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Polyhydroxybutyrate synthesis in Camelina: Towards coproduction of renewable feedstocks for bioplastics and fuels

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Summary

Plant-based co-production of polyhydroxyalkanoates (PHAs) and seed oil has the potential to create a viable domestic source of feedstocks for renewable fuels and plastics. PHAs, a class of biodegradable polyesters, can replace conventional plastics in many applications while providing full degradation in all biologically active environments. Here we report the production of the PHA poly[(*R*)-3-hydroxybutyrate] (PHB) in the seed cytosol of the emerging bioenergy crop *Camelina sativa* engineered with a bacterial PHB biosynthetic pathway. Two approaches were used: cytosolic localization of all three enzymes of the PHB pathway in the seed, or localization of the first two enzymes of the pathway in the cytosol and anchoring of the third enzyme required for polymerization to the cytosolic face of the endoplasmic reticulum (ER). The ER-targeted approach was found to provide more stable polymer production with PHB levels up to 10.2% of the mature seed weight achieved in seeds with good viability. These results mark a significant step forward towards engineering lines for commercial use. Plant-based PHA production would enable a direct link between low-cost large-scale agricultural production of biodegradable polymers and seed oil with the global plastics and renewable fuels markets.

Introduction

One of the most interesting classes of biomaterials are the polyhydroxyalkanoate (PHA) family of microbial biopolymers (Madison and Huisman, 1999; Suriyamongkol *et al*., 2007) that have unique properties depending on their monomer unit composition. PHAs are a class of polyesters produced by a biological polymerization process and are accumulated by some microbes as a carbon storage and energy reserve under nutrient-limiting conditions. The subsequent intracellular or extracellular consumption of PHAs by microbes upon the return of favourable growth conditions provides for their natural biodegradability in many environments (Madison and Huisman, 1999; Snell and Peoples, 2009; Suriyamongkol *et al*., 2007). A wide range of monomers can be incorporated into PHAs (Steinbuchel and Valentin, 1995) which most often consist of short-chain (3–5 carbon units) and/or medium-chain-length (6–14 carbon units) monomers. Polymers that have been produced in engineered bacteria by fermentation for commercial purposes include the homopolymers poly[(*R*)-3-hydroxybutyrate] (PHB) and poly(4-hydroxybutyrate) (P4HB), and copolymers poly[(*R*)-3-hydroxybutyrate-co-4-hydroxybutyrate] (PHB-4HB), poly[(*R*)-3-hydroxybutyrate-co-(*R*)-3-hydroxyvalerate] (PHBV) and poly[(*R*)-3-hydroxybutyrate-co-(*R*)-3-hydroxyhexanoate] (Koller and Mukherjee, 2022). Varying the monomer composition of PHAs yields materials with a range of properties such that suitable replacements for many petroleum-derived plastics can be obtained. Besides plastics, PHAs can also be used in medical applications (Chen and Zhang, 2018; Koller, 2018; Koller and Mukherjee, 2022; Lim *et al*., 2017), converted by thermolysis to small molecule (Kang and Yu, 2015; Mamat *et al*., 2014; Mullen *et al*., 2014) precursors to commodity chemicals (Schweitzer *et al*., 2015; Schweitzer and Snell, 2015), and used in wastewater treatment applications (Chu and Wang, 2016; Hiraishi and Khan, 2003; Wang and Chu, 2016). In the latter, degradation of PHAs by denitrifying bacteria supplies reducing power for reactions that convert nitrates and other nitrogen containing species to inert nitrogen gas (Hiraishi and Khan, 2003). PHAs produced by bacterial fermentation have successfully accessed markets in high-value medical applications, but the high capital and operating costs of fermentation have so far prohibited their widespread use in commodity applications. Regardless of the end-of-life environmental benefits enabled by biodegradability, cost remains the key factor for broad adoption of new products.

Engineering plants to produce PHAs enables polymer production from CO_2 at the capital efficiency and scale of agriculture. Extensive work has been performed to produce PHB in leaf chloroplasts (Poirier and Brumbley, 2010; Snell *et al*., 2015;

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Somleva *et al.*, 2013; Van Beilen and Poirier, 2012) by co-expressing genes encoding plastid-targeted β-ketothiolase (PhaA), NADPH-dependent reductase (PhaB) and PHA synthase (PhaC) enzymes to convert plastidial acetyl-CoA to polymer (Figure S1; Snell *et al.*, 2015). High level production of PHB in chloroplasts has been achieved, with up to ~40% dry cell weight PHB achieved in *Arabidopsis thaliana* (Bohmert *et al.*, 2000), but this has often been met with stunted plant phenotypes when significant levels (>3% dry cell weight) are produced (Bohmert *et al.*, 2000, 2002, 2004; Somleva *et al.*, 2013). Prior work in oilseeds has targeted PHB synthesis to seed plastids (Houmiel *et al.*, 1999; Malik *et al.*, 2015; Valentin *et al.*, 1999) by co-expressing genes encoding plastid-targeted PhaA, PhaB, and PhaC enzymes to divert acetyl-CoA from fatty acid biosynthesis to produce polymer. Up to 7.7% of the seed fresh weight was produced in seed plastids of *Brassica napus* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999) and up to 15.2% (mature seed weight) was obtained in *Camelina sativa* (Malik *et al.*, 2015). The high levels achieved in Camelina, however, resulted in chlorotic cotyledons, poor emergence and low-seedling survival (Malik *et al.*, 2015).

Despite the challenges, production of PHAs in oilseeds can be advantageous since it simplifies logistics and storage of the harvested product prior to processing, and would coproduce revenue generating seed oil and protein rich meal in a biorefinery (Snell and Peoples, 2013). In this study, we chose to investigate PHB production in the cytosol of seeds to determine if moving PHB production away from the developing photosynthetic machinery in the seed embryo plastid to the cytosol would yield seeds with high levels of PHB and good seedling emergence. PHB was chosen as the target with the goal of enabling production of a cheap source of homopolymer that could be blended with other biopolymers to create materials with improved properties (Koller and Mukherjee, 2022; Li *et al.*, 2016). Prior work with PHB production in the cytosol has been limited to leaves and has resulted in the production of low levels of polymer (Poirier *et al.*, 1992b) with significantly reduced fresh plant weight (Poirier *et al.*, 1992a,b; Snell *et al.*, 2015; Somleva *et al.*, 2013). To our knowledge, cytosolic production of PHB in seeds has not been previously reported. The highest reported level of cytosolic PHB produced in any organ to date is 0.34% dry weight (DW) PHB produced in cotton fibres (John and Keller, 1996). *C. sativa*, an annual plant from the Brassicaceae family, was chosen as the host for our work since its seeds contain high levels of oil and the plant can thrive in marginal growth conditions (Eynck *et al.*, 2013). Camelina also does not cross-pollinate with *B. napus* (FitzJohn *et al.*, 2007), an important edible oilseed crop. In addition to expressing the PHB biosynthesis pathway in the seed's cytosol, a key element of our strategy was to modify the last enzyme of the PHB biosynthetic pathway, PHA synthase (PhaC), with a C-terminal sequence that has been previously shown to tail-anchor (Abell and Mullen, 2011) recombinant proteins to the cytosolic face of the endoplasmic reticulum (ER) (Barbante *et al.*, 2008). Seed PHB levels, the viability and health of seedlings, and the partitioning of carbon between polymer, seed oil and seed protein are described.

