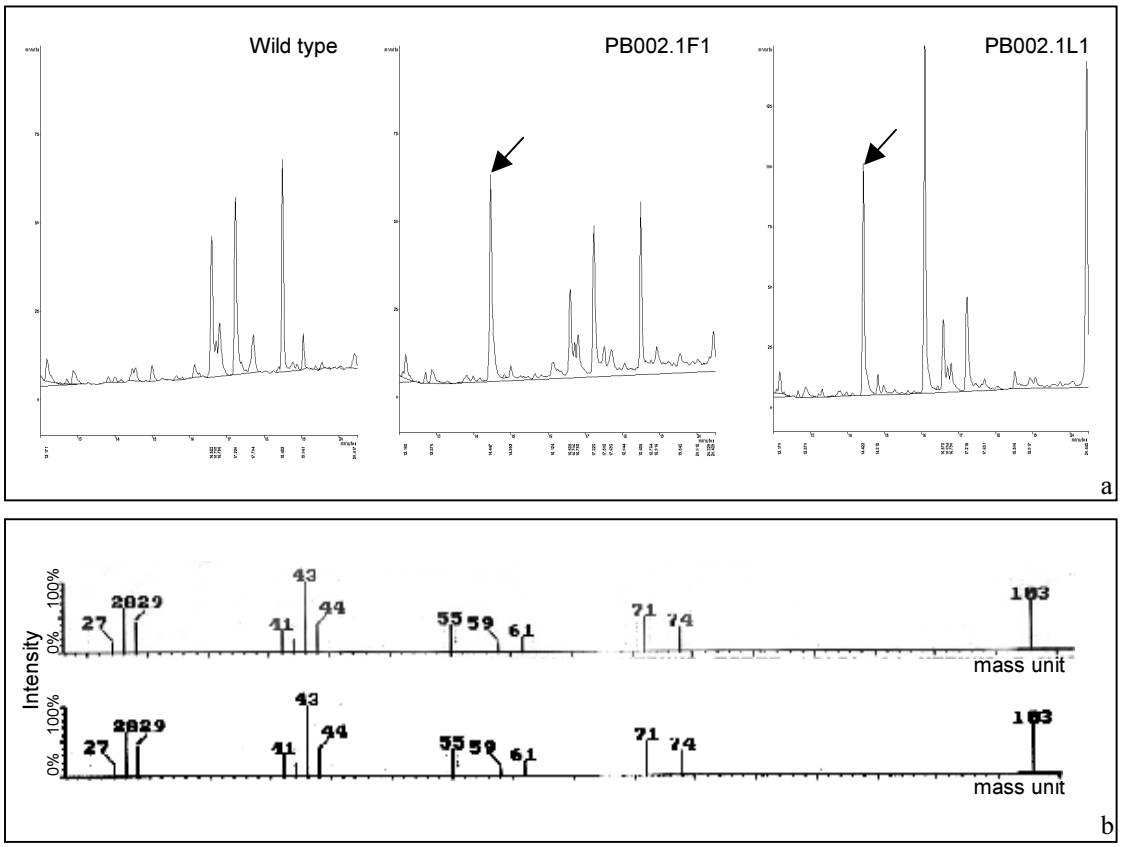


Figure 5. a) GC analysis of the methyl ester extracted from the wild type and two of the transgenic lines producing mcl-PHA. The arrows indicate 3-(*R*)-hydroxyoctanoate. b) GC-MS analyses to confirm the nature of the methyl ester observed by GC. Top: MS spectra of 3-(*R*)-hydroxyoctanoate. Bottom: MS spectra of the compound observed in the transgenic lines expressing the Pha-C1 polymerase eluted at the same retention time as 3-(*R*)-hydroxyoctanoate.

part degraded and in part polymerised to polyhydroxyoctanoate (Pha-C1 positive lines).

Discussion

This chapter describes, for the first time, the expression the Pha-C1 polymerase from *P. oleovorans* and the accumulation of mcl-PHA in transgenic potato lines. Without substrate feeding, expression of the Pha-C1 polymerase in the cytoplasm did not lead to the accumulation of mcl-PHA *in vivo*, presumably due to the lack of the substrate in this cell compartment. Thus, the feeding approach in cell suspensions (Figure 6a and Chapter 1, Figure 4, model 1) was attempted for two main reasons: on the one hand, to

study the minimum gene set required for mcl-PHA accumulation from fatty acids metabolism, on the other hand, to check whether the *pha*C1 gene from *P. oleovorans* would be expressed in potato. Low expression of prokaryotic genes in plants has been reported before (De Rocher et al., 1998; Diehn et al., 1998). The cytoplasm as target for the expression has the advantage that bacterial genes can be expressed with no further protein modification. During the course of these experiments, the first report of mcl-PHA polymerase gene expression in a plant was published (Mittendorf et al., 1998) but the source of the genes in this case was *P. aeruginosa*.

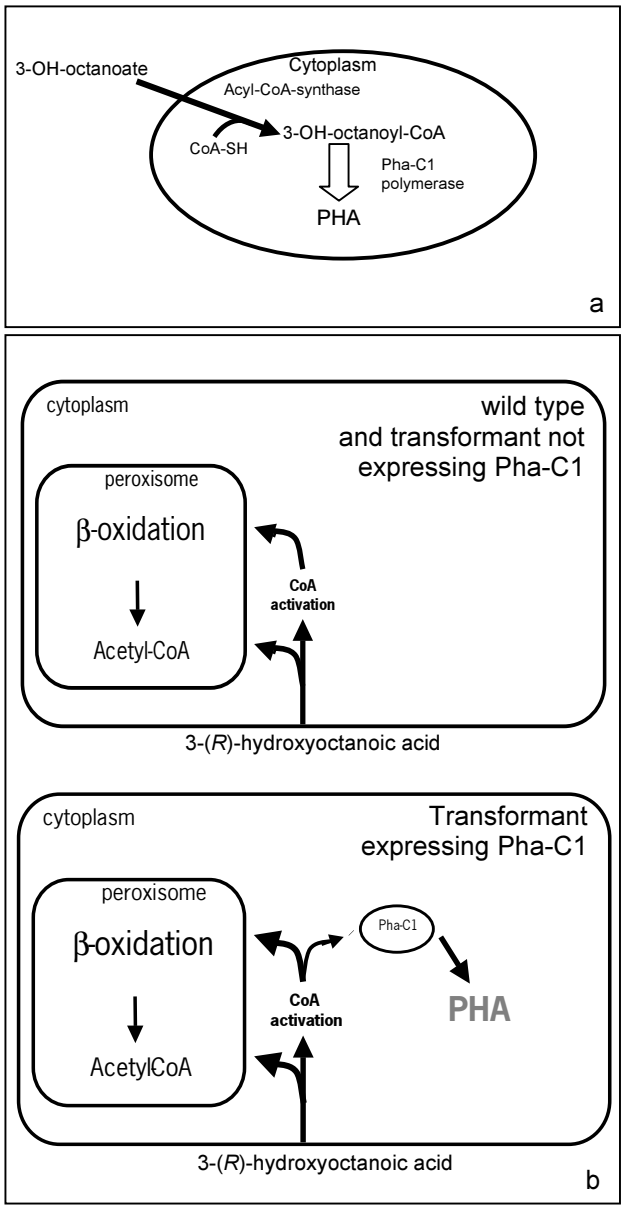


Figure 6. a) Scheme of the model implemented in this work to produce mcl-PHA. The Pha-C1 polymerase is expressed in the cytoplasm of transgenic potato lines and the substrate (3-OH-octanoate) is exogenously provided. See text for explanations. b) Proposed model to explain the observed data. Upon entrance into the cell of lines showing no Pha-C1 polymerase expression, the substrate is degraded through the β-oxidation (top; wild type and transgenic lines not expressing the Pha-C1 polymerase). In those lines in which the Pha-C1 polymerase is expressed, part of the substrate is converted into a polymer (bottom). See text for explanation.

Seven transgenic potato lines, 4 showing expression and 3 showing no expression of the Pha-C1 polymerase, and a wild type line were selected for further analyses. Mcl-PHA contents were analysed in corresponding cell suspensions lines, after they were provided with the substrate by feeding. Controls in which the same cell suspensions were not supplied with substrate were included. None of the lines analysed showed mcl-PHA accumulation if the substrate was not supplemented. However, only the 4 lines showing *pha*C1 gene expression, but not the wild type nor trangenic lines with no *pha*C1 expression, showed mcl-PHA accumulation after 3-(*R*)-hydroxyoctanoate feeding. The kind of analyses performed in this work does not demonstrate the presence of high-molecular-weight polymer (GC is based on monomer analysis after methanolysis). However, the correlation between the presence of the Pha-C1 polymerase (western blot) and the recovery of 3-(*R*)-hydroxyoctanoate-methyl esters by GC, and the absence of the same compound in the wild type or in the transgenic lines not expressing the polymerase, was considered proof for mcl-PHA synthesis.

A model is proposed to explain these data (Figure 6b). When supplemented in the medium, the substrate is taken up by the cell and eventually activated with CoA. Acyl-CoA synthase activity has been shown to be present in the plant cytoplasm [reviewed by (Ohlrogge & Browse, 1995)]. The presence of fatty acids which diverge from the normal pool of fatty acids present in the plant (i.e. palmitic, stearic, oleic, linoleic acids) activated the β-oxidation pathway (Eccleston, 1996; Mittendorf et al., 1999), thus, no 3-(*R*)-hydroxyoctanoate, although supplemented, could be detected in the transgenic lines not expressing the *pha*C1 gene and in the wild type (Figure 6b, top). In the transgenic lines expressing the Pha-C1 polymerase, 3-(*R*)-hydroxyoctanoate was converted to an inert polymer as terminal product and could subsequently be detected because potato has no bacterial enzymatic activity capable of degrading the polymer (Figure 6b, bottom). Thus, in the transgenic lines expressing the polymerase, β-oxidation and mcl-PHA biosynthesis may have competed for the utilisation of the substrate. Assuming that all the substrate entered the cells and was directed into β-oxidation or mcl-PHA (Figure 6b,

bottom), β-oxidation appeared to be 150 - 1800 times more efficient than the mcl-PHA biosynthesis.

In conclusion, this work demonstrates that the *pha*C1 gene from *P. oleovorans* is active and leads to the expression of active Pha-C1 polymerase and accumulation of mcl-PHA in transgenic potato. The feeding approach also showed to be a suitable system for precursor supply and for feeding-based studies.

Accumulation of mcl-PHA in plants has been reported only for *A. thaliana*. Moreover, accumulation of PHAs in general (mcl-PHA and PHB), has been reported predominantly in oil crops (Chapters 1 and 7) and only very recently the use of transgenic maize (Hahn et al., 1999) and potato (Bohmert et al., 2002) for the production of PHB has been published. Due to the generally larger productivity of starch crops, compared to oil crops, the formers are considered more suitable for PHA accumulation (Chapters 1 and 7). Therefore this work opens new promising prospects for mcl-PHA biosynthesis in plants (see Chapter 6).

Acknowledgement

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CHAPTER 6

Synthesis of medium-chain-length polyhydroxyalkanoate in intact transgenic potato plants[1]

[1] *A manuscript based on this chapter is in preparation*.

Abstract

Medium-chain-length poly-3-(*R*)-hydroxyalkanoate (mcl-PHA) belongs to the group of microbial polyesters. Mcl-PHA has thermal and physical properties that make them suitable for a number of possible applications, including biodegradable plastics, elastomeric rubbers, coatings and medical applications. In recent reports, the minimum gene-set for mcl-PHA accumulation from *de novo* fatty acid biosynthesis (FAB) has been identified in prokaryotes. The same set of genes has now been used to achieve the accumulation of mcl-PHA in transgenic potato. Transgenic potato lines were produced, after particle bombardment-mediated co-transformation, expressing in the plastids the *Pseudomonas oleovorans* Pha-C1 polymerase with a truncated form of the *Escherichia coli* thioesterase-I or with the *P. putida* ACP-CoA-acyltransferase. Theoretically, the two combinations of genes represent the minimum gene-set for mcl-PHA accumulation in prokaryotes. PHA contents of transgenic lines were analysed by gas chromatography. The double transformants expressing the Pha-C1 polymerase and the thioesterase-I did not show any polymer accumulation, most probably due to the requirement of a few β–oxidation steps that do not occur in the plastids. However, expression of the Pha-C1 polymerase and the ACP-CoA-acyltransferase led to the production of mcl-PHA in the leaves of the selected transgenic lines. This report establishes for the first time a possible route for the production of mcl-PHA from *de novo* FAB in plants.

