Engineering *Camelina sativa* (L.) Crantz for enhanced oil and seed yields by combining diacylglycerol acyltransferase1 and glycerol-3-phosphate dehydrogenase expression

Sudesh Chhikara¹,*,‡, Hesham M. Abdullah¹,²,‡, Parisa Akbari¹, Danny Schnell³ and Om Parkash Dhankher¹,*

¹Stockbridge School of Agriculture, University of Massachusetts Amherst, Amherst, MA, USA
²Biotechnology Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt
³Department of Plant Biology, Michigan State University, East Lansing, MI, USA

Received 25 May 2017; revised 14 September 2017; accepted 27 September 2017.
*Correspondence (Tel 413-545-0062; fax 413-545-1058; email parkash@umass.edu)
†Present address: Centre for Biotechnology, Maharshi Dayanand University, Rohtak, 124001, India.
‡These authors contributed equally to this work.

Keywords: *Camelina sativa*, triacylglycerols, biofuels, lipid metabolism, metabolic engineering.

**Summary**

Plant seed oil-based liquid transportation fuels (i.e., biodiesel and green diesel) have tremendous potential as environmentally, economically and technologically feasible alternatives to petroleum-derived fuels. Due to their nutritional and industrial importance, one of the major objectives is to increase the seed yield and oil production of oilseed crops via biotechnological approaches. *Camelina sativa*, an emerging oilseed crop, has been proposed as an ideal crop for biodiesel and bioproduct applications. Further increase in seed oil yield by increasing the flux of carbon from increased photosynthesis into triacylglycerol (TAG) synthesis will make this crop more profitable. To increase the oil yield, we engineered Camelina by co-expressing the *Arabidopsis thaliana* (L.) Heynh. diacylglycerol acyltransferase1 (DGAT1) and a yeast cytosolic glycerol-3-phosphate dehydrogenase (GPD1) genes under the control of seed-specific promoters. Plants co-expressing DGAT1 and GPD1 exhibited up to 13% higher seed oil content and up to 52% increase in seed mass compared to wild-type plants. Further, DGAT1- and GDP1-co-expressing lines showed significantly higher seed and oil yields on a dry weight basis than the wild-type controls or plants expressing DGAT1 and GPD1 alone. The oil harvest index (g oil per g total dry matter) for DGTA1- and GDP1-co-expressing lines was almost twofold higher as compared to wild type and the lines expressing DGAT1 and GDP1 alone. Therefore, combining the overexpression of TAG biosynthetic genes, DGAT1 and GPD1, appears to be a positive strategy to achieve a synergistic effect on the flux through the TAG synthesis pathway, and thereby further increase the oil yield.

**Introduction**

Vegetable oils, composed mainly of triacylglycerols (TAGs), are important nutritional and industrial commodities. They can provide the nutritional requirements of humans and animals and provide chemical feedstocks involved in various industrial products, including biofuels (van Erp et al., 2014; Vigeolás et al., 2007). The worldwide production of vegetable oils has increased dramatically in recent decades, and according to the last statistics of year 2015/2016, it is approximately 179.5 million metric tons (MMT) per year, with the majority produced from palm (65.5 MMT), soybean (53.7 MMT), rapeseed (26.6 MMT) (The statistics portal, Statista, www.statista.com). Despite this increased production of vegetable oils, a wider gap between the production and consumption exists. To overcome the societies’ ever-growing demands for vegetable oils, there is considerable interest in the metabolic engineering of improved seed oil yields and qualities (van Erp et al., 2014).

The application of genetic engineering approaches to boost the metabolic flux of carbon into seed oils has been limited mainly by the benightedness of how lipid metabolism is regulated (Taylor et al., 2009). The pathway for the metabolic flux of carbon into seed storage lipids is complex and a multigene process and involves various subcellular compartments (Durrett et al., 2008; van Erp et al., 2014; Schwender et al., 2004). Briefly, in the developing seeds, sucrose (Suc) is unloaded in the phloem and metabolized into glucose-6-phosphate (G6P) via either Suc synthase- or invertase-dependent pathways (Barratt et al., 2009; van Erp et al., 2014; Hills, 2004). In glycolysis pathway, G6P is then converted to pyruvate, which is subsequently imported into the chloroplast to synthesize acetyl-CoA, a precursor in fatty acid synthesis, via the activity of pyruvate dehydrogenase complex (Durrett et al., 2008; Vigeolás et al., 2007). The newly synthesized acetyl-CoAs are then converted to malonyl-CoA via the activity of acetyl-CoA carboxylase (ACCase-α) and then the fatty acid synthase enzymes, namely 3-ketoacyl Acyl carrier proteins (ACP) are involved to condensate acetyl-CoA and malonyl-ACP. Subsequently, the fatty acyl chains are released from ACP via hydrolysis catalysed by acyl-ACP thioesterases. The synthesized fatty acyl-CoAAs exported from the plastids are utilized in the stepwise esterification of the glycerol backbone via the Kennedy pathway to synthesize glycerolipids, including TAGs at the endoplasmic reticulum (Durrett et al., 2008; Kennedy, 1961). TAG assembly is initiated by acylation of glycerol-3-phosphate (Gly3P) by Gly3P acyltransferase (GPAT) to lysophosphatic acid, which is subsequently acylated to phosphatic acid (PA) by lysophosphatic acyltransferase (LPAT). PA is then dephosphorylated to form diacylglycerol (DAG) by phosphatic acid...
phosphohydrolase (PAH). DAG acyltransferases (DGAT) finally esterify DAG to produce TAG, which is ultimately stored in ER-derived oil bodies (Lacey et al., 1998; Vigeolas et al., 2007).

In developing seeds of oilseed crops, DGAT catalyses the acylation of the sn-1,2-diacylglycerol (DAG) to form TAG, which is the final committed step in the Kennedy pathway (Kennedy, 1961). Biochemical analysis and transgenic studies of lipid metabolism in several oilseed crops have evidenced that DGAT activity has a considerable effect on carbon flow into seed oil and appears to be crucial for mediating seed oil biosynthesis in quantitative and qualitative manners (Taylor et al., 2009; Wesełak et al., 2008). Compared with other enzymes in lipid biosynthesis, DGAT exhibited relatively low activity, and the accumulation of its substrate DAG in developing seeds suggests that DAG-to-TAG acylation reaction represents a rate-limiting step in seed oil formation (Perry et al., 1999; Taylor et al., 2009). Accordingly, cloning of genes encoding distinct oilseed DGATs has been reported, and seed-specific overexpression of DGATs has resulted in an increase in DGAT activity and subsequently seed oil content (Aznar-Moreno et al., 2015; van Erp et al., 2014; Jako et al., 2001; Kim et al., 2016; Taylor et al., 2009).