Results

Seed-specific cytosolic PHB production in Camelina seeds

Camelina oil contains multiple fatty acids of chain length ≥ 20 carbon units (Figure S2) that are formed by elongation of plastid-exported fatty acids using malonyl-CoA as a two-carbon donor and an ER-associated, multi-enzyme fatty acid elongase complex (Huai *et al.*, 2015; Joubès *et al.*, 2008; Li-Beisson *et al.*, 2010). Since malonyl-CoA is derived from acetyl-CoA, targeting the PHB biosynthetic pathway to the cytosol could allow the capture and conversion of a portion of the carbon designated for fatty acid elongation to polymer (Figure 1). A base transformation construct (pMBXS394) for testing cytosolic production of PHB in seeds was constructed with a gene encoding β-ketothiolase (PhaA) (Peoples and Sinskey, 1989) expressed from a seed-specific promoter from the soybean glycinin-1 gene (Iida *et al.*, 1995), and genes encoding an NADPH-dependent reductase (PhaB) (Peoples and Sinskey, 1989) and a hybrid PHA synthase (PhaC) (Kourtz *et al.*, 2005), each expressed from the seed-specific promoter from the soybean gene encoding oleosin isoform A (Rowley and Herman, 1997) (Figure S3A). These promoters have previously been used in our labs to produce high levels of PHB (15.2% mature seed weight) in plastids of Camelina seeds (Malik *et al.*, 2015). A cassette for constitutive expression of DsRed, a red fluorescent protein from the *Discosoma* genus of coral (Matz *et al.*, 1999) previously used as a visual marker to select transgenic Camelina seeds by several groups (Lu and Kang, 2008; Malik *et al.*, 2015, 2018), was also included. *Camelina sativa* line 10CS0043 (abbreviated WT43), a large seeded line previously used for plastid-targeted PHB production (Malik *et al.*, 2015), was transformed with genetic construct pMBXS394 using an *Agrobacterium*-mediated *in planta* transformation system (Lu and Kang, 2008). First-generation (T_1) seeds of putative transformed lines were identified by visualization of DsRed, sterilized, and germinated on MS medium containing sucrose. This strategy has previously allowed the rescue of seedlings obtained from high plastid-targeted PHB producing Camelina seeds that were compromised in their vigour (Malik *et al.*, 2015). After plating, 79% of pMBXS394 DsRed positive seeds germinated and formed seedlings on this media.

T_1 seedlings were transferred to soil and all plants grew normally, were healthy, and set seeds with a growth phenotype similar to WT43. A sample of DsRed-positive T_2 seeds was picked from the segregating seed population and used to determine PHB content using a simultaneous extraction and butanolysis procedure followed by gas chromatography (GC) (Kourtz *et al.*, 2007; Malik *et al.*, 2015). Second-generation (T_2) seeds from 56 of the 63 T_1 lines analysed produced detectable levels of PHB (Table S1). The highest PHB level obtained was 4.5% of the mature seed weight (Figure 2a; Table S1), a level significantly higher than the maximum levels of PHB previously achieved in the cytosol of a plant (0.34% DW in cotton fibres (John and Keller, 1996)). T_2 seeds that contained PHB levels ≥2% (mature seed weight) were germinated in soil, and the emergence and survival of each line was determined (Figure 2b). Lines were found to have varying levels of survival with the two highest polymer producing pMBXS394 lines (4.5% and 4.2% PHB in mature seed) possessing survival rates of 33% and 93%, respectively. After germination, most PHB-producing lines developed cotyledons that were visually approximately 30% narrower compared with the WT43 control at BBCH growth stage 10 (Martinelli and Galasso, 2011) but were otherwise green and healthy (Figure 3a,b). Analysis of the structural differences in cotyledons by light microscopy revealed that the typical expansion of cells observed in the palisade layer in WT43 controls (Figure 4a,b) was not visible in cotyledons of pMBXS394 PHB producing lines (Figure 4c,d). In addition, intercellular spaces were significantly reduced, if not absent, in spongy and palisade mesophyll of cotyledons of

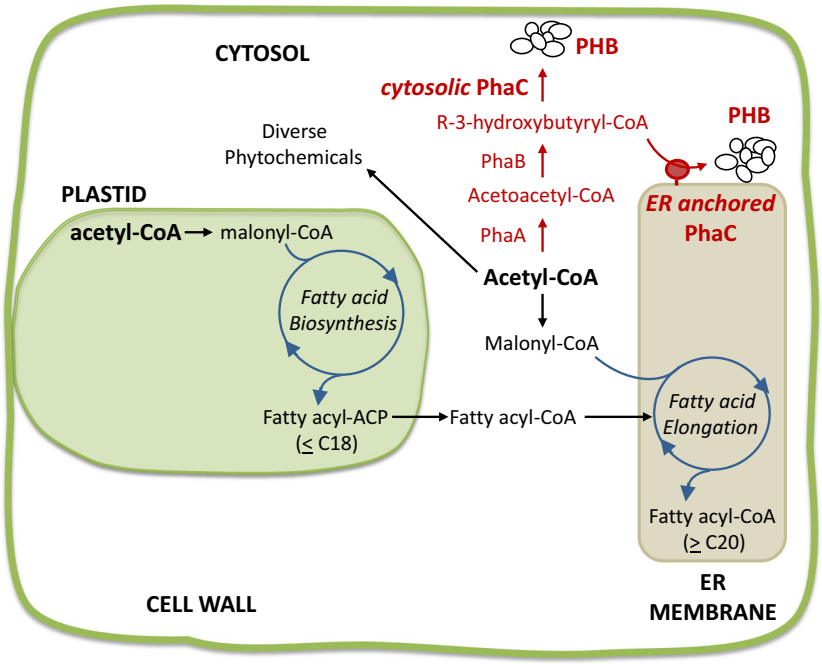

Figure 1 Strategies for cytosolic PHB production in developing seeds. Cytosolic PHB production using a three-enzyme pathway of β-ketothiolase (PhaA), NADPH-dependent reductase (PhaB), and PHA synthase (PhaC) captures a portion of the acetyl-CoA that could otherwise be used for fatty acid elongation or synthesis of an array of diverse phytochemicals. Strategies in which PhaC, the enzyme polymerizing substrate R-3-hydroxybutyryl-CoA to polymer, is targeted to the cytosol or anchored to the cytosolic face of the ER membrane are shown.

PHB-producing lines. Depending on the line, the development of seedlings from PHB-producing seeds was delayed 1–5 days compared to the WT43 control.

An endogenous depolymerase enzyme capable of degrading PHB is not known to be present in plants, therefore granules of PHB formed in the cotyledon tissue of the embryo are expected to be present in the seedling. Examination of thin sections of cotyledon tissue by transmission electron microscopy (TEM) confirmed the presence of granules in the cytosol and vacuoles of pMBXS394 cotyledons (Figure S5).

Targeting PHA synthase to the ER

In an attempt to increase polymer levels beyond the 4.5% PHB obtained with transformation construct pMBXS394, we developed an additional genetic construct (pMBXS763) in which the PHA synthase (PhaC) was fused at its C-terminus to a sequence from the *Arabidopsis thaliana* cytochrome B5 isoform D protein that has been previously shown to anchor recombinant proteins to the cytosolic face of the ER (Barbante *et al.*, 2008). Use of this sequence for ER-targeting of the Nef (human immunodeficiency virus protein Nef/negative factor) improved accumulation of the recombinant protein in transgenic tobacco, possibly by increasing the protein's stability and/or making it less susceptible to degradation. Construct pMBXS763 was prepared to stabilize the PhaC enzyme for improved PHB synthesis. The resulting plasmid pMBXS763 (Figure S3B) was transformed into WT43 and DsRed-positive T_1 seeds were isolated. Up to 4.9% PHB was obtained with T_2 seeds (Figure 2c). Detectable levels of PHB were produced in seeds of 74 of the 77 T_1 lines analysed (Table S1). The emergence and survival of the PHB-producing lines varied, with some lines producing >4% PHB with good survival (Figure 2c,d, Line 12-0978, 4.43% PHB, 74% survival; Line 12-0939, 4.39% PHB, 86% survival). Like lines transformed with pMBXS394, cotyledons of T_2 seedlings of pMBXS763 were narrower than those of WT43 (Figure 3c). When examined by light microscopy, the cell structures of the pMBXS763 cotyledons were similar to those obtained with pMBXS394 (Figure 4g,h) and TEM analysis confirmed that PHB was present in the vacuoles and cytoplasm of cotyledons (Figure S5E,F).

Spatial distribution of PHB granules, oil, and protein bodies in seeds

Ultra-thin sections of imbibed pMBXS394, pMBXS763 and control WT43 seeds from the cotyledon region were analysed by TEM to examine the spatial distribution of PHB, and oil and protein bodies. Granules of PHB were observed to be scattered throughout the cytosol in T_2 seeds from pMBXS394 and pMBXS763 (Figure 4k,l,o,p) but were absent in control WT43 seeds (Figure 4i,j,m,n). An ordered structure of oil bodies localized around protein storage vacuoles containing protein bodies was visible in WT43, whereas T_2 seeds from pMBXS763 line 12-0933, containing 4.9% PHB, had visibly fewer oil bodies than the control (Figure 4).

