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Trade-offs between biomass growth and inducible biosynthesis of polyhydroxybutyrate in transgenic poplar

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Summary

Polyhydroxybutyrate (PHB) is a bioplastic that can be produced in transgenic plants by the coexpression of three bacterial genes for its biosynthesis. PHB yields from plants have been constrained by the negative impacts on plant health that result from diversion of resources into PHB production; thus, we employed an ecdysone analogue–based system for induced gene expression. We characterized 49 insertion events in hybrid transgenic poplar (*Populus tremula x alba*) that were produced using *Agrobacterium* transformation and studied two high-producing events in detail. Regenerated plants contained up to 1–2% PHB (dry weight) in leaves after 6–8 weeks of induction. Strong induction was observed with 1–10 mM Intrepid and limited direct toxicity observed. Confocal fluorescence microscopy was used to visualize PHB granules in chloroplasts after chemical treatment to reduce autofluorescence. A greenhouse study indicated that there were no negative consequences of PHB production on growth unless the PHB content exceeded 1% of leaf weight; at PHB levels above 1%, growth (height, diameter and total mass) decreased by 10%–34%.

Introduction

Polyhydroxybutyrate (PHB) is a polyester that is produced by bacteria such as *Ralstonia*, *Azotobacter*, *Rhizobium* and many others. PHB consists of monomers of four carbon atoms and functions to store energy and carbon in an osmotically inert form, much as plants use starch for the same purpose. PHB is one example of the polyhydroxyalkanoate (PHA) class of polyesters that hold potential as a source for biodegradable and sustainable plastic to replace petroleum-based products. Plants do not produce PHAs naturally, but a number of species, beginning with *Arabidopsis* in 1992, have been genetically engineered to do so using the genes of the biosynthetic pathway from bacteria (Poirier *et al.*, 1992). Since then, at least 10 additional species of plants have been successfully engineered to produce PHB (van Beilen and Poirier, 2008; Somleva *et al.*, 2008).

The production of PHB in plants requires the introduction of transgenes from bacteria that can be introduced by standard transformation methods. The biosynthesis of PHB requires only three genes (*phbABC*). Yields vary considerably between plant species, with the most productive systems being targeted to plastids, presumably because of the large pool of acetyl CoA from which PHB is synthesized. Although PHB yields of up to 40% (w/w dry weight) have been observed in *Arabidopsis* (Bohmert *et al.*, 2000), these high yields are accompanied by severe reduction in growth such that the gross amount of PHB produced is small.

Considerable research has been directed towards exploring this technology, and successful commercial applications based on bacterial fermentation have already reached the market (e.g. http://www.metabolix.com). However, this approach is limited by the high cost of bacterial fermenters fuelled by large supplies of carbon substrates. These problems could be avoided by development of crop plants capable of producing PHB if the restrictions of low yield and growth retardation can be overcome. Additional benefits for PHB-producing crops would accrue if the plants were capable of producing coproducts, such as biomass for electricity or biofuel (e.g. cellulosic ethanol). In this regard, the recent report of PHB yields up to 3.7% (dry weight) in switchgrass (*Panicum virgatum*) is promising (Somleva *et al.*, 2008).

The potential for woody bioenergy crops to produce PHB has been little studied. Poplar (*Populus* spp.) is particularly promising because it is already cultivated under intensive management systems in many parts of the world and is widely regarded as the model system for functional genomics and biotechnology in woody plants. Poplars produce high biomass yields, often 9–16 Mg/ha/year in the Pacific Northwest USA (personal communication, Brian Stanton Greenwood Resources Inc.). The trees can be grown in plantations with 5–7 year cycles in which all above ground material is harvested and a new crop is allowed to resprout from roots—a practice known as coppicing. Unlike many woody plants, poplars are easy to transform, regenerate and propagate. Genomic resources in support of poplar biotechnology and scientific research are extensive and include large EST, genome and short-read sequence databases, as well as extensive microarray expression data (reviewed by Brunner *et al.*, 2004; Jansson and Douglas, 2007).

PHB production in transgenic plants is commonly achieved with the use of strong constitutive promoters such as the widely employed cauliflower mosaic virus 35S promoter. While this system provides for strong expression of transgenes, it provides no means of coordinating expression with the developmental stage of the plant to minimize negative effects on plant growth. It may be preferable for plants to devote resources to vegetative growth early in their development and then divert

metabolites to PHB production only once sufficient biomass and reserves of carbon have been obtained. This could be achieved by use of an inducible promoter in which at least one of the three necessary biosynthetic genes (*phbABC*) is not expressed until the appropriate inducing signal is applied. One of the most successful inducible promoters is the ecdysone-inducible promoter, which was initially employed in mammalian cells (No *et al.*, 1996), but has subsequently been used in a number of plant species (reviewed by Padidam, 2003; Wang *et al.*, 2003). The advantages of an ecdysone-based induction system include low toxicity to plants and nontarget organisms, full induction below phytotoxicity levels, efficient transport throughout the plant and the availability of commercial inducing agrochemicals that have been approved for field application to crops. Such an ecdysone-inducible system has been successfully used in *Arabidopsis* to produce plants capable of yielding up to 14.3% PHB per unit dry weight (Kourtz *et al.*, 2007). We report here the results from use of this system in transgenic poplar and the effects of induction on biomass productivity.

Results

Initial screening

A total of 2834 poplar explants were cocultivated with *Agrobacterium* of which 192 eventually produced shoots and roots in tissue culture. PCR confirmed that 49 of these plants contained the transgenes for PHB biosynthesis. In the initial screening for PHB, all of these events were screened for PHB content after 7–8 weeks of induction with 10 mM Intrepid. (Experiments to determine the optimal level of Intrepid for induction of gene expression are reported below.) A total of 18 of these events contained detectable levels of PHB (Figure 1). The highest levels of PHB were observed in events numbered 34 (1.9%) and 397 (2.1%); these were therefore selected for analysis of trade-off studies. For both of these events, the PHB content under noninducing conditions was approximately 20%–37% of that observed under inducing conditions (Figure 1).