DGAT1 enzyme was evidenced to be a major determining factor for oil quantity and fatty acid composition of seed oils in several crops, and manipulation of its gene expression has proven to be one of the successful approaches utilized to increase oil content and alter the fatty composition (Jako et al., 2001; Kim et al., 2016; Taylor et al., 2009; Xu et al., 2008). However, structural and functional motif analysis of DGAT1 from Tropaeolum majus L. and Arabidopsis also revealed multiple potential motifs as target sites of members of the sucrose non-fermenting (SNF)-related protein kinase 1 (SnRK1) family, suggesting the regulation of DGAT1 activity by post-translational modification (i.e. phosphorylation), which may down-regulate DGAT1 activity and affect seed oil production (Xu et al., 2008; Zou et al., 1999). It is suggested to enhance DGAT1 activity by blocking the SnRK1 target via site-directed mutagenesis (SDM), thus preventing phosphorylation. Accordingly, the substitution of Ser197 to Ala of SnRK1 site in T. majus DGAT1 has increased its activity by 38%–80%, and when the modified DGAT1 gene was expressed into Arabidopsis seeds, it enhanced the seed oil production in transgenic plants by 3%–10%, seed weight by 45% and total oil yield by 51% on dry weight basis (Xu et al., 2008). This crucial impact of SDM on DGAT1 activity would encourage researchers to undertake similar approach to up-regulate DGAT1 activity by targeting a putative SnRK1 site, thus encouraging DGAT1 impact in seed oil production. However, the negative regulation of DGAT1 activity via phosphorylation of Ser via SnRK1 has not been confirmed yet in planta, the point which requires further investigations.

Utilizing genetic and biochemical knowledge of lipid metabolism, several studies have established the possibility to increase seed oil contents and/or alter oil composition by manipulating the expression levels of individual enzymes involved in oil biosynthesis (Dalal et al., 2015; van Erp et al., 2014; Jako et al., 2001; Kelly et al., 2013; Li et al., 2015; Taylor et al., 2009; Vigeolas et al., 2007; Zou et al., 1997). Nevertheless, the multiple steps involved in the flow of carbon flux through oil metabolic pathways and the sensitivity of the seed oil content to multiple metabolic reactions render the impact of this approach theoretically limited (van Erp et al., 2014; Tang et al., 2012; Wesełak et al., 2008). Over the years, several attempts have been made to significantly boost the seed oil content in various oilseed crops via biotechnological approaches, and many of these attempts concentrated on increasing fatty acids production/use rates or TAG assembly. For instance, manipulating a key rate-limiting enzyme involved in fatty acid biosynthesis, ACCase-α resulted in 5% increase in seed oil in oilseed rape (Brassica napus L., Roesler et al., 1997). Further improvement in seed oil content has been reported through manipulating TAG synthesis pathways. Overexpressing a yeast sn-2 acyltransferase gene (namely, LPAT) has resulted in 8%–48% and 3.2% increases in seed oil content in oilseed rape and soybean (Glycine max (L.) Merr.; Zou et al., 1997; Taylor et al., 2002; Rao and Hillbrand, 2009), respectively. Further, targeting a diacylglycerol acyltransferase (DGAT1), seed oil content has substantially increased as reported in several transgenic crops, including Arabidopsis (~30%, Jako et al., 2001), oilseed rape (~3–8%, Taylor et al., 2009; Xu et al., 2008) and Camelina (~24%, Kim et al., 2016) as compared to nontransgenic plants, on a dry weight basis. Furthermore, a successful increase in overall TAG accumulation was accomplished mostly by targeting the WRINKLED 1 (WR1), a master transcriptional regulator of genes involved in FA synthesis and glycolysis (10%–20% increase compared to WT plants (van Erp et al., 2014).

Several oilseed crops have been investigated concerning oil metabolism and were targeted for improved seed and oil qualities. Among those crops, Camelina sativa (L.) Crantz, an oilseed crop, which belongs to Brassicaceae family, has attracted increasing interests in the last decades due to its positive agronomic attributes, geographic suitability, genetic engineering feasibility, availability of genetic information and the wide range of applications for its oil and its bioproducts (Putnam et al., 1993; Séguin-Swartz et al., 2009; Li et al., 2015; Abdullah et al., 2016; Kang et al., 2011; Lu and Kang, 2008; Kagale et al., 2014; Nguyen et al., 2013). Unlike the case in other oilseed crops, few reports on enhancement of seed yield and seed oil content via genetic engineering of C. sativa have been demonstrated (An and Suh, 2015; Dalal et al., 2015; Kim et al., 2016; Li et al., 2015; Zhang et al., 2012). These reports have mainly concentrated on genes involved in reducing photosynthesis and modulating carbon metabolism, which indirectly impacts seed oil content (Dalal et al., 2015; Zhang et al., 2012), a gene encodes the Arabidopsis WR1 (Kim et al., 2016) and a gene encodes a patatin-related phospholipase AIIb (pPLAIIb), which involved in phosphatidycholine turnover (Li et al., 2015).

In contrast to the several reports on DGAT1 and other TAG-related genes, only a few reports have studied the critical role of Gly3P, the second substrate needed for TAG synthesis. It was reported in developing seeds of Arabidopsis and oilseed rape that the rate of Gly3P supply is not sufficiently rapid to maintain high Gly3P levels during fast oil accumulation stage in seeds (Gibon et al., 2002; Vigeolas and Geigenberger, 2004). Crucially, increasing Gly3P levels in developing rape seeds fed with glycerol has resulted in an increase in the carbon flux to TAG, suggesting that Gly3P appears to co-limit the rate of TAG formation in seeds (Vigeolas and Geigenberger, 2004). Overexpression of a cytosolic glycero3-phosphate dehydrogenase (Gly3PDH) gene from Yeast (Saccharomyces cerevisiae) into oilseed rape has significantly increased Gly3P activity, resulting in up to 40% increase in seed fatty acid content (Vigeolas et al., 2007).

Therefore, to increase seed oil content in Camelina, we used a transgenic approach to investigate the importance of Gly3P supply for use as the backbone for TAG synthesis, and the importance of acylation of fatty acids in the downstream process for TAG synthesis. For this purpose, we overexpressed a yeast
gene coding for cytosolic Gly3PDH (GPD1) under the control of the seed-specific oleosin promoter, and the Arabidopsis DGAT1 gene, either in its native or modified form, to draw fatty acids into TAG, under the control of seed-specific glycinin promoter, in transgenic Camelina. Further, we investigated the effect of stacking these two genes on achieving a synergistic effect on the flux through the TAG synthesis pathway, and thereby further increase the oil yield.

**Results**

**Overexpression of ScGPD1 and AtDGAT1 into C. cativa**

For the metabolic engineering of Camelina seeds for increased levels of triacylglycerols, GPD1 and DGAT1 genes were introduced into Camelina nuclear genome under the control of oleosin and glycinin promoters from soybean, respectively. Further, to test whether the mutation in DGAT1, where serine205 was substituted with alanine, can enhance its enzymatic activity and positively impact seed oil content, the modified DGAT1S205A (referred here as DGAT1m) was also introduced into Camelina under soybean glycinin promoter, following similar approach reported in Xu et al. (2008). Furthermore, to test whether combined expression of GPD1 and DGAT1 or DGAT1m can further increase the oil yield, Camelina plants were co-transformed with DGAT1 or DGAT1m and GPD1 constructs (referred here as GPD1 + DGAT1 and GPD1 + DGAT1m, Figure 1).

The expression of transgenes in Camelina plants was verified by quantitative real-time qRT-PCR. T1 Transgenic plants showing DsRed fluorescence were sown into the soil to obtain future generations, and DsRed fluorescence was used to identify homozygous lines. T1 plants were also tested for the presence of oleosin and glycinin promoters in GPD1 and DGAT1 overexpressors, respectively, by PCR analysis (Figure S1A, B and C).