Polymer production in advanced generations

T_2 seeds of the most promising PHB-producing lines with one or two copies of T-DNA inserts and good survival were propagated for additional generations to produce homozygous lines. In lines of pMBXS394, PHB levels dropped from a high of 4.5% PHB in T_2 seeds to a high of 2.9% PHB in T_3 seeds (Table 1). In contrast, PHB production in lines of pMBXS763 was generally stable and in some instances higher than the previous generation. Homozygous lines of pMBXS763 were isolated that produced up to 6.8% and 9.1% PHB in T_3 seeds (Table 1). Homozygous lines were much easier to isolate from lines transformed with pMBXS763 and due to the instability of PHB production in pMBXS394 lines, only pMBXS763 lines were advanced to the next generation (see Figure S5.

T_3 homozygous pMBXS763 lines with one or two copies of the T-DNA insert were chosen for further propagation in 10-inch pots in a greenhouse or controlled environmental chamber. The chamber was set with variable conditions to simulate changes in temperature and day length that plants would encounter in fields around Saskatoon, SK, Canada if planted in early May and

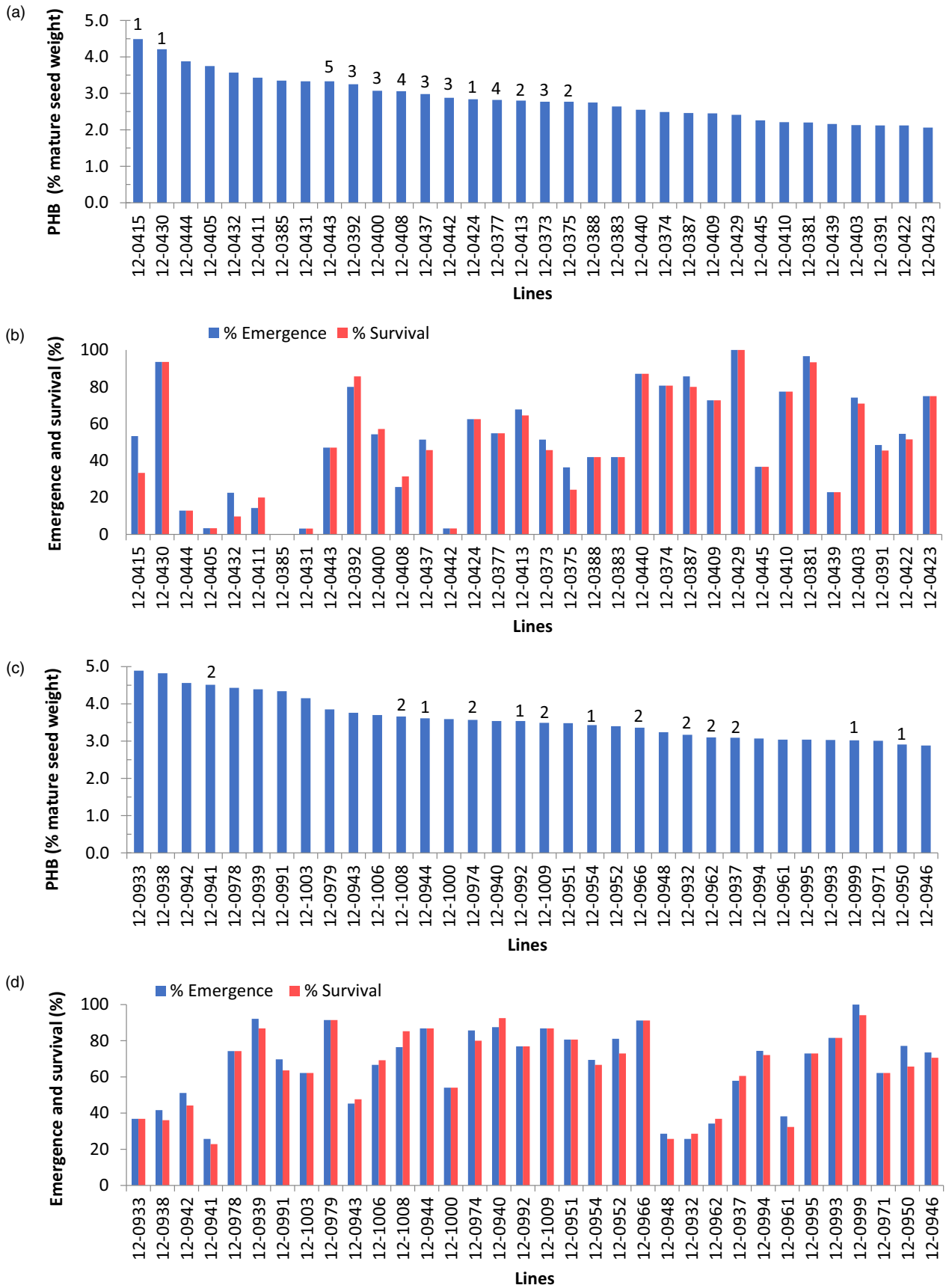

Figure 2 T_2 seed PHB content and T_2 seedling emergence and survival of select lines obtained from transformations of WT43 with pMBXS394 and pMBXS763. (a) PHB content in T_2 seeds of WT43/pMBXS394. (b) Emergence and survival of T_2 seedlings of WT43/pMBXS394. Lines producing ≥2% PHB in seeds were tested for emergence and survival. (c) PHB content in T_2 seeds of WT43/pMBXS763. 56 lines had ≥2% PHB, only the highest producing 34 lines are shown. (d) Emergence and survival of T_2 seedlings of WT43/pMBXS763. Select T_1 events obtained from pMBXS394 and pMBXS763 transformations were analysed for insert copy number by Southern blot analysis (Figure S4). Where analysed, the insert copy number of the line is shown above the PHB content bar in (a) and (c). Plants without a number were not tested.

Figure 3 Phenotype of *C. sativa* WT43 control and T_2 seedlings obtained from transformations of WT43 with pMBXS394 and pMBXS763. Seeds were germinated in soil, and seedlings were photographed at the fully expanded cotyledon stage with first true leaves emerging (BBCH stage 10, Martinelli and Galasso, 2011). Seedlings of (a) *C. sativa* WT43 control, (b) pMBXS394 T_2 line 12-0415 (4.5% PHB) and (c) pMBXS763 T_2 line 12-0939 (4.4% PHB) are shown.

harvested in late July (Table S2). T_3 siblings derived from the same T_2 lines performed differently when grown in the greenhouse than in the chamber. In general, higher yields of polymer were obtained in the controlled environmental chamber than in the greenhouse (Table 2) with maximum PHB levels obtained in T_4 seeds of line 12-0992, which produced up to 7.1% and 10.2% of the mature seed weight in the greenhouse and growth chamber, respectively. The average PHB production of this line was 5.2 ± 1.0% (n = 8) and 7.0 ± 2.4% (n = 4) in the greenhouse and growth chamber, respectively (Table 2).

T_4 seed yield per plant obtained from chamber grown T_3 plants often approached two times the yield obtained from greenhouse-grown siblings derived from the same T_2 line. Maximum levels of seed produced per plant were 18.6 ± 2.1 g (SD) for descendants of pMBXS763 transformed T_1 line 12-0944 in the controlled environmental chamber, whereas siblings produced 8.6 ± 2.1 g (SD) of seed in the greenhouse. In some lines, a decrease in average seed yield was observed upon PHB production but the difference was not statistically significant compared to the wild-type control (Table 2).

