Progress of PHA production in transgenic plants

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Abstract With its completely biodegradable ability, good physical and processing properties, and biocompatability, PHA has become the most attractive alternative to take the place of chemo-plastics and eliminate environmental pollution caused by chemo-plastics. Small-scale commercial production has been realized by bacterial fermentation, but thus produced PHA is too expensive to compete with chemo-plastics. However, it is very prospective to lower biodegradable plastic price by producing PHA in transgenic plants. Presently PHB/PHBV biosynthetic genes have been transferred into plants and expressed, but it is still far away from meeting the demands of commercial production. Enhancing research on the regulation of carbon and fatty acid metabolisms, and their biochemical and molecular mechanisms is the key to achieving this aim.

Keywords: **PHA, PHB, PHBV, transgenic plants, biodegradable plastics.**

PETROLEUM-DERIVED plastics have gradually become ideal and important high molecular materials for a wide variety of industrial and consumer products in our modern society, but its intrinsic durability and resistance to degradation have been causing severe pollution of environment. The low recycling rate of plastic discard also results in large waste of resources. Although the traditional methods of plastic waste management can reduce its pollution in some degree, they need high cost and cause second pollution. In present society, with our human's consciousness of environmental protection increasing day by day, it has become an urgent task to develop and produce biodegradable plastics to reduce and eliminate pollution. Now many developed countries are doing research work in this area, and some products have been produced, such as photodegradable plastics in which light-sensitive groups are incorporated into the backbone of the polymer; and partly biodegradable plastics in which starch, cellulose, or microbial polyester is mixed with chemical polymers. These kinds of plastics can be either photodegraded or non-enzymaticly or enzymaticly hydrolyzed into nondegradable smaller fragments and lost their structural integrity, which in some degree reduce environment pollution. But these small fragments can cause second pollution. So there exist disputes over these kinds of biodegradable plastics. Among the various biodegradable plastics available, there is a growing interest in the group of polyhydroxyalkanoates (PHA), a family of natural polyesters which can be completely degraded by some microorganisms. PHA combines biodegradability with biocompatability and good physical properties, making it suitable polymer for a wide variety of uses. Research on the microbiology, biochemistry, and molecular biology of PHA biosynthesis has dramatically increased in the last 10 years. Small-scale production has been realized by bacterial fermentation. Now the possibility of producing PHA on a large scale and at a cost comparable to synthetic plastics has arisen from the demonstration of PHA production in transgenic plants. Transgenic plant, one kind of new bioreactors shows us broad prospects in producing biodegradable plastics. In this review, we try to introduce the current advances of PHA synthesis in bacteria and plants.

1 Introduction to PHA

PHA is one family of storage polyesters synthesized in some bacterial cells under unbalanced nutrient conditions (e.g. short of nitrogen, or phosphorus, or magnesium), functions as starch in plants and fat in animals. It has been found to occur in over 300 genera of bacteria, encompassing Gram-positive and Gram-negative species. Over 90 kinds of PHA have been characterized, the majority of them are composed of D-(-)-poly-3-hydroxyalknoic acid monomers ranging from C3 to C14 (fig. 1). With different R groups, chain lengths, and site of hydroxyl group in monomers, various PHAs are formed[1]. PHA can be classified into three groups: short-chain-length PHA with C3-C5 monomers, such as poly-3-hydrobu-

tyrate (PHB); medium-chain-length PHA with C6-C14 monomers, such as poly-3-hydroxyoctanoate (PHO); and copolymer by different monomers, such as poly-3-hydroxybutyrarte-co-3-hydroxyvalerate (PHBV). Not all of the PHAs are suitable for materials of biodegradable plastics. So the first and important step to produce PHA in transgenic plants is to choose PHA of good properties. Because PHB is the first discovered, most widely existed, best understood PHA, also has good physical and processing properties, it certainly became the first PHA to be produced by bacterial fermentation and transgenic plants.