Developing seeds (16-21 DAF) of T3 homozygous lines were used to extract RNA for confirming the expression of DGAT1 and GDP1 transcripts using RT-PCR. The relative expression of DGAT1, DGAT1m and GDP1 transcripts was 10- to 40-fold higher in Camelina transgenic lines than in wild-type (WT) controls (Figure 2a and b). Similarly, the expression levels of DGAT1, DGAT1m and GDP1 in GDP1 + DGAT1 and GDP1 + DGAT1m lines were ~10-fold higher than WT controls (Figure 2c). These results confirmed that DGAT1, DGAT1m and GDP1 genes were successfully integrated and expressed at higher levels in transgenic lines.

**Seed-specific overexpression of AtDGAT1 and ScGPD1 increased seed mass, seed size and seed yield in transgenic C. sativa**

To examine whether overexpression of DGAT1 and GPD1 can cause morphologic changes in Camelina seeds, the size and weight of transgenic and WT seeds were measured. Analysis of 100-seed weight indicated that overexpression of GPD1, DGAT1 or DGAT1 m has altered seed morphology regarding significant increases ($P < 0.05$) in seed mass (Figure 3). Transgenic lines expressing GDP1, DGAT1 or DGAT1m, individually, showed 19%–30% increase in seed mass. The maximum increase in

![Figure 1](image)

**Figure 1** T-DNA insertions used to transform Camelina sativa. Shown here are the pBnRGW RedSeed binary vectors containing seed-specific cassettes for expression of the glycerol-3-phosphate dehydrogenase, GPD1 (a) and the diacylglycerol acyltransferase, DGAT1 and DGAT1m (b and c). DsRed fluorescence marker and the herbicide-resistant bar gene (Basta containing phosphinothricin) for selection of transformants are shown in the constructs.

© 2017 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 1034–1045
seed mass was observed in Camelina lines co-expressing GPD1 + DGAT1 (up to 52%) or GPD1 + DGAT1m (up to 33%), relative to the WT seeds (Figure 3a). This increase in seed weight in the co-transformed lines appears to be associated with a significant increase in their seed sizes as compared to WT seeds (Figure 3b).

Total per plant seed yield of T3 homozygous transgenic lines was measured relative to WT seeds (Figure 4a). Unlike the significant increases in seed mass seen in almost all of the transgenic lines overexpressing GPD1, DGAT1 or DGAT1m, individually, no significant difference in seed yield obtained in those lines, except the transgenic line DGAT1 #4, which attained 49% more seed yield as compared to WT, whereas the cotransformed GDP1 + DGAT1 and GPD1 + DGAT1m lines showed the maximum gain in per plant seed yield compared to WT plants. Both GPD1 + DGAT1 and GPD1 + DGAT1m lines exhibited 86%–88% gain in seed yield under glasshouse conditions (Figure 4a).

Seed-specific overexpression of ScGPD1 and AtDGAT1 increases seed oil content but causes no change in seed protein content

Seeds of T3 homozygous Camelina lines transformed individually with GPD1, DGAT1 and DGAT1m or co-transformed (GPD1 + DGAT1 and GPD1 + DGAT1m) were analysed for seed percentage oil content using Minispec mq-20 20 MHz NMR...
spectroscopy to quantify total seed oil content (wt./wt., Figure 4b). All Camelina lines co-expressing DGAT1 and GPD1 exhibited statistically significant \( P < 0.05 \) increases in oil content per dry seed weight compared to the WT controls. On average, seed oil content in those lines was approximately 8%–13% higher compared to that in WT (Figure 4b), and some individual lines exhibited up to 20% increase in seed oil content (seed oil content was 34.5% in transgenics vs. 28.5% in WT; line GPD1 + DGAT1 #12-3-11; see Table S1). Further, seed oil content in Camelina lines overexpressing the modified DGAT1 was also increased by ~8.5% compared to an increase of 5.6% in DGAT1 lines and an increase of 5.4% in GPD1 lines, as compared to WT plants, on average basis (Figure 4b and Table S1). Further, seeds of some of the individual transgenic lines overexpressing either GDP1, DGAT1 or modified DGAT1m also led up to an average 11% increase in oil contents as compared to WT (Figure 4b and Table S1).

Due to the finding that the seed weight of Camelina is more than 40% of the per plant total above-ground biomass, expression of percentage increase in seed oil yield as a percentage of total above-ground dry biomass severely underestimates the total oil yield. Therefore, we expressed the per plant total oil yield based on the % oil contents and total seed weight for all T3 homozygous transgenic Camelina lines. Determining the total seed yield in grams per plant (Figure 4a) helped us to derive total oil yield (grams per plant), and by multiplying the total seed oil content by the total seed yield per plant, it was possible to determine the total oil yield per plant basis (Figure 4c and Table S1). The results indicated that Camelina transgenic lines, which exhibited significant increases in total seed yield per plant (Figure 4a), also displayed significant increases \( P < 0.05 \) in total oil yield per plant as compared to the WT plants.

Interestingly, the highest oil yield was obtained in seeds of Camelina transgenic lines co-expressing GPD1 and DGAT1. The per plant oil yield was approximately doubled in GPD1 + DGAT1 and GPD1 + DGAT1m lines than that in WT (up to 3.0 g in transgenic lines vs. 1.44 g in WT, Figure 4c and Table S1). However, overexpressing DGAT1 or GDP1 alone, except line DGAT1 #4, observed slight, but not significant, increases in total oil yield per plant in GPD1, DGAT1, DGAT1m transgenics despite the increased percentage of seed oil content seen in their seeds compared to WT seeds. Our results suggest that an increase in seed oil content and seed yield in Camelina can lead to an overall increase in Camelina oil yield under glasshouse conditions.

Furthermore, the oil harvest index, which is used in agriculture to quantify the oil yield versus the total plant biomass (Vafaei...
et al., 2010), was determined in Camelina transgenic lines in comparison with WT plants (Figure 4d). The oil harvest index exhibited no changes in Camelina transgenic lines overexpressing GPD1, DGAT1 or DGAT1m alone compared to their relative WT. However, as expected, transgenic lines GPD1 + DGAT1 and GPD1 + DGAT1m exhibited significantly higher oil harvest index (up to twofold, \( P < 0.05 \)) in both GPD1 + DGAT1 and GPD1 + DGAT1m lines than that in WT. Altogether, these findings indicate that the seed-specific co-expression of GPD1 and DGAT1 enhanced the overall seed oil yield in Camelina plants.

Moreover, to investigate the influence of increased seed oil content in Camelina transgenic lines on the amount of seed storage proteins, transgenic lines and WT seeds were further analysed for protein content (Figure 5). The results indicated that the expression of GPD1 and DGAT1, either individually or in a combination, has led to no significant changes in the protein content in Camelina mature seeds.

### Overexpression of GDP1 and DGAT1 altered fatty acid composition

An increased expression of GDP1 and DGAT1 could affect the type of fatty acids (FAs) incorporated into the glycerol backbone via the activity of acylglycerol acyltransferase enzymes, that is GPATs, LPATs and DGATs. Therefore, to evaluate the effect of GPD1 and DGAT1 overexpression in Camelina, a FAME analysis was applied on mature seeds of transgenic and WT plants, and both the content and composition of FAs were determined (Figure 6 and Table 1). Fatty acid composition of the seed oil in WT plants revealed an average content of palmitic (C16:0, 7%), stearic (C18:0, 2.7%), oleic (C18:1, 14%), linoleic (C18:2, 16.5%), \( \alpha \)-linolenic (C18:3, 40%) and gondoic (C20:1, 11.5%) acids, which is in agreement with the FA profile previously reported in Camelina oil (Abdullah et al., 2016; Kim et al., 2016; Li et al., 2015; Rodriguez-Rodriguez et al., 2013).

