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Production of high levels of poly-3-hydroxybutyrate in plastids of *Camelina sativa* seeds

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Summary

Poly-3-hydroxybutyrate (PHB) production in plastids of *Camelina sativa* seeds was investigated by comparing levels of polymer produced upon transformation of plants with five different binary vectors containing combinations of five seed-specific promoters for expression of transgenes. Genes encoding PHB biosynthetic enzymes were modified at the N-terminus to encode a plastid targeting signal. PHB levels of up to 15% of the mature seed weight were measured in single sacrificed T_1 seeds with a genetic construct containing the oleosin and glycinin promoters. A more detailed analysis of the PHB production potential of two of the best performing binary vectors in a Camelina line bred for larger seed size yielded lines containing up to 15% polymer in mature T_2 seeds. Transmission electron microscopy showed the presence of distinct granules of PHB in the seeds. PHB production had varying effects on germination, emergence and survival of seedlings. Once true leaves formed, plants grew normally and were able to set seeds. PHB synthesis lowered the total oil but not the protein content of engineered seeds. A change in the oil fatty acid profile was also observed. High molecular weight polymer was produced with weight-averaged molecular weights varying between 600 000 and 1 500 000, depending on the line. Select lines were advanced to later generations yielding a line with 13.7% PHB in T_4 seeds. The levels of polymer produced in this study are the highest reported to date in a seed and are an important step forward for commercializing an oilseed-based platform for PHB production.

Introduction

Interest in producing fuels, chemicals and materials from plants has grown with concerns of diminishing fossil fuel feedstock availability and increased global climate change. Production of these industrial commodities from agriculture not only provides a renewable, often closed carbon cycle with the capture of CO_2 during photosynthesis, but also the opportunity to produce these goods on a large scale at low cost. Polyhydroxyalkanoates (PHAs) are polymers that have received considerable attention for production in crops because they have properties, depending on their monomer unit composition, that make them suitable renewable replacements for many petroleum derived plastics with an added benefit of biodegradability (Snell and Peoples, 2009; Tsui *et al.*, 2013). PHAs are polyesters that naturally accumulate as intracellular granules of stored carbon and energy in a number of bacteria when the organisms are faced with a nutrient limitation or imbalance (Madison and Huisman, 1999; Suriyamongkol *et al.*, 2007). These reserves can be mobilized at a later time to provide energy for growth when more favourable conditions return. The interest in PHAs has more recently moved beyond plastics to include applications in the chemicals and feed markets (Mullen *et al.*, 2014; Schweitzer *et al.*, 2015; Snell and Peoples, 2013; Somleva *et al.*, 2013), potentially broadening the market opportunities for these polymers.

The homopolymer R-poly-3-hydroxybutyrate (PHB), composed of repeating units of R-3-hydroxybutyrate, is the most studied member of the PHA family. It can be produced in plants as a co-product to lignocellulosic biomass (Snell and Peoples, 2009) or seed oil (Snell and Peoples, 2013) by expression of a bacterial three gene biosynthetic pathway (Poirier and Brumbley, 2010; Suriyamongkol *et al.*, 2007). Work in plants has focused primarily on the engineering of PHB biosynthesis in the green tissue of different plant species and has resulted in the production of a wide range of polymer levels depending on the host plant, mode of gene expression and the organelle site of synthesis (Poirier and Brumbley, 2010; Somleva *et al.*, 2013; Suriyamongkol *et al.*, 2007). Interest in plant-based production has been driven by the possibility of accumulating PHB within lignocellulosic material such that the resulting plant feedstock could be used for the co-production of polymer and energy (Snell and Peoples, 2009). The highest levels of polymer produced to date, up to approximately 40% dry weight (DW) in *Arabidopsis thaliana* (Bohmert *et al.*, 2000) and 18% DW in *Nicotiana tabacum* (Bohmert-Tatarev *et al.*, 2011), have been achieved when the production of polymer is directed to the leaf chloroplasts, likely due to the high

flux of acetyl-CoA in these organelles for fatty acid biosynthesis. Significant progress in engineering PHB production in agronomically relevant high yielding biomass crops, such as maize (Poirier and Gruys, 2002), sugarcane (Petrasovits *et al.*, 2007, 2012; Purnell *et al.*, 2007) and switchgrass (Somleva *et al.*, 2008), has also been made.

Work to produce PHAs in seeds of plants has been much more limited and to date has consisted of engineering *Brassica napus* to produce either PHB or the copolymer of R-3-hydroxybutyrate and R-3-hydroxyvalerate (PHBV) in seed plastids (Houmiel *et al.*, 1999; Valentin *et al.*, 1999), *Arabidopsis* to produce medium chain length PHAs in seed peroxisomes (Poirier *et al.*, 1999), tobacco to produce a mixture of short chain length PHAs in seed peroxisomes (Nakashita *et al.*, 2001) or soya bean to produce PHB in the vacuoles of the seed coat (Schnell *et al.*, 2012). Of these studies, only work in *B. napus* yielded significant levels with the production of up to 7.7% PHB fresh weight (FW) or 2.3% dry weight (DW) PHBV (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). Interest in seed-based PHA production is driven by the possibility of co-producing polymer and seed oil, which could be used to generate renewable liquid fuels or oleochemicals, from the same plant feedstock (Snell and Peoples, 2013). The use of the high protein PHB containing seed meal as an enhanced feed supplement is an alternative to extraction of the polymer for industrial uses. Multiple feed trials have shown that PHB is beneficial when added to feed delivering potential prebiotic effects, resistance to pathogenic bacteria and/or increased weight gain [(Somleva *et al.*, 2013; Thai *et al.*, 2014) and references within].

In an effort to further evaluate the potential for high level production of PHB in plastids of oilseeds, we constructed plant transformation vectors for seed-specific expression of genes encoding plastid targeted PHB biosynthetic enzymes. Vectors with combinations of five different seed-specific promoters were constructed and transformed into *Camelina sativa*. Camelina is an oilseed from the Brassicaceae family that is receiving considerable attention as an industrial crop due to its high oil content that is suitable for production of renewable aviation and other liquid biofuels (Biomass Advisors, 2010). It requires low inputs for cultivation and has a short growth cycle making it favourable for climates with shorter growing seasons (Eynck *et al.*, 2013; Moser, 2010). Camelina is also a versatile metabolic engineering platform because of its simple, nonlabour intensive *Agrobacterium*-based transformation protocol and availability of genetic resources (Collins-Silva *et al.*, 2011; Kagale *et al.*, 2014; Lu and Kang, 2008; Nguyen *et al.*, 2013). The best genetic constructs for PHB production were identified using a novel single-seed polymer screening approach, and two constructs were used to transform a larger seeded Camelina germplasm for detailed evaluation of polymer production. As PHB production competes with fatty acid biosynthesis in seed plastids for the common precursor acetyl-CoA, the partitioning of carbon between PHB, seed oil and seed protein was determined and the fatty acid composition of the oil was analysed. To date, possible changes in carbon partitioning and fatty acid composition upon PHB production in seeds have not been previously described.