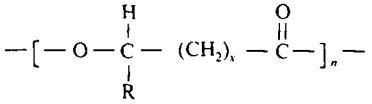

Fig. 1. Chemical structure of PHA. R = H or C1-C11; $x = 1—3$, $n = 100—30\,000$.

2 Biosynthesis and molecular mechanism of PHA production

The biochemical pathway of PHB synthesis is shown in fig. 2[2]. In most bacteria, such as *Alcaligenes eutrophus*, PHB is synthesized from acetyl-CoA by the sequential action of 3 enzymes. Two *molecules of acetyl-CoA are condensed by the 3-ketothiolase* (acetyl-CoA acetyl transferase; E.C. 2.3.1.9) to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase; E.C. 1.1.1.36) subsequently reduces acetoacetyl-CoA to D-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHB synthase to form high-molecular-weight PHB. However, there exists another pathway (five-step) in *Alcaligenes eutrophus*. NADH-dependent acetoacetyl-CoA reductase reduces acetoacetyl-CoA as formed in three-step pathway to L(+)-3-hydroxybutyayl-CoA, L(+)-3-hydroxybutyayl-CoA is then converted to D(-)-isomer form by the action of two enoyl hydrase, and finally polymerized to form PHB. The three-step pathway can also produce PHBV, one kind of PHAs with good physical and processing properties and chosen to be produced commercially much earlier.

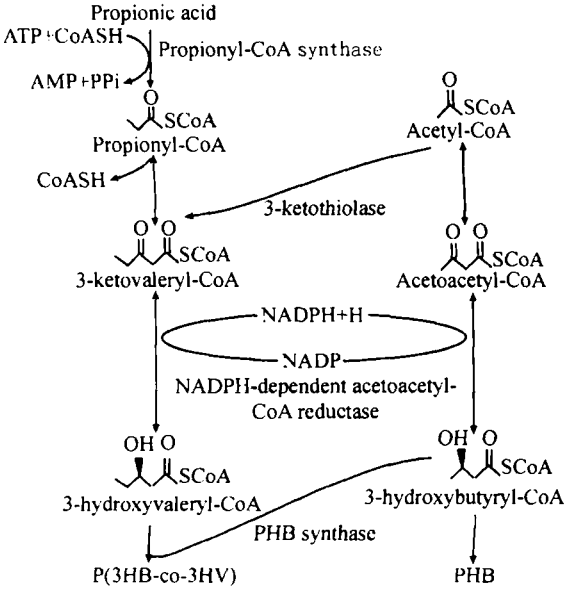

Fig. 2. Pathway of PHB and PHBV synthesis in *A. eutrophus*.

The chemical pathway of medium-chain-length PHA synthesis has not yet been well understood nowadays. PHO is synthesized in *Pseudomonas oleovorans*, its monomer comes from β-oxidation or *de novo* synthesis pathway of fatty acids. Many other *Pseudomonas* bacteria can produce poly-3-hydroxydecanoate (PHD), taking acetyl-CoA as the precursor[3].

Till now, more than 30 genes relevant to the synthesis and degradation of PHA have been cloned. Three research groups used different methods[4—7], such as scanning target gene fragments through enzyme activity, transponson caused mutant, and heterogeneous probe etc., to segregate DNA fragments encompassing *phbA* (encoding 3-ketothiolase), *phbB* (encoding acetoacetyl-CoA reductase), *phbC* (encoding PHB synthase) genes[8]. These three genes coexist in one operon, and are controlled by the same promoter[2].