Characterization of polymer produced in T_4 seeds

Polymer in seeds harvested from select homozygous T_3 lines of pMBXS763 was isolated and characterized by gel permeation chromatography and ^{1}H NMR to verify that the material was high molecular weight PHB. For ^{1}H NMR experiments, crushed seed

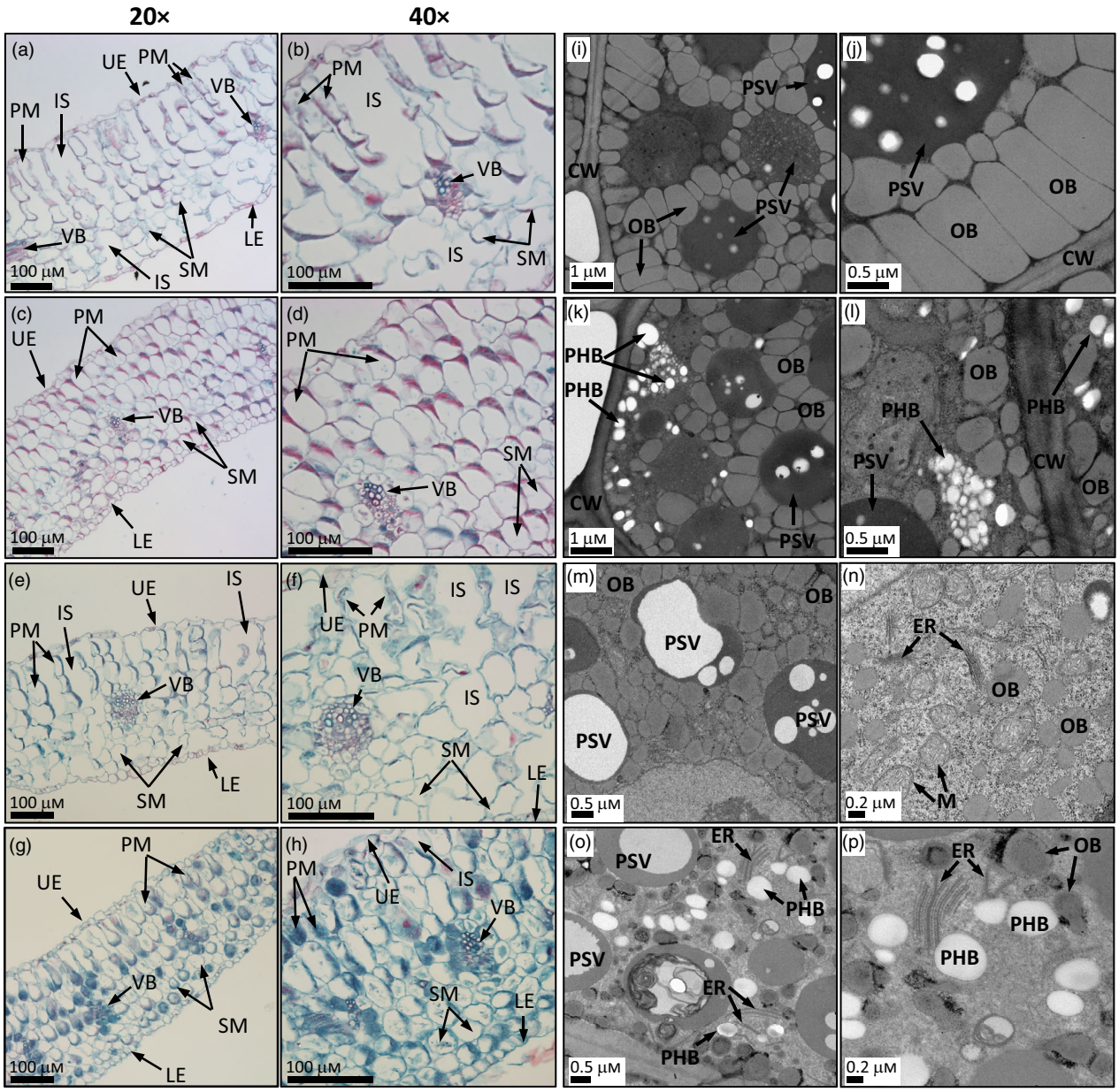

Figure 4 Microscopy of seedling cotyledons and imbibed seeds of *C. sativa* WT43 control and T_2 seedlings obtained from transformations of WT43 with pMBXS394 and pMBXS763. (a–h) Light microscopy images of cross sections of seedling cotyledons at 20× (a, c, e, g) and 40× (b, d, f, h) magnification, respectively. (a, b) *C. sativa* WT43 control grown alongside and imaged with (c, d) pMBXS394 transformed T_2 line 12-0415 containing 4.5% PHB in bulk T_2 seed samples. (e, f) *C. sativa* WT43 control grown alongside and imaged with (g, h) pMBXS763 transformed T_2 line 12-0933 containing 4.9% PHB in bulk T_2 seeds. Panels f and h are not magnified from panels e and g, respectively, but show a different view of the sample. T_2 seeds were germinated in soil and T_2 seedlings were sampled at the fully expanded cotyledon stage with first true leaves emerging. (i–p) Transmission electron microscopy (TEM) images of longitudinal sections of imbibed seeds. Longitudinal sections for analysis passed through cotyledonary region. (i, j) *C. sativa* WT43 control grown alongside and imaged with (k, l) pMBXS394 transformed line 12-0415 containing 4.5% PHB in bulk T_2 seeds. (m, n) *C. sativa* WT43 control grown alongside and imaged with (o, p) pMBXS763 transformed T_2 line 12-0933 containing 4.9% PHB in bulk T_2 seed. Seeds were imbibed for 5 h before processing for TEM. CW, cell wall; ER, endoplasmic reticulum; IS, intercellular space; LE, lower epidermis; M, mitochondria; OB, oil bodies; PM, palisade mesophyll; PSV, protein storage vesicles; SM, spongy mesophyll; UE, upper epidermis; VB, vascular bundle. Scale bars are provided at lower left of each image.

from greenhouse and chamber grown samples with the highest polymer content (7.1% and 10.2% mature seed weight, respectively; Table 2) were used and extracted polymer was confirmed to be PHB (Figures S6–S10). For molecular weight measurements, crushed seed from select plants were extracted with chloroform and gel permeation chromatography was performed (Malik *et al.*, 2015). Weight averaged molecular weights (Mw) from 800 000 to slightly under 1.8 million were observed with the polymer molecular weight inversely proportional to the seed polymer content (Figure S11A). The Mw of PHB polymer has previously been found to change with the levels of PHA synthase activity in bacterial systems (Sim *et al.*, 1997). The number average molecular weight (Mn) of lines ranged from 410 000 to slightly under 890 000 (Figure S11A).

Table 1 PHB content in T_2 and T_3 seed of select lines transformed with pMBXS394 and pMBXS763

T_2 generation				T_3 generation			
Genetic construct[†]	T_1 line	Copy number T_1 line[‡]	% PHB, bulk T_2 seed[§]	# lines tested	Range of PHB T_3 seed[§]	Avg PHB content[§,¶] T_3 seed	Highest PHB content in homozygous line
pMBXS394	12-0415	1	4.5	10	0.5–2.9	1.8 ± 0.9 (SD)	2.3
	12-0430	1	4.2	7	0.9–2.4	1.5 ± 0.6 (SD)	1.7
	12-0424	1	2.8	5	1.0–2.9	1.5 ± 0.8 (SD)	na
pMBX763	12-0944	1	3.6	2	3.6–4.3	3.9 ± 0.5 (SD)	4.3
	12-0950	1	2.9	3	2.3–5.3	3.6 ± 1.5 (SD)	5.3
	12-0954	1	3.4	4	3.5–4.4	3.9 ± 0.4 (SD)	4.4
	12-0962	2	3.1	8	1.5–9.1	4.1 ± 2.2 (SD)	9.1
	12-0974	2	3.6	7	1.5–6.8	4.2 ± 1.7 (SD)	6.8
	12-0992	1	3.5	2	3.5–3.7	3.6 ± 0.2 (SD)	3.6
	12-0999	1	3.0	6	3.2–5.6	4.5 ± 1.0 (SD)	5.6

[†]Genes in each construct are shown in Figure S3.

[‡]Representative Southern blots used to determine copy numbers are shown in Figure S4.

[§]Units for PHB content are % mature seed weight.

[¶]Average PHB content is calculated from lines containing detectable levels of PHB in seeds. All plants were grown in the greenhouse. Data points for individual plants used to calculate average PHB content in T_3 seed are shown in Table S5.