Results

Evaluation of different seed-specific promoters for PHB production

To test PHB production in Camelina seeds, we constructed five plant transformation vectors, each containing genes encoding the three enzymes of the PHB biosynthetic pathway, β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase, modified with an N-terminal plastid targeting signal. Vectors differed with respect to the seed-specific promoters driving the PHB transgenes. The promoters used in the binary vectors include the *Lesquerella fendeleri* oleate 12-hydroxylase (P-Lh, pMBXS364), the *B. napus* napin (pMBXS491), the soya bean glycinin-1 (pMBXS492), a combination of the soya bean oleosin and glycinin-1 (pMBXS490) and a combination of soya bean αβ-conglycinin and glycinin-1 (pMBXS493) (Figure 1). The P-Lh promoter was chosen because it has previously been used for PHB production in seeds of *B. napus* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). The napin, glycinin and β-conglycinin promoters were chosen because they are active during seed development for production of major seed storage proteins while the oleosin promoter was investigated because it is active during the formation of the oleosin oil body-associated protein in seeds. All transformation constructs also contained an expression cassette for DsRed, a red fluorescent protein from the *Discosoma* genus of coral (Matz *et al.*, 1999). This protein has previously been used as a visual marker to select transgenic Camelina seeds (Lu and Kang, 2008). *Agrobacterium*-mediated transformation of the five multigene constructs into *C. sativa* var. Suneson was performed using a previously described floral dip *in planta* transformation system (Lu and Kang, 2008). T_1 seeds of putative-transformed lines were identified by visualization of DsRed, and PHB levels in single T_1 seeds were determined by a destructive simultaneous extraction and butanolysis procedure followed by gas chromatography (GC). Individual seeds transformed with pMBXS364 containing the P-Lh promoter contained a range of PHB contents (Figure S1) with maximum levels of 6.3% of the mature seed weight (Table 1), a value that is close to the 7.7% observed previously in *B. napus* with a similar construct (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). The top performing construct in our studies was pMBXS490, containing the soya bean oleosin promoter driving expression of the *phaB* and *phaC* genes and the glycinin-1 promoter for the *phaA* gene, that produced PHB levels ranging from 0 to 15.2% of mature seed weight (Table 1, Figure S1). This level of PHB production is almost twice what has previously been reported in *B. napus* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). Constructs pMBXS491, pMBXS492 and pMBXS493 yielded seeds containing up to 10.9%, 7.8% and 1.0% PHB, respectively (Table 1, Figure S1). Among the T_1 seeds tested, the majority of DsRed positive seeds obtained from the plants transformed with the binary vectors produced some level of PHB (Table 1).

PHB production in Camelina line bred for larger seed size

Two of the constructs from initial small scale screening studies with *C. sativa* var. Suneson were chosen to transform into a Camelina genotype (*C. sativa* line 10CS0043) with a larger seed size that was isolated in a breeding program at Agriculture and Agri-Food Canada (AAFC). As Camelina seeds are small, a larger seed size is a desirable trait to increase emergence under nonoptimal conditions and for seed collection in the field and subsequent processing. The 10CS0043 Camelina line (subsequently abbreviated WT43) has been previously tested in field trials in several sites in Canada and found to provide good seed yield and oil content under field conditions (K. Falk, AAFC, personal communication). All transformations were performed at a scale to generate a large population of lines for evaluation. Plasmid pMBXS490 was chosen for these experiments because

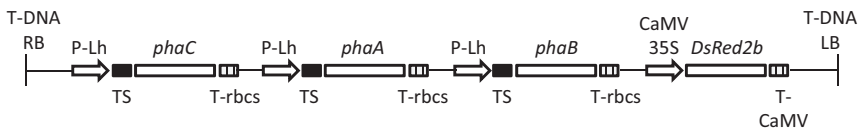
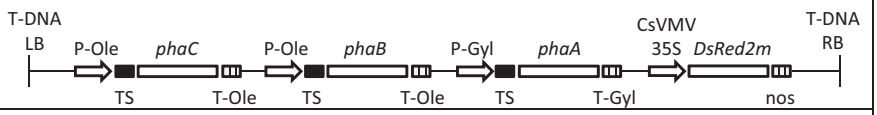
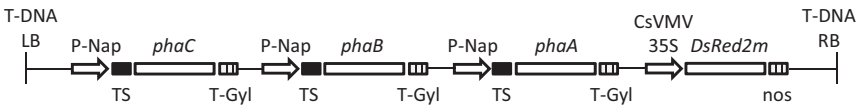
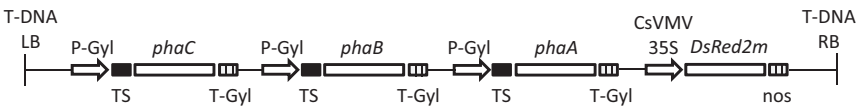
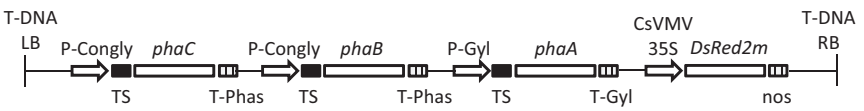
Plasmid	Size (kb)	Map
pMBXS364	20.47	
pMBXS490	15.89	
pMBXS491	17.39	
pMBXS492	16.02	
pMBXS493	17.34	

Figure 1 T-DNA structure of binary vectors used for transformation of Camelina plants. Abbreviations are as follows: RB and LB, right and left border of T-DNA; P-Lh, promoter from the *Lesquerella fendleri* oleate 12-hydroxylase gene (Broun *et al.*, 1998); TS, DNA encoding the signal peptide from ribulose-1,5-bisphosphatase carboxylase (Rubisco) small subunit from *Pisum sativum* including the first 24 amino acids of the mature protein (Cashmore, 1983) and a three amino acid linker containing an *Xba* I restriction site allowing fusion of the desired transgene (Kourtz *et al.*, 2005); *phaC*, DNA encoding a hybrid *Pseudomonas oleovorans/Zoogloea ramigera* PHA synthase (Kourtz *et al.*, 2005); *phaA,* gene encoding the β-ketothiolase from *Ralstonia eutropha* (Peoples and Sinskey, 1989); *phaB,* reductase from *R. eutropha* (Peoples and Sinskey, 1989); T-rbcs, 3′ termination sequence from the *P. sativum* Rubisco small subunit (*rbcs-E9*) gene (Coruzzi *et al.*, 1984); CaMV 35S, 35S promoter from the cauliflower mosaic virus (Odell *et al.*, 1985); CsVMV 35S, 35S promoter from the cassava vein mosaic virus (Verdaguer *et al.*, 1996); *DsRed2b*, DNA encoding a 233 amino acid red fluorescent protein from the *Discosoma* genus of coral (Matz *et al.*, 1999) with the first 225 amino acids equivalent to GenBank EF451141 followed by amino acid sequence VPMTRVSP; *DsRed2m*, DNA encoding a 228 amino acid red fluorescent protein from the *Discosoma* genus of coral (Matz *et al.*, 1999) with the first 225 amino equivalent to GenBank EF451141 followed by amino acid sequence VPM; *nos,* 3′ termination sequence from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene (Bevan *et al.*, 1983); P-Ole and T-Ole, promoter and 3′ termination sequence from the soya bean oleosin isoform A gene (Rowley and Herman, 1997); P-Gyl and T-Gyl, promoter and 3′ termination sequence from the soya bean glycinin (subunit G1) gene (Iida *et al.*, 1995); P-Nap, promoter from the *B. napus* 1.7S napin (*napA*) gene (Josefsson *et al.*, 1987), P-congly, promoter from the soya bean β-conglycinin gene (α subunit) (Yoshino *et al.*, 2001); T-Phas, terminator from the *Phaseolus vulgaris* phaseolin gene (Slightom *et al.*, 1983).

seeds from lines obtained from transformations of this vector produced the highest level of polymer in preliminary screens of transformation constructs in the Suneson germplasm background (Table 1). Plasmid pMBXS492 containing an intermediate value of PHB (up to 7.8% DW; Table 1, Figure S1) was also selected for transformation into WT43 for comparison purposes.

Initial germination tests of DsRed positive T_1 seeds obtained from transformations of WT43 with pMBXS490 and pMBXS492 suggested that seedling survival of some lines was impaired. In

Table 1 Comparison of PHB production in sacrificed single T_1 seeds of Suneson transformed with various constructs for seed-specific PHB production

Genetic construct*	No. seeds analysed	No. seeds with PHB	Average PHB content[†,‡]	Highest PHB producer[‡]	Lowest PHB producer[‡,§]
pMBXS364	22	22	3.4 ± 1.7	6.3	<0.1
pMBXS490	25	23	6.4 ± 3.9	15.2	0.3
pMBXS491	23	22	5.4 ± 3.2	10.9	0.4
pMBXS492	21	18	4.4 ± 2.6	7.8	<0.1
pMBXS493	17	15	0.5 ± 0.3	1.0	<0.1

*Promoters in each construct are shown in Figure 1.

[†]Average PHB content is calculated from lines containing detectable levels of PHB in seeds.

[‡]Units for PHB content are % weight of mature seeds as described in Experimental procedures.