Nevertheless, the expression of GPD1 and DGAT1 revealed significant changes in FA concentrations and their saturation levels, with similar patterns of changes observed in all examined transgenics (Figure 6 and Table 1). Expression of GPD1 resulted in significant increases in the levels of C16:0 and C18:2 acids (up to 17% and 31%, respectively), and this incline was associated with significant decreases in the levels of C18:1, C18:3 and C20:1 acids (up to 19%, 9% and 8%, respectively) in GPD1 lines as compared to WT. Similarly, the FA profile in DGAT1 lines showed an increased levels of C16:0 and C18:2 acids (up to 18% and 33%, respectively), and decreased levels of C18:1, C18:3 and C20:1 acids (up to 14%, 11% and 11%, respectively) compared to that in WT. Co-expression of GPD1 and DGAT1 also showed the same pattern of changes with even a further decrease in the level of C18:1 acid (up to 36%) as compared to WT.

![Figure 6](image_url) Relative change in FA composition in dried transgenic and WT Camelina seeds analysed by gas chromatography. The percentage relative increase or decrease in the levels of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), \( \alpha \)-linolenic acid (C18:3) and eicosenoic acid (C20:1) in transgenic Camelina lines overexpressing AtDGAT1 and ScGPD1, individually or in a combination, as compared to nontransgenic WT are shown. Values are means ± SE (\( n = 4 \)). WT values are normalized to the threshold 100. Asterisks denote significance of differences between WT and transgenic lines (Student’s \( t \)-test): **\( P < 0.01 \); *\( P < 0.05 \).
C24:1 nervonic acid. Others account for C14:0 myristic acid; C16:1 palmitoleic acid; C22:0; C20:3 mead acid; C22:2 docosadienoic acid; C20:5 eicosapentaenoic acid; C18:1 oleic acid; C18:2 linoleic acid; C18:3 linolenic acid; C20:0 arachidonic acid; C20:1 gondoic acid; C20:2 eicosadienoic acid; C20:4 arachidonic acid; and/or DGAT1 was associated with any negative or positive impacts on seed vigour, an important agronomic trait, seeds of the WT, and comparisons significant at the 0.05 level are highlighted in bold. 

Table 1 Fatty acid composition and content of Camelina oil of transgenic and wild-type plants

| Fatty acid | WT | GPD1 #2 | DGAT1 #4 | DGAT1 m #2 | DGAT1m #33 | GPD1 + DGAT1 #17 | GPD1 + DGAT1m #11 |
|------------|----|---------|----------|------------|------------|------------------|--------------------|
| 16:0       | %  | 7.19 ± 0.02 | 8.39 ± 0.05 | 8.46 ± 0.10 | 8.17 ± 0.15 | 8.02 ± 0.07 | 8.74 ± 0.41 | 8.03 ± 0.10 |
| nmol       |    | 99.14 | 118.36 | 128.09 | 119.27 | 126.03 | 104.35 | 113.87 |
| 18:0       | %  | 2.79 ± 0.04 | 3.14 ± 0.03 | 3.12 ± 0.11 | 2.88 ± 0.07 | 2.94 ± 0.09 | 2.89 ± 0.13 | 2.86 ± 0.06 |
| nmol       |    | 38.51 | 44.18 | 47.14 | 42.40 | 46.30 | 34.53 | 40.59 |
| 18:1       | %  | 14.07 ± 0.01 | 11.41 ± 0.12 | 12.12 ± 0.12 | 10.94 ± 0.06 | 12.48 ± 0.13 | 10.38 ± 0.08 | 12.61 ± 13.13 |
| nmol       |    | 193.88 | 160.83 | 184.09 | 160.18 | 195.95 | 126.47 | 179.75 |
| 18:2       | %  | 16.57 ± 0.02 | 21.62 ± 0.12 | 21.94 ± 0.16 | 21.31 ± 0.18 | 21.11 ± 0.07 | 20.41 ± 0.18 | 21.29 ± 0.14 |
| nmol       |    | 228.43 | 304.76 | 332.27 | 312.54 | 331.96 | 250.40 | 302.62 |
| 18:3       | %  | 39.67 ± 0.07 | 36.00 ± 0.14 | 35.38 ± 0.18 | 37.03 ± 0.09 | 36.30 ± 0.18 | 36.72 ± 0.85 | 36.03 ± 0.31 |
| nmol       |    | 546.86 | 507.65 | 536.50 | 541.88 | 570.72 | 453.89 | 512.78 |
| 20:0       | %  | 1.51 ± 0.01 | 1.87 ± 0.04 | 1.82 ± 0.03 | 1.72 ± 0.05 | 1.67 ± 0.05 | 1.78 ± 0.05 | 1.64 ± 0.05 |
| nmol       |    | 20.87 | 26.37 | 27.60 | 25.38 | 26.37 | 21.65 | 23.35 |
| 20:1       | %  | 11.53 ± 0.03 | 10.59 ± 0.01 | 10.47 ± 0.15 | 10.56 ± 0.05 | 10.67 ± 0.07 | 10.83 ± 0.09 | 10.72 ± 0.15 |
| nmol       |    | 158.93 | 149.35 | 158.99 | 154.71 | 167.97 | 131.96 | 152.96 |
| 20:2       | %  | 1.34 ± 0.0 | 1.91 ± 0.01 | 1.79 ± 0.02 | 1.98 ± 0.01 | 1.83 ± 0.01 | 2.01 ± 0.02 | 1.53 ± 0.34 |
| nmol       |    | 18.45 | 26.96 | 27.19 | 28.95 | 28.87 | 24.47 | 22.24 |
| 20:4       | %  | 2.64 ± 0.03 | 2.35 ± 0.02 | 2.26 ± 0.03 | 2.60 ± 0.02 | 2.31 ± 0.04 | 2.78 ± 0.04 | 2.29 ± 0.04 |
| nmol       |    | 36.31 | 33.10 | 34.29 | 38.03 | 36.51 | 33.80 | 32.68 |
| 24:1       | %  | 0.52 ± 0.01 | 0.50 ± 0.01 | 0.51 ± 0.01 | 0.54 ± 0.01 | 0.50 ± 0.01 | 0.67 ± 0.08 | 0.50 ± 0.02 |
| nmol       |    | 7.10 | 7.10 | 7.76 | 7.85 | 7.84 | 7.69 | 7.04 |
| Others     | %  | 2.18 | 2.22 | 2.12 | 2.28 | 2.17 | 2.78 | 2.49 |
| nmol       |    | 30.08 | 31.33 | 32.10 | 33.25 | 34.18 | 32.80 | 34.89 |

Data represent the mean of the three independent measurements ± standard errors, in nmol per dry weight and in percentage %. C16:0 palmitic acid; C18:0 stearic acid; C18:1 oleic acid; C18:2 linoleic acid; C18:3 γ-linolenic acid; C20:0 arachidonic acid; C20:1 gondoic acid; C20:2 eicosadienoic acid; C20:4 arachidonic acid; C24:1 nervonic acid. Others account for C14:0 myristic acid; C16:1 palmitoleic acid; C22:0; C20:3 mead acid; C22:2 docosadienoic acid; C20:5 eicosapentaenoic acid; and C24:0 tetracosanoic acid. The significance of the effect of the genotypes on FA profiles was tested statistically by Dunnett’s test at P < 0.05 compared with the WT, and comparisons significant at the 0.05 level are highlighted in bold.