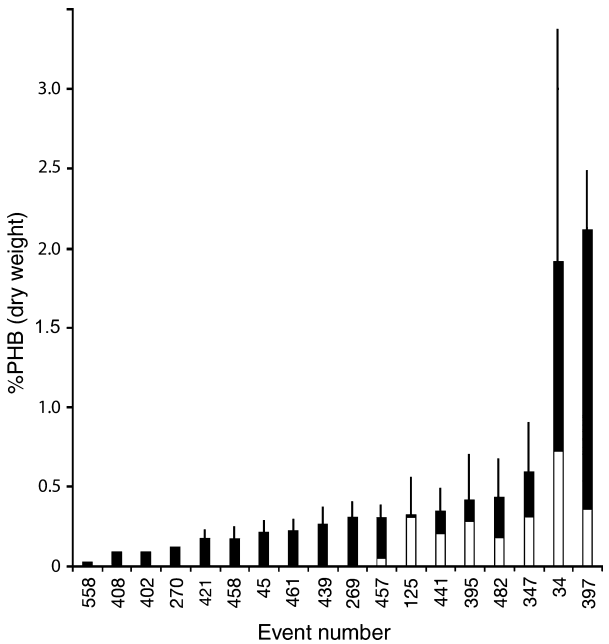

Figure 1 Concentration of PHB in poplar leaves from all events that contained detectable levels after 7–8 weeks of induction with 10 mM Intrepid. Open bars represent the levels in noninduced (no Intrepid) plants of the same age and event, but different ramets. Noninduced levels in weak events (nos. 558–269 from left to right) were not determined. Each value is the mean of 4–6 replicates (i.e. 4–6 individual plants or ramets from each event) +1 SEM (Standard Error of the Mean).

Temporal and spatial variation of PHB content

A detailed analysis of the time course of PHB content in leaves was conducted for four events: one weak (no. 270), one moderate (no. 125) and two strong (nos. 34 and 397, Figure 2). Induction was achieved by biweekly application of 10 mM Intrepid, and PHB content of leaves was determined at 1-week intervals up to a total of 8 weeks. PHB levels in events nos. 270 and 125 reached maximum levels by week no. 6 and then declined slightly. PHB content of strong events (nos. 34 and 397) continued to increase to the end of the experiment, although there was no statistical difference in the levels between weeks nos. 7 and 8. After 6–8 weeks of induction, the amount of PHB in leaves from 25 plants generated from event 34 varied between 0% and 2.5%, while it varied between 0% and 2.9% for 21 plants derived from event 397. The variation among ramets from a single transformation event is intriguing. It may be that slight differences in growth conditions could account for this despite the random block greenhouse design and twice-weekly rotation of position of individual pots. Variation in responses among ramets in poplar is often great. In the present case, this could be because of experimental error in PHB determination, plant physiology, concentration of inducer at point of sampling and response to inducer. Gene silencing among ramets is very rare in poplar and is not associated with gene copy number, so is not a likely cause (Li *et al.*, 2009). Stability of transgenes in poplar as well as spatiotemporal variation of expression has been reviewed by Brunner *et al.* (2007).

Repeated sampling of individual plants revealed that the levels in any one plant (leaves from the middle or bottom of canopy) did not vary greatly, whereas there were strong variations between individual plants and events. Maximum levels of PHB were observed at the highest concentration (10 mM) of Intrepid (Figure 3). PHB levels were 93% of the maximum when 1.0 mM Intrepid was used, and this amount was not significantly different from the value observed at 10 mM.

PHB content was less in younger leaves (top third of canopy, Table 1). There was no difference in PHB content between leaves of the middle and lower canopy positions. Similarly, there was no difference in PHB content within any single, mature leaf blade. Petioles contained about 48% of the PHB concentration found in leaf blades. No PHB was detected in bark (cambium) or stem wood that had been pulverized by a Wiley mill followed by butanol/HCl derivatization (event 34 only studied, $n = 3$ stems following induction with 10 mM Intrepid).

Visualization of PHB granules

Chloroplasts and cell walls in poplar leaves have strong autofluorescence, and therefore, we cleared the leaf samples in ethanol and sodium hypochlorite to visualize the fluorescently stained PHB. The agglomerations of PHB were specifically stained with Nile blue A after extraction of chlorophyll and bleaching of the leaves and were visualized in the palisade and spongy mesophyll in the events 34 and 397 but not in the control (Figure 4). In samples that were not treated with sodium

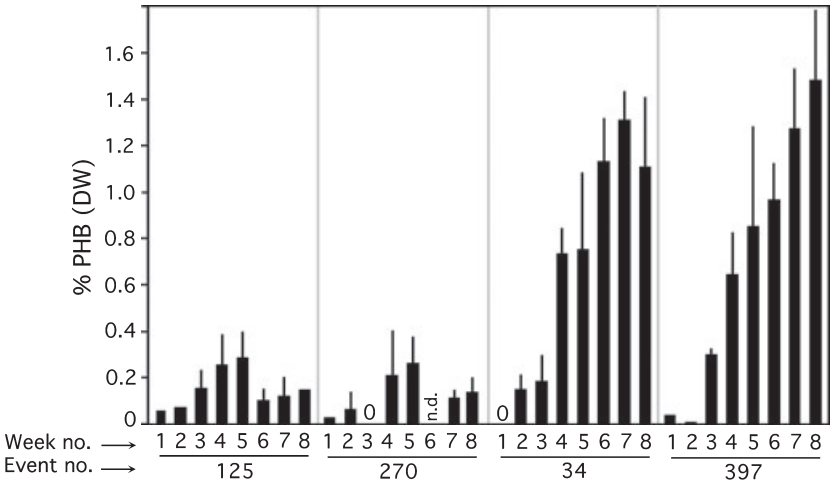

Figure 2 Time course of PHB content of poplar leaves of four events following induction with 10 mM Intrepid. At each time point, a single leaf was collected from the mid-canopy position. Each value is the mean of 4–8 replicates (i.e. 4–8 individual plants or ramets from each event) +1 SEM n.d., not determined.

