Bioplastic Production by Transgenic Poplar

David A. Dalton, Cathleen Ma, Ganti S. Murthy, Steven H. Strauss

Plants have long been the source of biopolymers of great economic importance to humans. Starch and cellulose – both biopolymers – have obvious roles as major of sources of food and fiber, respectively. It is increasingly likely that biotechnology can develop plant-based methods for the production of non-traditional biopolymers with profound benefit to humans. Bioplastics such as polyhydroxybutyrate (PHB) are particularly intriguing in this regard. Although plants do not produce PHB, many bacteria do, and plants can be engineered to produce PHB by transfer of the relevant bacterial genes. The ability of transgenic plants to produce PHB was first demonstrated about 20 years ago, and since that time at least 11 additional species of plants have been successfully engineered to do so[1,2]. PHB can potentially partially replace petroleum-based plastics and it has other compelling advantages, including biodegradability and possibly a neutral carbon footprint.

PHB is a polymer of 3-hydroxy butyrate that forms a linear polyester typically 10^3 to 10^4 units in length (**Fig. 1**). Just as with starch, PHB is osmotically and metabolically inert so it can be stored without hazard to the host. Its biosynthesis is a 3-step process starting with acetyl CoA[1]. In the first reaction, two molecules of acetyl CoA condense to form acetoacetyl CoA. In the second reaction the keto group on the number 3 carbon atom is oxidized to a hydroxyl group to form 3-hydroxyl butyryl–CoA. The third and final step involves polymerization via the enzyme PHB synthase.

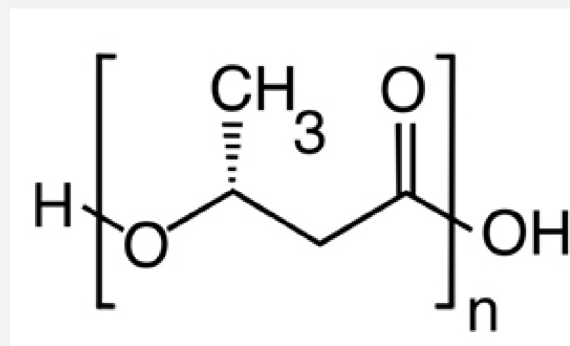

Figure 1. The chemical structure of PHB, which is a repeating polymer of 3-OH butyrate monomers

The precursor for PHB – acetyl CoA – is abundant in plants, which suggests that plants might be good platforms for making bioplastic, once the proper genes are introduced. The flux of acetyl CoA is particularly high in chloroplasts, because this organelle is the site of fatty acid synthesis, which also requires acetyl CoA as the precursor. For several years, the general strategy for creating plants capable of making PHB has used a strong constitutive promoter (usually the cauliflower mosaic virus 35S promoter) along with plastid targeting sequences for each of the three genes of PHB biosynthesis (*phbABC*) derived from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). While this approach has created plants capable of producing PHB, it has not yet resulted in plant systems that show commercial feasibility for production of PHB. The major underlying constraint seems to be that the diversion of resources away from growth and towards the production of PHB reduces plant vigor and yield. For example, in the most extreme example of this problem, Bohmert *et al*. (2000) reported yields as high as 40% (dry weight) in *Arabidopsis*, but the plants were extremely stunted and chlorotic[3]. Even fairly modest yields of PHB (< 3%) can have profound impacts on overall plant productivity.

New approaches for bioplastics production in plants

Recent work in our labs has focused on expanding the range of PHB-producing plants to include poplar (*Populus*)[2]. Poplar is a well-developed model for genetic studies; its high biomass and perennial growth make poplar attractive as a biofactory for products such as PHB, especially if PHB is considered a secondary co-product in addition to fiber or biofuel. A further advantage – mostly speculative – is that it might be eventually possible to target bioplastic production directly to woody tissue (i.e., xylem cell walls), thus creating an entirely new class of material with a novel structural or industrial applications.