GPD1 and DGAT1 overexpression has no effect on seed germination and early seedling growth

To determine whether the seed-specific overexpression of GPD1 and/or DGAT1 was associated with any negative or positive impacts on seed vigour, an important agronomic trait, seeds of transgenic lines and WT plants were germinated. The average time for 50% of seeds to germinate was recorded, and then the growth of 5-day-old seedlings was measured as mg per plant (Figure 7). These findings suggest that overexpression of DGAT1 and/or GPD1 has no detrimental effects to seed vigour under laboratory conditions.

Discussion

The present study shows that the seed-specific overexpression of the cytosolic GPD1 gene from S. cerevisiae, to provide Gly3P for

Figure 7 Early seedling growth rate of Camelina in the T3 transgenic and WT Plants. Values are means ± S.E. of measurements on seeds from individual plants (n = 12) of each genotype grown under controlled condition over germination papers.
TAG assembly, and A. thaliana DGAT1, which catalyses the final step in the TAG assembly from DAG and acyl-CoA, has positive effects on seed oil content, seed weight, and seed yield in C. sativa. Transgenic C. sativa plants overexpressing DGAT1 or DGAT1m gene under the control of glycinin promoter have observed averages of ~23% increase in seed mass and ~6%–11% increase in seed oil contents, whereas overexpression of GPD1 under the control of oleosin promoter has resulted in significant increases in seed mass (up to 30%) and seed oil contents (up to 10%). Co-expression of these two genes in Camelina further enhanced seed mass (up to ~52%), seed oil content (up to ~13%) and total seed yield per plant (up to ~46%–88%), as compared to the nontransformed WT plants. These significant increases in seed oil content and seed yield can be translated into a greater oil yield and oil harvest index, on a dry matter basis, although the relationship seems to be less than proportionate and is limited by the experimental conditions applied in this study. Our findings are in agreement with the previous reports, which investigated the positive effects on seed and oil yields in other oilseed crops via engineering multiple steps in the TAG metabolic pathway. Overexpression of A. thaliana DGAT1 under the control of seed-specific promoter has been reported to boost seed oil content in Arabidopsis and Canola (van Erp et al., 2014; Jako et al., 2001; Taylor et al., 2009). Further, DGAT1 from other plant species, when overexpressed, has also increased the storage oil content in transgenic oilseed rape (Brassica napus L., Weselake et al., 2008), maize (Zea mays L., Zheng et al., 2008), garden nasturtium (Tropaeolum majus L., Taylor et al., 2009) and Camelina (Kim et al., 2016).

By contrast, to our knowledge, only a unique study has investigated the overexpression of yeast cytosolic GPD1 in transgenic B. napus under the control of the seed-specific napin promoter, despite the importance of Gly3P supply as the second substrate essential for TAG assembly (Vigeolas et al., 2007). Furthermore, few studies have investigated the importance of stacking TAG pathway-related genes in order to enhance seed oil content in oilseed crops. Among those, a study by van Erp and his colleagues has showed that stacking Arabidopsis DGAT1 and WR1 genes, combined with suppression of the TAG lipase, SUGAR-DEPENDENT1 (SDP1) gene, observed a higher percentage seed oil content and a higher seed mass in transgenic Arabidopsis compared to WT plants (van Erp et al., 2014). Consistent with their findings, co-expression of GPD1 and DGAT1 genes herein has resulted in comparable further increases in seed oil content, seed mass and seed yield in Camelina transgenic lines.

Increasing the expression of cytosolic GPD1 in transgenic Camelina under the control of oleosin promoter has significantly altered the seed mass in all the lines tested, with three lines exhibited considerable increases in seed oil content (Figures 3 and 4). It was reported that GPD1, when overexpressed in oilseed rape, has led to a twofold increase in Gly3PDH activity, which significantly increases the supply of Gly3P as a precursor for TAG assembly (Vigeolas et al., 2007). This increase in Gly3P supply is correlated with the increase in FA content in mature seeds and the decrease in Gly3PDH’s potential substrate dihydroxyacetone phosphate (DHAP) by the direct conversion of DHAP into Gly3P catalysed by Gly3PDH activity (Vigeolas et al., 2007). We believe that a possible interpretation of increasing seed mass and stimulating TAG synthesis in Camelina seeds overexpressing GPD1 could be the increase in embryo weight, which is not measured herein, due to the increased lipid content and the continuous supply of Gly3P for TAG assembly.

Similarly, increasing seed oil content in Camelina lines overexpressing DGAT1 could be as a result of the correspondent increase in DGAT1 enzyme activity as previously discussed (Kim et al., 2016; Taylor et al., 2009). As DGAT1 catalyses the conversion of DAG and acyl-CoA into TAG in the ER, the increased levels of DGAT1 activity, while its substrate DAG is redundant, can extensively increase the amount of TAGs accumulated in mature seeds. However, the high expression of DGAT1 gene can also have an adverse effect, as it also affects the substrate-enzyme-product relationship, thus causing a limitation in certain substrates in TAG metabolic pathway than others (Taylor et al., 2009). Combining the expression of both GPD1 and DGAT1 in Camelina seeds stimulates oil synthesis, possibly through the increased supply of Gly3P, which can directly provide the required glycerol backbone for TAG assembly, and indirectly enrich the amount of acyl-CoA pools (Vigeolas et al., 2007). Therefore, G3P and acyl-CoAs would become more available to the downstream enzymes in TAG metabolic pathways, including DGAT1, which incorporate the acyl-CoAs into the final TAGs. This stimulation of oil synthesis appears to require the up-regulation of the enzymes involved in controlling the committed steps in TAG assembly. Relatively, in agreement with the previous report by Sharma et al. (2008), overexpression of Arabidopsis DGAT1 in B. napus was associated with increasing the transcription of several genes involved in the Kennedy pathway for TAG synthesis, including GPAT1, LPAT, PAH and DGAT1. Moreover, RNA-Seq analysis of Camelina transcriptome during seed development has indicated higher expressions for a GPAT family member (GPAT9), PP family members (PAH1 and PAH2) and DGAT1, but not LPATs (Abdullah et al., 2016). It could be the case here that these active enzymes are responsible for the committed flow of metabolites, by acting as sinks, towards increased levels of TAG in Camelina seeds overexpressing GPD1 and DGAT1. Furthermore, the increased seed weight and size in Camelina transgenics could be as a result of the growth of cotyledonal embryonic cells due to the accumulation of TAG molecules as previously reported (Kim et al., 2016).

Site-directed mutagenesis (SDM) has been applied in previous reports as an efficient tool to modify putative functional motifs in DGAT1 enzyme to up- or down-regulate its activity causing effective changes in seed and oil qualities (Katavic et al., 1995; Xu et al., 2008; Zou et al., 1999). As mentioned earlier in the introduction section, in an attempt to increase T. majus DGAT1 activity, Xu and his coworkers substituted Ser197 with Ala at the putative SnRK1 serine/threonine protein kinase target site for dephosphorylation. Overexpression of the mutated TmDGAT1 in Arabidopsis seeds resulted in ~38%–80% increase in DGAT1 activity and 3%–10% higher seed oil content in the transgenic seeds on a seed DW basis (Xu et al., 2008). Following similar approach, in the current research, we modified DGAT1 in the putative SnRK1 target site, and the Ser205 to Ala mutant caused a slight, but not significant, increase in the levels of seed oil content in the modified DGAT1m lines compared to that in the native DGAT1 lines. Unlike Xu et al. (2008) findings, we did not find any significant difference in seed yield, seed size or overall plant growth between DGAT1 and DGAT1m lines. The inconsistency between the impact of DGAT1 modification detected herein and the previous report by (Xu et al., 2008) could be due to various factors, including the different DGAT1 enzyme (A. thaliana vs. T. majus) used for mutagenesis in our study, the putative functional motif(s) targeted for phosphorylation, the structural integrity surrounding this motif as well as the factors that control
the enzymatic reactions, such as the availability and concentration of DGAT enzyme substrates, pH, temperature (Guo et al., 2001; Tang et al., 2005; Xu et al., 2008). Additionally, our analysis of the amino acid sequences of Arabidopsis DGAT1 (At3g54320) revealed several putative phosphorylation target motifs via kinases (data not shown). As we targeted only one serine at position 205 in the current study, there may be other sites as targets for kinases that could alter the activity of AtDGAT1. Further studies are needed to better understand the potential impact of post-translational modifications on AtDGAT1 enzyme activity and how it can be translated into effective improvement in seed and oil qualities.