PHA synthase is the key enzyme of PHA biosynthesis. It determines the composition of monomers in PHA. 17 gene clones encoding such enzyme have been acquired from different bacteria. According to their primary structures and substrate specificity, PHA synthases can be categorized into three classes[9]. The first one catalyzes the short-chain-length PHA formation, e.g. PHB synthase in *A. eutrophus* (encoded by *phbC*). There is 37%—38% amino acid homology among this kind of PHA synthases. The second one catalyzes the medium-chain-length PHA formation, such as in *Pseudomonas oleovorans* and

Pseudomonas putida (encoded by *phaC*1 and *phaC*2). 54%—80% amino acid homology exists among them. There is 34%—40% amino acid homology between these two kinds of PHA synthases. The third one was found in *Chromatium vinosum* and *Thiocapsa violacae*. They act on the polymerization of short-chain-length PHA, but their amino acid chain is 35%—40% shorter than that of the two groups mentioned above, and the homology among them is only 21%—27%. Krantz's study on *Rhodobacter capsulatus* D. indicated that the expression of *phaC* gene may not be doomed to PHA synthesis. This means that the regulation of PHA synthase may occur in the process of post translation[10].

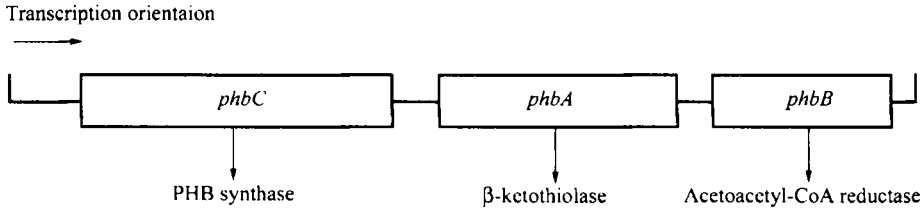

Fig. 3. Construct of *A. eutrophus phbC-phbB-phbA* operon.

Many bacteria can secrete PHA depolymerase to degrade PHA to monomers or oligomers and then absorb them into their bodies. PHA depolymerase is encoded by *phaD* gene[1)]. Readers of interests may refer to refs. [3, 10—13] to obtain more information on the biochemical and molecular mechanisms of PHA synthesis and degradation.

3 PHB/PHBV production by bacterial fermentation

Earlier in 1975 ICI Company (in England) began to produce PHB using a mutant strain of *A. eutrophus*. In 1981, Zeneca Company produced a random copolymer (PHBV) of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) by batch fermentation of *A. eutrophus* cultured on glucose and propionic acid medium. PHBV yield reached 70%—80% of bacterial cell dry weight. Lately Monsanto Company bought the patent of PHBV production, and turned out plastic product commercially named Biopol, with the yield of 1 000 t per year[1]. Berlin Company of America began to sale making-up bottles made of Biopol. In Austria, Chemie Linz Gmbh Company produced PHB using the continual fermentation method with a yield of one ton per week.

The production cost of biodegradable plastics by bacteria fermentation is much higher than chemically synthesized one, due to its expensive fermentation substrates and complicated fermentation process. Researchers are taking various measures to enhance yield, reduce production cost, and search for other PHA of better physical properties or of special uses in order to improve the market competent ability of biodegradable plastics produced by bacteria fermentation. These measures include altering hereditary structures of bacteria strains by transferring PHB biosynthetic genes to those which can use cheap substrates as nutrients, or transferring genes whose expression products can take low-price substrates as growth nutrients to bacteria strains producing PHA[1,14—16].

Despite of those endeavors as mentioned above, it is still unlikely that the cost of microbially produced PHA is reduced to one-fourth that of petroleum-derived plastics and able to compete with it[1)]. While in recent years, transgenic technique in plants has become more and more mature, there have been no many problems for foreign genes to incorporate into plant genomes and get expressed. In comparison with bacterial or yeast fermentation, plants are capable of using abundant carbon pool to produce large amounts of a number of useful chemicals and active proteins, these advantages are showing us great potential for plants to become cheap bioreactors. Plant gene engineering has allowed us not only to increase crop yield, enhance insect- and pathogen- resistant ability, and improve crop products quality, but also to produce bioactive macromolecules, such as antigen and antibody, etc.[17, 18]. Plant transgenic

1) Huisman, G. W., Poly(3-hydroxyalkanoates) from *Pseudomonas putida*: from DNA to plastic, PhD. thesis, Groniengen University, The Netherlands, 1991.

technique throws bright light on the prospects of reducing PHA price to the same level of starch and fatty acids[19] and making it possible for biodegradable plastics to replace chemically synthesized plastics gradually and completely at last.

