Plant Biotechnology Journal (2007) **5**, pp. 431–441 doi: 10.1111/j.1467-7652.2007.00252.x

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

Helene Vigeolas[1,†], Peter Waldeck[1,†], Thorsten Zank[2] and Peter Geigenberger[1,*]

[1]*Max-Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany*
[2]*BASF Plant Science GmbH, Carl-Bosch-Strasse 64, 67117 Limburgerhof, Germany*

Received 20 November 2006;
revised 31 January 2007;
accepted 5 February 2007.
**Correspondence* (fax +49-(0)-331-5678408;
e-mail geigenberger@mpimp-golm.mpg.de)
†These authors contributed equally to this article.

Keywords: Biofuel, oil content, transgenic crops, metabolic regulation.

Summary

Previous attempts to manipulate oil synthesis in plants have mainly concentrated on the genes involved in the biosynthesis and use of fatty acids, neglecting the possible role of glycerol-3-phosphate supply on the rate of triacylglycerol synthesis. In this study, a yeast gene coding for cytosolic glycerol-3-phosphate dehydrogenase (*gpd*1) was expressed in transgenic oil-seed rape under the control of the seed-specific napin promoter. It was found that a twofold increase in glycerol-3-phosphate dehydrogenase activity led to a three- to fourfold increase in the level of glycerol-3-phosphate in developing seeds, resulting in a 40% increase in the final lipid content of the seed, with the protein content remaining substantially unchanged. This was accompanied by a decrease in the glycolytic intermediate dihydroxyacetone phosphate, the direct precursor of glycerol-3-phosphate dehydrogenase. The levels of sucrose and various metabolites in the pathway from sucrose to fatty acids remained unaltered. The results show that glycerol-3-phosphate supply co-limits oil accumulation in developing seeds. This has important implications for strategies that aim to increase the overall level of oil in commercial oil-seed crops for use as a renewable alternative to petrol.

Introduction

Plants use photosynthetically fixed carbon to support growth and to build up reserve products, such as starch or lipids. Storage oil (triacylglycerol, TAG) is a major plant product with great economical importance in human nutrition and as a renewable feedstock for various industrial products and bio-fuels (Murphy, 1994; Broun *et al*., 1999; Thelen and Ohlrogge, 2002a). The world-wide production of vegetable oil is approximately 87 million metric tons in total per year, which mainly consists of soybean, oil palm, rapeseed and sunflower oil (Gunstone, 2001). Rapeseed production is increasing world-wide, with yields of 36 million tons in 2003–04 and 46 million tons in 2004–05 (CRB Commodity Yearbook, 2005, 2006). The crop is mainly used for feed and food, but, increasingly, is being used in bio-diesel production. As a result of the great economic importance of vegetable oils and their expanded use as a renewable feedstock, there is considerable interest in the metabolic engineering of increased seed oil content (Thelen and Ohlrogge, 2002a).

In seeds of developing oil-seed rape (*Brassica napus* L.), sucrose is unloaded from the phloem and metabolized to glycolytic intermediates, such as hexose-phosphates, phosphoenolpyruvate and pyruvate, which are subsequently imported into the plastid and used for fatty acid synthesis (Rawsthorne, 2002; Hills, 2004). Free fatty acids are activated to coenzyme A (CoA) esters, exported from the plastid and used for the stepwise acylation of the glycerol backbone to synthesize TAG in the endoplasmic reticulum (Kennedy, 1961; Ohlrogge *et al*., 1979; Cao and Huang, 1986; Stymne and Stobart, 1987; Lacey and Hills, 1996). In the first two steps of TAG assembly, glycerol-3-phosphate (Gly3P) is acylated by

Gly3P acyltransferase (GPAT) to lysophosphatidic acid, which is then acylated further by lysophosphatidic acid acyltransferase (LPAT) to phosphatidic acid. This is followed by dephosphorylation of phosphatidic acid by phosphatidic acid phosphohydrolase to release diacylglycerol (DAG), and the final acylation of DAG by DAG acyltransferase (DAGAT). Final storage of TAG occurs in endoplasmic reticulum-derived oil bodies (Lacey *et al*., 1998).