hyperchlorite, fluorescence signals from chloroplasts in the red, blue and green spectra were detectable in all controls and transgenic plants, but no specific signal from PHB could be distinguished. Three-dimensional (3-D) movie of PHB localization in palisade mesophyll cells of event 34 can be seen online (Movie S1).

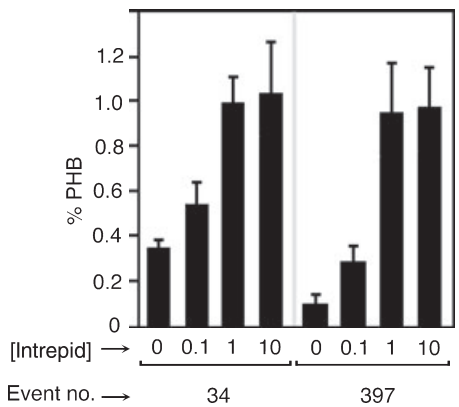

Figure 3 Effects of concentration of Intrepid on PHB content of poplar leaves following 6 weeks of bi-weekly application. Values for 1 and 10 mM Intrepid were not significantly different ($P = 0.87$). Data are presented as the mean of 12 replicates (i.e. 12 individual plants representing four ramets from each event) ±1 SEM.

Table 1 Concentration of PHB in poplar leaves from three positions in the canopy at 7–8 weeks after induction

Event no.	Position	Mean ± SE	Maximum
34	Top	0.341 ± 0.24	0.584
	Middle	1.356 ± 0.31	2.270
	Bottom	1.267 ± 0.247	1.813
397-1	Top	0.997 ± 0.412	2.475
	Middle	2.567 ± 0.454	3.690
	Bottom	1.565 ± 0.254	2.307
397-2	Top	0.473 ± 0.094	0.668
	Middle	0.943 ± 0.150	1.340
	Bottom	0.849 ± 0.109	0.957

All values are % (w/w dry weight). Leaves were sampled individually without pooling, with 3–6 replicates for each position. Numbers after the hyphen indicate ramet number. $n = 3–6$.
PHB, polyhydroxybutyrate.

The absence of a PHB signal outside of the chloroplast stroma strongly suggests that PHB is synthesized within the chloroplasts, as expected because of the presence of plastid targeting sequences in our genetic constructs. The absence of PHB granules in the epidermis (Figure 4c) is consistent with this interpretation because epidermal cells lack chloroplasts.

The size of the chloroplasts (length/width 5.49/1.69 μm; SD = 0.41/0.24; $n = 30$) was statistically different from the size of the PHB agglomerations (3.6 μm; SD = 1.12; $n = 30$) as visualized in single optical sections (one-way ANOVA; $F_{2,87} = 219.4339$; $P < 0.0001$, Figure S2).

Effect on plant growth and development

Growth parameters (height, diameter, volume index (height × diameter^2), dry weight (leaves, stem, roots—separately and totalled), chlorophyll fluorescence (F_v/F_m) and stem wood density were analysed to determine the effects of PHB production on plants (Figure 7 and Figure S3). Because there was no statistical difference in the slopes of the PHB/growth trait regressions when events 34 and 397 were compared (data not shown), the data from the two events were pooled for further analyses. However, because the events did differ in their average growth for most of the traits (Table 2, Figure 6), we used standardized values in graphical representations (i.e. event means were subtracted from individual plant values). Although the effect of Intrepid level on growth was not statistically significant (Table 2), because of its negative trend with most growth traits as Intrepid levels were increased (Figure 7 and Figure S3), we also included it as a covariable in some analyses and graphical summaries to separate its possible effect on growth from that of PHB production itself.

Nearly all of the growth parameters were statistically significantly ($P < 0.05$) and strongly negatively correlated with PHB content (Table 3, Figure 7). The only exceptions were leaf mass, which was also negatively correlated but had a P value of 0.085, and stem wood density, which showed no evidence of statistical association. When Intrepid level was added to the regression model, the negative relationship with all of the previously significant growth traits persisted, but was reduced in statistical strength. The regression coefficient remained significant