Key Words. Particle bombardment, co-transformation, medium-chain-length poly-3-(*R*)-hydroxyalkanoate, Pha-C1 polymerase, ACP-CoA-3-(*R*)-hydroxyalkanoate transacylase, thioesterase-I

Introduction

Medium-chain-length poly-3-(*R*)-hydroxyalkanoate (mcl-PHA) is a polyester 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 (see Chapter 1; Huisman et al., 1989; Timm & Steinbüchel, 1990). The key enzyme for mcl-PHA formation is the PHA-polymerase, which catalyses the esterification of the hydroxy group at the 3rd carbon with the carboxyl group of a separate CoA activated 3-(*R*)-hydroxy fatty acid.

The *pha*-operon (Figure 1) contains the genes involved in the PHA metabolism: *pha*C1 and *pha*C2 genes code for the Pha-C1 and Pha-C2 polymerases, respectively. The *pha*C1 and *pha*C2 genes are spatially separated by the *pha*Z gene which codes for the depolymerase (Steinbüchel et al., 1992). Several extra genes are present which code for proteins involved in different aspects of the mcl-PHA metabolism, such as major PHA granule-binding proteins (named phasins) and gene expression regulators (Prieto et al., 1999a; Prieto et al., 1999b; Klinke et al., 2000; York et al., 2002). Given the variety of genes and proteins involved, mcl-PHA metabolism is rather complex (Prieto et al., 1999a; York et al., 2001; York et al., 2002) and its physiology and genetics partly still unknown. In the past, this has hampered the use of genetic engineering approaches for the accumulation of mcl-PHA in heterologous hosts. Recent studies attempting at mcl-PHA accumulation in recombinant bacteria led to the establishment of a possible minimum gene-set for mcl-PHA production. In its natural host, mcl-PHA can be accumulated from intermediates derived from β-oxidation or from 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. Thus the concerted actions of an hydratase, an epimerase, or a reductase (reviewed by Madison & Huisman, 1999; see Chapter 1) have been postulated for the epimerisation of the –*S*- to –*R*-enantiomer. Under unbalanced conditions (limiting ammonia and excess of carbon) *P. putida* and *P. aeruginosa* may accumulate mcl-PHA from not related sources (i.e. sucrose, gluconate, acetone; Timm & Steinbüchel, 1990; Huijberts et al., 1992) and supported by

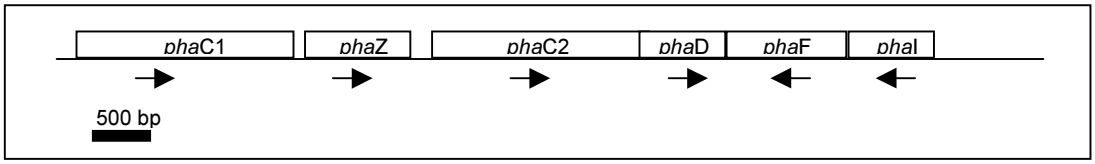

Figure 1. *pha*-operon from *P. oleovorans* (reviewed by Madison & Huisman, 1999). *pha*C1, *pha*Z, *pha*C2, *pha*D, *pha*F, *pha*I indicate the genes comprised in the *pha*-operon and coding for Pha-C1 polymerase, depolymerase, Pha-C2 polymerase and PhaD, PhaF and PhaI proteins. Arrow: direction of transcription.

FAB. FAB proceeds through the –(*R*)-enantiomer. However, in this case, the thio-ester activation takes place *via* the ACP molecule, but not *via* CoA. Mutant strains of *P. putida* which were not able to accumulate mcl-PHA from not related sources, led to the identification and cloning of the *pha*G gene, coding for an ACP-CoA-hydroxyacyl transacylase (Rehm, 1998. Figure 2). The analogue 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, it has no affinity for straight-chained fatty acids (C-8, C10) and the purified enzyme requires $MgCl_2$ as a co-factor for its catalytic activity (Rehm, 1998). Expression of the *pha*G gene in a number of recombinant prokaryotic hosts led to the accumulation of mcl-PHA from FAB. For example, expression of *pha*G in combination with the *pha*C1 genes in the non-mcl-PHA accumulating *P. fragi*, led to the accumulation of mcl-PHA from non related sources (Fiedler et al., 2000). *P. oleovorans* contains an inactive *pha*G gene and is not capable of mcl-PHA accumulation from FAB (Hoffmann et al., 2000a). However, recombinant *P. oleovorans*, expressing the *pha*G gene, accumulated mcl-PHA from non-related sources (Hoffmann et al., 2000a). Furthermore, Hoffman and co-workers (Hoffmann et al., 2000a), using heterologous DNA hybridisation, showed that *pha*G analogues are present in all bacteria accumulating mcl-PHA from FAB.

Not only the *pha*G gene has been shown to create a link between FAB and mcl-PHA biosynthesis. Klinke et al. (1999) achieved the accumulation of mcl-PHA from FAB in recombinant *E. coli* expressing the *pha*C1 gene from *P. oleovorans* in combination with a truncated form of the *E. coli tes*A (*'tes*A) gene leading to cytoplasmic expression of the thioesterase-I (Cho & Cronan, 1993 and 1995).

In the present report, the models developed in prokaryotes to produce mcl-PHA from FAB (PHA-polymerase + ACP-CoA transacylase or PHA-polymerase + thioesterase) have been implemented in potato (Chapter 1, Figure 4). FAB in plants occurs in the plastids. The potato small subunit ribulose bisphosphate carboxylase transit peptide (TPrbcS) was used to target the protein into the plastids. The Pha-C1 polymerase from *P. oleovorans* was expressed in the plastids of transgenic potato in combination with the ACP-CoA transacylase (*pha*G gene product) from *P. putida* 2442 or with the truncated thioesterase-I (*'tes*A gene product) from *E. coli*. The accumulation of mcl-PHA was monitored in leaves of transgenic potato lines. The expression of the Pha-C1 in combination with the truncated thioesterase-I did not lead to the production of mcl-PHA. On the contrary, the expression of the Pha-C1 and the ACP-CoA transacylase did lead to the production of mcl-PHA with a maximum yield of 0.26 mg of mcl-PHA / g of dry weight. The monomer composition indicated that the precursors were derived from the FAB.

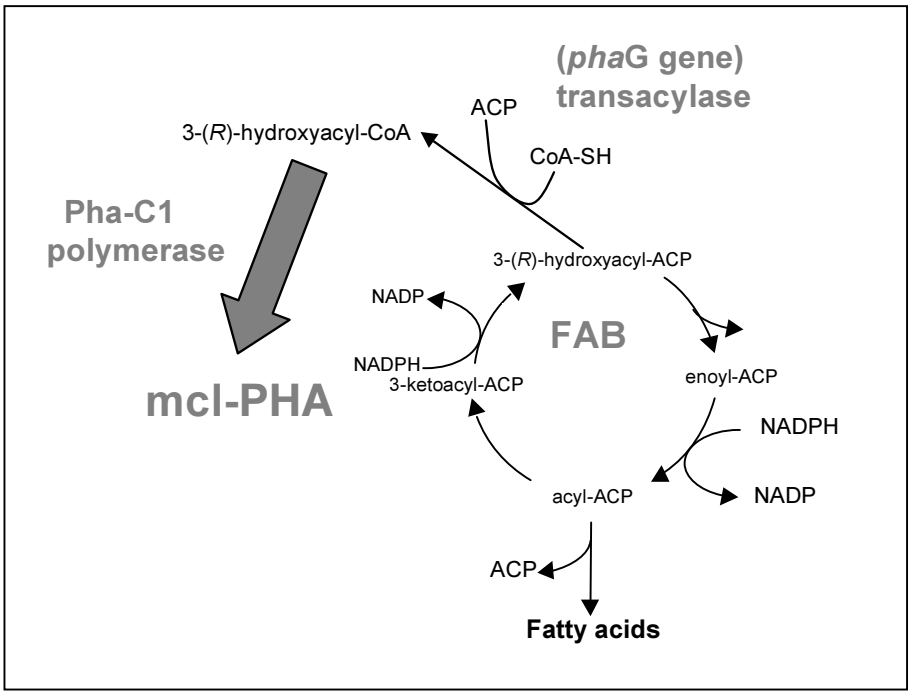

Figure 2. Scheme for the synthesis of mcl-PHA using intermediates from FAB and based on the expression of the Pha-C1 polymerase and the ACP-CoA-transacylase (*pha*G gene product). See text for further explanations

Material and methods

Plant Material and transformation.

In vitro grown *Solanum tuberosum*, genotype 1024-2 (amf, diploid; Jacobsen et al., 1989) was used as starting material for particle bombardment-mediated co-transformation as described in Chapters 2 and 3. Wild type and transgenic plants were cultivated in pots at 20 °C, 16 hours photoperiod, to accumulate material enough for analyses.

DNA constructs.

Plasmid 35-S-Kan containing the *npt*II selectable marker under the control of the 35-S promoter and terminator was kindly received from the John Innes Centre (Norwich, UK). The '*tes*A gene was amplified by PCR using the Pwo DNA polymerase as described by the manufacturer (Eurogentec) and plasmid pCY323 (‘*tes*A gene; Klinke et al., 1999) as template. Plasmids pCY232 and pET701 (see below) were kindly given by Prof. Witholt (ETH, Zurich, CH). Primers 5'-CCATGGCGGACACGTTATTGATTCT-3' and 5'-CGGGATCCTTATGAGTCATGATTTAC-3' were used to introduce *Nco*I and *Bam*HI restriction sites at the 5' and 3' of the gene, respectively. The *pha*G gene was cloned by PCR (Pwo DNA polymerase) from genomic DNA of *P. putida* 2442. Primers 5'-CCATGGGGCCAGAAATCGCTGTACTTG-3' and 5'-CGGGATCCTCAGATGGCAAATGCATGC-3' were used and *Nco*I and *Bam*HI restriction sites were introduced at the 5' and 3' ends of the gene, respectively. PCR products were cloned into pGEM-T-easy vectors (Promega), sequenced and subcloned into *Nco*I – *Bam*HI of pAMV-1 (Rouwendal et al., 1997). The *Nco*I fragment containing the plastidial transit peptide sequence (TPrbsC) was amplified from genomic DNA from cv. Saturna by Rouwendal et al. (unpublished) using primers 5’-GCGGACCATGGCTTCCTCAATTGTC and 5’-CGCCTCCATGGGCCACAC CTGCAT and cloned into the *Nco*I sites of pAMV-1-‘*tes*A, pAMV-1-*pha*G and pAPP62 (pAMV-1-*pha*C1; Chapter 5) giving plasmids pAPP59, pAPP58 and pAPP64, respectively. A fragment of 207 bp at the 3’ of the *pha*C1 gene in plasmid pAPP64 was replaced with the corresponding sequence from plasmid pET701 (Ren, 1997) in which the 3’ end of the *pha*C1 gene includes 36 bp coding for 12 aminoacids at the C-terminus of the vesicular stomatitis

virus glycoprotein (VSV-G. Ren, 1997; Ren et al., 2000c). Thus, the resulting gene product corresponded to the Pha-C1 polymerase fused at the C-terminus with VSV-G. Primers 5'-AGGCAACCCCAAGGCGCGCTT-3' (including site *Bss*HII at position 1474 of the *pha*C1 gene) and 5'-CGGCCGCACGGATCCTTTATTTG-3' annealing at the 3' of the phaC1-VSV-G in pET701 and introducing the *Bam*HI restriction site at the 3', were used for PCR amplification using the Pwo DNA polymerase and pET701 as a template. The resulting fragment was sequenced and cloned into the *Bss*HII – *Bam*HI sites of pAPP64 giving plasmid pAPP65. The latter contained the synthetic *pha*C1-VSV-G gene, 1722 bp. *Bgl*II-*Bam*HI fragments from pAPP59 (*amv*-TPrbcS-'*tes*A), pAPP58 (*amv*-TPrbcS-*pha*G) and pAPP65 (*amv*-TPrbcS-*pha*C1-VSV-G) were cloned into pAPP23 (Chapter 2) containing the E-35-S, *nos* terminator, giving rise to the plant expression vectors pAPP100, pAPP101 and pAPP102, respectively.