Table 2 PHB production in T_3 homozygous lines of pMBXS763 with high-survival under greenhouse[†] and controlled environmental chamber[‡] conditions designed to simulate spring planting on The Canadian Prairies

T_1 line[§]	% PHB, bulk T_3 seed[¶]	Growth method	# of plants[††]	% emergence T_3 seedlings	% survival T_3 seedlings	T_4 PHB range[¶,‡‡] (%)	T_4 PHB average[¶] (%)	Avg T_4 seed yield (g)[§§]
12-0944	4.3	GH	8	86	80	2.9–5.3	4.2 ± 0.7 (SD)	8.6 ± 2.1 (SD)
		CEC	5	83	69	4.0–7.0	5.3 ± 1.1 (SD)	18.6 ± 2.3 (SD)
12-0954	3.8	GH	8	33	36	2.3–4.3	3.3 ± 0.9 (SD)	8.1 ± 1.8 (SD)
		CEC	5	50	47	4.6–6.1	5.1 ± 0.7 (SD)	15.2 ± 3.2 (SD)
12-0992	3.5	GH	8	86	86	3.9–7.1	5.2 ± 1.0 (SD)	8.2 ± 1.8 (SD)
		CEC	4	81	78	4.6–10.2	7.0 ± 2.4 (SD)	14.7 ± 3.9 (SD)
WT43	–	GH	8	92	94	–		8.5 ± 1.2 (SD)
		CEC	5	97	97	–		17.8 ± 2.2 (SD)

[†]Growth conditions in greenhouse (GH) were 22/18 °C day/night and a photoperiod of 16 h under a supplemental light intensity of ~900 μmol/m^2/s during the daytime.

[‡]Temperature settings in the controlled environmental chamber (CEC) were adapted from averages of weekly historical data between early May and late July for Saskatoon, Saskatchewan, Canada and were obtained from https://climate.weather.gc.ca/climate_data/almanac_e.html?StationID=3333&period=30&searchMethod=contains&txtStationName=Saskatoon&month=5&day=22. Detailed settings for the CEC are shown in Table S2.

[§]All T_1 lines in table are single copy events.

[¶]Units for PHB content are % mature seed weight.

[††]Number of T_3 plants grown for analysis. A reduced number of plants was grown in the CEC due to space restrictions of the chamber.

[‡‡]PHB in T_4 bulk seed from individual plants. Individual data points for plants used to calculate average PHB content in T_4 seed are shown in Table S6.

[§§]Individual data points for plants used to calculate average T_4 seed yield are shown in Table S7. Difference in yield of wild-type and PHB lines for a given growth condition are not significant as determined by Student *t*-test (two sided, $P < 0.05$). Abbreviations are as follows. AVG, average; GH, greenhouse; CEC, controlled environmental chamber; WT43, wild-type control line.

Partitioning of carbon in PHB-producing lines

T_4 seed samples from select pMBXS763 lines, grown under standard greenhouse or controlled environmental conditions, were tested for oil and protein content to determine if cytosolic PHB production affected the amount of these seed storage products. A decrease in oil was observed upon PHB production in plants grown in both the greenhouse and growth chamber. Plants grown in the controlled environmental chamber consistently produced more oil than plants grown in the greenhouse (Figure S11B). Significant changes in fatty acid profile of oil were also observed, with increases in 16 : 0, 18 : 0, 18 : 3, 20 : 3 and 22 : 0 and decreases in 18:2 and 20:2 upon PHB production in greenhouse grown plants (Figure S12). In chamber grown plants, the significant differences included increases in 16 : 0, 18 : 3 and 20 : 3 and decreases in 18 : 1, 18 : 2, 20 : 1, and 20 : 2 upon PHB production (Figure S12). Seed protein level remained essentially constant with PHB production (Figure S13) and protein content in seeds harvested from chamber and greenhouse grown plants was similar.

Discussion

Interest in renewable sources of biodegradable plastics has increased with the closing of overseas markets for plastic waste and recent public awareness that recycling is not economically viable (Sullivan, 2020). PHAs are a leading solution to the world's plastics problem since they are renewable, biodegradable materials with properties that can replace petroleum-based plastics in many applications. In fact, PHA bioplastics were named one of the 25 ideas that will shape this decade (Fortune, 2019) because of their unique biodegradability in all biologically active environments, including soil, rivers, oceans, compost and sewage (Snell and Peoples, 2009). The widespread use of PHAs produced by large-scale bacterial fermentations has been hindered by the high capital and operating costs of production. Today, starches and vegetable oils are produced on an enormous scale at very low cost through agriculture. Similarly, plant-based production of PHAs could significantly decrease costs of production. The concept of plant-based PHA production was first described over 30 years ago (Pool, 1989), and despite much effort by academic and industrial researchers, primarily targeted towards the production of PHB (Snell *et al*., 2015; Somleva *et al*., 2013; Suriyamongkol *et al*., 2007), industrially relevant success has not yet been achieved. Production of PHAs in plants has often led to negative agronomic effects when the biosynthetic pathways are expressed, including impaired plant growth when pathways are expressed in vegetative tissue (Bohmert *et al*., 2000, 2004; Somleva *et al*., 2013) or impaired seedling survival with seed-specific expression (Malik *et al*., 2015).

Oilseeds such as *Camelina sativa* are ideal for biosynthesis of PHB and its copolymers, since acetyl-CoA is a common substrate for both PHB and seed oil biosynthesis (Figure 1). Previous work in our labs was successful in producing high levels of polymer in Camelina seed plastids, reaching up to 15.2% of the mature seed weight, but cotyledons were chlorotic and a significant negative impact on seedling emergence and viability was observed (Malik *et al*., 2015). We reasoned that production of PHB in an alternate location, away from the developing photosynthetic machinery of the seedling, might increase seedling vigour. In this study, we evaluated the cytosol as an alternative seed-specific production site for PHB. At the onset of our work, production of PHB in the cytosol of plants was considered problematic as prior attempts in leaves yielded only low levels of PHB (highest level in a plant, 0.34% DW PHB, cotton fibres (John and Keller, 1996)) with often stunted plant phenotypes (Poirier and Brumbley, 2010; Snell *et al*., 2015; Somleva *et al*., 2013). To our knowledge, no efforts to produce PHB in the cytosol of seeds have been reported. Since the cytosol of seeds supplies malonyl-CoA for ER-associated fatty acid elongation reactions (Figure 1), we reasoned that a greater pool of accessible acetyl-CoA might be available in the cytosol of developing seeds than in leaves for production of PHB. Cytosolic production of PHB using strong seed-specific promoters (transformation construct pMBXS394) yielded PHB levels of up to 4.5% of the mature seed weight in T_2 seeds, 13 times higher than the previously reported highest amounts of cytosolic PHA produced in plants (0.34% DW PHB, cotton fibres; John and Keller, 1996). Surprisingly, PHB levels dropped in subsequent generations suggesting that cytosolic PHB production was not stable.

We next pursued a strategy of anchoring PHA synthase to the cytosolic face of the ER to attempt to stabilize the PHA synthase and thus polymer production. ER targeting has previously been used to increase the production of human immunodeficiency virus proteins Nef (Barbante *et al*., 2008) and p24 (Virgili-López *et al*., 2013) in tobacco, possibly by increasing the stability of the proteins and/or making them less susceptible to degradation by proteases (Pedrazzini, 2009). Fusion of the gene encoding PHA synthase (PhaC) to a similar ER tail-anchoring sequence and co-expression with the other genes of the PHB biosynthetic pathway (transformation construct pMBXS763) yielded lines that produced PHB up to 4.9% of the mature seed weight in T_2 seeds. Importantly, these lines showed stable PHB production through multiple generations with some lines showing increased polymer levels producing up 7.1% PHB in homozygous T_4 seeds in the greenhouse (Table 2). While targeting of the PHA synthase (PhaC) to the ER was not experimentally confirmed, our results suggest that PhaC fused to the ER targeting signal in transformation construct pMBXS763 provides more stable PHB synthesis than transformation construct pMBXS394 containing PhaC without an ER targeting signal. Several independent ER targeted events showed more stable PHB production through multiple generations and, in some cases, produced higher polymer levels. Additional work is required to understand the factors contributing to increased stability of product formation in ER-targeted lines compared to their cytosolic counterparts.

Use of a controlled environmental chamber to simulate spring growth conditions produced higher levels of PHB with up to 10.2% PHB observed in T_4 seeds with good emergence and survival of T3 seedlings (78%, Table 2). ER-targeted PHB producing seedlings were green and healthy but possessed cotyledons that were narrower than wild-type controls (Figure 3). The chlorotic phenotype previously observed in seedlings of seed-specific plastid PHB producers (Malik *et al*., 2015) was not observed.