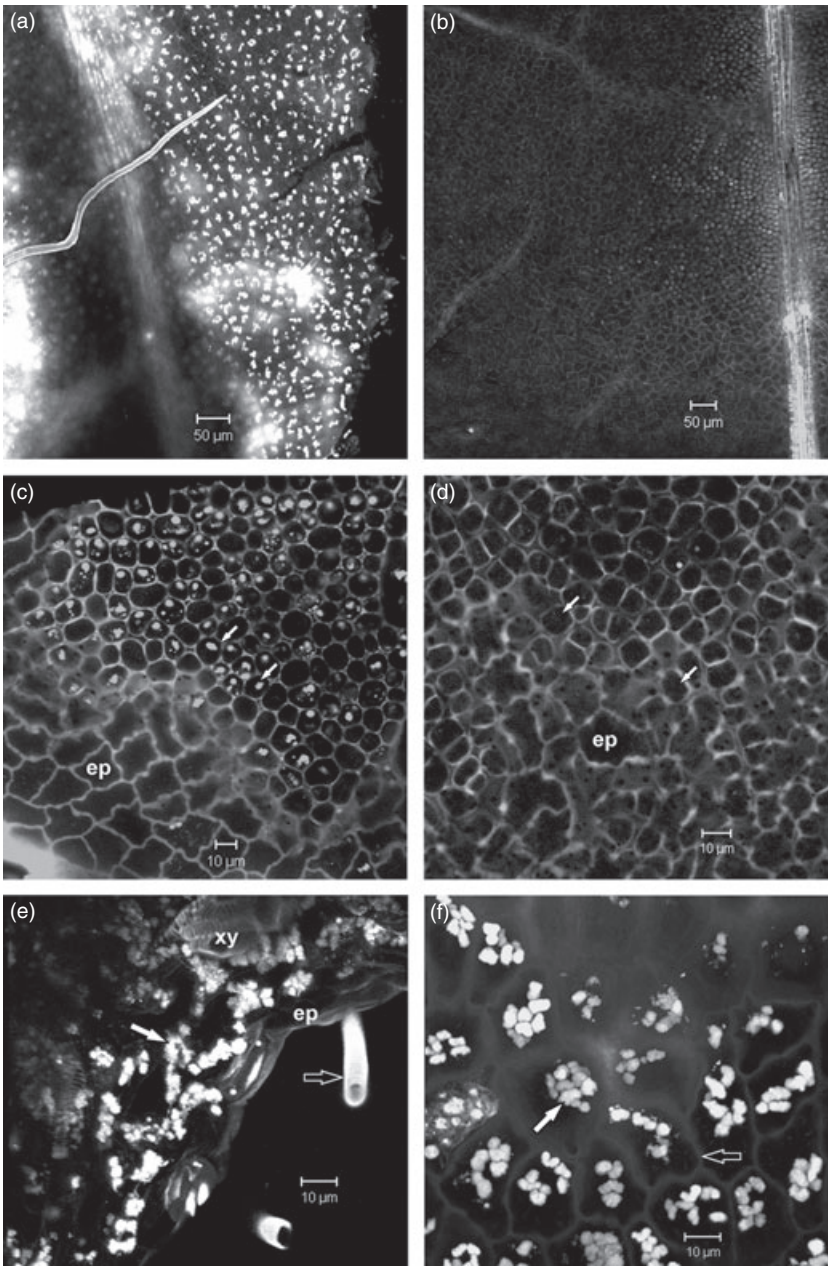

Figure 4 Leaves of transgenic and control plants visualized by confocal microscopy. (a) and (b) Optical sections (z resolution, approximately 7.5 µm) parallel to the leaf blade just below the adaxial epidermis of event 34 (a) and control (b). PHB granules are seen in the mesophyll of the event 34 but not in the control. (c) and (d) Oblique optical sections (z resolution, approximately 1.0 µm) through the adaxial epidermis (ep) and palisade mesophyll (arrows) of event 397 (c) and control (d). PHB granules (bright dots) are seen in the palisade mesophyll of the event 397 but not in the control. The complete series of optical sections from the samples in (c) and (d) are shown in Figure S1. Close-up views of PHB granules in the spongy and palisade mesophyll of event 34. (e) A cross section at the abaxial epidermis (ep) showing PHB granules (white arrow), trichome (black arrow) and xylem elements of a leaf vein (xy). The image in (e) is a maximum projection of 36 optical sections at 0.39-µm intervals. (f) A section parallel to the leaf blade showing PHB granules in the palisade mesophyll (white arrow). Epidermal cells (black arrow) are visible below the plane of the PHB granules. The cell walls of the palisade mesophyll are not visualized in this image because of their considerably lower intensity of fluorescence compared to the PHB granules. The image in (f) is a maximum projection of 20 optical sections at 0.39-µm intervals. Objective lens, Plan-Neofluar 10×/0.3 (a) and (b); Plan-Neofluar 40×/1.3 Oil DIC (c); Plan-Apochromat 63×/1.4 Oil DIC (c, e) and (f).

below the 0.05 probability level for diameter, volume index, root mass, total mass and chlorophyll fluorescence, but exceeded this probability level for height, leaf mass, stem mass and shoot mass.

Graphical analysis (Figure 7) suggested that a PHB content above 1% presented an approximate threshold above which negative effects on growth accrued; when only those plants with a PHB content of <1% were analysed, there was no statistical relationship between PHB content and growth parameters except for chlorophyll fluorescence ($P = 0.0431$). When the PHB content exceeded 1%, there were reductions of 26% in volume index, 34% in root mass and 32% in total plant mass,

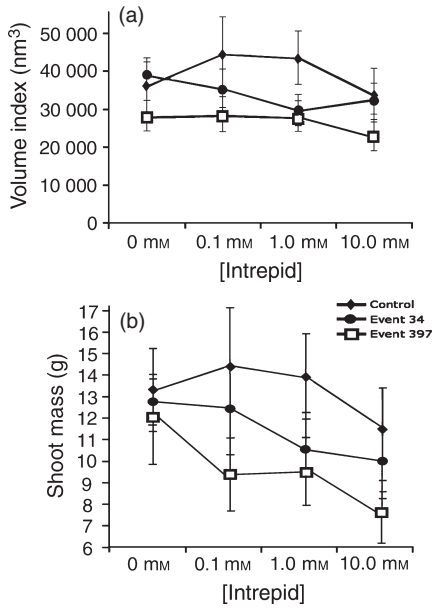

Figure 5 Volume index (a) and shoot mass (b) of all plants following 6 weeks of induction with various concentrations of Intrepid. There were no significant differences between treatments. Event 397 had values significantly lower than the control for both parameters. Data are presented as means of 12 replicates (i.e. 12 individual plants representing four ramets from each event) ± 1 SEM.