In oilseed crops, the fatty acid composition of oils is determined by the substrate preference of DGATs and the availability of the acyl-CoA species in the ER lumen (Kim et al., 2016). Analysis of seed FAs in Camelina WT plants revealed FAME contents and composition similar to the previous reports (Rodríguez-Rodríguez et al., 2013; Kim et al., 2016; Li et al., 2015). The FAME analysis of the selected GPDI- and DGAT1-overexpressing lines has indicated significant increases in C16:0 and 18:2 levels and significant decreases in C18:1, C18:3 and C20:1 levels as compared to nontransformed WT. This FAME profile is in contrast to the FAME profile reported in Camelina transgenics overexpressing CsDGAT1B, which accumulated higher C18:1 and C18:3 while the level of C18:2 was lower as compared to nontransgenic plants (Kim et al., 2016). This disagreement could be due to the varied redundancy of acyl-CoA species and/or substrate preferences for Arabidopsis DGAT1 used in this study, compared to the indigenous Camelina CsDGAT1B used in Kim et al. (2016). Conversely, unlike the previous reports on the impact of DGAT1 overexpression on FA profile, there are no data of altering GPDI expression in Camelina to compare with; however, the yeast GPDI overexpression did not change the fatty acid composition of the seed oil in transgenic oilseed rape (Vigeolas et al., 2007). Further, the observed increase in the levels of linoleic acid (up to 33%) and the decrease in α-linolenic acid (up to 11%) in seed oils of Camelina transgensics would further improve the effectiveness of Camelina oil as a healthier ingredient in food and feedstocks as well as for a relatively better biodiesel blend (Rodríguez-Rodríguez et al., 2013).

The oil content of Camelina mature seeds ranges from 30% to 40% of the seed weight, and this wide range appears to be dependent on several factors, including genetic background, geographic and climatic conditions, and soil quality (Rodríguez-Rodríguez et al., 2013). In the present study, the levels of seed oil production and overall plant growth for Camelina transgensics and WT plants were determined from plants grown in pots under glasshouse conditions. The variations between individual plants could be due to the variations in growth conditions, that is soil water content and nutrient levels. We observed consistent results obtained from T3 and T4 homozygous seed generations of plants grown under similar controlled conditions and using random block design experiment.

The percentage of seed oil content of WT plants was about 28.5% on average, which was determined using the NMR spectroscopy. This obtained percentage of seed oil coincides with the previous reports where Camelina seed oil was quantified as the total FAME content, using completely different parameters (Kim et al., 2016; Li et al., 2015; Rodríguez-Rodríguez et al., 2013).

In a survey conducted in 2007, it was reported that the percentage of oil content of various oilseeds crops is ranging from 20% to 50%, with the oil fruit coconut accumulating the highest amount (65%). The transgenic approach appears to be a positive strategy to boost seed oil content to the upper limit, which still not known in many of the oilseed crops, including Camelina (Taylor et al., 2009). By manipulating the expression of DGAT1 and GPDI in the present study, we were able to increase the seed oil content from 28.5% in WT to 33% in the transgenics, on an average basis (see Table S1). The highest increase in seed mass, seed yield, oil contents and total oil yields was observed in the lines co-expressing the native DGAT1 with GPDI (GPDI + DGAT1 #12 and #17). These Camelina plants can produce an average of ~11.0 g/plant seed yield and 3.8 g/plant oil yield, when grown under glasshouse conditions, thus exhibiting the highest harvest index (Table S1). This reflects their ability to convert photosynthesized products into an economically valuable form, that is seed oil content, under the applied growth conditions. However, it remains to be established whether a similar improvement in seed and oil qualities can be achieved in the natural field conditions.

Therefore, to boost seed oil content in Camelina to the upper limit, we suggest that it may be helpful to combine overexpression of multiple enzymes to further improve seed and oil yields in Camelina and other commercial oilseed crops although it is not quite obvious that this combination approach would be feasible and efficient in other oil seed crops. The levels of seed oils in Camelina or other oilseed crops could be further increased if the factors that limit the production of oils in the developing seeds are illustrated. It was reported that the seed oil content is controlled by several constraints, including the bottlenecks in the TAG metabolic pathways, the oxygen and light availability in seed tissues, and the developmental regulation of TAG biosynthesis (Abdullah et al., 2016; Baud and Lepiniec, 2010). To further increase the seed oil content in Camelina, we need to identify the rate-limiting step(s) that affect the TAG synthesis and accumulation in seeds. To this end, we plan to carry out comprehensive RNA-Seq, metabolome and lipidome analysis of the Camelina transgensics co-expressing GPDI1 and DGAT1 in comparison with WT. By integrating these ‘omics’ approaches, we will be able to identify the bottlenecks that can be targeted to increase the TAG accumulation further.

Experimental procedures

Plant material

Camelina sativa (L.) Crantz cultivar ‘Suneson’ was grown in the glasshouse at 22°C under natural light conditions supplemented with high-pressure sodium lights (light threshold of 566 μmol/m²/s) with a 16-h photoperiod (16 h of light and 8 h of darkness), and a 50% minimum humidity. Plants were watered regularly and were fertilized with 200 ppm N of Peters Professional 20-10-20 Peat-lite water-soluble fertilizer.

Generation of GmGly::DGAT1, GmGly::DGAT1S250A and GmOle::GPDI constructs for plant transformation

To overexpress Arabidopsis DGAT1 and Yeast GPDI genes into Camelina seeds, the coding sequences for DGAT1 (TAIR ID: At3g54320) and GPDI1 (GenBank ID: AY588965) were synthesized using codon-optimized genes for expression in Camelina by GenScript (GenScript, Piscataway NJ, http://www.genscript.com/). The glycain promoter from soybean (Glycine max) was selected to drive the expression of DGAT1 (Fathi et al., 2013), and the oleosin promoter from soybean was chosen to drive the
expression of GPD1. To test whether a mutant DGAT1 can have an additive effect on seed oil content, a phosphorylation site was abolished by changing serine205 to alanine for increased DGAT1 activity as per Xu et al. (2008). The three individual gene constructs, GmGly::DGAT1 (referred here as DGAT1), GmGly::DGAT1S205A (referred here as DGAT1 m) and GmOle::DGAT1 (referred here as DGAT1), were cloned into a multi-site Gateway system (Invitrogen, Carlsbad) and then transferred into a binary destination pBnRGW vector, which has DsRed fluorescence marker driven by Napin promoter (Figure 1). The sequences of the constructs were verified before they were introduced into the Agrobacterium tumefaciens strain GV3101. Six-week-old Camelina plants were transformed using the floral dip method (Lu and Kang, 2008). For cotransformation, cultures of Agrobacterium containing DGAT1 and those containing GPD1 constructs were blended in equivalent concentrations and used to transform Camelina. T1 seed generation of the transgenic plants were harvested and screened for the expression of DsRed, in which the seeds were illuminated by a green LED flashlight attached to a DsRed filter (Pearstone Inc.) as previously described (Lu and Kang, 2008). T1 transgenic plants were further screened for the expression of the herbicide-resistant bar gene by spraying seedlings grown in soil-filled pots with the herbicide Basta (containing phosphinothricin) and observed over a 1-week period for survival as by Kang et al. (2011).