PCR and Southern blot RT-PCR and northern blot analysis.

For these analyses, see Chapters 2, 3 and 4.

PHA isolation, Gas Chromatography (GC) analysis.

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 hours to remove fatty acids, and PHA was eventually solubilised from the de-fatted powder with chloroform at 55 °C for 48 hours. The volume of the chloroform fraction containing PHA was reduced with a rotavapor, and concentrated PHA was analysed by GC after methanolysis (Lageveen et al., 1988). Methylesters were analysed by GC using a Carlo-Erba GC6000 apparatus (Carlo Erba) equipped with a 25 m CP-Sil5CB capillary column (Chrompack).

Results

Cloning of the phaG gene.

The *pha*G gene was cloned from *P. putida* strain 2442 by PCR using primers designed on the sequence of *P. putida* 2440 (Rehm, 1998). *P. putida* 2442 also accumulates mcl-PHA from FAB (Huijberts et al., 1992). The 8.88 Kb gene cloned in this work codes for a 295-amino acids polypeptide with a

mass of 33.8 Kda. The translated protein shares high homology with the other cloned *pha*G genes. Compared to the *pha*G gene from *P. putida* 2440 (Rehm, 1998), one nucleotide (C-17-A) and the corresponding aminoacid (Ala-6-Asp) deviated compared to the sequence of strain 2440. This residue is not included in any conserved region neither in sequences known to be involved in the catalysis. The consensus motif identified in all *pha*G genes, H X_4 D, is present in the *pha*G gene product from *P. putida* 2442 (Figure 3). This motif has been proposed to be associated with enzymatic catalysis of the ACP-CoA transacylase (Rehm, 1998).

P. putida 2440	KRFNYRHVSSLDSHE	this work
P. aeruginosa	KRYNFRHVSSLDEHE	Hoffmann et al., 2000b
P. oleovorans	KRFNYRHVSSLDSHE	Hoffmann et al., 2000a
P. putida 2442	KRFNYRHVSSLDSHE	Rehm et al., 1998
Consensus	_____HXXXXD____	

Figure 3. H X_4 D domain present in the ACP-CoA-hydroxyacyl transacylase from some related *Pseudomonas* species, presumably essential for the enzymatic catalysis.

Transformation and selection of transgenic plants.

More than 500 internodes were bombarded with plasmids pAPP100 (*pha*C1) and pAPP101 (*pha*G) and 14 plants resistant to kanamycin were obtained (lines PB005). Twenty-seven plants resistant to kanamycin (lines PB006) were obtained after bombardment of 560 internodes with plasmids pAPP100 and pAPP102 (*'tes*A). In each case, plasmid 35-S-Kan was co-bombarded in combination with the other two constructs containing genes relevant for mcl-PHA biosynthesis, and selection of transgenic plants was based on kanamycin. The efficiency of transformation using the *pha*C1 and *pha*G (2.8 plants rooting on kanamycin per 100 bombarded internodes) was lower than for the *pha*C1 and '*tes*A gene combination (4.8 plants rooting on kanamycin per 100 bombarded internodes).

Southern blot or PCR analyses (Figure 4) were used to check in which lines resistant to kanamycin the non-selected transgenes were integrated (Table

1). Northern blot or RT-PCR (Figure 4) analyses were used to check which lines transcribed mRNAs of the expected size.

Table 1. List of transgenic lines studied. C, G and T indicate *pha*C1, *pha*G and *'tes*A genes, respectively. Column "DNA": C, G and T indicate integration into the plant genome of the respective genes. Column "RNA": + and – indicate the presence or not of the mRNA for the respective gene. Column "mcl-PHA": + and – indicate the presence or not of the polymer. nd: not determined.

Experiment: *pha*C1-*pha*G				Experiment: *pha*C1-*'tes*A			
Line	DNA	RNA	mcl-PHA	line	DNA	RNA	mcl-PHA
PBOO5.1	C / -	$-^{C}$	-	PB006.2	C / -	$+^{C}$	-
PBOO5.2	C / -	$-^{C}$	-	PB006.3	C / T	$-^{C}/+^{T}$	-
PBOO5.4	C / -	$-^{C}$	nd	PB006.5	C / -	nd	nd
PBOO5.5	C / -	$+^{C}$	-	PB006.8	C / -	$+^{C}$	-
PBOO5.10	C / G	$+^{C}/+^{G}$	+	PB006.9	- / T	$+^{T}$	nd
PBOO5.11	C / G	$+^{C}/+^{G}$	+	PB006.10	C / T	$+^{C}/+^{T}$	-
PBOO5.13	C / -	nd	nd	PB006.11	- / T	$-^{T}$	nd
PBOO5.14	C / G	nd	nd	PB006.12	C / T	$+^{C}/+^{T}$	-
				PB006.13	- / T	$-^{C}$	nd
				PB006.16	C / T	$+^{C}/+^{T}$	-
				PB006.18	C / T	$+^{C}/+^{T}$	nd
				PB006.19	C / -	$-^{C}$	nd
				PB006.22	C / T	$+^{C}/+^{T}$	nd
				PB006.24	C / T	nd	nd

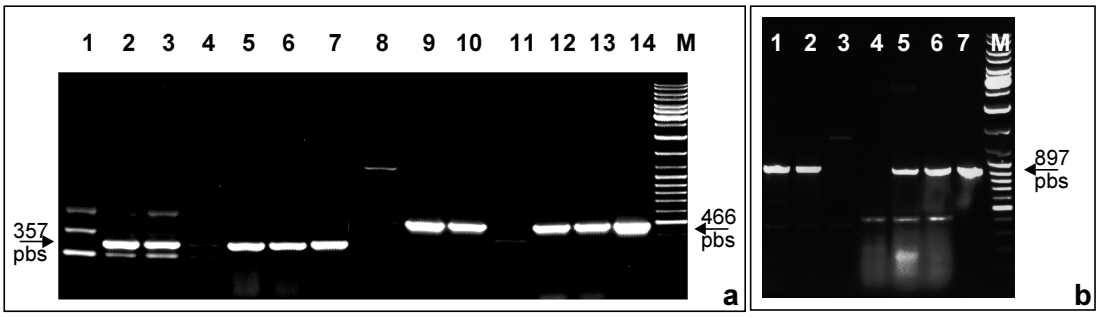

Figure 4. a) PCR analysis on plant genomic DNA and cDNA using primers specific for the 357 bps 5' end of the *pha*C1 gene (lanes 1 - 7) and primers specific for the 466 bp 3' end of the *pha*C1 gene (lanes 8 – 14). Lane 1, 2 and 3: PCR on genomic DNA from the wild type, PB005.10 and PB005.11 lines, respectively. Lanes 4, 5 and 6: PCR on cDNA from the wild type, PB005.10 and PB005.11 lines, respectively. Lanes 8, 9 and 10: PCR on genomic DNA from the wild type, PB005.10 and PB005.11 lines, respectively. Lanes 11, 12 and 13: PCR on cDNA from the wild type, PB005.10 and PB005.11 lines, respectively. Lanes 7 and 14: plasmid positive control. b) PCR analysis on plant genomic DNA and cDNA using primers specific for the amplification of the complete 897 bps *pha*G gene. Lanes 1, 2 and 3: PCR on genomic DNA from PB005.10, PB005.11 and the wild type lines, respectively. Lanes 4, 5 and 6: PCR on cDNA from the wild type, PB005.10 and PB005.11 lines, respectively. Lane 7: plasmid positive control. Lane M: molecular weight marker.

Plant morphology.

The presence of the phaG gene strongly affected the morphology of the transgenic plants (Figure 5). Lines PB005.10, PB005.11 (phaC1 and phaG co-transformants) showed a dwarfed phenotype with curled and brittle leaves and strongly retarded growth. Transgenic plants transformed only with plasmid 35-S-Kan were never affected to this extent, indicating that the phenotypes observed in lines expressing the phaC1 and phaG genes were not a result of the transformation procedure. Also plants containing the phaC1 gene alone or in combination with 'tesA (Figure 5), appeared normal or only slightly affected and the same was observed in plants transformed with cytoplasmic expression vectors containing the phaC1 gene (Chapter 5), the 'tesA and the phaG genes (results not shown). Line PB005.14 (the third line co-transformed with phaC1 and phaG. Table 1) grew very slowly and did not root on soil, but only in vitro, yielding too little material for analysis.

Figure 5. Examples of some transgenic and wild type plants rooted on soil. a, b) transgenic line PB006.16 (*pha*C1 / '*tes*A double transformant). c, d) transgenic line PB006.8 (*pha*C1 transformant). e, f) on the left, transgenic line PB005.10, on the right, transgenic line PB005.11 (*pha*C1 / *pha*G double transformants). g) wild type line. Bar: 5 cm.

PHA analysis.

A number of extraction protocols used for PHA extraction from bacteria were adapted for use on plants and tested for their efficiency. Lyophilised powder derived from ground leaves was first washed with ethanol. This first step extracted fatty acids which solubilised in ethanol. The de-fatted powder could be directly analysed by GC after methanolysis (as described in Chapter 5). However, since only small amounts of powder could be methanolysed, all mcl-PHA present was extracted with chloroform. In this way, also small amounts of mcl-PHA could be detected. A precipitation step with methanol after mcl-PHA had been solubilised (as described by Hahn et al., 1999 and Nakashita et al., 1999) was also used, but results were not completely conclusive. The extraction procedure of mcl-PHA from leaf material is described in Figure 6a. As shown in Figure 6a & b the extraction protocol based on ethanol washing / chloroform extraction / analysis of the concentrated polymer solution appeared more efficient and reproducible. All fractions (ethanol fraction, chloroform fraction, ethanol-chloroform extracted powder) were analysed by GC after methanolysis of each sample. The ethanol fraction contained fatty acids (Figure 6b chromatograms on the left). As expected, in the chloroform fraction (central column of chromatograms) of the wild type line no mcl-PHA was detected. However, the two transgenic lines analysed, expressing the *pha*C1 and the *pha*G genes, accumulated mcl-PHA consisting of 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate with monomer compositions as indicated in Table 2. The chromatographic mcl-PHA profiles of transgenic potato lines were compared with that of *P. putida* to confirm the presence of the polymer. Line PB005.10 accumulated 0.26 mg / g of dry mass of mcl-PHA and line PB005.11 accumulated 0.017 mg / g of dry mass (Table 2). Expression of only the *pha*C1 gene did not yield any mcl-PHA (Table 1). The same was observed when *pha*C1 and *'tes*A were co-expressed. Although thioesterase activity was detected in transgenic lines transformed with and transcribing the '*tes*A gene (results not shown), no mcl-PHA was detectable when *pha*C1 and *'tes*A were co-expressed.