We have attempted to minimize the deleterious effects of PHB production on plant vigor by engineering plants in which the genes for biosynthesis of PHB remain silent until expression is induced by addition of a chemical agent. Thus the plants are allowed to reach a size and stage of development at which the diversion of resources to PHB production is potentially less damaging. The control of gene expression through inducing chemicals uses appropriate promoters upstream of one or more of the structural genes for PHB biosynthesis. We chose to use an ecdysone-based system that achieves PHB yields of up to 14.3% in *Arabidopsis*[4]. The inducing agent, known generically as methoxyfenozide, is available commercially from Dow

AgroSciences under the brand name Intrepid®. Among the advantages of this compound is that it is already licensed for field use (as an insecticide) and has limited toxicity to non-target organisms.

The genetic construct for PHB production in transgenic poplar includes a kanamycin resistance marker and all three structural genes for phb biosynthesis (*phbABC*) under control of the ecdysone-based promoter (**Fig. 2**). A plastid-targeting sequence is included for each gene. This construct was introduced into poplar (*Populus tremula* x *alba* clone 717-1B4) using *Agrobacterium*-mediated transformation that is routine for our labs. Following tissue culture, we eventually recovered plants from 49 events in which PCR confirmed the presence of the transgenes for PHB biosynthesis. Of these 49 events, 18 contained detectable levels of PHB (**Fig. 3**) with the highest level (~2%) in events nos. 34 and 397. We also used fluorescence dyes to detect the presence of PHB granules in induced leaves (**Fig. 4**). Control (non-transformed) plants lacked these fluorescent granules. For quantification, PHB was first converted to the butyl ester form of 3-OH butyrate monomers and then quantified by gas chromatography. Typical chromatograms are also shown in **Fig. 4**.

Metabolic tradeoff studies

We selected the two strongest events (nos. 34 and 397) for detailed studies to determine the metabolic expense of PHB production. These studies also addressed the possible direct phytotoxicity effect of Intrepid. We determined that a concentration of 0.5 to 1 mM Intrepid is sufficient to obtain maximum induction. Statistical analyses of growth parameters revealed that there is no direct toxicity from Intrepid unless the concentrations exceeded 20 mM – a concentration far above that required for induction.

Production of PHB is a metabolic expense that causes reduced growth. This expense most likely explains our inability to recover viable plants when we used the constitutive 35S promoter to drive expression of the *phb* genes (unpublished data). This negative impact of PHB production is typified by a decline in volume index (diameter2 x height), a frequently used means of representing cumulative growth in trees (**Fig. 5**). We also observed negative effects of PHB production on plant mass (dry weight) and on chlorophyll fluorescence (an indicator of plant stress). Plants that produced more than 1% PHB (dry weight in leaves) grew 20 to 30% less than those that produced less than 1% PHB.

Extraction of PHB

Several extraction methods for PHB recovery have been developed. These involve centrifugation, filtration, extraction with organic solvents (chloroform and methanol), treatment with sodium hypochlorite, and digestion with enzymes. These methods are targeted for extraction of PHB from bacterial cells, as historically most of the research has been focused on microbial PHB production, which is already a commercial success. Extraction from plant leaves is more problematic due to the lower concentrations of PHB and the presence of many interfering substances, such as chlorophyll. Recently we used a modified solvent sequential extraction method to extract the PHB from genetically modified hybrid poplar leaves[5]. The process involved grinding leaves to a fine mesh, extracting with ethanol to remove chlorophyll, and then extracting the leaf residue with chloroform to recover the PHB. In addition, it is possible to quantify extracted PHB with a spectrophotometric method based on the conversion of PHB into crotonic acid by H_2SO_4. This method is quicker and less technically demanding than other methods based on gas chromatography that have been previously used in plant studies.

Although the technical feasibility of PHB production and extraction have been demonstrated, economic viability, process scalability, and safety of the process are equally critical for commercial success. There is a need to develop additional extraction methods that are specifically optimized for PHB extraction from plant biomass.