4 PHB production in transgenic plants

(ⅰ) First generation transgenic plant——transgenic *Arabidopsis* producing PHB. Synthesis of PHB in plants was initially explored by transferring two PHB biosynthetic genes of the bacterium *Alcaligenes eutrophus*, *phbB* and *phbC*, under the control of constitutive CaMV 35S promoter to model plant *Arabidopsis thaliana*[20]. Thus acquired transgenic *Arabidopsis* plants can use endogenous 3-ketothiolase and foreign *phbB* and *phbC* gene products——acetoacetyl-CoA reductase and PHB synthase to produce PHB with 0.14% of plant dry weight yield. To obtain enough PHB to be used for analysis, Poirier *et al*.[21] cultured transgenic *Arabidopsis* cells by suspension culture technique. PHB content in transgenic cells was five times higher than that in transgenic phants. Gas chromatography, mass spectrometry, transmission electron micrographs analysis and visualization by epifluorescence microscopy showed that the electron density, size, form and composition of PHB inclusions in transgenic *Arabidopsis* cells were very similar to those in bacteria. PHB in transgenic plants exists in roots, stems, leaves and seeds. While on cellular level, it distributes in vacuole, nucleus, and cytoplasm, none could be detected in chloroplast and mitocondria. It may be the hydrophobility of PHB inclusions that makes them not be able to enter into organelles of lipid bilayer membrane. Expression of *phbB* gene in transgenic plants caused significant reduction in growth and 45% reduction in seed production relative to wild-type plants. Transgenic plants containing both genes were more severely stunted in growth[21], which most probably resulted from too much acetyl-CoA being diverted from the mevalonate pathway to PHB biosynthesis, or a noxious effect of the PHB inclusions, especially the possible interaction between the PHB granules and DNA in nucleus.

The flux to acetyl-CoA is high in plant plastid since it is the site of fatty acid biosynthesis for membrane and storage lipids. Plastids are also known to have a high storage capacity. Starch, a kind of high molecular weight polysaccharide, normally accumulates to a high extent in the plastids of many storage tissues. It also transiently stores in chloroplasts during day time of high CO_2 exchange rate without noxious effect on the function of chloroplasts. In addition, the first generation of PHB-producing transgenic plants indicated that PHB inclusions cannot penetrate bilayer envelope of plastids, which means an added advantage of avoiding potential mechanical disruption of other organelles by PHB production. Based on the consideration, Nawrath *et al*.[22,23] inserted the DNA fragment of the transit peptide from the small subunit of pea Rubisco into three expression vectors containing *phbA*, *phbB*, *phbC* respectively under the control of CaMV35S promoter. This transit peptide can lead PHB synthetic gene products to the plastids. These three expression vectors were transferred to *Arabidopsis* plants mediated by agrobacterium respectively. Transgenic plants containing three genes together were thereby subsequently obtained by continual crossing. PHB was observed to accumulate in plastids under a transmission electron microscope, not detected in any other organelles. PHB content increases gradually following plant growth. It is ten times higher in full-expanded leaves than in unfledged leaves. The final content can reach 10 mg/g fresh weight, or 14% of leaf dry weight, 100 times higher than in the first generation of PHB-producing transgenic plants. Except a bit etiolation in old leaves, no significant effect on plant growth was observed. Enzyme activity analysis indicates that it is the key enzyme activity of PHB biosynthesis, not the lack of acetyl-CoA that limits further accumulation of PHB. It seems that PHB content could be further increased.