Table 2 Summary statistical analyses of the growth/PHB trade-off experiment when analysed by one-way ANOVA for group (event number) and inducer treatment (Intrepid concentration) effects

Response variable	Treatment (inducer) *P* value	Treatment (inducer) significant trend	Group (event) *P* value	Group significant trend
Height	0.8057	None	0.0341	Cont > event 397
Diameter	0.3753	None	0.0070	Cont > event 397
			0.0175	event 34 > event 397
Volume index	0.55633	None	0.0018	Cont > event 397
Chlorophyll fluorescence	0.5009	None	0.8204	None
Leaf mass	0.5808	None	0.0135	Cont > event 397
Stem mass	0.2680	None	0.0035	Cont > event 397
Root mass	0.7101	None	0.0163	Cont > event 397
Stem wood density	0.7026	None	0.4252	None

PHB, polyhydroxybutyrate.

compared to plants in which the PHB content was <1% (Table 3). Controlling for Intrepid effects did not substantially alter these decreases. Detailed responses of growth parameters grouped by treatment [(Intrepid)] can be seen in Figure S3.

Discussion

The ecdysone-inducible promoter was shown to be an effective means of elevating PHB production in transgenic poplar, with induced yields of up to 2.4% PHB (w/w dry weight). Yields in this range, however, were obtained with only two transgenic lines out of a total of 49 PCR-verified events, and both of these also had significant background (noninduced) expression (Figure 2). Several additional lines were generated that appeared to have a lower proportion of noninduced expression, but were not analysed in depth because their PHB yields were small (data not shown; a reporter gene study of this ecdysone induction and its background expression is currently being prepared for publication). Nonetheless, because strong production of PHB was largely postponed until application of the inducing agent, the plants all looked very healthy, having had a chance to grow and become well established before its resources, especially acetyl CoA (the precursor of PHB biosynthesis), was diverted away from normal growth.

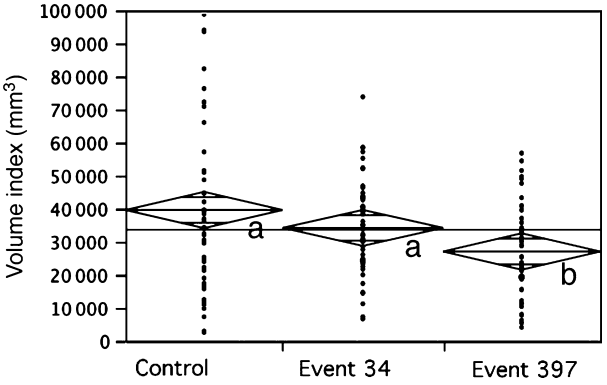

Figure 6 Volume index of all plants regardless of Intrepid treatment at 6 weeks after beginning induction. The horizontal line that extends fully across the figure is the grand mean of all data. The line in the centre of diamonds is the group mean. The upper and lower horizontal lines within the diamonds are 95% confidence interval limits. Unmatched letters indicate significant differences ($P < 0.05$).

The two high PHB yielding events studied differed in most growth traits, but did not differ in their response to inducer (Table 2). Thus, it is likely to be a somaclonal or gene insertion effect that is not directly related to the production of PHB itself. Such effects, although rarely reported for poplar (Strauss *et al.*, 2004), are not uncommon in the plant transformation literature and are not surprising given the very large size of the multigene insert employed.

The procedures we developed for clearing and bleaching of leaf tissue along with the application of confocal microscopy were successful in providing high-quality images of PHB granules in leaves. Staining with Nile blue A has been previously used for fluorescence imaging of PHB in bacteria by Ostle and Holt (1982), in transgenic tobacco leaves (Nakashita *et al.*, 2001), in cotton fibres (John and Keller, 1996) and in alfalfa (Saruul *et al.*, 2002). Two-dimensional images by transmission electron microscopy of thin sections of chloroplasts in transgenic *Arabidopsis*, cotton, alfalfa and flax revealed PHB granules with diameters much smaller than 1 μm (Nawrath *et al.*, 1994; John and Keller, 1996; and Saruul *et al.*, 2002; Wróbel *et al.*, 2004). Therefore, the PHB granules in our samples of poplar leaves that were visualized by confocal microscopy, which averaged 3.6 μm in size, might be agglomerations of smaller PHB particles. Further improvement of the method of imaging of PHB, possibly by the use of both confocal and electron microscopy, would be needed for revealing the actual size of individual PHB particles that are synthesized in poplar leaves.

Table 3 Relationship of induced PHB content to growth traits under two regression models, A and B (see first two columns). Leaves were sampled individually without pooling with 3–6 replicates for each position

Parameter	(A) *P* value for PHB effect with % PHB as explanatory variable (Regression coeff., SE)	(B) *P* value for PHB effect with [Intrepid] & PHB % as explanatory variables (Regression coeff., SE)	Mean for all plants	Mean for plants with <1% PHB in model A	Mean for plants with >1% PHB in model A	% decrease if PHB >1% vs. <1% in model A	% decrease if PHB >1% when [Intrepid] included (model B)
Height (cm)	0.046 (–5.400, 2.66)	0.093 (–5.04, 2.96)	62.51	63.84	57.38	10.1	9.6
Diameter (mm)	0.006 (–0.644, 0.229)	0.021 (–0.593, 0.253)	6.69	6.87	6.06	11.8	11.0
Volume index (mm^3)	0.016 (–6660, 2700)	0.048 (–6110, 3040)	30 459	32 635	24 147	26.0	24.7
Leaf mass (g)	0.085 (–1.017, 0.584)	0.149 (–0.940, 0.645)	6.44	6.71	5.35	20.3	20.3
Stem mass (g)	0.023 (–1.09, 0.471)	0.065 (–0.959, 0.512)	4.57	4.88	3.35	31.4	31.2
Root mass (g)	0.024 (–0.767, 0.334)	0.046 (–0.747,0.369)	3.07	3.29	2.16	34.2	36.8
Shoot (leaf + stem) mass (g)	0.022 (–2.43, 1.04)	0.137 (–1.72, 1.15)	9.679	10.893	8.089	25.7	24.8
Total mass (g)	0.015 (–3.35, 1.35)	0.042 (–3.02, 1.46)	14.11	15.22	10.42	31.5	30.9
Chlorophyll fluorescence	<0.001 (–0.020, 0.0056)	<0.001 (–0.0224, 0.0062)	0.733	0.736	0.713	3.1	3.7
Wood density (g/cm^3)	0.355 (–0.0136, 0.0146)	0.658 (–0.00709, 0.0160)	0.327	0.328	0.321	2.1	0.9
PHB (%)	–	–	0.659	0.394	1.495	–	–