Figure 2. T-DNA region of PHB expression cassette used to transform poplar. Pnos, promoter for nopaline synthase; NPTII, kanamycin resistance gene; NT, terminator for nopaline synthase gene; 35S, promoter for 35S cauliflower mosaic virus; GRvH, glucocorticoid response element; greMP, minimal promoter with a glucocorticoid response element binding site; TS, plastic targeting sequence; phbABC, genes for biosynthesis of PHB.

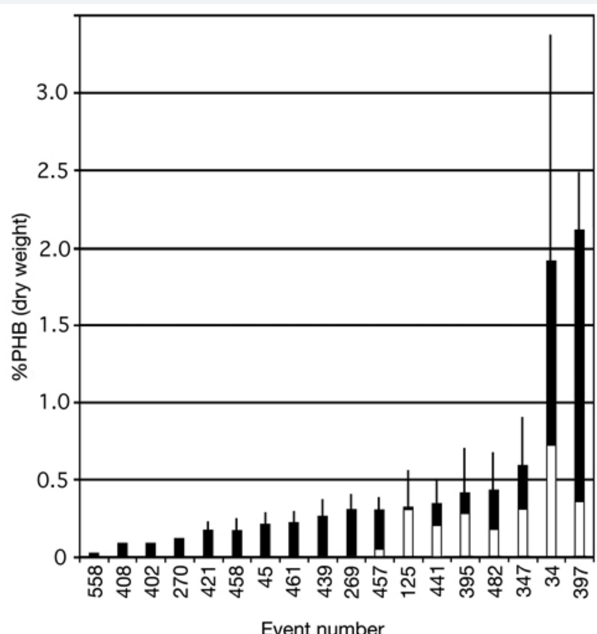

Figure 3. Concentration of PHB in poplar leaves after 7–8 weeks of induction with 10 mM Intrepid. Open bars represent the levels in noninduced (no Intrepid) plants. Noninduced levels in weak events (nos. 558–269 from left to right) were not determined. Each value is the mean of 4–6 replicates +1 standard error of the mean (reproduced from Dalton et al., *Plant Biotechnol J* 9:1-9, 2011)

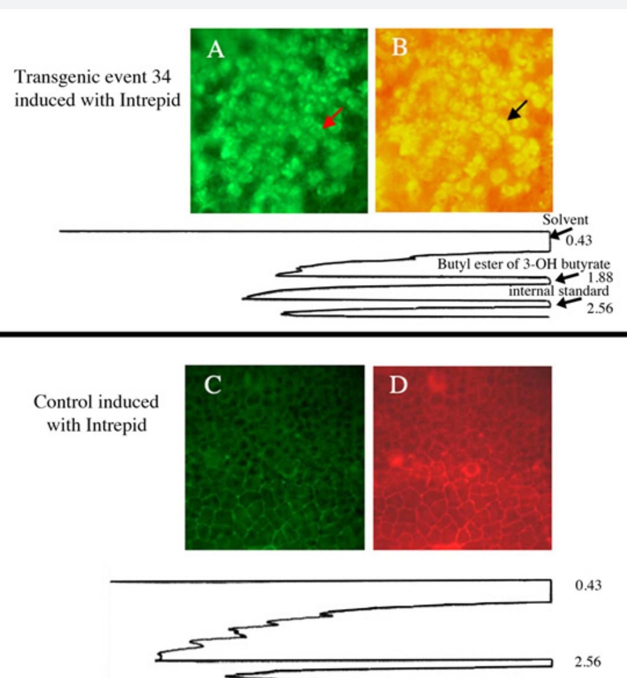

Figure 4. Demonstration of PHB in poplar leaves. Microphotographs are epifluorescence micrographs of poplar leaves stained with Nile Blue A. A and C; excitation wavelength of 450-490 nm. B and D; excitation wavelength of 510-560nm. PHB granules are evident as fluorescence granules (arrows) within the chloroplasts of the transgenic event. The chromatogram beneath each photo confirms the presence of the derivatized form of PHB in the transgenic event and its absence in the control. Numbers on the chromatograms indicate retention times in minutes.