(ⅱ) PHB production in transgenic rape seed. PHB production in model plant *Arabidopsis thaliana* showed the feasibility of producing PHA in transgenic plants, but there is no producing value in *Arabidopsis* itself. As the first largest oil crop, rape seed has storage lipids content up to 40% of seed dry weight, the flux through acetyl-CoA in plastids is especially high. Rape seed should be one of the most suitable target to produce PHA. When Zeneca Company's researchers transformed rape seed plants with the same strategy as in *Arabidopsis*, they got the same results: plant growth was severely stunted when PHB production was located in cytoplasm; while PHB yield could reach 0.01% of leaf fresh weight when

in plastids[24—26].

PHB distributes in most parts of plant when PHB biosynthesis is located in plastids. This would make PHB extraction become more difficult and thereafter increase the production cost. As mentioned above, with the high flux through acetyl-CoA to fatty acids in seed plastids, not only the noxious effects of PHB on plant growth could be reduced to a less degree, but also extraction of PHB can be much more easier. Nawrath *et al.*[23] modified the coding sequence of each corresponding *phb* gene by inserting, at the N-terminal end of the protein, the transit peptide from the small subunit of rubisco and the seed-specific promoter isolated from the CRB gene of the 12S seed storage protein of *Arabidopsis thaliana*, thereby localized PHB production in seed plumule. A PHB yield of 1% seed fresh weight was obtained.

Our research group cloned the three *phb* genes by PCR technique from *A. eutrophus*, and constructed expression vector pKCB containing *phbB* and *phbC* genes. When it was transferred to *E. coli*, *phbB* gene product and PHB were detected, which means the cloned *phbB* and *phbC* genes can work normally[27,28]. In order to introduce PHB biosynthesis into seed plastids, we also cloned transit peptide gene *ctp* and 7S seed-specific promoter sequence. Firstly constitutive expression vector pKA containing *phbA* gene was constructed and transferred to tobacco plants. High activity of ketothiolase detected in transgenic tobacco chloroplasts proved that *phbA* and *ctp* genes can work well[29]. On these basis, we constructed seed-specific expression vectors containing one, two and three *phb* genes respectively, then used them to transform rape seed plants. Some transgenic plants have been acquired at present. Further detection work is underway.

(ⅲ) PHB production in transgenic potato. Potato has also been considered to be the subject of PHB production because of its high yield, wide growth area, asexual propagation way, and mature transformation system. Our research group constructed tuber-specific expression vectors by inserting whole-length Patatin Ⅰ, a promoter induced to express by high concentration sucrose, to the 5′-terminal of *phbB* and *phbC* genes, transferred it to potato plants and obtained a batch of transgenic plants. But PHB content was very low, the most probable reason is the low acetyl-CoA content in tuber cells. Inhibiting starch biosynthesis to divert more carbon source to acetyl-CoA may be the key step to increase PHB biosynthesis. Müller-Robert[30] transferred the antisense gene of AGPase, a key enzyme in starch biosynthesis, to potato plants. The starch content in transgenic plants was reduced to 5% that of wild type, and sucrose content increased greatly. Fructokinase catalyzes the conversion of sucrose to fructose-6-phosphate in the glycolysis pathway. It is very likely that the flux through acetyl-CoA from sucrose is enhanced if fructokinase gene expression is increased. This gene has been cloned from plants and bacteria[31]. However, regulation of plant carbohydrate metabolism is proved extremely complex. It would not only be very difficult to reach the expected results, but also most probably disturb carbohydrate metabolism, if only one or a few enzymes are inhibited or enhanced[32]. Quite a long time will have to be taken to accomplish precisely regulating carbon metabolism.

Additionally, the respiration rate of potato tubers during storage increases relatively, the substrates of PHB biosynthesis would increase accordingly. It may allow us to produce PHA through stored potato tubers.

The most direct method would be to locate PHA production in mitochondria. Trethway[1] is now studying this feasibility. Mitochondria are one of the most important organelles. It is not like plastid which can contain starch granules much bigger than PHB granules. There would be more problems to produce PHA in mitochondria than in plastids.