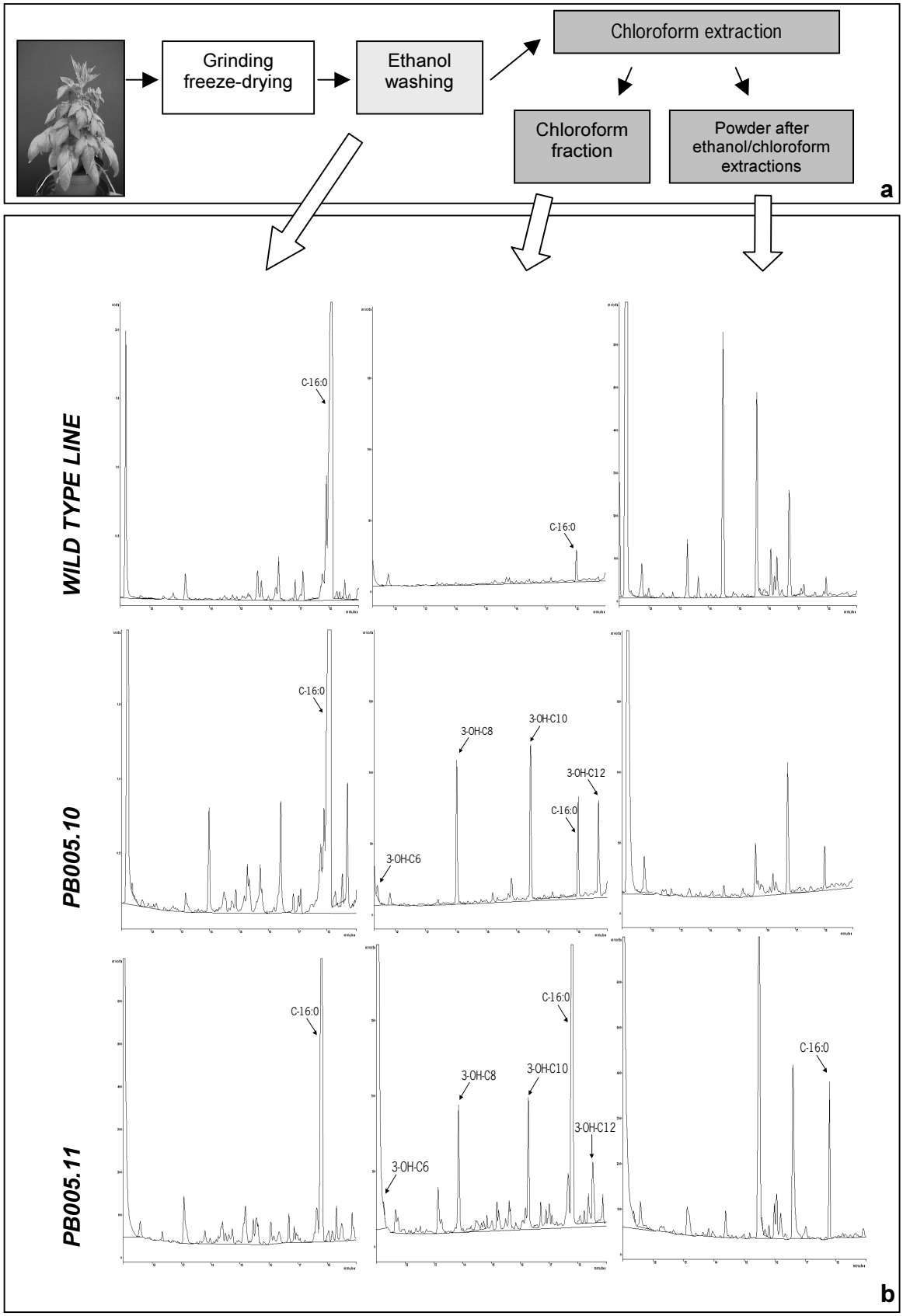
Grinding
freeze-drying
Ethanol
washing
Chloroform extraction
Chloroform
fraction
Powder after
ethanol/chloroform
extractions
a
WILD TYPE LINE
PB005.10
PB005.11
C-16:0
3-OH-C6
3-OH-C8
3-OH-C10
3-OH-C12
b

Figure 6. Mcl-PHA extraction and analysis on transgenic PB005.10, PB0010.11 and wild type lines. a) Mcl-PHA extraction procedure; plant material was first freeze dried, then washed with ethanol, and then mcl-PHA was solubilised in chloroform. b) Overview of the chromatographic analyses performed: residual ethanol of the washing steps containing fatty acids, chloroform containing mcl-PHA and residual material after the whole procedure were methanolysed and analysed by GC. Empty arrows indicate methanolyses. 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: palmitic acid. See text for explanation

Discussion

In recent years, mcl-PHAs gained considerable world wide interest because of their large number of possible applications (van der Walle et al., 1999 and 2001; see Chapter 7, general discussion). 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 plant genetic engineering work aimed at the production of PHAs has focussed on PHB biosynthesis. The gene-set required for the production of PHB in transgenic plants is well known (see Chapter 4). On the other hand, the incomplete knowledge of mcl-PHA biosynthesis, hampered the identification of the minimum-gene-set required for its production. It has been shown that the PHA-polymerase alone was able to sustain mcl-PHA synthesis in *E. coli*, deriving precursors directly from β–oxidation (Langenbach, 1997). The same model has been implemented in *Saccharomyces cerevisiae* (Poirier et al., 2001a), *Pichia pastoris* (Poirier et al., 2002b) and *A. thaliana* (Mittendorf et al., 1998). In all three cases, the mcl-PHA-polymerase was expressed in peroxisomes of the heterologous host, because in yeast and plants β–oxidation occurs in this particular cell compartment. Transgenic hosts were able to accumulate mcl-PHA re-directing intermediates from β–oxidation. However, the production of mcl-PHA from β–oxidation has some disadvantages in plants. Degradation of fatty acids by β–oxidation is predominantly active during seed germination (or senescence). Thus, mcl-PHA accumulation would compete with the energy supply needed for the germinative process. Mcl-PHA accumulation from FAB is therefore more desirable for a number of reasons. Being continuously active, FAB would be a preferred source of mcl-PHA precursors and larger

amounts would be expected. It is also envisaged that the double layer-membrane, which surrounds plastids (where FAB occurs), would stand easily the accumulation of the polymer and would be suitable for bulk PHA production. Many pseudomonad strains naturally produce mcl-PHA from FAB (Timm & Steinbüchel, 1990; Huijberts et al., 1992), and recombinant bacteria capable to accumulate mcl-PHA from FAB have been obtained as well (Rehm, 1998; Klinke et al., 1999; Fiedler et al., 2000; Hoffmann et al., 2000a; Hoffmann et al., 2000b). The link between FAB and mcl-PHA synthesis in these prokaryotes is supported by the *pha*G gene coding for the ACP-CoA-transacylase or the '*tes*A gene coding for a truncated form of the *E. coli* thioesterase-I (Cho & Cronan, 1993 and 1995). In the present report, potato was used as a host for the implementation of the two prokaryotic models to accumulate mcl-PHA from FAB (Figure 2; Chapter 1, Figure 4). One approach was based on the simultaneous expression of the Pha-C1 polymerase (*pha*C1 gene) and the ACP-CoA-transacylase (*pha*G gene) in plastids of transgenic potato lines (Figure 2). In a second approach, plastids were the expression compartment of the Pha-C1 polymerase and the truncated thioesterase-I ('*tes*A gene). This latter approach did not result in mcl-PHA accumulation. As postulated by Klinke and coworkers (1999), the truncated thioesterase-I is active on straight chain fatty acids but not hydroxy fatty acids. *In vitro* studies (Cho & Cronan, 1993) had shown as well that the thioesterase-I is not active on hydroxylated moieties. This implies that, before being polymerised, fatty acids had to be oxidised to hydroxyacyl-CoA *via* β–oxidation steps (Klinke et al., 1999). Because FAB and β–oxidation in plants occur in separate cell compartments, the truncated thioesterase-I would not be able to provide precursors for mcl-PHA biosynthesis in plastids. Co-transformation with the *pha*C1 and *pha*G genes resulted in low efficiencies of transformation and abnormal phenotypes were observed. Most probably, the transacylase activity interferes with fatty acid metabolism. Similarly, expression of the *pha*G in *A. thaliana* led to marked deleterious effects on plant growth (Poirier, 2002a). No *pha*G single transformant was obtained in the present work. However, the co-ordinated expression of the Pha-C1 polymerase and the ACP-CoA-transacylase directed the accumulation of

mcl-PHA from FAB in the two plants analysed (Table 2). A third line also expressing the *pha*C1 and *pha*G genes, consistently showed retarded growth and was not further analysed. Up to 0.017 mg / g and 0.26 mg of polymer / g of cell dry weight were accumulated in the two transgenic plants, respectively (Table 2). Transgenic *A. thaliana* expressing the mcl-PHA-polymerase in the peroxisomes accumulated similar amounts of polymer 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 mcl-PHA accumulated in the two transgenic potato lines analysed is shown in Table 2. Potato lines accumulated 3-(*R*)-hydroxyalkanoate with carbon chains ranging from C-6 to C-12. No unsatured monomers were formed, as can be expected if the FAB had provided the precursors. Double bonds are indeed introduced in the elongating fatty acid only after palmitic acid is formed (Ohlrogge & Browse, 1995). The same situation has been reported in natural or recombinant hosts accumulating mcl-PHA from FAB (Table 2). On the other hand, when β–oxidation intermediates were directed into mcl-PHA biosynthesis, the monomer composition included also unsatured moieties. The relative abundance of each monomer in the polymer was directly related to the abundance of the degradation intermediates during β–oxidation (Mittendorf et al., 1998 and 1999; Allenbach & Poirier, 2000). Table 2 shows the monomer composition of transgenic *A. thaliana* (Mittendorf et al., 1998), which is similar to the composition reported in transgenic yeast (Poirier et al., 2001a and 2002b) and natural hosts (De Waard et al., 1993) when β–oxidation sustains mcl-PHA biosynthesis.

The use of potato for the production of mcl-PHA now has gained new prospects. The use of tuber specific promoters may lead to the accumulation of mcl-PHA in tubers. Large-scale extraction protocols able to separate PHA from starch in maize have been patented (reviewed by Poirier, 2001b) and could be exploited for bulk extraction of polymer. Still, the main problem of the use of plants for bulk PHAs accumulation remains the low yield. The potato lines described here, and other plant species as well, accumulated very low amounts of mcl-PHA. It is not yet possible to pinpoint any definitive bottlenecks. The Pha-C1 polymerase abundance or its activity may be too low

HOST	%PHA	H6	H8	H8:1	H10	H12	H12:1	H12:2	H14	H14:1	H14:2	H14:3	H16	H16:2	H16:3
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[1]	14.6	1.8	47.2	--	45.9	5.1	--	--	--	--	--	--	--	--	--
P. oleovorans[2]	46		1	7.5	78	13.5	--	--	--	--	--	--	--	--	--
P. putida PHAG$_N$-2[3]	50	3.1	14.2	--	76.6	6.1	--	--	--	--	--	--	--	--	--
P. fragi[4]	10	1	16	--	69	10	4	--	--	--	--	--	--	--	--
P. aeruginosa PAO1[5]	16.8	3.3	23.7	--	63.5	9.6	--	--	--	--	--	--	--	--	--
P. putida KT2440[6]	54	3.1	24.2	--	66.4	6.3	--	--	--	--	--	--	--	--	--
P. putida KT2442[7]	16-27	1.7	21.4	--	63.6	3.8	8.6	--	0.1	0.8	0	--	--	--	--
A. thaliana[8]	0.02	1.1	23	18	4.7	5.8	4.3	5	4.2	6.7	7.5	11	2	2	5.6

Table 2. Yields and monomer composition of mcl-PHA accumulated by transgenic potato lines, and other recombinant and natural hosts using intermediates from FAB. Yields and monomer composition of mcl-PHA accumulated by recombinant *A. thaliana* using intermediates from the β-oxidation. Monomers derived from FAB are shaded. % PHA: 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 acid. H14:2) 3-(*R*)-hydroxytetradecadienoic acid. H14:3) 3-(*R*)-hydroxytetradecatrienoic acid. H16) 3-(*R*)-hydroxyhexadecanoic acid. H16:1) 3-(*R*)-hydroxyhexadecenoic acid. H16:2) 3-(*R*)-hydroxyhexadecadienoic acid. H16:3) 3-(*R*)-hydroxyhexadecatrienoic acid. nd: not detectable. --) not determined.

[1]: recombinant *pha*G-negative mutant harbouring an active *pha*G gene from the wild type strain and grown on gluconate (Hoffmann et al., 2000b). [2]: wild type strain, not-accumulating mcl-PHA from FAB, harbouring the *pha*G gene from *P. putida* KT2440 and grown on gluconate (Hoffmann et al., 2000a). [3]: *P. putida pha*G negative mutant harbouring an active *pha*G gene from the wild type and grown on gluconate (Hoffmann et al., 2000a). [4]: wild type (non-accumulating mcl-PHA) strain harbouring an active *pha*G from *P. putida* KT2440 and grown on gluconate (Fiedler et al., 2000). [5]: wild type strain grown on gluconate (Hoffmann et al., 2000b). [6]: wild type strain grown on gluconate (Hoffmann et al., 2000a). [7]: wild type strain grown on glycerol (Huijberts et al., 1992). [8]: leaves of recombinant *A. thaliana* harbouring the Pha-C1 or Pha-C2 polymerase in the peroxisomes (Mittendorf et al., 1998).