[§]Lowest PHB producing seed, some seeds with no PHB were obtained. More detailed data for these experiments are shown in Figure S1.

an attempt to recover all lines to determine maximum PHB production potential with the 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). Seedlings with a range of cotyledon phenotypes, including those with chlorotic, variegated and green cotyledons, were isolated. 90% of the pMBXS490 and pMBXS492 seeds germinated on tissue culture media. Of 150 seeds of pMBXS490 that were plated on tissue culture media, 118 T_1 seedlings were obtained and transferred to six-inch pots in the greenhouse and 105 of these plantlets survived. Of 145 seeds of pMBXS492 that were plated on tissue culture media, 112 T_1 seedlings were obtained and transferred to pots in the greenhouse and 108 of these plantlets survived. Once the first true leaves of the seedlings formed, all plants grew normally, were healthy and set normal seeds. T_2 seeds were harvested and a sample of DsRed positive seeds (approximately 30 mg) was picked from the segregating seed population and used to determine PHB content by GC/MS.

From pMBXS490 transformations, 78 of the 83 T_1 lines analysed produced detectable amounts of PHB in their T_2 seeds (Table 2). The highest levels observed were 15.2% (Table 2, Figure 2a), a value similar to the T_1 values obtained in individual seeds with Suneson transformations (Table 1). To evaluate germination, seedling emergence and survival, T_2 DsRed positive seeds from 43 lines containing >6% PHB were germinated on wet filter paper and seedlings were transferred to soil under greenhouse conditions. Seedling survival rates of various PHB lines ranged from 0 to 40%, with the highest survival observed with a line that contained 8.6% PHB in T_2 seeds (Line 11-2438, Figure 2). Additional lines of interest include a line with 10.7% PHB that had a 26% survival rate (Line 11-2440, Figure 2) and a line containing 12.8% PHB with a 19% survival rate (Line 11-2439, Figure 2). Wild-type control WT43 line had 100% germination, seedling emergence and survival.

From pMBXS492 transformations, 60 of 65 T_1 lines analysed contained some level of PHB (Table 2). The highest PHB level observed in DsRed positive T_2 seeds from these transformations was 13.5% (Figure 3a), a value higher than the approximately 8% produced in preliminary T_1 seed screens of the construct in *C. sativa* Suneson (Table 1, Figure S1). Germination, emergence and survival tests on T_2 DsRed positive seeds from 22 lines that contained >6% PHB were performed, as described for pMBXS490 lines, and a survival rate of 0–30% was observed (Figure 3b). The maximum 30% survival was obtained for a line that contained 6.2% PHB in T_2 seeds (Line 11-2646, Figure 3)

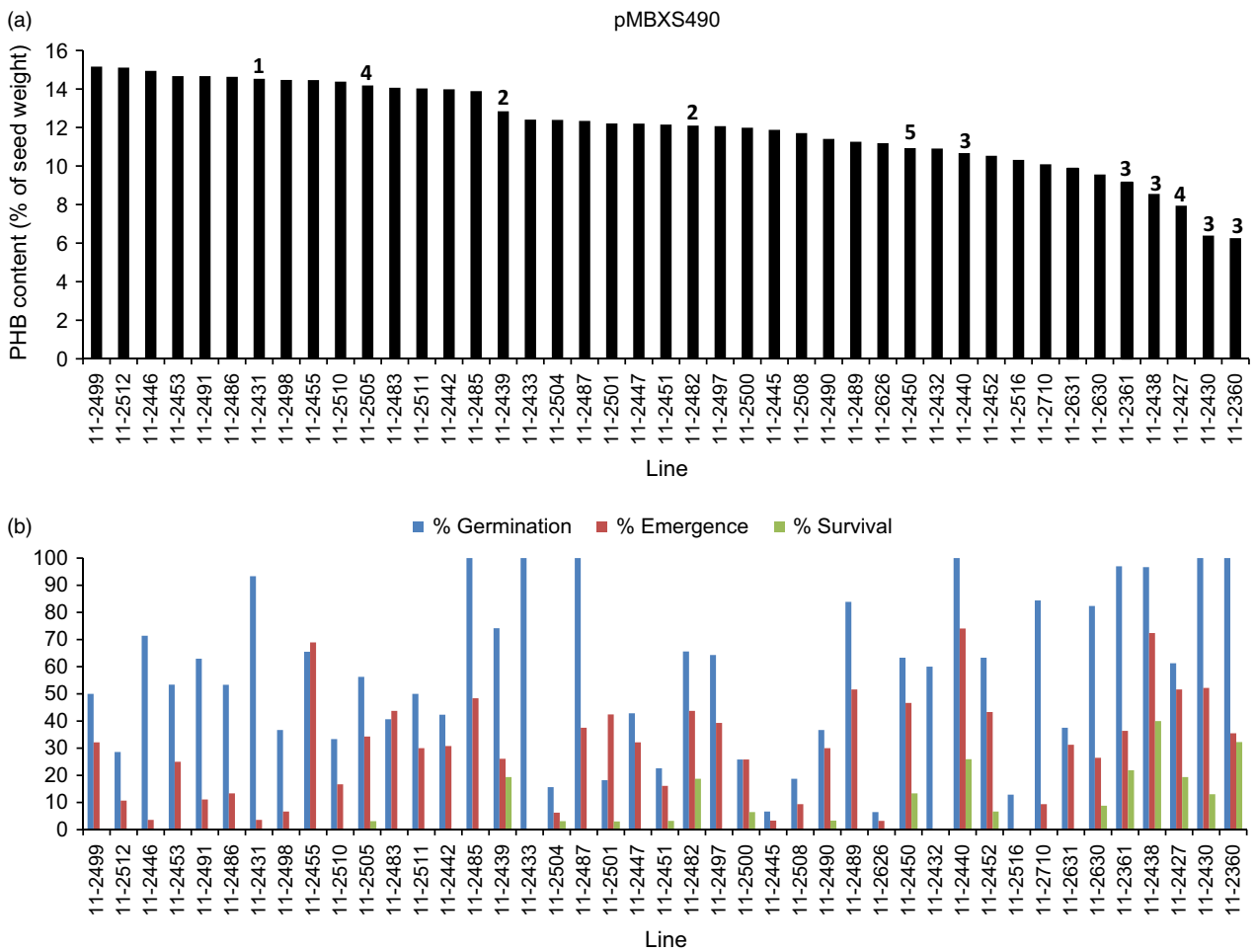


Figure 2 PHB content and health of select T_2 seeds and seedlings obtained from transformations of WT43 with pMBX490. (a) PHB content in T_2 seeds. Number above select bars indicates copy number of T-DNA insert as determined by Southern analysis. (b) Germination, emergence and survival of T_2 seeds and seedlings. Lines producing ≥6% PHB in seeds were tested.

Table 2 Comparison of PHB production in T_2 seeds of WT43 transformed with pMBXS490 and pMBXS492

Genetic construct*	No. lines analysed	No. lines with PHB	Average PHB content†,‡	Highest PHB producer‡	Lowest PHB producer‡,§
pMBXS490	83	78	11.6 ± 2.8	15.2	1.1
pMBXS492	65	60	9.6 ± 2.7	13.5	0.4

*Promoters in each construct are shown in Figure 1.

†Average PHB content is calculated from lines containing detectable levels of PHB in seeds.

‡Units for PHB content are % mature seed weight as described in Experimental procedures.

§Lowest PHB producing seed, some seeds with no PHB were obtained.

as compared to 100% seedling survival for the wild-type WT43 line.

Determination of insert copy number of select lines

The number of T-DNA inserts in a subset of T_1 transgenic lines was determined by Southern blot analysis. Representative Southern blots obtained from these analyses are shown in Figure S2. The insert copy number of analysed lines is shown above the PHB content bar in Figures 2a and 3a. Although some lines with a single copy insert were identified, insert copies of between two and five were also observed. There was no obvious correlation between insert copy number and PHB level.

Spatial distribution of granules and oil bodies in wild-type and PHB producing seeds

T_2 seeds from pMBXS490 PHB producing line 11-2431 (14.5% PHB, Figure 2) and wild-type control WT43 were analysed by transmission electron microscopy (TEM) to visualize possible impacts of high level PHB production on seed morphology. Ultrathin sections of whole mature imbibed seeds were prepared by longitudinal sectioning passing through the seed cotyledon tissue. Wild-type control seeds were found to have a very ordered structure of oil bodies localized around protein storage vacuoles containing protein bodies (Figure 4a,b). In PHB producing seeds (Figure 4c,d), discrete granules of PHB that varied in size were observed with visibly fewer oil bodies and apparently smaller protein bodies. All the plastids in the sections of transgenic seeds analysed appeared to be full of PHB.