(ⅳ) Improvement of cotton filament in transgenic cotton. John *et al.*[33] constructed constitutive expression vector with *phbB* and *phbC* genes and transferred it to cotton plants by the bombardment method. Under the action of endogenous ketothiolase and exogenous acetoacetyl-CoA reductase and PHB

1) Tretheway, R., Metabolic engineering of potato for industrial applications, China-EU Workshop on Manipulation and Agrobiotechnology, October 27—31, 1997.

synthase, transgenic cotton plants accumulated PHB in filament with a yield of 0.34% of dry weight, heat absorbing and releasing rate reduced, and thermal capacity increased. While the filament quality was not affected. This provides an altering method for developing new generation filament products.

(Ⅴ) Production of other PHAs in transgenic plants. PHB is not a perfect material for producing biodergradable plastics. Stiffness and relative brittleness limit its commercial application in some degree. Fox reported that Slater and his collaborators[34] have been trying to search for PHA of better physical properties for more than ten years. Recently they acquired a genetically modified strain of *Ralstonia eutropha*. When fed with propionic acid, it can produce PHBV. PHBV has good flexibility and relative low melting point (130℃). It is more suitable for producing biodegradable plastics than PHB. One substrate for PHBV biosynthesis, β-hydroxyvalerate, comes from propionate (fig. 2), which is of low content in plants. The closest biochemical entity to propionate for this synthetic purpose is the biosynthetic pathway in which several amino acids, including threonine and isoleucine, are produced, and from which intermediate product α-ketobutyrate can be diverted to C5 monomer biosynthesis. They firstly introduced the genes of the artificially assembled pathway into *E. coli*. When the cells were fed with threonine, C5 monomer production was as high as 3.5%, and when the enzyme threonine deaminase was activated, C5 production rose to 8%. These same manipulations also work when the genes are moved into the plastids of *Arabidopsis*. Transgenic *Arabidopsis* plants overproduce α-ketobutyrate, and with production of PHBV up to 0.84% of the dry weight. Padgette *et al.*[35] are doing such research work too. Because *phbA* gene products cannot catalyze propionyl-CoA and acetyl-CoA to form ketovaleryl-CoA efficiently, they cloned another gene encoding ketothiolase——*bktB*. Propionyl-CoA comes from threonine biosynthetic pathway. In plants, threonine is deaminated to form α-ketobutyrate by the action of threonine deaminase, endogenous pyruvate dehydrogenase then catalyze α-ketobutyrate to become propionyl-CoA.

5 Problems and prospects of PHA production in transgenic plants

Although research on PHA production in transgenic plants has made a great progress, PHA yield is still very low, and with very limited varieties. To meet the demands of commercial production, we should not only continue the study on increasing PHA production in transgenic plants, but also pay attention to picking out PHA with better physical properties. This may be considered to set out from the following several respects:

(ⅰ) Avoiding transgene silence as far as possible, and improving the expression stability of PHA biosynthetic genes in transgenic plants. Because of DNA methylation and cosuppression[17], foreign genes are often found to be inactivated in transgenic plants, especially when several genes are transferred into plants simultaneously, such as PHA production in transgenic plants. Under the condition that PHA production is located in seed plastids, seed-specific promoter, transit peptide gene, polyA sequence, resistant gene, and PHA biosynthetic genes should be fused together. If we use the same constructs, most probably it will cause gene silence, then cause low expression of PHA products. It might be practicable that we transfer PHA biosynthetic genes respectively and use different expression construct frames.