Past attempts at commercialization of microbial based PHAs have been difficult, primarily due to the high cost of advanced fermentation-produced copolymers that have restricted entry into many markets. We believe crop-based production will enable an advantaged cost structure, thereby eliminating the remaining barrier to entry for broad adoption of these materials. The multiple co-products harvested in a PHB-producing seed, including oil, polymer and protein-rich seed meal, increase the seed value, providing additional revenue streams to support a business (Snell and Peoples, 2013). We have demonstrated the production of up to 10.2% PHB in seeds of Camelina with good seedling viability. This is a significant step towards producing field-ready lines for a commercial pipeline. This study is the first report of cytosolic production of PHB in seeds, and is the highest level of cytosolic PHB production in a plant to date at 30 times the previously reported highest amounts of cytosolic PHA produced (John and Keller, 1996).

Materials and methods

Plant material and growth

Camelina sativa line 10CS0043 was obtained from Kevin Falk at Agriculture and Agri-Food Canada (AAFC). This line has been reported to have a larger seed size than other lines of Camelina and has previously been used in studies to produce PHB in Camelina seed plastids (Malik *et al*., 2015). *C. sativa* line 10CS0043 (abbreviated WT43) and T_1 and T_2 plants were grown in 6-inch pots in a greenhouse at 22/18 °C day/night and a photoperiod of 16 h under supplemental light intensity of

650 μmol/s/m^2 during the daytime. T3 lines were grown in 10-inch pots in the greenhouse under the conditions described above, as well as in a BDR16 controlled environmental chamber from Conviron (Winnipeg, MB, Canada). The BDR16 chamber was set with variable conditions to simulate changes in temperature and day length that plants would encounter in fields around Saskatoon, SK, Canada if planted in early May and harvested in late July. Conditions are listed in Table S2. During day hours, the maximum light capability of the BRD16 chamber (900 μmol/m^2/s) was used.

Construction of binary vectors for PHB production

All transformation constructs used in this study are derivatives of pCAMBIA binary vectors (Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia) and are shown in Figure S3. PHB biosynthetic pathway genes used in transformation constructs included a gene encoding β-ketothiolase (PhaA) from *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) (Peoples and Sinskey, 1989), an NADPH-dependent reductase (PhaB) from *C. necator* (Peoples and Sinskey, 1989), and a hybrid *Pseudomonas oleovorans/Zoogloea ramigera* PHA synthase (PhaC) (Huisman *et al.*, 2001; Kourtz *et al.*, 2005).

Plant transformations

For transformation of WT43, genetic constructs were inserted into *Agrobacterium tumefaciens* strain GV3101(pMP90). The transformation of Camelina plants was performed by floral dip methods as described previously (Lu and Kang, 2008). An expression cassette for DsRed was included in all constructs to allow the visual detection of transgenic seeds. DsRed seeds were visually identified by fluorescent microscopy using a Nikon AZ100 microscope with a TRITC-HQ(RHOD)2 filter module (HQ545/30X, Q570LP, HQ610/75M). In an attempt to recover all transformants and to determine the maximum PHB production potential with cytosolic constructs, T_1 seeds were sterilized and germinated on half strength MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose and 1 μM gibberellic acid (GA_3).

Measurement of PHB content

Seeds of transgenic plants containing PHB were analysed using a previously described simultaneous extraction and butanolysis procedure that converts PHB polymer into butyl esters of monomeric units that were quantified using gas chromatography with FID detection (Kourtz *et al.*, 2007; Malik *et al.*, 2015). Calibration curves were made with purified PHB (Sigma-Aldrich). PHB levels were calculated as percent of mature seed weight.

Measurement of total oil content, fatty acid profile and protein content

The total oil content and fatty acid profile of Camelina seeds was determined as previously described (Malik *et al.*, 2015). Differences in oil content and oil profile of PHB lines and WT43 were determined by the Student's *t*-test (two sided). A value of $P < 0.05$ was considered significant. Protein content in Camelina seeds was measured by POS-Biosciences in Saskatoon, Saskatchewan using the American Oil Chemists Society (AOCS) Ba 4e-93 method (Generic Combustion Method for Determination of Crude Protein).

Copy number analysis of T_1 plants

Leaf samples were collected from T_1 plants for transgene copy number analysis and stored at −80 °C. Genomic DNA extraction was carried out with Nucleon PhytoPure DNA extraction system (GE Healthcare Lifesciences) and quantified using a Nanodrop instrument (Thermo Scientific). Southern blots were prepared with 10 μg *Bam*HI digested genomic DNA of T_1 plants and hybridized with a 400-bp *phaC* PCR-labelled DIG probe (Figure S4) as previously described (Malik *et al.*, 2015).

Molecular weight determination

The weight averaged molecular weight (Mw) and number averaged molecular weight (M_n) of polymer extracted from select seed samples was determined by gel-permeation chromatography in chloroform as previously described (Malik *et al.*, 2015).

^{1}H NMR analysis

Ground seeds (~0.3 g) were extracted with $CDCl_3$ (~15 g, 24 h stirring at RT), filtered (0.22 μm syringe filter), and the organic filtrate solution was analysed by ^{1}H NMR (400 MHz, Spectral Data Services, Inc., Champaign, IL, USA; Figures S7 and S8). A control sample of pure PHB in $CDCl_3$ was also analysed (Figure S6). Since the spectra from extracted seed material predominantly contained peaks consistent with the presence of lipids (Barison *et al.*, 2010; Figures S7 and S8), approximately 15 g of additional $CHCl_3$ was added to each sample and the extraction was performed for 24 h at room temperature. The suspensions were filtered, and the residues washed with additional $CHCl_3$. Concentration of the filtrates followed by high vacuum (~5 Torr, 1 h) to remove all volatiles yielded a weakly brown, sticky solid (~80 mg) for each sample. Addition of isopropanol (~6 g) to these residues resulted in the formation of a weakly yellow liquid organic phase, in which a white solid was suspended. Isopropanol has previously been described as a good solvent for extraction of oil from mustard (Sinichi and Diosady, 2014). The organic liquid phase was removed using a pipette, evaporated to dryness, and dissolved in $CDCl_3$. The isopropanol-insoluble fraction, a white solid, was washed with additional isopropanol (~5 g). The combined organic liquid phases were concentrated under high vacuum yielding a weakly brown oil (~60 mg) which was analysed by ^{1}H NMR and found to contain peaks consistent with seed lipids (Barison *et al.*, 2010; Figure S9). The isopropanol-insoluble white solids (~20 mg) were also analysed by ^{1}H NMR, yielding purified PHB (Figure S10; ($CDCl_3$; 400 MHz) δ = 1.27 (d, J = 6.3 Hz, 3H), 2.47 (dd, J = 5.8, 15.5 Hz, 1H), 2.60 (dd, J = 7.4, 15.5 Hz, 1 H), 5.26 (m, 1H)) that contained only small amounts of unidentified materials (Figure S10; t at 4.12 ppm, d at 2.06 ppm, and p at 1.97 ppm).

Electron microscopy

Seed and cotyledon samples were processed for TEM according to previously described procedures (Malik *et al.*, 2015). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a FEI transmission electron microscope (Model-Morgagni 268, operating at 80 kV).

Light microscopy

Fully expanded cotyledons were fixed in a modified Karnovsky's fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Tissues were loaded into histology cassettes, and dehydration of tissue was initiated manually in 50% and 70% ethanol. Subsequent dehydration and infiltration steps were performed in a LEICA TP1020-Automatic Tissue Processor through a gradual alcohol-toluene series (70%, 90%, 100% ethanol, 1 : 1 ethanol:toluene followed by toluene and then paraffin). Cotyledons were oriented and

embedded in paraffin blocks. 7–10 μm thick sections were cut with a Leica RM2125 RTS microtome and placed on glass slides that were air dried overnight at 37°C. Staining with 1% Safranin O and 0.67% Fast Green FCF was performed according to a previously described protocol (Clark and Bartholomew, 1981) with minor modifications. Sections were viewed and digitally photographed with a Zeiss AXIO Scope.A1 compound microscope equipped with an Optronics digital camera.