(see also Chapter 4 for analogous evidence on PhbC polymerase). In Chapter 5, it was hypothesised that when Pha-C1 polymerase competed with β–oxidation for 3-(*R*)-hydroxyocanoate, β–oxidation was 150 - 1800 times more efficient than polymerisation. *In vitro* competition studies of Pha-C1 polymerase and ACP-CoA-transacylase would be an approach to test this hypothesis. Finally, redirecting the carbon flux from starch to mcl-PHA synthesis, or using low-starch potato cultivars / transformants as hosts, may further improve the mcl-PHA production efficiency. In Chapters 1 and 7 it is speculated on this point.

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CHAPTER 7
General discussion

Polyhydroxyalkanoates (PHAs) represent a large class of polymers with molecular weights between 50-100 KDa. PHAs are divided in two classes: short-chain-length-PHA (scl-PHA) and medium-chain-length-PHA (mcl-PHA). Scl-PHA comprises the homopolymer polyhydroxybutyrate (PHB) and the heteropolymer polyhydroxybutyrate-hydroxyvalerate (PHB / HV) and are produced by a large number of bacteria [including *Ralstonia eutropha*, *Rhodococcus ruber, Chromatium vinosum* (Madison & Huisman, 1999)]. Mcl-PHA is a heteropolymer with monomer carbon-chain compositions ranging between hydroxyhexanoate – hydroxytetradecanoate and are produced by fluorescent presudomonads (De Smet et al., 1983; Huisman et al., 1989; Timm & Steinbüchel, 1992). More than 300 bacterial species possess the ability to synthesise PHAs as storage compounds when micronutrients become limiting (Bebel et al., 2001). Both scl-PHA and mcl-PHA are stored in intracellular granules separated from the aqueous cell environment by a monolayer consisting of phospholipids and a number of non-enzymatic structural proteins associated to the PHA-granules and called phasins (Stuart et al., 1998; Fuller, 1999).

PHAs gained considerable interest for their large number of possible applications. The first applications developed for PHB / HV and mcl-PHA were thermoplastics, materials for the food packaging industry, biodegradable elastomers and rubber-like materials (De Koning, 1995; van der Walle et al., 2001).

It is generally accepted that fossil fuel reserves are limited (Gerngross & Slater, 2000) and the waste disposal of non-degradable synthetic plastics is a problem for the environment. Therefore, a considerable effort has been directed at developing systems and biotechnological strategies for replacing non-renewable and non-degradable oil-derived plastics with PHA-based plastics, which rely on renewable resources and are biodegradable. In this context, the use of transgenic plants for the accumulation of bulk PHAs is considered a promising strategy especially because fermentative based-PHAs are confronted with high production costs. During the course of this project, a number of methods have been developed to produce PHAs in potato that have been described in this thesis.

A series of drawbacks have been identified that hampered the use of bulk PHAs as biodegradable plastics produced both in plants and microorganisms. This chapter reviews and compares the production of PHAs by microbial fermentation and the production in potato and in other transgenic plants. Finally, the obstacles encountered in the exploitation of natural and recombinant prokaryotes and transgenic plants for bulk PHA production are reviewed. A basic economical evaluation is given as well.

In parallel, during the last decade, numerous novel applications for PHAs have been developed as well (see below). These novel applications certainly have opened new prospects for the exploitation of PHAs not only as bulk biodegradable plastics, but also for a number of other formulations.

Fermentative production in natural and recombinant hosts

PHAs can be produced by bacterial fermentation. In response to the world oil crisis, Imperial Chemical Industries established an industrial scale (200,000 litres) fermentative production of PHB using glucose and propionic acid as substrates (reviewed by Gerngross & Slater, 2000 and by Kessler et al., 2001b).

Numerous studies have been dedicated to the isolation of new bacterial strains able to accumulate the polymer more efficiently, with higher productivity or with an aberrant composition that will allow the production of PHAs with programmable properties. However, conventional microbial fermentations are based on the use of expensive carbon sources, which makes the final production costs by fermentation non-competitive, especially for bulk production. The use of cheap substrates in those strains that naturally accumulate PHAs is accompanied with a strong reduction in productivity (Lee & Choi, 2001 and references therein). Genetics and biochemistry of PHA metabolism, both anabolism and catabolism, have been subjects of investigation of several research groups. Until now more than 40 PHA synthases from 38 different organisms have been cloned (Qi et al., 2000). The increased understanding of the biochemistry and the molecular biology of PHA biosynthesis allowed the development of recombinant bacteria with a

high productivity and growing on cheap substrates (Lee & Choi, 2001; Solaiman et al., 2001; Kessler et al., 2001b and references therein).
The production of different heteropolymers, with different physical properties, has also been expanded. Recently, an industrial scale procedure for the accumulation of polyhydroxybutyrate / hydroxyhexanoate in *Aeromonas hydrophyla* has been developed (Chen et al., 2001).
Despite these achievements, the current fermentative production of PHAs is still confronted with high costs (Gerngross & Slater, 2000). Choi and Lee (Choi & Lee, 2000) evaluated that the cost of PHB / HV in recombinant *E. coli* at a scale of 100,000 tons per year would be 3.95 U.S. $ / Kg PHB / HV using glucose and propionic acid as carbon sources. This price cannot yet compete with the price of oil derived plastics which is 0.6 – 1.0 U.S. $ / Kg polypropylene and 0.8 – 1.8 U.S. $ / Kg polyethylene (Poirier, 1999; Kessler et al., 2001b).

PHAs in transgenic plants: from *Arabidopsis* to potato

Given the aforementioned high investment and production costs, the challenge to produce PHAs in transgenic plants has received world-wide attention. The use of sun light and atmospheric CO_2 enables a cheap and sustainable production of the polymer from renewable resources. In this context, the use of carbohydrate crops, *versus* oil crops, appeared significantly more amenable. Starch crops accumulate 5 tons of dry material / year / ha while oil crops produce only 2.5 tons / year / ha (van der Leij & Witholt, 1995). Estimates based on a PHA accumulation of 2.5 tons / year / ha suggested that 4 X 10^3 ha of farmland would have been required for 10^6 tons of PHAs and the price of these plant-based-PHAs were expected to ultimately drop to 0.5 – 1 U.S. $ / Kg of PHAs (van der Leij & Witholt, 1995). Analogous estimates of fermentation-based PHAs in the same period were considerable less optimistic, and the expected price of fermentation-based-PHAs was not lower than 5 U.S. $ (Nawrath et al., 1995). During the last decade, a series of publications demonstrated that plants like *Arabidopsis* (Nawrath et al., 1994; Mittendorf et al., 1998), *Brassica* (Houmiel et al., 1999; Slater et al., 1999), cotton (John & Keller, 1996), maize (Hahn et al., 1999) and tobacco

(Nakashita et al., 1999) can accumulate both scl-PHA and mcl-PHA. In the work described in this thesis and in the recent work by Bohmert and coworkers (2002), the feasibility of potato to produce PHAs has been shown using a number of metabolic engineering strategies.

Particle bombardment mediated transformation. The production of PHAs in heterologous hosts, like plants, requires several genes to be expressed in a coordinate manner. In addition, a selectable marker is needed for improved selection of transgenic plants. When this project started, the minimum gene set for PHA accumulation was not completely known in prokaryotes, and certainly not in plants. Thus, a considerable advantage would be achieved by the ability to introduce simultaneously all candidate genes comprised in separate DNA molecules into the potato genome. This would result in a large flexibility in combining different sets of genes in order to define the necessary gene-set. *Agrobacterium*-mediated transformation of potato is a well established technique. Nevertheless, preliminary experiments of simultaneous co-transformation by *Agrobacterium* using separate plasmids failed (Romano et al. unpublished). Based on *Agrobacterium*-mediated transformation, the need to test different combinations of genes would have required the laborious construction of multi-gene plant vectors for each combination of genes to be tested. On the contrary, high frequencies of co-transformation have been reported for particle bombardment of plants (Aragao et al., 1996; Bower et al., 1996; Chen et al., 1998). In the first part of this project, efficient particle bombardment-mediated procedures have been developed for simultaneous gene transfer from separate plasmids or separate linear DNA fragments. Moreover, also other important features appeared to be associated with the use of particle bombardment in potato. In contrast to previous reports on *Agrobacterium*- and particle bombardment-mediated transformation of several plant species, including potato (Cooley et al., 1995; Chen et al., 1998; Kohli et al., 1998; Wolters et al., 1998), our transgenic potato plants produced by particle bombardment showed simple patterns of integration often with single copy of the transgenes and spatial separation of the sites of integration of the selectable marker and the non-selected genes (Chapter 3). Thus, primary

transformants could be theoretically improved by elimination *via* out-crossing of undesired integration sites including the site of integration of the selectable marker. The gene cassette (i.e. DNA fragment comprising promoter – gene – terminator) co-transformation resulted in the elimination of redundant plasmid sequences comprising antibiotic resistance genes, plasmid replication sites, etc. These are important aspects considering that the presence of redundant bacterial vector DNA and antibiotic or herbicide resistance markers are under debate and subject to new government regulations.

PHAs in potato. Particle bombardment-mediated co-transformation was subsequently used to introduce the candidate genes responsible for the production of PHAs into the potato genome. In total, 4 methods to produce PHAs, using different combinations of the candidate genes, have been considered and 3 of these led to the formation of detectable amounts of PHAs (Table 1).

The simplest method to produce PHAs was the accumulation of PHB. PHB production in other plants is well documented (Nawrath et al., 1994; John & Keller, 1996; Hahn et al., 1999; Nakashita et al., 1999) and the initial substrate is acetyl-CoA, which is available in several cell compartments due to its central role in a number of plant metabolic pathways. The formation of PHB was achieved after *R. eutropha phb*B and *phb*C genes coding for an NADPH-dependent acetoacetyl-CoA reductase and for the PHB-polymerase, respectively, were expressed in transgenic potato lines (Chapter 4). PHB was synthesised using the cytoplasmic pool of acetyl-CoA and exploiting the action of the endogenous ketothiolase activity for the condensation of 2 molecules of acetyl-CoA to form acetoacetyl-CoA. The highest amount of PHB detected was 0.7 μg / g of cell dry weight. In other reports, the content of PHB in transgenic plants expressing the acetoacetyl-CoA reductase and the PHB-polymerase in the cytoplasm ranged between 10 – 1000 μg / g of cell dry weight (Chapter 1, Table 2. Poirier et al., 1992; Nakashita et al., 1999). When the *R. eutropha* ketothiolase, acetoacetyl-CoA reductase and the PHB-polymerase were expressed in the plastids of transgenic plants, 3.2 – 400 mg

Table 1. Methods implemented during the course of this project (and described in this thesis) and the method used by Bohmert et al. (2002) to produce PHAs in transgenic potato. [1]: 3-hydroxyoctanoate. [2]: polyhydroxyoctanoate.

aim	gene	source	target	polymer	reference
PHB	*phb*B *phb*C	*R. eutropha* *R. eutropha*	cytoplasm	PHB	*This thesis Chapter 4*
PHB	*phb*A *phb*B *phb*C	*R. eutropha* *R. eutropha* *R. eutropha*	plastid	PHB	*Bohmert et al. 2002*
mcl-PHA (HO[1] feeding)	*pha*C1	*P. oleovorans*	cytoplasm	PHO[2]	*This thesis Chapter 5*
mcl-PHA from FAB	*pha*C1 *'tes*A	*P. oleovorans* *E. coli*	plastid	No polymer	*This thesis Chapter 6*
mcl-PHA from FAB	*pha*C1 *pha*G	*P. oleovorans* *P. putida*	plastid	mcl-PHA	*This thesis Chapter 6*

of PHB / g of cell dry weight was observed (Chapter 1, Table 2. Nawrath et al., 1994; Bohmert et al., 2000 and 2002). These data indicate the presence of differences in the flux of acetyl-CoA between cytoplasm and plastids and also large differences between different plant species. A feeding experiment in which cell suspension cultures were derived from transgenic potato plants synthesising PHB and were exposed to different substrate intermediates of the PHB biosynthetic pathway (i.e. acetate and 3-hydroxybutyrate), did not result in any increase of the PHB content, indicating that the substrate availability is not the only bottleneck which hampers the production of high amounts of polymer. Low expression of the PHB-polymerase was observed in potato and tobacco cytoplasm (Chapter 4 and Nakashita et al., 1999) and can be considered as one important obstacle.