Previous attempts to manipulate lipid levels in plants have mainly concentrated on the genes involved in the biosynthesis and use of fatty acids for TAG assembly (Ohlrogge and Jaworski, 1997; Ramli *et al*., 2002; Thelen and Ohlrogge, 2002a; Hills, 2004). Over-expression of the individual genes involved in *de novo* fatty acid synthesis in the plastid, such as acetyl-CoA carboxylase (ACCase) and fatty acid synthase, did not substantially alter the amount of lipids accumulated (Roesler *et al*., 1997; Shintani *et al*., 1997; Thelen and Ohlrogge, 2002a,b); however, studies investigating the over-expression of LPAT (Zou *et al*., 1997; Taylor *et al*., 2002) and DAGAT (Jako *et al*., 2001) showed that the final steps acylating the glycerol backbone exert significant control over the flux to lipids in seeds.

By contrast, only few studies have investigated the importance of Gly3P, the second substrate required for TAG assembly. Recent measurements of Gly3P levels during rape seed (Vigeolas and Geigenberger, 2004) and *Arabidopsis* seed (Gibon *et al*., 2002) development have indicated that the rate of Gly3P provision is not sufficiently rapid to maintain high Gly3P levels during the rapid oil accumulation phase in seeds. Crucially, when Gly3P levels were increased after *in planta* feeding of glycerol to developing seeds of oil-seed rape, there was an increase in the carbon flux to TAG, providing physiological evidence that Gly3P supply may co-limit the rate of TAG synthesis in seeds (Vigeolas and Geigenberger, 2004).

Plants can use two different routes to synthesize Gly3P. In the first route, Gly3P is formed directly from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) via Gly3P dehydrogenase (Gly3PDH), catalysing the conversion of DHAP and NADH (reduced form of nicotinamide adenine dinucleotide) to Gly3P and NAD^+ (oxidized form of nicotinamide adenine dinucleotide). In leaves and developing seeds, two different Gly3PDH isoforms have been identified, one in the cytosol and one in the plastid (Finlayson and Dennis, 1980; Gee *et al*., 1988; Kirsch *et al*., 1992; Sharma *et al*., 2001). In the second route, Gly3P is synthesized from glycerol via glycerol kinase (GlyK), which is ubiquitous in plant tissues and confined to the cytosol (Huang, 1975; Hippman and Heinz, 1976; Sadava and Moore, 1987). On the basis of physiological studies, it has been suggested that GlyK has a role in assimilating glycerol, whereas Gly3PDH is involved in Gly3P synthesis from glycolytic intermediates (Finlayson and Dennis, 1980; Singh, 1998; Sharma *et al*., 2001). This is supported by analyses of *Arabidopsis* mutants that lack GlyK, which show impaired glycerol metabolism during seed germination (Eastmond, 2004), and mutants defective in *sfd*1 and *gly*1 encoding Gly3PDH in the plastid, which show changes in plastid localized glycerolipids in leaves (Miquel *et al*., 1998; Kachroo *et al*., 2004; Nandi *et al*., 2004). The latter mutants showed no visible phenotype and the composition of cytosolic glycerolipids remained unchanged, indicating that changes in plastid Gly3PDH did not affect TAG synthesis in the cytosol. However, few studies have investigated the contribution and importance of cytosolic Gly3PDH for TAG synthesis in developing seeds.

In this study, we used a transgenic approach to investigate the importance of Gly3P supply for oil accumulation in developing seeds of oil-seed rape. For this purpose, we expressed a yeast gene coding for cytosolic Gly3PDH (*gpd*1) in transgenic oil-seed rape using the seed-specific napin promoter. It was shown that increased Gly3PDH activity leads to a strong increase in Gly3P levels in seeds, and a significant and substantial increase in the final lipid content of seed. This provides evidence that Gly3P supply co-limits the rate of lipid synthesis in seeds, and has obvious implications for biotechnological strategies to increase the overall level of oil in commercial oil-seed crops.

Results

Generation of transgenic plants expressing GPD1, a Gly3PDH from yeast

The gene *gpd*1, encoding cytosolic Gly3PDH (EC1.1.1.8) from yeast (Larsson *et al*., 1993), was cloned in the sense orientation into the plant transformation vector (pGPTV) containing the napin promoter from *Brassica napus*, identified to be seed specific in transgenic oil-seed rape (Stalberg *et al*., 1996). The petioles of the cotyledons from oil-seed rape plants (cv. Westar) were transformed with this construct using an *Agrobacterium*-mediated protocol, as in Moloney *et al*. (1989). Transgenic plants were selected on kanamycin-containing medium, and nine independent events were selected and confirmed by polymerase chain reaction (PCR) and Southern blot analysis. From these nine independent lines, four different lines (3, 6, 8 and 9) were chosen for further analysis and grown to the T4 generation. In the T3 (Figure S1a, see 'Supplementary material') and T4 (Figure 1a)