Partitioning of carbon in PHB producing lines

The total oil content and the oil fatty acid profile of both low and high PHB producers was measured and compared to the wild-type line. Increased PHB production resulted in decreased oil content in the seed (Figure 5) and a change in the fatty acid profile of the oil (Figure 6). In general, an increase in the levels of

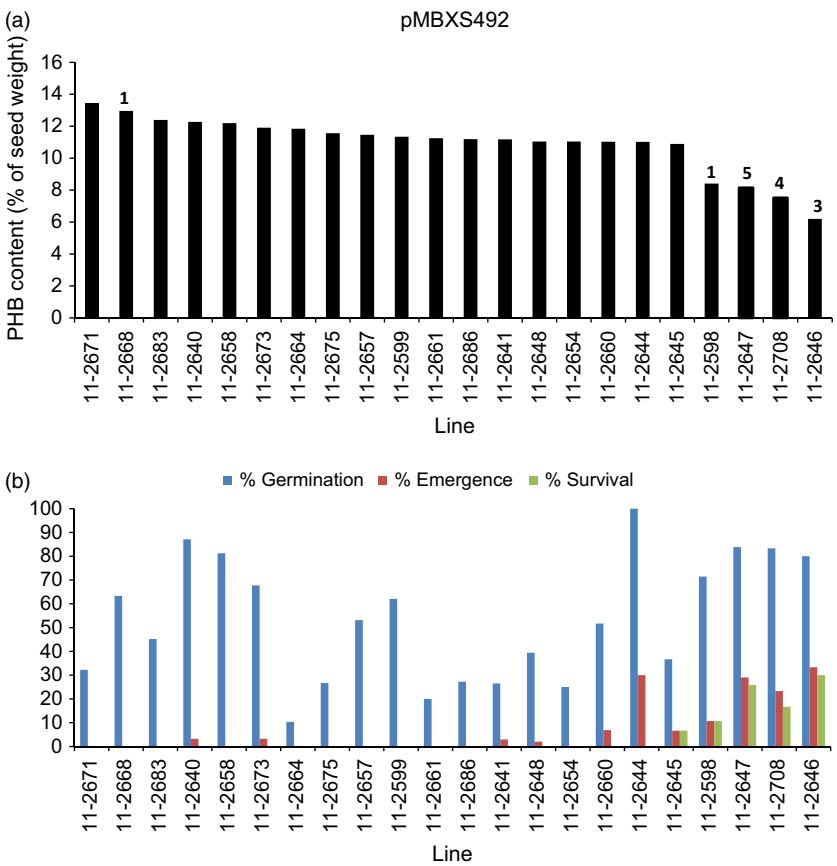


Figure 3 PHB content and health of select T_2 seeds and seedlings obtained from transformations of WT43 with pMBX492. (a) PHB content in T_2 seeds. Number above select bars indicates copy number of T-DNA insert as determined by Southern analysis. (b) Germination, emergence and survival of T_2 seeds and seedlings. Lines producing ≥6% PHB in seeds were tested.

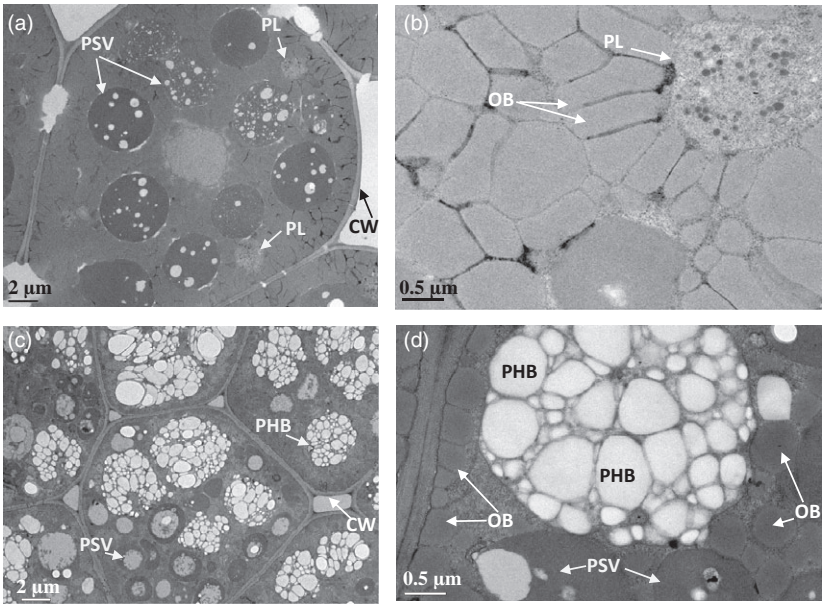


Figure 4 Transmission electron micrographs of mature seeds of wild-type and PHB producing Camelina. (a, b) wild-type line WT43. (c, d) T_2 line 11-243 (14.5% PHB in mature T_2 seeds, Figure 2) obtained from transformation of pMBXS490. CW, cell wall; OB, oil body; PSV, protein storage vacuoles; PHB, granule of polyhydroxybutyrate; PL, plastid.

saturated fatty acids (16:0, 18:0, 20:0, 22:0) was observed with increases in PHB production. For unsaturated fatty acids, levels of 18:1 increased whereas levels of 18:3 and 20:1 decreased (Figure 6). Total protein content in seeds from a selected number of lines was also measured to determine whether PHB production impacted its accumulation in the seed. Little to no impact on levels of seed protein was found upon PHB production (Figure 7).

Size of PHB polymer chain

As molecular weight is an important property of polymers that can change their suitability for different applications, the molecular weight of PHB produced in seeds of select lines containing different levels of polymer was measured by gel-permeation chromatography (Figure 8). In all samples measured, a relatively high molecular weight polymer was present. The weight-averaged molecular weight (M_w) varied depending on the line and construct used for transformation. In lines of pMBXS490, a trend of M_w change with polymer level in the seed was not apparent (Figure 8a,b). Seeds from the pMBXS490 lines possessed a PHB M_w of between 1×10^6 and 1.3×10^6. In lines of pMBXS492, polymer M_w decreased with increasing PHB level in seeds (Figure 8c,d). PHB M_w ranged from 1.5×10^6 in seeds that contained 1.5% PHB to 630 000 in seeds that produced 13.4% PHB. The molecular weight of PHB in bacterial systems has previously been shown to change with varying PHA synthase activity (Sim *et al.*, 1997) or by the presence of chain transfer agents such as alcohols (Hiroe *et al.*, 2013; Tomizawa *et al.*, 2010). The polydispersity index (PDI), equivalent to M_w/M_n where M_n is the number average molecular weight, ranged from 1.48 to 1.81. PDI provides an indication of the size distribution within the population of polymer chains in a sample which can have an effect on the properties of the polymer (Gilbert, 1995).

PHB production in advanced generations

Select lines of WT43 transformed with either pMBXS490 or pMBXS492 were propagated to produce T_3 seed. Seeds from between five and seven individual progenies were tested for PHB level, germination, emergence and seedling survival. In general, there was some decrease in PHB formation in the T_3 generation that depended on the line analysed (Table 3). Seedling germination, emergence and survival was also line dependent with some lines displaying slightly increased or decreased survival compared to the previous generation. One line each of pMBXS490 and pMBXS492

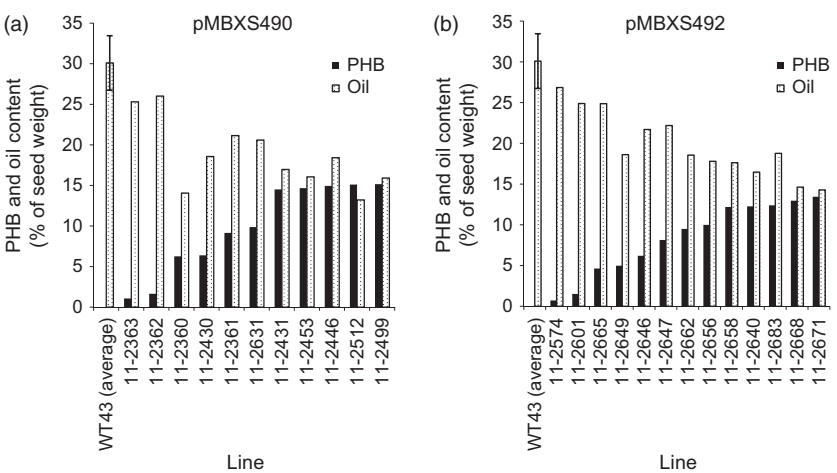


Figure 5 PHB and oil content in T_2 seeds of select lines of WT43 transformed with (a) pMBXS490 or (b) pMBXS492. Wild-type line data is the average of nine different separately grown seed samples of line WT43. Standard deviations for WT43 values are shown on the graph. Data for pMBXS490 lines (11-2363, 11-2362 and 11-2431) and pMBXS492 lines (11-2668 and 11-2671) are the average of two separate measurements. Data for all other transgenic lines are from one measurement.