(ⅱ) Diverting (D)-hydroxyacyl-CoA from fatty acid metabolic pathway into the biosynthesis of PHA with better physical and processing properties. The intermediate metabolites of fatty acid *de novo* biosynthesis pathway are (D)-hydroxyacyl-ACP. The gene of (D)-hydroxyacyl-ACP——CoA transferrase, by the action of which (D)-hydroxyacyl-ACP is converted to (D)-hydroxyacyl-CoA, should be cloned and introduced to fatty acid *de novo* synthesis pathway if its intermediate metabolites are extracted to produce PHA. Fatty acid β-oxidation pathway also can be used to produce PHA. In doing so, we can introduce fatty acid β-oxidation pathway into the chloroplast, and make the abundant fatty acids in the chloroplast be degraded into (D)-hydroxyacyl-CoA. However, β-oxidation enzyme system in plant peroxisome cannot degrade fatty acids into (D)-hydroxyacyl-CoA, but (L)-hydroxyacyl-CoA, while that of fungi can do. So we can inactivate 3-hydroxyacyl-CoA dehydrogenase of fungi fatty acid β-oxidation pathway to stop the reaction steps after 3-hydroxyacyl-CoA, and introduce thus reformed fungiβ-oxidation pathway into plant chloroplasts. Fatty acids thereby can be degraded into 3-hydroxyacyl-CoA of various chain length, and PHAs are formed finally.

In addition, there exist intact glycolysis pathway and other enzymes in seed plastid. Pyruvate, glucose-6-phosphate, acetate, etc. in cytoplasm can be absorbed and converted to fatty acids through various pathways. Kang's *in vitro* study indicated that these enzymes' activity is beyond that of needed[36]. Over expressing the carrier proteins of these substrates can increase their transport and subsequently the biosynthesis of fatty acids. More substrates of PHA production can be provided.

(ⅲ) Oil body is the storage site for oil crops seed fat. It is formed by phospholipid unilayer wrapping around tri-acylglycerol, with the help of surface-bound protein-oleosin. When tri-acylglycerol is synthesized in endoplasmic reticulum, oleosin inserts into the wrapping phospholipid unilayer to inhibit their cluster and fusion. PHA inclusions also have a unilayer membrane, very similar to oil bodies in structure. If we introduce PHA synthase into the endoplasmic reticulum and let oleosin join the formation of PHA inclusions, there may be oil body-like PHA granules formed. They would exist in cytoplasm only, not penetrating into other organelles. Inhibiting tri-acylglycerol biosynthesis by anti-sense gene technique can increase the concentration of cytoplasmic fatty acids and supply more substrates for PHA biosynthesis. Knutzon *et al.*[37] thus increased the cytoplasmic fatty acids concentration to 25 times higher in transgenic plants than in wild type.

(ⅳ) On the one hand, we always hope to acquire higher PHA yield to reduce producing cost and increase competent ability with petroleum-derived plastics; on the other hand, seed germination is most probably affected by high-content PHA in seed plastids, because PHA cannot be degraded in plants naturally. This problem can be solved by transferring PHA biosynthetic genes into three lines, sterile line, maintainer line and restorer line, respectively. In this way, the hereditary stability of PHA biosynthetic genes can be maintained, and when needed, PHA biosynthetic pathway can be restored in F_1 generation. We may introduce inducible promoters to regulate PHA biosynthetic genes not to express in F_1 seeds, but in F_2 seeds; or introduce inducible PHA depolymerase gene to let F_1 seed be able to use PHA as energy source for seed germination. F_1 heterosis can result in higher PHA yield. At present there have been several chemical-induced promoters. We can use them to control the opening and closing of transgenes quite conveniently. But their practical application has not been reported.

(ⅴ) From the long view, with its far higher yield than that of oil crops, low market price, asexual propagation way, and mature transformation technique, potato tuber is still a very hopeful subject for PHA production. Although presently we do not understand well acetyl-CoA metabolism in potato tuber, and know even less on its regulation, it is very likely that there would be more advantages to produce PHA in potato tubers than in oil crops' seed plastids, if we found out the regulation method on the flux to acetyl-CoA.

Acknowledgement This work was supported by the National Natural Science Foundation of China (Grant No. 59673008) and the State 863 High Technology Program (Grant No. 101-04-03-04).

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(*Received April* 15, 1999)