Germination of PHB-producing lines on tissue culture media

To ensure rescue of high PHB-producing lines, a tissue culture medium was used for germination and visualization of early seedling development as previously described (Malik *et al*., 2015).

Emergence and survival testing of PHB-producing lines in soil

Thirty DsRed seeds from an individual PHB line were planted in 6-inch pots filled with soil (Sunshine Mix #4 saturated with water containing NPK 20-20-20 fertilizer) and a top layer of vermiculite. The plants were grown in the greenhouse with supplemental lighting (16 h photoperiod, 22 °C, typical light intensity of 900 μmol/s/m^2 during daytime). The pots were moistened daily with fertilized water (NPK-20-20-20) and percent emergence was determined 1 week after transfer of the seeds to pots. Percent survival was determined after an additional week under ambient greenhouse conditions. Percent emergence and survival were calculated based on 30 seeds.

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Conflict of interest

All the authors are current or former employees of Yield10 Bioscience or Yield10 Oilseeds.

Author contributions

M. R. M. participated in formulating the strategy for the study, performed data analyses and line selection, supervised research and participated in writing the manuscript; N. P. participated in formulating the strategy for the study, supervised research, performed cloning and molecular biology procedures and performed plant molecular biology tasks including plant transformations; N. S. participated in plant transformations and the design and performance of plant molecular biology work and participated in writing the manuscript; J. T. participated in formulating the strategy for the study and performed plant molecular biology tasks; C. B. participated in plant transformations and supervised and participated in large scale greenhouse and controlled environmental chamber growth and line screening; Y. J. performed plant molecular biology work; M. M. performed cloning and molecular biology procedures, and performed plant molecular biology tasks; A. H. performed cloning and molecular biology procedures, and performed plant molecular biology tasks; D. S. participated in polymer characterization including purification and ^{1}H NMR verification of PHB samples; O. P. participated in formulating the strategy for the study and supervised research; K. D. S. participated in formulating the strategy for the study, supervised research and participated in writing the manuscript.

References

Abell, B. and Mullen, R. (2011) Tail-anchored membrane proteins: exploring the complex diversity of tail-anchored-protein targeting in plant cells. *Plant Cell Rep.* **30**, 137–151.

Barbante, A., Irons, S., Hawes, C., Frigerio, L., Vitale, A. and Pedrazzini, E. (2008) Anchorage to the cytosolic face of the endoplasmic reticulum membrane: a new strategy to stabilize a cytosolic recombinant antigen in plants. *Plant Biotechnol. J.* **6**, 560–575.

Barison, A., da Silva, C.W., Campos, F.R., Simonelli, F., Lenz, C.A. and Ferreira, A.G. (2010) A simple methodology for the determination of fatty acid composition in edible oils through ^{1}H NMR spectroscopy. *Magn. Reson. Chem.* **48**, 642–650.

Bohmert, K., Balbo, I., Kopka, J., Mittendorf, V., Nawrath, C., Poirier, Y., Tischendorf, G. *et al.* (2000) Transgenic *Arabidopsis* plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. *Planta*, **211**, 841–845.

Bohmert, K., Balbo, I., Steinbuchel, A., Tischendorf, G. and Willmitzer, L. (2002) Constitutive expression of the b-ketothiolase gene in transgenic plants. A major obstacle for obtaining polyhydroxybutyrate-producing plants. *Plant Physiol.* **128**, 1282–1290.

Bohmert, K., Peoples, O.P. and Snell, K.D. (2004) Metabolic engineering: Plastids as bioreactors. In *Molecular Biology and Biotechnology of Plant Organelles*(Daniell, H. and Chase, C.D., eds), pp. 559–585. Netherlands: Kluwer Academic Publishers.

Chen, G.-Q. and Zhang, J. (2018) Microbial polyhydroxyalkanoates as medical implant biomaterials. *Artif. Cell Nanomed. B.* **46**, 1–18.

Chu, L. and Wang, J. (2016) Denitrification of groundwater using PHBV blends in packed bed reactors and the microbial diversity. *Chemosphere*, **155**, 463–470.

Clark, G. and Bartholomew, J.W. (1981) *Staining Procedures Used by the Biological Stain Commission: Published for the Biological Stain Commission*. Baltimore, London: Williams & Wilkins.

Eynck, C., Shrestha, D., Vollmann, J., Falk, K.C., Friedt, W., Singh, H.P. and Obeng, E. (2013) Sustainable oil crops production. In *Biofuel Crop Sustainability* (Singh, B.P., ed), pp. 165–204. Ames, Iowa: John Wiley & Sons, Ltd.

FitzJohn, R., Armstrong, T., Newstrom-Lloyd, L., Wilton, A. and Cochrane, M. (2007) Hybridisation within Brassica and allied genera: evaluation of potential for transgene escape. *Euphytica*, **158**, 209–230.

Fortune. (2019) *25 ideas that will shape the 2020s*. https://fortune.com/longform/ideas-shape-2020s-tech-economy-markets-ai-health-work-society/

Hiraishi, A. and Khan, S.T. (2003) Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. *Appl. Microbiol. Biotechnol.* **61**, 103–109.

Houmiel, K.L., Slater, S., Broyles, D., Casagrande, L., Colburn, S., Gonzalez, K., Mitsky, T.A. *et al.* (1999) Poly(beta-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*. *Planta*, **209**, 547–550.

Huai, D., Zhang, Y., Zhang, C., Cahoon, E.B. and Zhou, Y. (2015) Combinatorial effects of fatty acid elongase enzymes on nervonic acid production in *Camelina sativa*. *PloS One*, **10**, e0131755.

Huisman, G.W., Skraly, F., Martin, D.P. and Peoples, O.P. (2001) *Biological systems for manufacture of polyhydroxyalkanoate polymers containing 4-hydroxyacids. US Patent No: 6,316,262*.

Iida, A., Nagasawa, A. and Oeda, K. (1995) Positive and negative *cis*-regulatory regions in the soybean glycinin promoter identified by quantitative transient gene expression. *Plant Cell Rep.* **14**, 539–544.