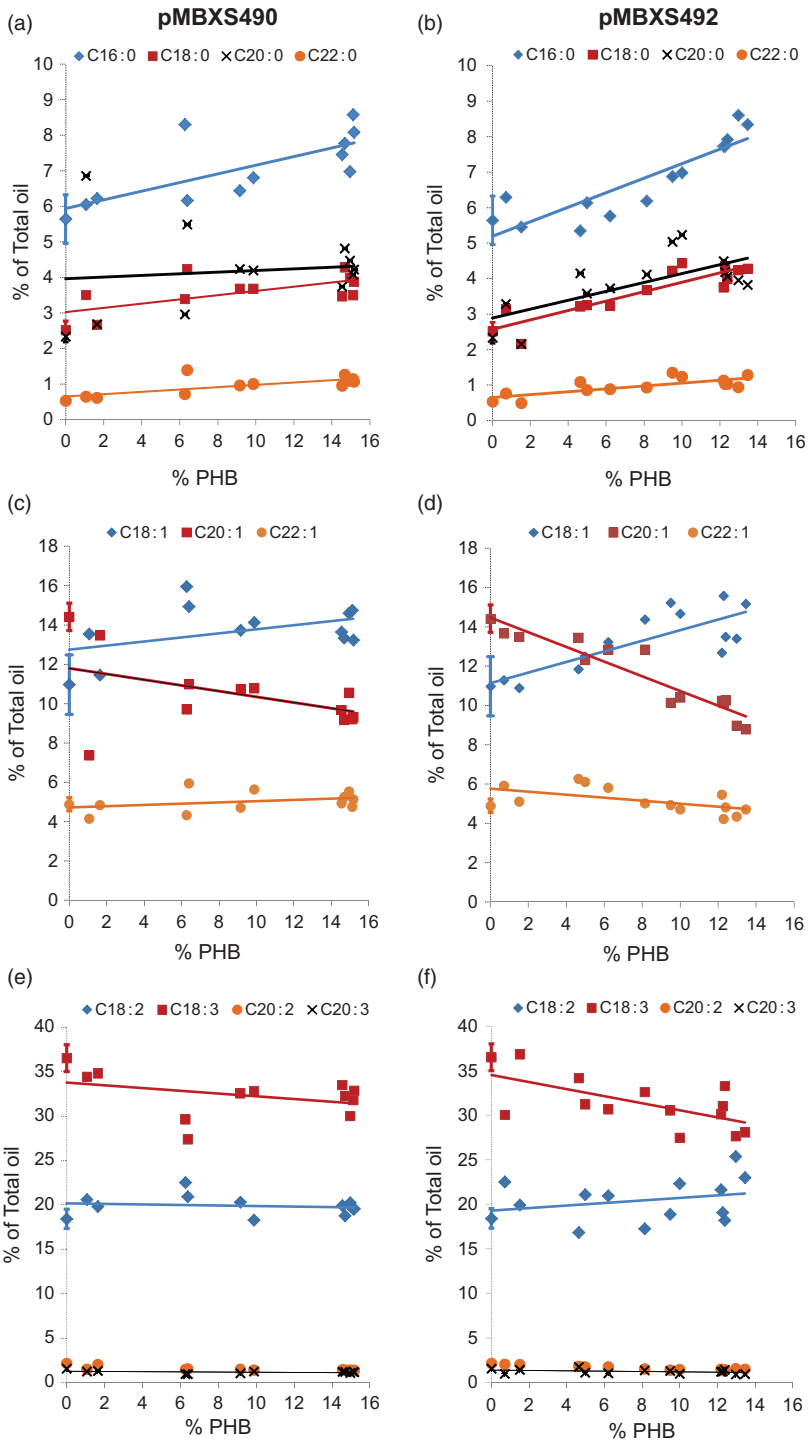


Figure 6 Fatty acid profile of T_2 seeds of WT43 lines transformed with pMBXS490 or pMBXS492. Total oil content of lines is shown in Figure 5. (a, b) Fatty acid profile of saturated fatty acids for (a) pMBXS490 and (b) pMBXS492 transformants; (c, d) Fatty acid profile of mono-unsaturated fatty acids for (c) pMBXS490 and (d) pMBXS492 transformants; (e, f) Fatty acid profile of poly-unsaturated fatty acids for (e) pMBXS490 and (f) pMBXS492 transformants. Wild-type line data (0% PHB on graphs) is the average of nine different, separately grown, seed samples of line WT43. Data for pMBXS490 lines (11-2363, 11-2362 and 11-2431) and pMBXS492 lines (11-2668 and 11-2671) are the average of two separate seed measurements. Data for all other transgenic lines are from one measurement.

was further propagated to produce T_4 seed. PHB levels increased slightly from the T_3 to T_4 generation with T_4 lines producing up to 13.7% (pMBXS490) and 11.5% (pMBXS492) PHB (Table 4). For these two lines, seedling survival was very low and remained essentially constant in the T_3 and T_4 generations. In this experiment, seedling survival of the control wild-type WT43 line was 97%.

Transcript levels of *phaA*, *phaB* and *phaC* genes in homozygous lines

Transcript levels from all three PHB genes were determined by RT-PCR in developing seeds at 12 and 18 days after flowering (DAF) for several homozygous lines produced from transformations of WT43 with pMBXS490 and pMBXS492. At 12 DAF in pMBXS490 lines, there was some correlation between increasing PHB content and higher transcript levels of *phaC* and *phaB* that were both expressed from the oleosin promoter (Figure 9). Thiolase transcript (*phaA*) expressed from the glycinin promoter was much less abundant and appeared to have little or no correlation to PHB content. For pMBXS492 lines, transcript abundance for *phaC* and *phaB* expressed from the glycinin promoter was much weaker at 12 DAF than that obtained with the oleosin promoter in the pMBXS490 lines. The pMBXS492 line

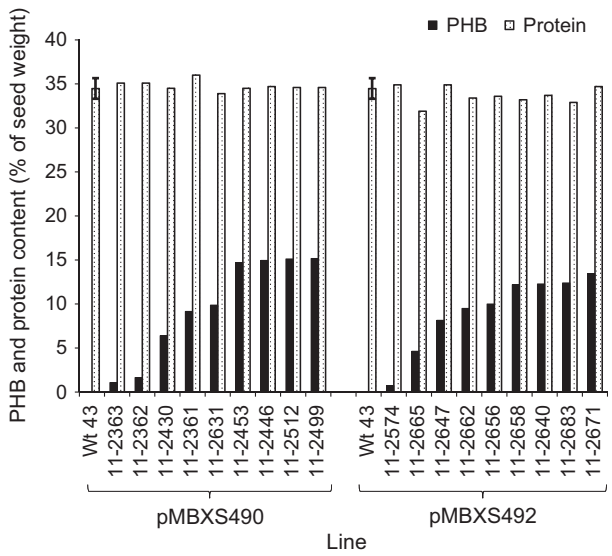


Figure 7 PHB and protein content of T_2 seeds of WT43 transformed with pMBXS490 or pMBXS492. Average protein contents were calculated for four separately grown wild-type WT43 plants. Data for transgenic samples are from one measurement.

with the lowest PHB value (Figure 9, line 14-0397) had the lowest transcript abundance for *phaC* and *phaB*. Higher PHB producing pMBXS492 lines generally had higher transcript levels for *phaC* and *phaB* albeit with no apparent correlation to PHB level. Thiolase transcript abundance was in general lower than *phaC* and *phaB* in pMBX492 lines. In developing seeds at 18 DAF, transcript levels were higher and expressed at similar levels in all pMBXS490 and pMBXS492 lines (Figure S3). Based on these observed transcript profiles in lines derived from two constructs, differing only in the promoter, the glycinin promoter appears to be noticeably weaker than the oleosin promoter at 12 DAF but of similar strength at 18 DAF.