Subsequently, the production of mcl-PHA was attempted in transgenic potato. The *Pseudomonas oleovorans* mcl-PHA-polymerase was first expressed in the cytoplasm and evidences for heterologous gene and protein expression were obtained (Chapter 5). Mcl-PHA were produced at amounts ranging from 0.02 – 9.7 mg / g cell dry weight after cell suspension cultures expressing the Pha-C1 polymerase were exposed to the monomeric substrate (3-hydroxyoctanoate). The cell suspension approach provided an excellent tool for testing heterologous gene expression and precursor supply for the Pha-C1 polymerase. Using this approach it may be also possible to control the monomer composition of the polymer, by exposing cell suspensions to different monomeric substrates. This will allow the production of polymers with

specific desired physical properties. Afterwards, two methods designed to re-direct endogenous hydroxy fatty acids from the FAB into mcl-PHA biosynthesis were tested (Chapter 6). A truncated form of *E. coli* thioesterase-I (‘*tes*A gene) and *P. putida* ACP-CoA-transacylase (*pha*G gene) were expressed in combination with the *P. oleovorans* Pha-C1 polymerase and targeted to the plastid by the Rubisco transit peptide. The combination of these genes represents the minimum gene-set for mcl-PHA accumulation deriving precursors from FAB in prokaryotes (Rehm, 1998; Klinke et al., 1999). The expression of the Pha-C1 polymerase and the ACP-CoA-transacylase resulted in the synthesis of 17 – 260 μg of mcl-PHA / g of cell dry weight. The double transformants which expressed the Pha-C1 polymerase and the thioesterase-I did not synthesise mcl-PHA, most probably due to the requirement of a few steps of the β-oxidation to convert FAB precursors into mcl-PHA monomers (Klinke et al., 1999).

Potato appears to be a suitable heterologous host for the formation of PHB (this work and Bohmert et al., 2002) and also mcl-PHA using intermediates derived from FAB. Although scl-PHA (i.e. PHB and PHB / HV) has been accumulated in transgenic *Arabidopsis*, cotton, *Brassica*, maize and tobacco (Nawrath et al., 1994; John & Keller, 1996; Hahn et al., 1999; Houmiel et al., 1999; Nakashita et al., 1999), mcl-PHA has been detected only in *Arabidopsis* while deriving precursors from β-oxidation (Mittendorf et al., 1998). Thus, the exploitation of FAB as a supplier of precursors for mcl-PHA biosynthesis in transgenic plants is completely innovative. In contrast to β-oxidation which is mainly active during seedling development and senescence, FAB, active during the whole plant life cycle, is considered a preferred approach to provide precursors for PHA biosynthesis.

The problem of the low yield of PHAs in transgenic plants

Despite the successful achievements with PHA production in plants, a number of obstacles have emerged. The minimum content suitable for commercialisation is expected to be 15 % of polymer per cell dry weight. (Houmiel et al., 1999; Slater et al., 1999). The yield of PHAs appears to be still too low to imagine the production of plastics in transgenic crops in the near

future at an industrial and competitive way. Consistently low rates of PHA accumulation were observed in this work and in previous reports (Nawrath et al., 1994), compared to other endogenous polymers, e.g. starch which accumulates to 12 mg / g fresh weight in a 12 hours photoperiod. In contrast, the formation of detectable amounts of PHAs in transgenic plants required weeks. In addition, the presence of high amounts of PHAs, especially in leaves, were coupled to morphological aberrations and a considerable retardation in growth (Nawrath et al., 1994; Bohmert et al., 2000, Chapter 6). Targeting PHA accumulation to storage organs like tubers or seeds, rather than leaves, may be more convenient and the use of tissue-specific promoters is a preferred approach. However, preliminary experiments in which the genes of interest were under the control of the tubes-specific GBSS promoter, and were targeted to the amyloplasts did not result in the synthesis of detectable amounts of PHAs. On the one hand, the use of starch crops like potato may require, as described in Chapter 1, the redirection of the carbon flux from carbohydrates to fatty acid metabolism. Theoretically, this could be achieved by increasing FAB *via* overexpression of an acyl-CoA-carboxylase (the key enzyme controlling FAB; Chapter 1 Figure 4). Starch-less or low-starch potato lines could be suitable candidates for PHA accumulation. Müller-Röber and coworkers (Müller-Röber et al., 1992) showed that antisensing ADP-glucose-pyrophosphorylase resulted in accumulation of free sugars in tubers. Overexpression of phospho-fructokinase would enhance the glycolysis and convert these sugars to acetyl-CoA and subsequently into FAB. On the other hand, the accumulation of PHAs in oilseeds is amenable because FAB is highly active during seed development. Subsequently, the flux of PHA substrates is high as well, but the depletion of fatty acids as energy supply for germination (triacylglycerols) in favor of PHA biosynthesis is expected to have undesirable effects on the germinative process.

Species-specific obstacles must be overcome as well: the high polymer content obtained in some species, like *Arabidopsis* and *Brassica*, has not yet been achieved in, agronomically more important, crops like potato. PHA formation is still insufficient in the two solanaceous species tested (i.e. tobacco and potato. Bohmert et al., 2002 and this work). In recent years,

important roles in PHA metabolism have been ascribed to phasins (Jossek et al., 1998; Valentin et al., 1998; York et al., 2001) and other regulatory proteins like PhaR and PhaF (Prieto et al., 1999a; Qi et al., 2000; York et al., 2002). These proteins, which are also present in the prokaryotic PHA granules, may also turn out to be essential for efficient PHA accumulation in eukaryotes.

"Green" plastics is a long term prospect

The fossil fuel energy demand. The main advantages of PHA-based plastics *versus* oil-derived plastics were originally identified as follows: a) PHAs would have been based on renewable resources, thus reducing the consumption of the limited oil reserves; b) PHAs are biodegradable, and would contribute to solving the problem of waste disposal. However, the optimistic scenario was cut down on the one hand because of the high costs of fermentation-based PHAs, and on the other hand because of the low yields of PHAs in transgenic plants. Nonetheless, these are not the only obstacles encountered. A recent study, estimates the greenhouse gas emission of plant-based PHB production in the stover (i.e. stalks, leaves and cobs) of transgenic corn plants (Gerngross & Slater, 2000; Kurdikar et al., 2001), with the grains of the same plants being destined to a traditional use. The costs in terms of energy demand related to cropping, harvesting, separation of grain from stover, drying, extraction of PHA from corn stover, recycling of the solvent and blending the plastic to produce resins, were evaluated using and comparing a number of models and considering a PHB yield of 1 Kg / 6.8 Kg of stover dry mass (about 15 %). The consumption of fossil fuel for the manufacturing of plant-based PHAs was compared with the energy demand of fermentative-based PHAs, synthetic petrochemical plastic and poly-lactic-acid (PLA). PLA is a polymer similar to polyethylene terephthalene (PET) and represents the only plastic derived from plant-row material currently commercialised. It is derived by microorganism fermentation of bulk agricultural products to lactate and subsequent chemical polymerisation. The energy demand and the subsequent greenhouse gas emission for the manufacturing of plant-based PHAs (Table 2) is estimated to be higher than the emission required for manufacturing the corresponding amount of oil derived plastic like PE

(polyethylene) and PET (Gerngross & Slater, 2000; Kurdikar et al., 2001). Also the energy demand by fermentation facilities is higher, in terms of fossil fuel requirement, than by the synthetic plastic manufacturing industry (Table 2. Gerngross & Slater, 2000; Kurdikar et al., 2001).

Thus, in conclusion, the environmental benefits of plant-based (and fermentative-based) PHAs are overshadowed by a high energy demand and subsequent greenhouse gas emission.

Table 2. Energy demand of some oil-derived plastics, PLA and PHAs (Gerngross & Slater, 2000). PE: polyethylene. PET: polyethylene terephthalene. PLA: polylactic acid. [1]: heteropolymer produced by *E. coli* fermentation using glucose and propionic acid as carbon source. [2]: PHB produced in stover of transgenic corn plants.

Polymer	Kg fossil oil per kg polymer	
	Oil as supply of energy	Oil for raw material
PE	1.3	0.9
Nylon	2.5	1.4
PET	1.0	1.1
PLA	1.5	0.0
Fermentation PHB / HV [1]	2.39	0.0
Plant PHB [2]	2.65	0.0

Renewable energy or reduction of energy demand. The only conceivable way to make “green” plastic using plant-based (and fermentation-based) PHAs requires that PHA production is uncoupled from the current need of fossil energy supply. Kurdikar and coworkers (2001) and Gerngross & Slater (2000), based on their modelling studies, suggested that burning plant material for energy production would result in no net CO_2 emission into the atmosphere. Furthermore, it is envisaged that burning part of the biomass, while leaving the other part for fertilisation purposes, would result in a surplus of energy which could be sold (Kurdikar et al., 2001). To-date, burning plant material for energy supply, replacing fossil fuel, requires the development of a completely new power generation infrastructure and large investments. This hampers the realisation of such plans in the near future. Nevertheless, it does not exclude

the occurrence of such a scenario in the future. In Brazilian sugarcane mills, biomass is used as a self-sufficient energy supply for several years already. A recent pilot test has been conducted using these pre-existing facilities in the sugarcane industry, and the sugarcane processing was integrated with the production of ethanol and fermentation-based PHB (Nonato et al., 2001).
Nevertheless, it should be considered that oil-based-plastic manufacturing has been developed and improved during several decades to reach the actual optimised situation. Thus, the use of renewable energy is certainly one possible solution and will benefit not only plastic manufacturing, but all energy requiring processes. However, at this moment, the energy demand for PHA extraction is very high (Kurdikar et al, 2001). Therefore, it cannot be excluded that the development of new optimised extraction and PHA manufacturing procedures will be economically and environmentally competitive and they are a logic answer to the current problem of plant-based PHAs.

What is the final choice? Taking into account all advantages and disadvantages of oil-derived and PHA-based and other biodegradable plastics, it is difficult to end up with a final evaluation. Which plastics will be produced in the future will ultimately depend on how society and politics will prioritise several aspects like greenhouse gas emission, depletion of fossil resources, use of farmland, waste disposal and economy and how much investment will be put in developing new plant-based PHA processing systems. The estimated oil reserve is for another 80 years, 70 years for natural gas, 700 years for coal. Synthetic plastic manufacturing uses around 10 % of the output of crude oil refineries, which corresponds, in the U.S., to 80 million tons of crude oil per year (Kurdikar et al., 2001). Manufacturing PHA-based plastic, not based on petroleum as raw material, and fueled by coal would slow down the oil depletion. However, the increased need of fossil fuels for PHA-based plastic production would contribute to, rather than solve, the global climate problem of greenhouse gas emissions. In this context, keeping on producing plastics from non-renewable petroleum using renewable biomass as a fuel would be a good strategy with a net null greenhouse gas emission (in theory all CO_2 released by burning biomass will be re-fixed by

plants during the following generation). However, the problem of solid waste disposal would remain unsolved. Fermentation-based PHA and PLA powered by renewable energy would not increase greenhouse gas emission, but it would compete for the farmland which has to be used to produce the row material. Production of PHAs in stover of corn plants used also for grain production (like in the model described by Gerngross and colleagues, 2000 and Kurdikar et al., 2001) or in leaves / stems of potato plants whose tubers can be used for traditional (non-food) use, or in potato tubers where PHAs and starch granules would be formed, would theoretically not (or only minimally) compete for farmland.

The near future of PHAs

The extensive studies that took place in the past decade allowed the development of a considerable number of novel applications for PHAs. These exploit unique properties of PHAs like biodegradability, hydrophobicity and optical activity.