PHB, polyhydroxybutyrate.

Even after statistical removal of the effect of inducer, there was clear evidence that PHB production had substantial impacts on most aspects of plant growth, with the exception of stem wood density (Table 2). Plants that produced more than 1% dry weight of PHB in leaves over several weeks of induction often grew one-quarter to one-third less than those that produced <1% PHB. This effect might be a result of interference with photosynthesis or other aspects of chloroplast function. F_v/F_m values decreased significantly in correlation with PHB content (Table 3, Figure 7c), and its *P* value of 0.0007 was the strongest of any in this experiment. F_v/F_m is a measure of the maximum quantum efficiency of Photosystem II photochemistry and is widely considered to be a reliable indicator of biotic and abiotic stress in ecophysiological studies (Mohammed *et al.*, 1995). This suggests that production of PHB in poplars led to stress conditions that directly affected chloroplast function and photosynthesis and was thus the indirect cause of the reduced growth that we observed.

The growth reductions we observed are in accord with prior reports of the effects of PHB production in plants. For example, yields as high as 40% have been reported for *Arabidopsis* when driven by a constitutive 35S promoter, but the resulting plants were severely stunted and the yields in terms of total mass correspondingly slight (Bohmert *et al.*, 2000). More typically, yields as low as 3% have been reported to result in a chlorotic and stunted phenotype (Bohmert *et al.*, 2000, 2002). In our laboratory, despite cocultivation and intensive selection of more than 2600 explants, we have been unable to recover viable transformants when using a 35S promoter to drive PHB production in poplar even though our construct (pBI ABC) was identical to that used by Bohmert *et al.* (2000) for *Arabidopsis*. This suggests that constitutive expression during plant regeneration may be lethal in poplar.

Economic models indicate that PHB contents must be at least 7.5% in switchgrass (Somleva *et al.*, 2008) and 12.5% in poplar (Murthy *et al.*, 2010) to be economically feasible. Thus, further improvements to PHB yield, and perhaps also in reduced impacts on growth, are likely to be necessary if this system is to be commercially useful. An Intrepid concentration of 1 mM appears to be optimal in poplar because this gave expression levels comparable to 10 mM (Figure 3). Concentrations higher than 1 mM should be avoided if possible because of possible negative growth effects, greater nontarget organism impacts and higher cost. Further experiments are needed to examine if lower concentrations might also be effective, such as the 0.5 mM levels used by Kourtz *et al.* (2007). Induction performance under field conditions also needs evaluation, as it could vary substantially compared to that in the greenhouse because of differences in plant physiology and chemical uptake.

Several additional lines of research are desirable for future development of PHB production in poplar. Other promoters should be examined, especially if promoters can be identified that provide for stronger expression with minimal impact on plant health. In this regard, our group is evaluating the potential of promoters associated with leaf senescence such that PHB production might be delayed until late in the growing season, when photosynthesis is diminishing. Although early work clearly showed that localization to chloroplasts was more desirable than a cytoplasmic location (Nawrath *et al.*, 1994), there are other possibilities that should be examined, such as the recent attempt to localize PHB production in peroxisomes (Tilbrook *et al.*, 2010). Although we were unable to find measurable quantities of PHB in woody tissues, if PHB could be directed to storage cells within woody tissues, this problem might be overcome. In addition, promoters that direct expression to developing xylem cells could lead to the production of wood fibres with modified properties, such as was shown for transgenic PHB-producing flax (Wróbel-Kwiatkowska *et al.*, 2007). Finally, we note that other sources of the *phb* genes might be more desirable. Almost all of the PHB-producing plants developed to date have been based on the genes from *Ralstonia* (previously *Alcaligenes*) originally described by Peoples and Sinskey (1989). A systematic screening of genera of bacteria for PHB production was conducted more than 15 years ago to optimize commercial production in bacteria (Lee, 1996), but to our knowledge such a screening has not been undertaken to identify promising *phb* genes for use in transgenic plants.