Discussion

In this study, we investigated the use of Camelina as a platform for seed-based production of the polymer PHB. Our interest in PHB stems from its unique attributes that allow it to be used in multiple applications in different market segments including bioplastics, enhanced feed supplements and renewable chemicals (Snell and Peoples, 2013; Somleva *et al.*, 2013). Five genetic constructs containing the PHB genes under the control of different combinations of five seed-specific promoters were evaluated for production of polymer in seed plastids using a novel single T_1 seed screening method. The screening was enabled by the use of the DsRed visual marker to identify transgenic seeds in the T_1 generation. One construct pMBXS490, containing a combination of the oleosin and glycinin-1 promoters driving the expression of the PHB transgenes, resulted in production of polymer in T_1 seeds at levels of up to 15% of the mature seed weight. This PHB level is almost twofold higher than what has previously been reported in a seed (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). The use of other seed-specific promoters for expression of the PHB transgenes led to lower levels of PHB ranging from 10.9% with the napin promoter to approximately 1% with the α subunit of β-conglycinin promoter. One of these additional promoters (P-Lh) has been used

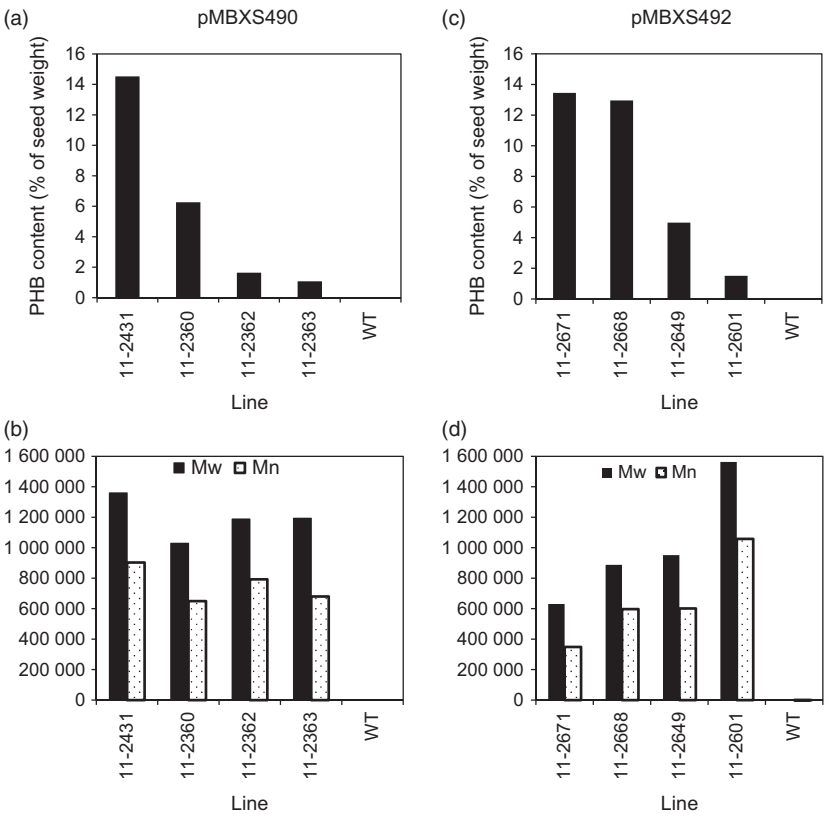


Figure 8 PHB content and molecular weight of polymer in T_2 seeds. (a, c) PHB levels in T_2 seeds from (a) pMBXS490 and (c) pMBXS492. (b, d) M_w and M_n values of polymer from (b) pMBXS490 and (d) pMBXS492.

Table 3 PHB content and health of select T_3 seeds and seedlings obtained from transformation of WT43 with pMBXS490 and pMBXS492

			T_2 generation				T_3 generation					
Genetic construct*	T_1 line	Copy number T_1 line	% PHB, bulk T_2 seed†	% germination T_2 seed	% emergence T_2 seedling	% survival T_2 seedling	No. lines tested	Range of % PHB T_3 seed†	Avg PHB content†,‡ T_3 seed	Avg % germination T_3 seed	Avg % emergence T_3 seedling	Avg % survival T_3 seedling
pMBXS490	11-2431	1	14.5	93	4	0	6	7.2–12.8	10.1 ± 2.1	72 ± 37	15 ± 13	4 ± 8
	11-2440	3	10.7	100	74	26	6	5.2–9.4	7.1 ± 1.5	73 ± 38	30 ± 21	26 ± 20
	11-2361	3	9.2	97	36	22	5	4.9–9.4	7.0 ± 2.2	95 ± 12	51 ± 18	37 ± 21
	11-2438	3	8.6	97	72	40	7	3.0 –5.5	4.3 ± 0.9	94 ± 10	85 ± 13	81 ± 20
	11-2360	3	6.3	100	35	32	7	5.3–7.0	6.3 ± 0.7	59 ± 43	70 ± 22	57 ± 25
pMBXS492	11-2668	1	13.0	63	0	0	5	9.4–11.7	10.4 ± 0.8	61 ± 42	5 ± 9	2 ± 4
	11-2647	5	8.1	84	29	26	7	5.9–8.4	7.0 ± 0.9	39 ± 40	7 ± 12	6 ± 11

*Promoters in each construct are shown in Figure 1.

†Units for PHB content are % mature seed weight as described in Experimental procedures.

‡Average PHB content is calculated from lines containing detectable levels of PHB in seeds.

Table 4 PHB content and health of select T_4 seeds and seedlings obtained from transformation of WT43 with pMBXS490 and pMBXS492

Genetic construct*	T_1 Source Lines	No. of lines tested	Range of %PHB T_4 seed†	Avg PHB content T_4 seed†,‡	Avg % germination T_4 seed	Avg % emergence T_4 seedling	Avg % survival T_4 seedling
pMBXS490	11-2431	5	10.1–13.7	12.1 ± 1.7	69 ± 40	3 ± 6	1 ± 2
pMBXS492	11-2668	2	9.0–11.5	10.2 ± 1.8	88 ± 17	18 ± 16	3 ± 5

*Promoters in each construct are shown in Figure 1.

†Units for PHB content are % mature seed weight as described in Experimental procedures.

‡Average PHB content is calculated from lines containing detectable levels of PHB in seeds.