Applications have been developed for PHB as latex coatings, adhesives, as biomedical disposable material, as osteosynthetic materials, in surgery and tissue engineering (Lee & Choi, 2001; van der Walle et al., 2001 and references therein). Mcl-PHA can be used as pressure sensitive adhesive, binder in paints, coating materials, cheese coating, elastomers and rubber-like materials, and in agricultural industry (van der Walle et al., 1999 and 2001) and references therein), drug delivery (Pouton & Akhtar, 1996) and tissue engineering (Sodian et al., 2000). Furthermore, due to the increasing awareness that chirality is important for biological activity, because of their chiral nature, PHA can be used as starting materials for (stereoscopic) drug industry (van der Walle et al., 2001; Kessler et al., 2001b). Those PHAs that comprise functional groups in the side chain of the monomers (phenyl, methyl-branched, nitrophenyl, cyano, halogenated etc.) are called functional PHAs (Kessler et al., 2001b). The production of functional PHAs in fermentation procedures in which a “functionalising” substrate is added, allows the production of polymers with desired properties and thus allows to tailor the new material to the need and demands of specific applications (Kessler et al.,

2001b). Nevertheless, controlling the monomer composition could in principle be done in transgenic plant cell suspension cultures, but not (or very unlikely) in whole transgenic plants, because that would require a large number of metabolic engineering steps.

Some applications of PHAs in plants are not based on bulk PHA production but on added value to plant components. One example of such an application is the modification of the physical properties of cotton fibre achieved by John and coworkers (John & Keller, 1996) through the incorporation of PHB into the fibres. Similar manipulations of other plant polymers could lead to novel blends and products with novel properties.

Conclusions

The production of bulk PHAs is still controversial and the question whether fermentative-based or plant-based-PHAs are preferred cannot be answered at the moment. It also appears that not one single possible strategy (e.g. fermentative-based-PHAs, plant-based-PHAs, oil-based plastics or other biodegradable polymers), will be able to overcome all technical, environmental and economical problems outlined above.

The use of PHAs for other aims than for bulk productions has expanded in the last years. Although a market must be established for the large number of novel applications developed for PHAs, they certainly opened interesting and optimistic future prospects. Also in this case, however, no choice can be easily made between microbial fermentation and plant as a biofactory for PHA accumulation. On the one hand, some applications will be possible only with one approach, like functional PHAs, which are not feasible in plants. On the other hand, some other applications, like the production of blends of PHAs and existing plant materials, will be only feasible in plants.

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Summary

Polyhydroxyalkanoates (PHAs) represent a large class of microbial polyesters which are widely distributed in prokaryotes. Because of the current environmental concerns related to the use of mineral-oil-based plastics, PHAs gained a considerable interest for their possible use as biodegradable polymers. In recent years the possible fields of application for PHAs have broadened and a considerable number of novel uses of PHAs as rubber material, coating material, binder in paints and several medical applications have been developed. However, high costs related to the fermentative production of PHAs in natural or recombinant microorganisms hampered the production of PHAs on large scale and the expansion of the utilisation of PHAs in society. The use of transgenic plants for the production of PHAs has been considered an excellent and elegant strategy to accumulate bulk amounts of PHAs at low costs. In this thesis, the possibility to produce PHAs in transgenic potato was investigated. A number of metabolic engineering strategies has been considered and the accumulation of low amounts of PHAs in transgenic potato lines has been achieved.

PHA production is one of those traits that require the coordinate expression of several genes. Because the minimum gene set required for PHA accumulation was not completely known at the beginning of the experiments described in this thesis, a flexible system was required for gene-transfer into the potato genome in order to avoid time-consuming or technically difficult approaches (multiple genes plant vector construction, crossing, re-transformation). It was advantageous to introduce simultaneously into the potato genome all the genes of interest delivered *via* separate DNA molecules. This allowed the easy combination of different sets of genes to establish the minimum gene-set required for PHA accumulation in potato. Although *Agrobacterium*-mediated transformation is a well established technique to deliver alien genes into potato, our preliminary analyses showed that it was not suitable for simultaneous co-transformation using separate plasmids. Thus, we developed a particle bombardment-mediated procedure to introduce simultaneously into the potato genome several genes from separate plasmids or separate linear DNA fragments. The particle bombardment protocol established during the course of this project proved to be very

efficient and comparable with *Agrobacterium*-mediated transformation. We observed that transgenic potato plants obtained by particle bombardment showed relatively simple patterns of integration of the transgenes and integration of different transgenes at independent sites into the plant genome. The system also proved applicable for co-transformation of potato using gene cassettes, i.e. DNA fragments comprising promoter – gene – terminator.

Particle bombardment-mediated co-transformation was subsequently used to introduce the genes involved in the PHA biosynthetic pathways. Four methods were considered for the production of PHAs in potato.

Method 1. The accumulation of the homopolymer polyhydroxybutyrate (PHB) was achieved after *Ralstonia eutropha phb*B and *phb*C genes coding for an NADPH-dependent acetoacetyl-CoA reductase and for the PHB-polymerase, respectively, were expressed in transgenic potato lines. Because the minimum gene set for PHB production in plants was well documented when this project started, the possible production of PHB seemed to be the simplest model to test the ability of potato to accumulate PHAs. Indeed, PHB formation in small amounts was observed.

Subsequently, we attempted the production of the heteropolymer medium-chain-length (mcl)-PHA in potato.

Method 2. To study the expression of the *Pseudomonas oleovorans* mcl-PHA-polymerase in potato, the polymerase was first expressed in the cytoplasm, where no post-transcriptional modification of the polypeptide is needed. Proper expression of the mcl-PHA polymerase was indeed observed in selected transgenic lines. However, because the cytoplasm of potato does not contain suitable substrates for the polymerase (mcl-hydroxy fatty acids), substrate feeding experiments were performed. These experiments resulted in the accumulation of low amounts of mcl-PHA.

Methods 3 and 4. Subsequently, mcl-PHA accumulation was attempted in plastids, by re-directing hydroxy fatty acid precursors from FAB to mcl-PHA biosynthesis. A truncated form of *Escherichia coli* thioesterase-I (*tes*A gene. Method 3) and *P. putida* ACP-CoA-transacylase (*pha*G gene. Method 4) were expressed in combination with the Pha-C1 polymerase. All heterologous proteins were targeted to the plastid by N terminal fusion with the Rubisco

transit peptide. The two combinations of genes (Pha-C1 plus thioesterase, and Pha-C1 plus transacylase) represent theoretically the minimum gene set for mcl-PHA accumulation deriving precursors from FAB. The double transformants which expressed the Pha-C1 polymerase and the thioesterase-I did not accumulate mcl-PHA because a few β-oxidation steps may be required to provide the proper precursor to the polymerase. However, simultaneous expression of the Pha-C1 polymerase and the ACP-CoA-transacylase did result in the synthesis of low amounts of mcl-PHA in leaves of intact plants. Transformation with the combination of *pha*C1 / *phaG* genes under the control of the GBSS (granule-bound-starch-synthase) promoter, which is highly expressed in tubers, did not result in detectable amounts of mcl-PHA in tubers.

We established several possible routes to achieve the accumulation of PHAs in transgenic potato, based on the use of the endogenous pool of acetyl-CoA, on feeding approaches of cell suspension cultures or on using precursors derived from *de novo* FAB. Although improvements in these systems are still required in order to increase the yields of PHAs, the results open new prospects for the accumulation of PHAs in potato. Final conclusions on the future development of production of PHAs in plants are overviewed. Advantages and disadvantages of fermentation-based-PHAs, plant-based-PHAs, other biodegradable polymers and oil-based-plastics are compared. Novel and future possible interesting applications of PHAs not based on bulk productions, are also described.

Samenvatting

Polyhydroxyalkanoaten (PHA's) zijn een grote groep van microbiële polyesters die wijd verbreid zijn binnen de prokaryoten. Gedurende de oliecrisis in de jaren 70 en vanwege de huidige zorg met betrekking tot het milieu, met name het gebruik van plastics op basis van minerale olie, staan PHA's steeds meer in de belangstelling voor mogelijke toepassing in biologisch afbreekbare polymeren. Vooral gedurende de laatste jaren is het aantal mogelijke toepassingen van PHA's enorm toegenomen, waaronder geheel nieuwe zoals voor rubber materiaal, coatings, bindmiddel in verf en een aantal medische applicaties.
Echter, de hoge kosten die verbonden zijn aan de productie van PHA's via fermentatieve productie, zowel in natuurlijke als in recombinante micro-organismen hebben de grootschalige productie van PHA's en penetratie van de markt belemmerd. Daarom wordt de toepassing van transgene planten beschouwd als een uitstekende en elegante strategie om *bulk*-hoeveelheden aan PHA's tegen lage kosten te produceren. In dit proefschrift is deze mogelijkheid, met name de productie van PHA's in transgene aardappel bestudeerd. Er werd een aantal mogelijke strategieën voor *metabolic engineering* in ogenschouw genomen en de vorming van kleine hoeveelheden PHA's in transgene aardappellijnen kon inderdaad worden vastgesteld.

De productie van PHA's is een eigenschap die een gecoördineerde expressie van verschillende genen (én van de selecteerbare merker die nodig is voor de primaire selectie van transgene planten) noodzakelijk maakt. Omdat de minimum genen-set voor PHA-accumulatie aan het begin van dit project nog niet geheel bekend was, niet voor prokaryoten, maar zeker niet voor recombinante eukaryoten, was een flexibel systeem vereist om genen naar het aardappel-genoom te kunnen overbrengen in plaats van de toepassing van tijdrovende of technisch moeilijke procedures (zoals de constructie van multigen-vectoren, kruising, re-transformatie). Hierom was het van belang een nieuw systeem te ontwikkelen, waarbij tegelijkertijd alle benodigde genen via verschillende DNA-fragmenten in het aardappel-genoom kunnen worden ingebracht. Dit zou het inbrengen van verschillende combinaties van genen vergemakkelijken om vast te kunnen stellen, welke genen-set minimaal nodig

is voor de biosynthese van PHA's in aardappel. Hoewel de transformatie via *Agrobacterium* een goed werkende techniek is voor gen-overdracht naar aardappel, hebben onze analyses aangetoond dat dit systeem niet geschikt is voor gelijktijdige gen-overdracht via meerdere afzonderlijke plasmiden. De oplossing werd geboden door de ontwikkeling van efficiënte procedures via de techniek van *particle bombardment*, waarmee tegelijkertijd verschillende genen gelegen op afzonderlijke plasmiden of zelfs lineaire DNA-fragmenten in het aardappel-genoom kunnen worden ingebracht. De *particle bombardment* protocols die gedurende dit project werden ontwikkeld, bleken voor wat betreft hun efficiëntie vergelijkbaar met die van de transformatie via *Agrobacterium*. We hebben ook kunnen vaststellen dat de via *particle bombardment* verkregen transgene aardappel-lijnen eenvoudige integratie-patronen vertonen van het donor-DNA en ook integratie laten zien op meerdere plaatsen in het aardappel-genoom. Het nu ontwikkelde systeem bleek ook toepasbaar voor de gelijktijdige overdracht van gen-cassettes, i.e. lineaire DNA-fragmenten met uitsluitend promoter – structureel gen – terminator.

Vervolgens werd particle bombardment toegepast om via co-transformatie genen in te brengen die deel uitmaken van de PHA-biosynthese-route. Vier verschillende methoden werden geëvalueerd voor de productie van PHA's in aardappel.

Methode 1. De vorming van het homopolymeer polyhydroxybutyraat (PHB) werd gerealiseerd na de succesvolle expressie in aardappel van de *Ralstonia eutropha phb*B and *phb*C genen die coderen voor een NADPH-afhankelijke acetoacetyl-CoA reductase en voor het PHB-polymerase, respectievelijk, Aangezien de minimum genen-set voor de productie van PHB in andere planten al goed was beschreven bij aanvang van dit project, was productie van PHB het eenvoudigste model om de vorming van PHA's in aardappel vast te stellen.