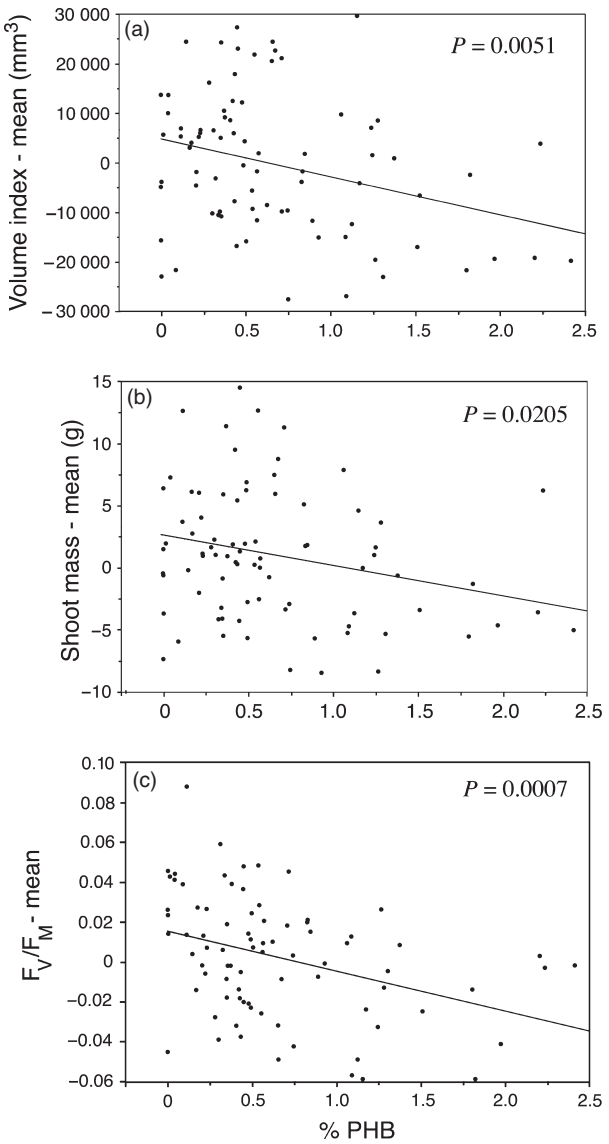

Figure 7 (a) Negative correlation between PHB content of leaves and volume index (a), shoot mass (b) and chlorophyll fluorescence (c) of all plants from events nos. 34 and 397 following 6 weeks of induction with four different concentrations of Intrepid. Each point is a single observation from an individual plant corrected for event (group effect) by subtracting the mean value for that event. Equation for linear fit: (a) y = 4711−7575x; (b) y = 2.586−2.435x; (c) y = 0.0150−0.0199x.

Experimental procedures

Plants and bacteria

Hybrid poplar (*Populus tremula x alba*) clone 717-1B4 was maintained under tissue culture or greenhouse conditions as described elsewhere (Filichkin *et al.*, 2005). Plants were transformed with *Agrobacterium tumefaciens* strain AGL1 by cocultivation of internodal stem segments (3–4 mm in length) or leaf discs (4 mm diameter) with selection for kanamycin resistance (Han *et al.*, 2000). Following selection, regeneration, PCR verification and propagation, plants were transferred to 6-cm diameter pots containing potting soil (Sunshine Professional Growing Mix, Sungro, Bellevue, WA) and maintained under a 16 h photoperiod with temperatures of 25–27 °C days and 19–21 °C night. Each plant was generated by micropropagation from separate ramets in tissue culture and thus represents replicates from the same transformation event. The plants were supplied twice weekly with 50 mL of a fertilizer solution consisting of 20-10-20 NPK (400–500 ppm). Once plants had achieved a height of approximately 30–50 cm (about 3–4 months after transferring to pots), induction was initiated by application of 50 mL 1.0 mM Intrepid® (methoxyfenozide; Dow AgroSciences, Indianapolis, IN, USA). An identical solution of Intrepid was also applied as a foliar spray to uniformly cover all foliage to the point where excess solution began to drip off. The application of inducing chemical was repeated twice weekly for up to 4 weeks. Those plants were then transferred into 15-cm diameter pots, grown for 4 months and then subjected to another round of induction. The second induction was executed by applying 200 mL of 10 mM Intrepid to each pot for root drench and also foliar spray (as above). Application was repeated twice weekly for 8 weeks.

Following an initial screening of all events, two events (numbers 34 and 397) were identified as most promising based on PHB content. These events, along with a nontransformed control, were selected for analysis of growth/biosynthesis trade-offs to determine the consequences of PHB production and exposure to Intrepid. New plants were generated and grown in the greenhouse as before. After 3 months, the plants had achieved a height of approximately 20 cm and were transferred to 10-cm diameter pots. These plants were allowed to acclimate to the new pots for 2 weeks, at which time induction began. The experiment consisted of a randomized block × 4 design with each block consisting of 12 trees: one tree of each of three genotypes (control, event 34, event 397), and four levels of Intrepid (0, 0.1, 1.0 and 10 mM). Intrepid was applied twice weekly by root drench (200 mL per pot) and a saturating leaf spray for a total of 6 weeks. The treatments also included 0.05% Tween 20 as a surfactant. Controls (0 mM Intrepid) consisted of an aqueous solution of 0.05% Tween 20. To accommodate the plants' rapid growth, all were transferred to 20-cm diameter pots after 4 weeks.

PHB-growth trade-off measurements

Measurements were taken of plant height and diameter (1 cm above crown) at time 0 and weeks 2, 4 and 6. An index of final volumetric growth of the tree stem (mm^3) was calculated using the formula: height × diameter^2. As an indication of stress and photosynthetic capacity, chlorophyll fluorescence (F_v/F_m) was also measured at the same times as above using a portable fluorometer (Opti-Science model OS1-FL; Opti-Sciences, Inc., Hudson, NH, USA). Determinations of final dry weight were made by harvesting at week 6, separating root, stem and leaves, and oven drying overnight at 60 °C. To calculate stem wood density, 30-cm sections of dried stems were submerged in water and the volume of displaced water measured.

Vectors

The binary vector pCAM(greCgreAgreB) was the kind gift of Kristi Snell (Metabolix, Kourtz *et al.*, 2007). This vector contains expression cassettes for all three of the genes for PHB biosynthesis (*phbABC*) under control of the glucocorticoid receptor response element minimal promoter gre MP (Martinez *et al.*, 1999). Each cassette also included a plastid targeting sequence and the nos 3′ terminator of the nopaline synthase gene (Bevan

et al., 1983). 'The vector used for PHB synthesis was described in detail by Kourtz *et al.* (2007). Each cassette for the genes of PHB biosynthesis (*PHBABC*) contained an upstream glucocorticoid minimal response promoter and a plastid targeting sequence that codes for the signal peptide of the small subunit of Rubisco from pea followed by the initial 24 amino acid residues of the mature protein. Each cassette also contained the *nos* 3′ terminator of nopaline synthase from *Agrobacterium*.