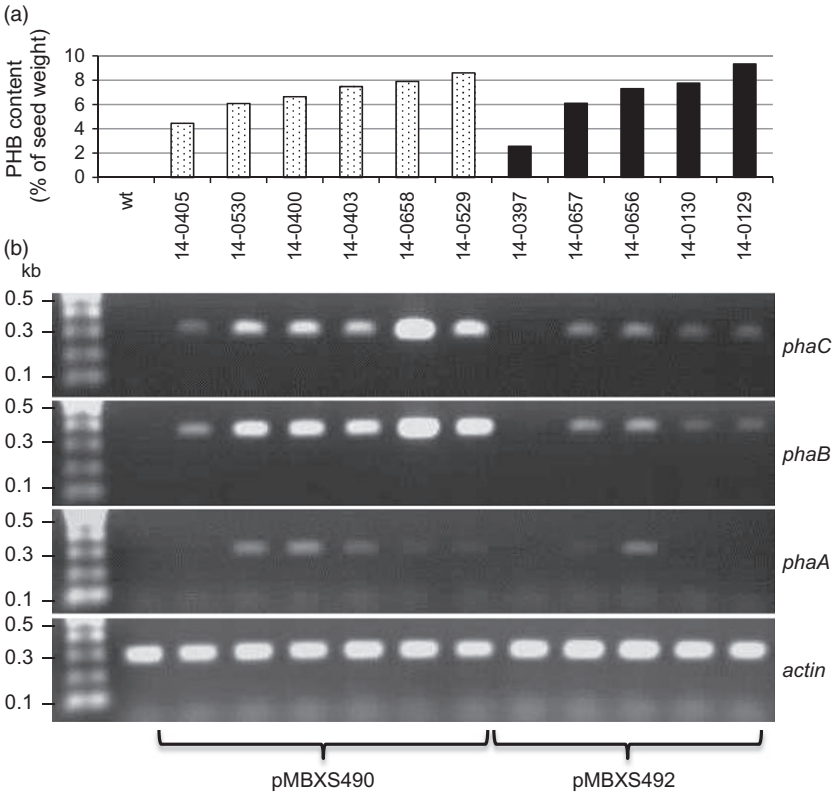


Figure 9 Transcript analysis in developing seeds of homozygous lines of pMBXS490 and pMBXS492 at 12 days after flowering (DAF). (a) PHB content (% mature seed weight) in seeds of homozygous lines. All pMBXS490 lines are T_3 seeds except for line 14-0658 which is T_5 seed. All pMBXS492 lines are T_4 seeds except for lines 14-0129 and 14-0130 which are T_5 seed. (b) Semi-quantitative analysis of *phaC*, *phaB* and *phaA* transcripts was performed by RT-PCR using actin as the control. Primers for the RT-PCRs are listed in Table S1. The following lines were derived from the same T_1 event: 14-0405 & 14-0530; 14-0400 & 14-0403; 14-0657 & 14-0656; 14-0130 & 14-0129. PHB content (a) was measured in mature seeds harvested from the same plant for which developing seeds were harvested for RNA extraction for RT-PCR (b).

previously to produce up to 7.7% PHB in seeds of *B. napus* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999). In screening efforts that measured PHB content in sacrificed T_1 seeds, a P-Lh promoter construct in our study reached similar levels of up to 6.3% PHB.

Increased seed size is an important trait for Camelina, a naturally small seeded plant, as larger seeds may help improve emergence in dry, windy environments (Enjalbert *et al.*, 2013) and improve seed recovery with mechanized harvesting. Two of the genetic constructs that were characterized in the single-seed screening experiments were transformed into WT43, a large seeded line isolated in a Canadian Camelina breeding program. Lines transformed with pMBXS490 and pMBXS492 producing up to 15.2% and 13.5% PHB of the mature seed weight, respectively, in T_2 seeds were identified. Varying levels of seedling vigour and cotyledon chlorosis were observed in PHB producing lines. Cotyledon phenotypes ranged from green to yellowish, to severely chlorotic or variegated, depending on the level of PHB in seeds. Often the most severe phenotypes were observed in seedlings obtained from seeds with the highest PHB levels, and the survival of these seedlings in soil was reduced. Thus our use of a seed-based DsRed visual selection strategy for identification of transgenic lines, combined with rescue of impaired seedlings on tissue culture medium supplemented with a carbon source, may have allowed the isolation of high PHB producing lines. Impaired seedlings may not have been identified as transgenic using traditional herbicide or antibiotic selectable markers, for which screening is often performed by germination on antibiotic or herbicide containing medium. Additional work to investigate factors contributing to reduced seedling vigour associated with high levels of PHB production, and to stack additional genes into transgenic lines to produce more vigorous seedlings, is currently in progress.

While the low survival rate of the highest PHB producers in soil is a challenge that needs to be overcome, it is also an opportunity for added gene containment when coupled with an inducible promoter or gene switch. In such a scenario, impaired seedlings from high PHB producing seeds inadvertently lost in the field during harvest would not be viable. With the proper gene switch, reduction of PHB gene expression by treatment of the seeds during seed formation or prior to sowing could be used to increase seedling vigour and survival in the field. Controlled polymer production in leaf tissue of *Arabidopsis* has been previously demonstrated using an ecdysone inducible promoter that is induced by an agricultural chemical (Kourtz *et al.*, 2007). Ecdysone inducible promoters have also been used in seeds to overproduce abscisic acid, by inducing expression of a gene cassette encoding a rate limiting enzyme in its biosynthesis, resulting in seed dormancy (Martínez-Andújar *et al.*, 2011).

Increased PHB production in seeds resulted in a concomitant decrease in total oil content with no detectable change in protein levels. As acetyl-CoA is the common precursor for both fatty acid biosynthesis and PHB synthesis, the drop in total oil content in PHB producing seeds appears reasonable. However, it is interesting that no detectable drop in protein content was observed because predictive modelling studies of developing seeds of *B. napus* have shown precursor demands, albeit small compared to fatty acid biosynthesis, for plastidial acetyl-CoA, pyruvate and phosphoenolpyruvate for synthesis of some amino acids (Schwender and Hay, 2012). The decrease in seed oil content with PHB polymer production suggests that developing seeds engineered with our constructs are not able to compensate for

diversion of carbon away from triacylglycerol synthesis. This contrasts to previous efforts to produce lauric acid (C12:0) in *B. napus* where total seed oil levels were maintained even though ^{14}C labelling studies indicated a significant amount of fatty acid produced in the seeds was diverted to breakdown by β-oxidation (Eccleston and Ohlrogge, 1998). Interestingly, PHB production in leaf tissue of *Arabidopsis* at levels of approximately 40% DW has not been observed to affect total levels of fatty acids (Bohmert *et al.*, 2000), suggesting that changes in carbon partitioning upon engineering a novel carbon sink may be different in leaves and seeds.

Poly-3-hydroxybutyrate production in Camelina seeds also resulted in a change in the fatty acid profile of the seed oil. Increased amounts of saturated fatty acids were observed with increased levels of PHB production. This increase in saturation may be reflective of a change in redox state within the seeds because desaturases require reducing equivalents as electron donors for the NAD(P)H/ferredoxin oxidoreductase or the NADH/cytochrome *b5* oxidoreductase systems (Murphy and Piffanelli, 1998). If cells are low in NAD(P)H, as might occur after the production of large amounts of PHB, this may prevent normal desaturation from occurring. More detailed studies are required to understand the change in fatty acid saturation upon PHB production. The altered oil profile in many of our high PHB producing lines is however likely more desirable for industrial end-use applications than oil isolated from wild-type plants. Researchers have noted that the high content of unsaturated fatty acids, in particular the high 18:3 content, is one of the major drawbacks of oil from Camelina for fuel use (Ciubota-Rosie *et al.*, 2013).

Our results show that production of high levels of PHB in Camelina oilseeds is feasible with expression of the basic three gene bacterial PHB biosynthetic pathway. Additional increases in total polymer levels may be possible with additional metabolic engineering. Further work is underway to increase seedling emergence and vigour in lines containing high levels of PHB in order to enable field cultivation. The demonstrated production of 15% PHB in a seed is an important step forward towards commercialization of a seed-based production platform for PHB.

Experimental procedures

Plant material

Camelina sativa var. Suneson was obtained from Montana State University and grown in a greenhouse at 21/20 °C day/night with a relative humidity of 40%, photoperiod of 16 h and light intensity of 650 μmol/m^2/s. Large seeded *C. sativa* germplasm 10CS0043 (abbreviated WT43) was obtained from Kevin Falk at Agriculture and Agri-Food Canada and grown in a greenhouse at 22/18 °C day/night and a photoperiod of 16 h under supplemental light intensity of 900 μmol/m^2/s during the day time.

Construction of binary vectors for PHB production

All genetic constructs are derivatives of pCAMBIA binary vectors (Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia) and are shown in Figure 1.

Plant transformations

For transformations of Suneson, binary vectors were introduced into *Agrobacterium* strain C58. For transformations of WT43, *Agrobacterium tumefaciens* strain GV3101(pMP90) was used. Transformation was performed by floral dip methods as described previously (Lu and Kang, 2008). DsRed seeds were visually identified with a green flashlight (INOVA LED green X5, Emissive Energy Corp, North Kingston, RI, USA) with an approximate excitation wavelength of 550 nm and a red filter with an approximate emission wavelength of 580 nm (Jach *et al.*, 2001) or by fluorescent microscopy using a Nikon AZ100 microscope with a TRITC-HQ(RHOD)2 filter module (HQ545/30X, Q570LP, HQ610/75M).