John, M.E. and Keller, G. (1996) Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. *Proc. Natl. Acad. Sci. USA*, **93**, 12768–12773.
Joubès, J., Raffaele, S., Bourdenx, B., Garcia, C., Laroche-Traineau, J., Moreau, P., Domergue, F. *et al*. (2008) The VLCFA elongase gene family in Arabidopsis thaliana: phylogenetic analysis, 3D modelling and expression profiling. *Plant Mol. Biol.* **67**, 547–566.
Kang, S. and Yu, J. (2015) Reaction routes in catalytic reforming of poly(3-hydroxybutyrate) into renewable hydrocarbon oil. *RSC Adv.* **5**, 30005–30013.
Koller, M. (2018) Biodegradable and biocompatible polyhydroxyalkanoates (PHA): Auspicious microbial macromolecules for pharmaceutical and therapeutic applications. *Molecules*, **23**, 362.
Koller, M. and Mukherjee, A. (2022) A New Wave of Industrialization of PHA Biopolyesters. *Bioengineering*, **9**, 74.
Kourtz, L., Dillon, K., Daughtry, S., Madison, L.L., Peoples, O. and Snell, K.D. (2005) A novel thiolase-reductase gene fusion promotes the production of polyhydroxybutyrate in *Arabidopsis*. *Plant Biotechnol. J.* **3**, 435–447.
Kourtz, L., Dillon, K., Daughtry, S., Peoples, O.P. and Snell, K.D. (2007) Chemically inducible expression of the PHB biosynthetic pathway in *Arabidopsis*. *Transgenic Res.* **16**, 759–769.
Li, Z., Yang, J. and Loh, X.J. (2016) Polyhydroxyalkanoates: opening doors for a sustainable future. *NPG Asia Mater.* **8**, e265.
Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S. *et al*. (2010) Acyl-lipid metabolism. *Arabidopsis Book*, **8**, e0133.
Lim, J., You, M., Li, J. and Li, Z. (2017) Emerging bone tissue engineering via polyhydroxyalkanoate (PHA)-based scaffolds. *Mater. Sci. Eng. C Mater. Biol. Appl.* **79**, 917–929.
Lu, C. and Kang, J. (2008) Generation of transgenic plants of a potential oilseed crop *Camelina sativa* by *Agrobacterium*-mediated transformation. *Plant Cell Rep.* **27**, 273–278.
Madison, L.L. and Huisman, G.W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. *Microbiol. Mol. Biol. Rev.* **63**, 21–53.
Malik, M.R., Yang, W., Patterson, N., Tang, J., Wellinghoff, R.L., Preuss, M.L., Burkitt, C. *et al*. (2015) Production of high levels of poly-3-hydroxybutyrate in plastids of *Camelina sativa* seeds. *Plant Biotechnol. J.* **13**, 675–688.
Malik, M.R., Tang, J., Sharma, N., Burkitt, C., Ji, Y., Mykytyshyn, M., Bohmert-Tatarev, K. *et al*. (2018) Camelina sativa, an oilseed at the nexus between model system and commercial crop. *Plant Cell Rep.* **37**, 1367–1381.
Mamat, M.R.Z., Ariffin, H., Hassan, M.A. and Mohd Zahari, M.A.K. (2014) Bio-based production of crotonic acid by pyrolysis of poly(3-hydroxybutyrate) inclusions. *J. Clean. Prod.* **83**, 463–472.
Martinelli, T. and Galasso, I. (2011) Phenological growth stages of Camelina sativa according to the extended BBCH scale. *Ann. Appl. Biol.* **158**, 87–94.
Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L. and Lukyanov, S.A. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17**, 969–973.
Mullen, C.A., Boateng, A.A., Schweitzer, D., Sparks, K. and Snell, K.D. (2014) Mild pyrolysis of P3HB/switchgrass blends for the production of bio-oil enriched with crotonic acid. *J. Anal. Appl. Pyrol.* **107**, 40–45.
Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
Pedrazzini, E. (2009) Tail-Anchored Proteins in Plants. *J. Plant Biol.* **52**, 88–101.
Peoples, O.P. and Sinskey, A.J. (1989) Poly-beta-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16. Characterization of the genes encoding beta-ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.* **264**, 15293–15297.
Poirier, Y. and Brumbley, S. (2010) Metabolic engineering of plants for the synthesis of polyhydroxyalkanaotes. In *Plastics from Bacteria: Natural Functions and Applications* (Chen, G.G.-Q., ed), pp. 187–211. London: Springer Verlag.
Poirier, Y., Dennis, D., Klomparens, K., Nawrath, C. and Somerville, C. (1992a) Perspectives on the production of polyhydroxyalkanoates in plants. *FEMS Microbiol. Rev.* **103**, 237–246.
Poirier, Y., Dennis, D.E., Klomparens, K. and Somerville, C. (1992b) Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. *Science*, **256**, 520–523.
Pool, R. (1989) In search of the plastic potato. *Science*, **245**, 1187–1189.
Rowley, D.L. and Herman, E.M. (1997) The upstream domain of soybean oleosin genes contains regulatory elements similar to those of legume storage proteins. *Biochim. Biophys. Acta*, **1345**, 1–4.
Schweitzer, D. and Snell, K.D. (2015) Acrylates via metathesis of crotonates. *Org. Process Res. Dev.* **19**, 715.
Schweitzer, D., Mullen, C.A., Boateng, A.A. and Snell, K.D. (2015) Bio-based n-butanol prepared from poly-3-hydroxybutyrate: optimization of the reduction of n-butyl crotonate to n-butanol. *Org. Process Res. Dev.* **19**, 710–714.
Sim, S., Snell, K., Hogan, S., Stubbe, J., Rha, C. and Sinskey, A. (1997) PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate in vivo. *Nat. Biotechnol.* **15**, 63–67.
Sinichi, S. and Diosady, L.L. (2014) Isopropyl alcohol extraction of de-hulled yellow mustard flour. *J. Am. Oil Chem. Soc.* **91**, 2143–2153.
Snell, K.D. and Peoples, O.P. (2009) PHA bioplastic: a value-added coproduct for biomass biorefineries. *Biofuels Bioprod. Bioref.* **3**, 456–467.
Snell, K.D. and Peoples, O.P. (2013) Production of value-added co-products in industrial oilseeds. *Inform*, **24**, 640–643.
Snell, K.D., Singh, V. and Brumbley, S.M. (2015) Production of novel biopolymers in plants: recent technological advances and future prospects. *Curr. Opin. Biotech.* **32C**, 68–75.
Somleva, M.N., Peoples, O.P. and Snell, K.D. (2013) PHA bioplastics, biochemicals, and energy from crops. *Plant Biotechnol. J.* **11**, 233–252.
Steinbuchel, A. and Valentin, H.E. (1995) Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiol. Lett.* **128**, 219–228.
Sullivan, L. (2020) How big oil misled the public into believing plastic would be recycled. *NPR*, **11**, 2020.
Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M. and Shah, S. (2007) Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants – a review. *Biotechnol. Adv.* **25**, 148–175.
Valentin, H.E., Broyles, D.L., Casagrande, L.A., Colburn, S.M., Creely, W.L., DeLaquil, P.A., Felton, H.M. *et al*. (1999) PHA production, from bacteria to plants. *Int. J. Biol. Macromol.* **25**, 303–306.
Van Beilen, J.B. and Poirier, Y. (2012) Plants as factories for bioplastics and other biomaterials. In *Plant Biotechnology and Agriculture: Prospects for the 21st Century*(Altman, A. and Hasegawa, P.M., eds), pp. 481–494. London: Academic Press.
Virgili-López, G., Langhans, M., Bubeck, J., Pedrazzini, E., Gouzerh, G., Neuhaus, J.-M., Robinson, D.G. *et al*. (2013) Comparison of Membrane Targeting Strategies for the Accumulation of the Human Immunodeficiency Virus p24 Protein in Transgenic Tobacco. *Int. J. Mol. Sci.* **14**, 13241–13265.
Wang, J. and Chu, L. (2016) Biological nitrate removal from water and wastewater by solid-phase denitrification process. *Biotechnol. Adv.* **34**, 1103–1112.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 PHB biosynthetic pathway used in this study.
Figure S2 Fatty acid profile of WT43 Camelina line grown under greenhouse conditions.
Figure S3 T-DNA structure of binary vectors pMBXS394 (A) and pMBXS763 (B) used for plant transformation.
Figure S4 Representative Southern blots of select *C. sativa* WT43 lines transformed with (A, B) pMBXS394 and (C) pMBXS763.
Figure S5 TEM images of cross sections of cotyledons from *C. sativa* WT43 control and T_2 seedlings obtained from transformations of *C. sativa* WT43 with pMBXS394 and pMBXS763.
Figure S6 ^{1}H NMR of pure poly[(*R*)-3-hydroxybutyrate] (PHB) standard in $CDCl_3$.
Figure S7 ^{1}H NMR spectra of chloroform extracts from PHB containing Camelina seed samples.
Figure S8 ^{1}H NMR spectra of chloroform extracts of crushed wild-type Camelina seed samples.
Figure S9 Removal of lipids from crude extracts of PHB producing seeds: isopropanol-soluble lipid fraction.

Figure S10 Removal of lipids from crude extracts of PHB producing seeds: isopropanol-insoluble PHB fraction.
Figure S11 Comparison of (A) PHB polymer molecular weight to PHB seed content and (B) seed oil content to PHB seed content in T_4 seeds obtained from transformations of *C. sativa* WT43 with pMBXS763.
Figure S12 Fatty acid profile in oil from T_4 seeds obtained from transformations of *C. sativa* WT43 with pMBXS763.
Figure S13 PHB and protein content in T_4 seeds harvested from individual plants of pMBXS763 grown in the (A) greenhouse or (B) a controlled environmental chamber simulating spring conditions.
Table S1 Comparison of PHB production in T_2 seeds of WT43 transformed with constructs for PHB production.
Table S2 Growth conditions in controlled environmental chamber simulating spring conditions on the Canadian Prairies.
Table S3 Data points for fatty acid profiles of wild-type biological replicates grown in the greenhouse.
Table S4 Data points for fatty acid profiles of wild-type biological replicates grown in the controlled environmental chamber.
Table S5 Data points for PHB content of biological replicates of T_2 Camelina lines transformed with pMBXS394 and pMBXS763.
Table S6 Data points for PHB content of biological replicates of T_3 homozygous Camelina lines transformed with pMBXS763.
Table S7 Data points for seed yield of biological replicates from T_3 homozygous Camelina lines transformed with pMBXS763.