Genotyping of transgenic plants by PCR and confirmation of gene expression using qRT-PCR

To verify the integration of the transgenes into the genomes of the transgenic plants, PCR was performed on genomic DNA obtained from leaves of selected T1 plants using the standard CTAB protocol, and the presence of promoter-transgene fragments was confirmed. To investigate the expression of the transgenes, total RNA was extracted from developing seeds at 16–21 days after flowering (DAF) of transgencs and WT plants using Spectrum Plant Total RNA kit (Sigma–Aldrich) following manufacturer’s instructions. RNA concentrations were measured in ng/μL, and purity ratios (260/280 nm and 260/230 nm) were calculated using NanoDrop 2000 spectrophotometer (Thermo Scientific) following manufacturer’s instructions. The cDNA pools were quantified and then diluted to a final concentration of 100 ng/μL and were used as templates for qRT-PCR. Camelina Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference in comparisons of gene expression data. All qRT-PCRs were performed in Eppendorf Mastercycler® ep realplex thermal cycler using the intercalation dye ABSolute Blue qPCR SYBR Green master mix kit (Thermo Scientific) as a fluorescent reporter. All PCRs were performed in triplicate. The cDNAs were amplified, and PCR products were quantified using the 2–ΔΔCt method (Schmittgen and Livak, 2004). The error bars represent the standard errors for the fold changes of relative gene expression. WT samples were used as calibrators. The PCR primers for genes and promoter fragments are summarized in Table S2.

Oil content determinations and FAME analysis

Fatty acid methyl esters (FAMEs) were prepared from Camelina mature seeds of transgenics and WT plants according to the Kansas Lipidomics Research Center standard protocols, and the method modified from Iven et al. (2013). The extracted FAMEs were quantified using 6890N GC (Agilent Technologies) coupled to a flame ionization detector (FID) on HP-88 capillary column (column length—100 m, internal diameter—250 μm, film thickness—0.20 μm) with helium as a carrier gas. Seed oil content was measured by the low-resolution time domain NMR spectroscopy using a Burkert Minispec MQ20 (Burkert Optik, GmbH, 76275 Ettingen, Germany). The oil calibration was constructed according to the manufacturer’s instructions using pure Camelina oil. The total oil yield was calculated by multiplying oil content and seed yield, and the values were expressed as gram oil per plant. The oil harvest index was expressed as mg oil per mg of total plant dry matter as described by (Li et al., 2015). At maturation, seed pods were harvested, and then the plant biomass was dried and weighted, and harvest index was determined according to the formula: Harvest index = (economical yield/biological yield) × 100 (Vafaei et al., 2010). Samples were analysed in triplicates unless otherwise mentioned.

Seed attributes analysis

The seeds were harvested when plants matured and the seed pods were dry. The seeds were cleaned using a sieve and dried at room temperature for a week. Seeds harvested from each plant were weighed to determine the seed yield. The weight of 100 Camelina seeds was used to indicate the seed mass. For total plant biomass measurements, the shoots and roots, after seed harvesting, were dried at 60°C for 14 days before weighing.

Protein content determination in Camelina seeds

Total protein content in seeds was measured according to (Vigeolas et al., 2007). Briefly, 10 mg Camelina seeds from WT and transgenic plants were homogenized in 1 ml of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.4, using a polytron. The protein content in the homogenate was quantified using the dye-binding assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

Seedling growth assay

A total of 100 Camelina seeds were germinated over moist papers containing enough moisture in Petri plates. The plates were then placed in a growth chamber set at 24°C, and the moisture was maintained. The weights of 5-day-old seedlings were measured from the germinated seedlings, and the data were recorded as mg per plant. The measurements were done in triplicates for each genotype.

Statistical analysis

The number of replicates (n) and the standard error (SE) are shown for most measurements. The seed and oil quality data were analysed with the SAS version 9.1 (www.sas.com) using ANOVA (P < 0.05) on the corresponding degrees of freedom (df), followed by Dunnett’s procedure for pairwise comparisons of all treatments to an untransformed WT control. The authors declare no conflict of interest.

Acknowledgments

The authors thank the Kansas Lipidomics Research Center (KLRC) for performing lipid analysis. This research was supported by a research grant from the Advance Research & Product Agency for Energy (ARPAe) via grant # DE-AR0000200 to OPD and DS, a partial funding support from USDA-AFRI Hatch Program (MAS00508) and a research support from the Cultural and Educational Bureau of the Egyptian Embassy—Washington DC (reference No: GM-0976) to HMA and OPD.
Authors’ contributions

OPD and DS conceived the study and oversaw its design and coordination. SC and HMA performed the experiments and collected and analysed the data. PA helped with growing plants and harvesting tissue samples. HMA and OPD drafted the manuscript. All authors have been involved in revising the manuscript critically for important intellectual contents. All authors have read and approved the final manuscript.

References

Abdullah, H.M., Akkari, P., Paulose, B., Schnell, D., Qi, W., Park, Y., Pareek, A. et al. (2016) Transcriptome profiling of Camelina sativa to identify genes involved in triacylglycerol biosynthesis and accumulation in the developing seeds. Biotechnol. Biofuels, 9, 136.

An, D. and Suh, M.C. (2015) Overexpression of Arabidopsis WRN1 enhanced seed mass and storage oil content in Camelina sativa. Plant Biotechnol. Rep. 9, 137.

Aznar-Moreno, J., Denolf, P., Van Audenhove, K., De Bodt, S., Engelen, S., Fahy, D. and Browse, J. (2015) Type 1 diacylglycerol acyltransferases of Brassica napus preferentially incorporate oleic acid into triacylglycerol. J. Exp. Bot. 66, 6497–6506.

Barratt, D.H.P., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J. and Smith, A.M. (2009) Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. Proc. Natl. Acad. Sci. USA, 106, 13124–13129.

Baud, S. and Lepincic, L. (2010) Physiological and developmental regulation of seed oil production. Prog. Lipid Res. 49, 235–249.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Dalal, J., Lopez, H., Vasani, N.B., Hu, Z., Swift, J.E., Yalamanchili, R. and Smyth, G. (2015) A photosynthetic bypass increases plant growth and seed yield in biofuel crop Camelina sativa. Biotechnol. Biofuels, 8, 175.

Durrett, T.P., Benning, C. and Ohlrogge, J. (2008) Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. 54, 593–607.

van Erp, H., Kelly, A.A., Memard, G. and Eastmond, P.J. (2014) Multigene engineering of triacylglycerol metabolism boosts seed oil content in arabidopsis. Plant Physiol. 165, 30–36.

Fatih, A., Zberzak, A.M. and Dörmann, P. (2013) Alterations in seed development gene expression affect size and oil content of arabidopsis seeds. Plant Physiol. 163, 973–985.