Expression of the gene for the chimeric ecdysone receptor GRvH was under control of the double enhanced promoter e35S. This receptor is composed of the hinge and binding domains of the ecdysone receptor from tobacco budworm (*Heliothis virescens*), and the transactivation domain of the *Herpes simplex* VP16 protein fused to the DNA binding domain and transactivation domain of a mammalian glucocorticoid receptor (Martinez *et al.*, 1999). This system is capable of inducing reporter gene activity up to 420-fold in transgenic tobacco (Martinez *et al.*, 1999) and has also been shown to be effective in controlling production of PHB in transgenic *Arabidopsis* (Kourtz *et al.*, 2007).

Staining of PHB

A novel procedure was developed that featured removal of chlorophyll followed by bleaching to reduce autofluorescence. Prior to the preparation of samples for microscopy, we examined the solubility of PHB (Aldrich, St Louis, MO, USA) in ethanol and sodium hyperchlorite. PHB powder and small granules were immersed for prolonged periods of time (more than 24 h) in ethanol, and 6% or 12% sodium hyperchlorite solution and no changes of the quantity or appearance of the PHB were noticed. Leaf discs were cut from controls and from selected transgenic events 34 and 397 that had higher concentrations of PHB and were stored in glycerine/30% ethanol (1 : 3 v/v). The leaf discs were further cut into 200- to 300-μm-thick sections or 1-mm narrow segments, then passed through an ethanol series (25%, 50%, 75% and 96%) and stored in 100% ethanol until the leaf segments completely lost their green colour because of extraction of chlorophyll. Then, the leaf segments were bleached with 6% sodium hyperchlorite for 10 min for reduction in the autofluorescence of cell walls and cellular membranes. After washing several times in distilled water, the leaf segments were stained with Nile blue A (1% aqueous) at 55 °C for 10 min. After staining, the leaf segments were washed several times in distilled water and immersed in 8% acetic acid for 10 min. Finally, the leaf segments were washed again in distilled water, immersed in water or glycerine and observed by wide-field epifluorescence or confocal microscopy with a LSM 510 META microscope (Carl Zeiss Microimaging Gmbh, Oberkochen, Germany) using 488, 514 and 543 laser lines, and emission filter LP 560.

Measurement of PHB

A protocol for gas chromatographic determination of the butyl ester derivative of PHB was developed by modifying the method described by Kourtz *et al.* (2007). Plant material was harvested, lyophilized and stored at −80 °C. Unless otherwise stated, only mature leaves (middle or bottom of canopy) were used. Samples were weighed into aliquots of 40–80 mg and placed into microfuge tubes with petioles and mid-veins excluded. Eight hundred microlitres of extraction solvent (90% 1-butanol and 10% concentrated HCl) was added to each tube and then quick frozen by submersion in liquid N_2. The samples were ground with a small plastic pestle attached to an electric drill until the extract had thawed, and the tissue was thoroughly macerated. The contents of each tube were transferred to a 20-mL round-bottom flask. An additional 2.0 mL of extraction solvent was used to rinse the microfuge tubes and to transfer the sample residue to the round-bottom flask. The samples were refluxed gently for 60 min to convert the PHB into the butyl ester of 3-OH butyrate. The derivatized sample was transferred to 15-mL plastic centrifuge tube. Additional extraction solvent was added to adjust the final volume to 3.0 mL, and 3.0 mL of aqueous 5% Na_2CO_3 was added to neutralize the acid. The tubes were shaken briefly, vented to relieve pressure and centrifuged at 1000 ***g*** for 5 min to facilitate phase separation. The upper (organic) phase was used directly for analysis with a Hewlitt Packard model 5890 gas chromatograph equipped with a 6 ft × 1/8 inch stainless steel column of 10% Rtx-1 100/120 silcoport (Restek, Bellefonte, PA, USA). The column temperature was held at 150 °C for the first 1.5 min and then increased to 270 °C at 45° min^{-1}. The injector and detector temperature was 200 °C. Diphenyl methane (0.1%) was included in the extraction solvent as an internal standard, and reagent grade PHB (Aldrich) was used as an authentic standard. Retention times were 14.9 min for the butyl ester of 3-OH butyrate and 21.0 min for the internal standard. This was well separated from the peaks for solvent and for unidentified compounds from leaf samples. Authentic PHB that was refluxed along with leaf tissue consistently produced a peak of the expected retention time and area.

Slight corrections were made for traces of an unidentified compound that matched the retention time for the butyl ester derivative that was present in leaves of the control (nontransformed) plants and in leaves of native *Populus trichocarpa* collected from the field. This correction amounted to about 5% (less in leaves younger than 6 weeks) of the levels of PHB observed in events 34 and 397. This unidentified peak was not present in solvent blanks, but was detected when chloroform rinses of fresh intact leaves of control plants were processed through the butanol/HCl derivatization procedure. This suggests that the compound may be a derivative of cutin degradation.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 PHB granules (red dots) in the mesophyll of event 397.

Figure S2 A single optical section by confocal microscopy showing a close-up view of chloroplasts in the mesophyll of transgenic event 34.

Figure S3 Growth parameters of all plants following 6 weeks of induction with various concentrations of Intrepid.

Movie S1 Three-dimensional movie showing a close-up view of PHB agglomerations in the mesophyll of transgenic event 34.

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