Methode 2. Vervolgens hebben we getracht de vorming van heteropolymeer *medium-chain-length* (mcl)-PHA in aardappel te realiseren. Om de mogelijke expressie van het *Pseudomonas oleovorans* mcl-PHA-polymerase in aardappel te onderzoeken, werd het polymerase eerst tot expressie gebracht in het cytoplasma waar geen post-transcriptionele modificatie van het eiwit

nodig is. Inderdaad werd expressie van het mcl-PHA polymerase vastgesteld in een aantal geselecteerde transgene lijnen. Echter, omdat het cytoplasma van aardappel van nature geen geschikte substraten bevat voor het polymerase-enzym (mcl-hydroxy-vetzuren), werden substraat-*feeding* experimenten uitgevoerd. Deze experimenten hebben geresulteerd in de vorming van kleine hoeveelheden mcl-PHA.

Methoden 3 and 4. Aansluitend werd getracht de vorming van mcl-PHA in plastiden (cel-organellen waarin normaal de synthese van chlorofyl of zetmeel plaatsvindt) te verkrijgen, waarbij de hydroxy-vetzuur-*precursors* voor de vorming van mcl-PHA worden “afgetapt” van de vetzuur-biosynthese (FAB, fatty acid biosynthesis). Een verkorte, cytoplasmatische vorm van het *Escherichia coli* thioesterase-I (*tes*A-gen; Methode 3) of het *P. putida* ACP-CoA-transacylase (*pha*G-gen; Methode 4) werd tot expressie gebracht in combinatie met het Pha-C1 polymerase. Alle bij de PHA-synthese betrokken heterologe eiwitten werden gedirigeerd naar de plastiden door middel van een N-terminale fusie met het Rubisco transit-peptide. Deze twee combinaties van genen (*pha*C1 plus *‘tes*A, of *pha*C1 plus *pha*G) zijn theoretisch de minimum genen-sets voor de vorming van mcl-PHA uit *precursors* van de vetzuur-synthese. De dubbel-transformanten die het Pha-C1 polymerase en het thioesterase-I tot expressie brachten waren echter niet in staat mcl-PHA te synthetiseren, waarschijnlijk omdat in dit geval een aantal extra β-oxidatie-stappen nodig zijn om de juiste *precursor* voor het polymerase-enzym te genereren. De gelijktijdige expressie van Pha-C1 polymerase en het ACP-CoA-transacylase daarentegen hebben wel geleid tot de vorming van mcl-PHA in bladeren van intacte planten. Transformatie met de *pha*G/*pha*C1-combinatie achter de GBSS (granule-bound-starch-synthase) promoter, die een relatief hoge expressie in knollen mogelijk maakt, heeft niet geleid tot de vorming van detecteerbare hoeveelheden PHA in (micro)knollen.

In dit proefschrift hebben we verschillende mogelijke benaderingen getest voor de vorming van PHA’s in transgene aardappel, waarbij gebruik werd gemaakt van de endogene *pool* van acetyl-CoA, van *feeding*-experimenten met celsuspensie-cultures of van *precursors* die waren afgeleid van de *de novo* vetzuur-synthese. Hoewel nog verbeteringen in deze systemen nodig

zijn, vooral om de hoeveelheid gevormd PHA te verhogen, bieden de resultaten aanknopingspunten voor de uiteindelijke ophoping van PHA's in aardappel.

Tenslotte worden in de algemene discussie de mogelijkheden van de toekomstige productie van PHA's in planten besproken. Voor- en nadelen van PHA's geproduceerd via microbiële fermentatie, PHA's in planten, andere bioafbreekbare polymeren en plastics op basis van minerale olie worden vergeleken. Nieuwe interessante toepassingen van PHA's die niet zijn gebaseerd op bulk productie-systemen worden ook genoemd.

Riassunto

I poli-idrossi-alcanoati (PHA) appartengono ad un gruppo di poliesteri di origine microbica e sono largamente distribuiti in natura. Le crescenti preoccupazioni di natura ambientale legate all'uso delle plastiche di origine petrolchimica hanno contribuito ad aumentare l'interesse, da parte di scienziati e istituti di ricerca, per i PHA a causa del loro possibile utilizzo come polimeri biodegradabili. Inoltre, recenti sviluppi della ricerca sui PHA permettono oggi di usare questi polimeri in un vasto numero di applicazioni come materiale elastico, gomme, materiale di rivestimento, ed é stato anche sviluppato un rilevante numero di applicazioni nel settore medico. Gli elevati costi necessari per il mantenimento e il funzionamento delle strutture per la coltivazione batterica (fermentatori) hanno tuttavia impedito la produzione e l'utilizzazione su larga scala dei PHA, mediante l'utilizzo di quei microrganismi (selvatici o ricombinanti) che li producono e li accumulano. La produzione dei PHA ad opera di piante transgeniche é stata considerata, fin dall'inizio degli anni 90, un'elegante e promettente strategia per produrre questi poliesteri in grande quantità e a bassi costi. Durante il corso di questa tesi, abbiamo investigato sulla possibilità di produrre i PHA nella patata. A tale scopo, sono state prese in considerazione diverse modelli di ingegneria metabolica, ed é stata ottenuta la sintesi di PHA in diverse linee transgeniche di patata.

La produzione dei PHA é uno di quei caratteri i quali richiedono che un numero elevato di geni sia espresso in maniera coordinata. Inoltre, dato che il set minimo di geni per la produzione dei PHA non era noto quando gli esperimenti descritti in questa tesi iniziarono, era necessario utilizzare un sistema di trasformazione della patata che permettesse una certa flessibilitá, onde evitare di dover utilizzare approcci laboriosi e tecnicamente complicati (ad esempio, l'utilizzo di plasmidi di espressione per le piante conteneti molti geni, l'uso di incroci e di ri-transformazioni). Un notevole vantaggio sarebbe derivato dalla capacità di poter introdurre simultaneamente nel genoma della patata i geni "candidati" per la produzione dei PHA, clonati in molecole di DNA separate. Ciò avrebbe permesso di combinare facilmente set di geni diversi e di stabilire il set di geni minimo per l'accumulo dei PHA. Sebbene la tecnica di trasformazione della patata basata su *Agrobacterium* sia molto usata ed efficiente, durante una serie di analisi preliminari avevamo dimostrato che

questa tecnica non era tuttavia efficiente per introdurre simultaneamente plasmidi separati. Per questa ragione, abbiamo sviluppato un protocollo di trasformazione della patata basato sul "particle bombardment" e capace di introdurre diversi geni contenuti in molecole di DNA separate. Questo protocollo si é dimostrato essere molto efficiente e paragonabile alla tecnica tradizionale di trasformazione della patata basata su *Agrobacterium*. Nelle linee transgeniche di patata prodotte, abbiamo constatato che l'organizzazione dei transgeni é risultata relativamente semplice a l'integrazione di molecole di DNA separate nel genome é avvenuta, in un numero elevato di casi, in punti del genoma spazialmente separati. Inoltre, il protocollo sviluppato ci ha permesso di trasformare e co-trasformare la patata usando "gene-cassette", cioè frammenti di DNA contenenti esclusivamente la combinazione di promotore – gene – terminatore.

I geni responsabili per a produzione dei PHA sono stati quindi introdotti simultaneamente nel genoma della patata usando la tecnica del "particle bombardment". Sono stati studiati 4 metodi diversi per produrre i PHA.

Metodo 1. I geni *phb*B e *phb*C del batterio *Ralstonia eutropha*, che codificano rispettivamente per un'acetoacetil-CoA riduttasi NADPH-dipendente e la poli-idrossi-butirrato (PHB)-polimerasi, sono stati espressi in linee transgeniche di patata e la sintesi dell'omopolimero PHB é stata ottenuta. Considerando che il set minimo di geni per la sintesi di PHB nelle piante transgeniche era noto quando questo progetto iniziava, la possibile produzione di PHB risultò essere il modello più semplice per stabilire la capacità o meno della patata di accumulare i PHA.

Successivamente abbiamo tentato di produrre l'eteropolimero "medium-chain-length" (mcl)-PHA.

Metodo 2. Per poter studiare l'espressione nella patata della polimerasi Pha-C1 del batterio *Pseudomonas oleovorans*, specifica per i mcl-PHA, la polimerasi stessa é stata prima espressa nel citoplasma di linee transgeniche di patata. L'espressione di geni eterologhi nel citoplasma delle piante non richiede nessuna modificazione post-trascrizionale. Questi esperimenti hanno dimostrato che la polimerasi può essere espressa correttamente nella patata. Siccome tuttavia il citoplasma della patata non contiene i substrati che la polimerasi Pha-C1 può utilizzare (cioè mcl-acidi grassi idrossilati), abbiamo

eseguito degli esperimenti in cui il substrato veniva fornito nel terreno di crescita delle linee di patata selezionate, e al termine di questi esperimenti, abbiamo osservato la sintesi di mcl-PHA in alcune di queste linee transgeniche.

Metodi 3 e 4. Successivamente, abbiamo tentato di ottenere la sintesi di mcl-PHA nei plastidi utilizzando gli acidi grassi idrossilati, intermedi della sintesi degli acidi grassi, come precursori dei mcl-PHA. Per fare ció, abbiamo espresso, in combinazione con la mcl-PHA-polimerasi, una forma troncata della tioesterasi-I del batterio *Escherichia coli* (gene *tes*A. Metodo 3) o la ACP-CoA-transacilasi del batterio *P. putida* (gene *pha*G. Metodo 4). Tutte le proteine eterologhe sono state espresse nei cloroplasti utilizzando la sequenza di transito per i cloroplasti della Rubisco. Queste due combinazioni di geni (codificanti per la polimerasi Pha-C1 e la tioesterasi, o per la polimerasi Pha-C1 e la transacilasi) rappresentano in teoria il set minimo di geni per sintetizzare i PHA derivando i precursori da intermedi della sintesi degli acidi grassi. I doppi trasformanti che esprimevano la polimerasi Pha-C1 e la tioesterasi-I non hanno mostrato accumulo di mcl-PHA probabilmente perché l'utilizzo di questi due transgeni per la sintesi dei PHA necessita di ulteriori passaggi della β-ossidazione, per convertire intermedi della sintesi degli acidi grassi in precursori dei PHA. L'espressione simultanea della polimerasi Pha-C1 e della ACP-CoA-transacilasi é risultata invece nella sintesi di mcl-PHA nelle foglie delle patate transgeniche selezionate. Precedentemente, la produzione dei PHA era stata anche tentata nei tuberi di linee transgeniche di patata usando un promotore tubero-specifico per il controllo dei geni responsabili per la sintesi dei poliesteri, ma inutilmente.

Durante il corso di questo lavoro, abbiamo stabilito diversi sistemi possibili per sintetizzare i PHA nella patata. Essi si basano sia sul fornire il substrato necessario alla sintesi dei PHA nel mezzo di crescita, sia sull'utilizzo del pool di acetil-CoA endogeno, sia sull'utilizzo di intermedi della sintesi degli acidi grassi. Questi sistemi richiedono dei miglioramenti soprattutto per il basso livello di accumulo del polimero che é stato ottenuto nelle linee transgeniche studiate. Tuttavia, il lavoro descritto apre nuove possibili e future aspettative nell'uso della patata per l'accumulo dei PHA. In questa tesi, é anche descritta

una serie di conclusioni finali e speculazioni sull'utilizzo delle piante transgeniche per la produzione dei PHA. I vantaggi e gli svantaggi di una produzione dei PHA basata sulle piante o sui batteri sono analizzati, confrontati e discussi. Infine, sono descritte nuove applicazioni possibile per i PHA non legate alla produzione di plastiche.

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Curriculum vitae

Andrea Romano was born on August the 26th 1970 in Vittorio Veneto (TV), Italy. In 1985 he entered the High School "Liceo Scientifico". Between 1990 and 1995 he studied at the University in Bologna and got the degree in Molecular Biology. For the thesis he has been working at the Institute of Biochemistry and Biophysics of the Bologna University, on sequencing of the ATP operon of the bacterium *R. capsulatus*. After he graduated, he worked at the same institute for 6 months at the biochemical characterisation of the ATPase from *R. capsulatus*. Subsequently he moved to England, funded by a "Leonardo" grant, and he was involved in a project aimed at monitoring the DNA transfer between food micro-organisms and bacteria from the gut micro-flora at the Bibra International, Carshalton (Surrey).

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