Gibon, Y., Vigeolas, H., Tiessen, A., Geigenberger, P. and Stitt, M. (2002) Sensitive analysis of potential substrate-binding sites in yeast and human acyl-CoA sterol acyltransferases by mutagenesis of conserved sequences. J. Biol. Chem. 277, 446–452.

Harwood, J.L. (2012) Metabolic control analysis of developing oilseed rape (Brassica napus L.) seed development enhances oil yield in oilseed rape (Brassica napus L.). Plant Biotechnol. J. 11, 355–361.

Kennedy, E.P. (1961) Biosynthesis of complex lipids. Fed. Proc. 20, 934–940.

Kim, H., Park, J.H., Kim, D.J., Kim, A.Y. and Suh, M.C. (2016) Functional analysis of diacylglycerol acyltransferase1 genes from Camelina sativa and effects of DGAT1B overexpression on seed mass and storage oil content in C. sativa. Plant Biotechnol. Rep. 10, 141–153.

Li, M., Wei, F., Tawfall, A., Tang, M., Saetete, A. and Wang, X. (2015) Overexpression of patatin-related phospholipase AII altered plant growth and increased seed oil content in Camelina. Plant Biotechnol. J. 13, 766–778.

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.

Nguyen, H.T., Silva, J.E., Podicheti, R., Macrander, J., Yang, W., Nazarens, T.J. and Cahoon, E.B. (2013) Camelina seed transcriptome: a tool for malting and oil improvement and translational research. Plant Biotechnol. J. 11, 759–769.

Perry, H.J., Bligny, R., Gout, E. and Harwood, J.L. (1999) Changes in Kennedy pathway intermediates associated with increased triacylglycerol synthesis in oil-seed rape. Phytochemistry, 52, 799–804.

Putnam, D.H., Budin, J.T., Field, L.A. and Breene, W.M. (1993) Camelina: a promising low-input oilseed. In New Crops (Janick, J. and Simon, J.E., eds), pp. 314–322. New York: Wiley.

Rao, S.S. and Hilderbrand, D. (2009) Changes in oil content of transgenic soybeans expressing the yeast SLC1 gene. Lipids, 44, 945–951.

Rodriguez-Rodrigo, M.F., Sánchez-García, A., Salas, J.J., García, R. and Martínez-Force, E. (2013) Characterization of the morphological changes and fatty acid profile of developing Camelina sativa seeds. Ind. Crops Prod. 50, 673–679.

Roesler, K., Shintani, D., Savage, L., Boddupalli, S. and Ohlrogge, J. (1997) Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseed. Plant Physiol. 113, 75–81.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108.

Schwender, J., Goffman, F., Ohlrogge, J.B. and Shachar-Hill, Y. (2004) Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature, 432, 779–782.

Seguin-Swartz, G., Eynck, C., Gugel, R.K., Strelkov, S.E., Olivier, C.Y., Li, J.L. and Falk, K.C. (2009) Diseases of Camelina sativa (false flax). Can. J. Plant Path. 31, 375–386.

Sharma, N., Anderson, M., Kumar, A., Zhang, Y., Giblin, E.M., Abrams, S.R. and Fobert, P.R. (2008) Transgenic increases in seed oil content are associated with the differential expression of novel Brassica-specific transcripts. BMC Genom. 9, 619.

Tang, G.Q., Novitzky, W.P., Carolin, H., Huber, S.C. and Devwey, R.E. (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. Plant J. 44, 433–446.

Tang, M., Guschina, I.A., O’Hara, P., Slabas, A.R., Quant, P.A., Fawcett, T. and Harwood, J.L. (2012) Metabolic control analysis of developing oilseed rape (Brassica napus cv Westar) embryos shows that lipid assembly exerts significant control over oil accumulation. New Phytol. 196, 414–426.

Taylor, D., Katavic, V., Zou, J., MacKenzie, S., Keller, W., An, J., Friesen, W. et al. (2002) Field testing of transgenic rapeseed cv. Hero transformed with a yeast sn-2 acyltransferase results in increased oil content, erucic acid content and seed yield. Mol. Breeding, 8, 317–322.

Taylor, D.C., Zhang, Y., Kumar, A., Francis, T., Giblin, E.M., Barton, D.L. and Zhu, W. (2009) Molecular modification of triacylglycerol accumulation by over-expression of DGAT1 to produce canola with increased seed oil content under field conditions. Botany, 87, 533–543.

© 2017 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 1034–1045.
Vafaei, S.N., Tobeh, A., Taee, A. and Jamaati-E-Somarin, S. (2010) Study of phenology, harvest index, yield, yield components and oil content of different cultivars of rain-fed safflower. *World Appl. Sci. J.* **8**, 820–827.

Vigeolas, H. and Geigenberger, P. (2004) Increased levels of glycerol-3-phosphate lead to a stimulation of flux into triacylglycerol synthesis after supplying glycerol to developing seeds of *Brassica napus* L. in planta. *Planta* **219**, 827–835.

Vigeolas, H., Waldeck, P., Zank, T. and Geigenberger, P. (2007) Increasing seed oil content in oil-seed rape (*Brassica napus* L.) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. *Plant Biotechnol. J.* **5**, 431–441.

Weselake, R.J., Shah, S., Tang, M., Quant, P.A., Snyder, C.L., Furukawa-Stoffer, T.L. and Harwood, J.L. (2008) Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. *J. Exp. Bot.* **59**, 3543–3549.

Xu, J., Francis, T., Mietkiewska, E., Giblin, E.M., Barton, D.L., Zhang, Y., Zhang, M. et al. (2008) Cloning and characterization of an acyl-CoA-dependent diacylglycerol acyltransferase 1 (DGAT1) gene from *Tropaeolum majus*, and a study of the functional motifs of the DGAT protein using site-directed mutagenesis to modify enzyme activity and oil content. *Plant Biotechnol.* **6**, 799–818.

Zhang, Y., Yu, L., Yung, K.-F., Leung, D.Y.C., Sun, F. and Lim, B.L. (2012) Over-expression of AtPAP2 in *Camelina sativa* leads to faster plant growth and higher seed yield. *Biotechnol. Biofuels* **5**, 19.

Zheng, P., Allen, W.B., Roesler, K., Williams, M.E., Zhang, S., Li, J., Glassman, K. et al. (2008) A phenylalanine in DGAT is a key determinant of oil content and composition in maize. *Nat. Genet.* **40**, 367–372.

Zou, J., Katavic, V., Giblin, E.M., Barton, D.L., MacKenzie, S.L., Keller, W.A. and Taylor, D.C. (1997) Modification of seed oil content and acyl composition in the brassicaceae by expression of a yeast sn-2 acyltransferase gene. *Plant Cell* **9**, 909–923.

Zou, J., Wei, Y., Jako, C., Kumar, A., Selvaraj, G. and Taylor, D.C. (1999) The Arabidopsis thaliana TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene. *Plant J.* **19**, 645–653.

**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** PCR genotyping of T1 generation developing seeds to confirm the integration of transgenes.

**Figure S2** Individual and combined effects of GPD1 and DGAT1 expression on seed germination rate after 14 h (A) and early seedling growth rate at 38 h (B).

**Table S1** Seed attributes (seed yield, seeds mass, %oil contents, oil yield oil harvest index, seed husk weight, and plant biomass) of Camelina transgenic lines.

**Table S2** PCR primers designed to investigate integration and expression of the transgenes into transgenic Camelina seeds.