Measurement of PHB content

Poly-3-hydroxybutyrate content was measured using a previously described simultaneous extraction and butanolysis procedure that converts PHB into butyl esters of monomeric units (Kourtz *et al.*, 2007). For Suneson transformations, mature seeds were harvested and stored in paper envelopes at room temperature for about 2 weeks prior to analysis. For seeds from WT43 transformations, harvested mature seeds were dried in an oven with mechanical convection set at 22 °C and stored in paper envelopes at room temperature (22 ± 2 °C) for at least 4 weeks prior to analysis. For bulk seed analysis, approximately 30 mg of mature seeds and 3 mL of butanolysis reagent (Kourtz *et al.*, 2007) was used. For single-seed analysis, 200 μL of butanolysis reagent was used. Total polymer content was quantified using either an Agilent 6890 gas chromatograph with flame ionization detection (GC-FID) and an HP-INNOWax column (30 m length × 0.25 mm inner diameter, 0.25-μm film thickness; Agilent Technologies, Santa Clara, CA, USA) or an Agilent 6890/5973 GC/MS equipped with a DB-225MS column and guard. For GC/MS analysis, a selected ion monitoring mode was used (87, 89 and 43.1 amu) as previously described (Kourtz *et al.*, 2007). Calibration curves were made with purified PHB (Sigma-Aldrich, St. Louis, MO, USA). PHB levels were calculated as per cent of mature seed weight.

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

Total seed oil content and oil fatty acid profile were determined using published procedures for preparation of fatty acid methyl esters (Li *et al.*, 2006) with some modifications. Briefly, 10–15 mg of mature seeds were placed in 13 × 100 mm screw-cap test tubes. To each tube, 1.5 mL of 2.5% (v/v) sulphuric acid in methanol (w/0.01% w/v BHT), 400 μL toluene and 500 μg of a triheptadecanoin (Nu-Chek Prep, Elysian, MN) solution (10 mg/mL in toluene) as internal standard were added. Tubes were purged with nitrogen, capped and heated at 90 °C for 1 h. Upon cooling, 1 mL of 1 M sodium chloride and 1 mL of heptane were added to each tube. Following mixing and centrifugation, the heptane layer containing fatty acid methyl esters was analysed with an Agilent 7890A gas chromatograph with a 30 m × 0.25 mm (inner diameter) INNOWax column (Agilent) and flame ionization detection. The oven temperature was programmed from 185 °C (1 min hold) to 235 °C (1 min hold) at a rate of 10 °C min^{-1} (11 min total run time), and the front inlet pressure was 35.8 psi of He. The oil content (% of seed weight) was determined by comparison of the detector response from seed-derived fatty acid methyl esters relative to methyl heptadecanoate from the triheptadecanoin internal standard. Protein content in bulk Camelina seeds was measured by POS-Biosciences, Saskatoon, SK, Canada using the American Oil Chemists Society (AOCS, Urbana, IL, USA) Ba 4e-93 method (Generic Combustion Method for Determination of Crude Protein).

Molecular weight determination

Samples of crushed Camelina seeds (approximately 140 mg) were heated in 5 mL of chloroform at 61 °C for 3 h. Cooled samples were triple filtered with 2-mm Teflon filters. The weight-averaged molecular weight (M_w) and number averaged molecular weight (M_n) of the extracted polymer was determined by gel-permeation chromatography in chloroform using a Waters 2414 Refractive Index detector, three Mixed B columns (300 × 7.5 mm; Polymer Laboratories, part of Varian Inc., Amherst, MA, USA) in series and monodisperse polystyrene standards for calibration. The polydispersity index, equivalent to M_w/M_n and an indication of the size distribution within the population of polymer chains, was calculated from M_w and M_n values.

Transmission electron microscopy

Samples were processed for TEM according to a previously described protocol (Bozzola and Russell, 1992) with minor modifications. Seeds were imbibed for 6 h in water and fixed in modified Karnovsky's fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After washing with phosphate buffer (0.1 M), samples were post-fixed in 1% osmium tetroxide, pH 7.4 (buffered with 0.1 M phosphate) for 2–2.5 h. Samples were washed in 0.1 M phosphate buffer. Specimens were dehydrated in a graded series of ethanol followed by propylene oxide and embedded in Spurr's resin. 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).

Germination of PHB producing lines on tissue culture media

To rescue very high PHB producing lines, a tissue culture media was used for germination and early seedling development. Seeds were rinsed for 30 s in 70% ethanol followed by 10 min gentle agitation in 20% commercial bleach (Javex). Seeds were then rinsed three times in sterile distilled water and plated on ½ strength MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose and 1 μM GA_3. As soon as the first true leaf was visible, seedlings were transferred to Jiffy peat pellets in Phyta jars (Sigma-Aldrich, Oakville, ON, Canada) and plantlets with 3–4 leaves were transplanted in 6″ pots in the greenhouse. The tissue culture plates and Phyta jars were maintained in a tissue culture chamber set at a 16 h photoperiod at 24 °C with a light intensity of 200 μmol/m^2/s.

Determination of germination, emergence and survival

Thirty DsRed positive seeds from an individual PHB line were surface sterilized with 70% ethanol, rinsed in sterile water and plated on 100 × 15 mm Petri plates lined with filter paper (Whatman # 1) that had been wetted with sterile distilled water. A plate with 30 wild-type Camelina seeds was included as a control in each experiment. Petri plates were sealed with Parafilm and kept in the cold (4–8 °C) in the dark for 3 days, after which time they were transferred to a lab bench (22 °C, light intensity of 20 μmol/m^2/s). After 24 h, the seeds with radicles were scored as germinated and both germinated and nongerminated seeds were transferred to 6-inch pots filled with soil (Sunshine Mix #4 saturated with water containing NPK 20-20-20 fertilizer) and a top layer of vermiculite. Pots were placed in the greenhouse with supplemental lighting (16-h photoperiod, 22 °C, typical light intensity of 900 μmol/m^2/s during day time) and were moistened daily with fertilized water (NPK-20-20-20). Emergence of the seedling above the surface of soil was scored as emerged after 1 week of transfer of seeds/seedlings to the pot. Seedlings that were alive after 2 weeks of transfer to pots were scored as positive for survival. Per cent germination, emergence and survival were calculated based on 30 seeds.

Transcript analyses of PHB biosynthesis genes in developing seeds

Total RNA was isolated from developing seeds at 12 and 18 days after flowering (DAF) using the Plant RNA Isolation Mini Kit (Agilent). For semi-quantitative RT-PCR, 1 μg of total RNA was used for first-strand cDNA synthesis with oligo $(dT)_{20}$ primers and Omniscript reverse transcriptase (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. The first-strand cDNA synthesis reaction was diluted 10 times and 1 μL of template cDNA was used for the PCR. Gene-specific primer pairs (Table S1) were designed for PHB biosynthetic genes *phaA*, *phaB* and *phaC* in pMBXS490 and pMBX492 constructs as well as a Camelina internal control gene *actin* (GenBank accession # GAFB01011390.1) so that amplified products were of comparable size. PCR cycling parameters for amplification of all four genes were one cycle at 95 °C for 5 min, 27 cycles at 95 °C for 1 min, 63 °C for 30 s and 72 °C for 30 s followed by a final extension at 72 °C of 10 min. Samples were run on a 1.2% agarose gel using 10 μL (12 DAF; Figure 9) or 7 μL (18 DAF; Figure S3) of the PCR mixture.

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Conflict of interest

Jihong Tang, Oliver Peoples and Kristi Snell are employees of Metabolix Inc. Nii Patterson is a former employee of Metabolix Inc. Meghna Malik, Claire Burkitt, Nirmala Sharma and Yuanyuan Ji are employees of Metabolix Oilseeds, a wholly owned subsidiary of Metabolix Inc.

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Supporting information

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

Figure S1 PHB content in individual T_1 seeds from *C. sativa* Suneson plants transformed with multi-gene constructs for seed-specific PHB production.

Figure S2 Southern analysis of select Camelina lines transformed with pMBXS490 and pMBXS492.

Figure S3 Transcript analysis in developing seeds of homozygous lines of pMBXS490 and pMBXS492 at 18 days after flowering (DAF).

Table S1 Primers used for semi-quantitative RT-PCR.