Future directions

While yields of 1–2% are encouraging, these levels fall considerably short of commercial goals of 12.5%, which may be necessary if PHB production in poplar is to be commercially feasible as a stand-alone product[5]. We are engaged in further studies aimed to increase yield by using native promoters associated with senescence to see if postponement of PHB biosynthesis until leaf senescence begins might enable high levels of production with acceptable effects on plant growth and health.

Transcriptome studies in our laboratory, extending the work by Andersson *et al.*[6], have identified dozens of genes with very strong upregulation during the early stages of senescence, when leaves still appear green. These might be useful for triggering senescence-associated biosynthesis. However, many questions still remain about the feasibility of this system. Will the senescing chloroplasts still be able to support PHB biosynthesis? Will the PHB remain intact as general cellular contents are degraded and readied for transport into stems and leaves? Will there be adequate levels of PHB produced? And can economic systems for leaf collection and processing,

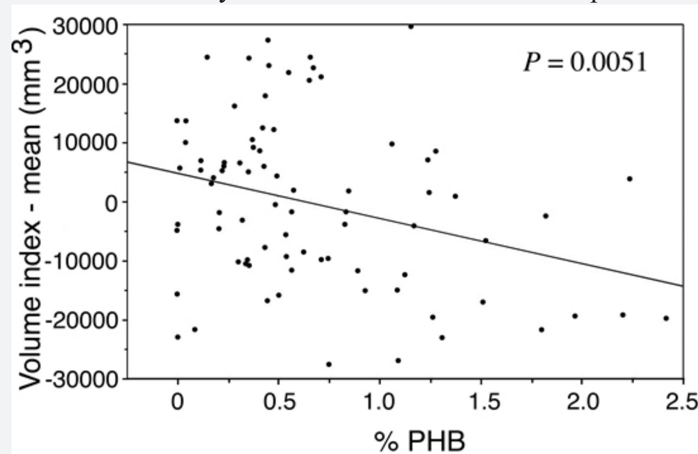

Figure 5. Negative correlation between PHB content of leaves and volume index (diameter2 x height) following 6 weeks of induction with four different concentrations of Intrepid. Each point is a single observation from an individual plant corrected for event (group effect) by subtracting the mean value for that event. (reproduced from Dalton et al., Plant Biotechnol J 9:1-9, 2011)

perhaps as part of coppice biomass harvest, be developed? As a first step, we have begun to test candidate promoter strength and specificity. If yields can be increased as hoped, then we will address the remaining issues related to harvest and extraction.

References

1. van Beilen JB, Poirier Y. Production of renewable polymers from crop plants. *Plant J* 54:684-701 (2008).
2. Dalton DA, et al. Trade-offs between biomass growth and inducible biosynthesis of polyhydroxybutyrate in transgenic poplar. *Plant Biotechnol J* 9:1-9 (2011).
3. Bohmert K, et al. Transgenic Arabidopsis plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. *Planta* 211:841–845 (2000).
4. Kourtz L, et al. Chemically inducible expression of the PHB biosynthetic pathway in Arabidopsis. *Transgenic Res* 16:759–769 (2007).
5. Murthy GS, et al. Extraction analysis of poly-β-hydroxybutyrate (PHB) from hybrid poplar leaves. ASABE Paper No. 1009380. ASABE, St.Joseph, MI. (2010)
6. Andersson A, et al. A transcriptional timetable of autumn scenescence. *Genome Biol* 5 R24.1-R24.13 (2004)

David Dalton
david.dalton@reed.edu
Biology Department, Reed College, Portland OR 97202 USA

Cathleen Ma
caiping.ma@oregonstate.edu
Steven Strauss
steve.strauss@oregonstate.edu
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR, USA

Ganti Murthy
murthy@engr.orst.edu
Department of Biological and Ecological Engineering, Oregon State University, Corvallis, OR, USA