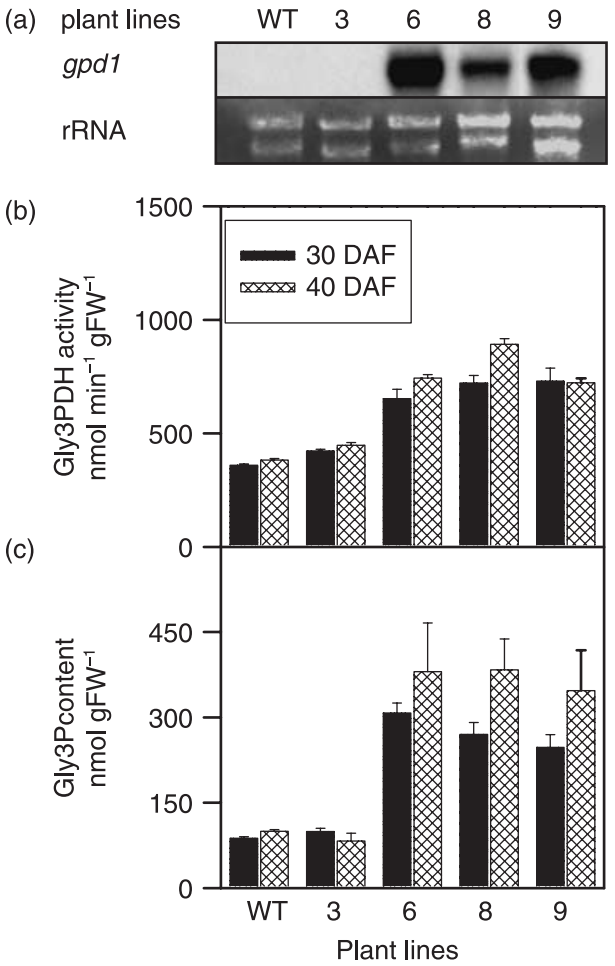

Figure 1 Increase in *gpd*1 transcript, glycerol-3-phosphate dehydrogenase (Gly3PDH) activity and glycerol-3-phosphate (Gly3P) content in developing seeds of transgenic oil-seed rape in the T4 generation. To select transgenic lines expressing *gpd*1, developing seeds [30 days after flowering (DAF)] were analysed for: (a) steady-state *gpd*1 mRNA levels using the full-length cDNA coding for GPD1 as hybridization probe; (b) Gly3PDH activity; and (c) Gly3P content. Values are the means ± standard error (n = 6). Control plants were planted randomly intermixed with the transgenics. FW, fresh weight.

generations, the abundance of *gpd*1 transcript was determined in developing seeds using Northern blot analysis. *gpd*1 transcript was detectable as a strong signal in lines 6, 8 and 9, but was not detectable in line 3 or the wild-type.

GPD1 expression leads to increased Gly3PDH activity and increased levels of Gly3P in developing seeds

It was confirmed that the expression of the yeast *gpd*1 gene resulted in an increase in enzyme activity by assaying Gly3PDH activity using a sensitive enzymatic cycling assay. As the yeast Gly3PDH does not differ significantly from the plant enzymes with respect to substrate specificity (Albertyn *et al*., 1992), it cannot be distinguished using enzyme assays. Consistent with the time course of napin promoter expression during seed development (Josefsson *et al*., 1987), Gly3PDH activity was assayed in soluble protein extracts of seeds from the mid- [30 days after flowering (DAF)] to later (40 DAF) stages in the T4 generation (Figure 1b). In the three transgenic lines 6, 8 and 9 showing significant *gpd1* expression, Gly3PDH activity increased 1.6–2.5-fold relative to that of the wild-type. Consistently, in line 3, showing no detectable *gpd*1 transcript level, the Gly3PDH activity was similar to that of the wild-type (cf. Figure 1a and 1b). Similar changes in Gly3PDH activity were observed in a further trial using the T3 generation (Figure S1b, see 'Supplementary material').

To investigate whether increased Gly3PDH activity resulted in an increase in Gly3P content, metabolite levels were analysed in the same seed material at 30 and 40 DAF in the T4 generation. Figure 1c shows that increased Gly3PDH activity resulted in a three- and fourfold increase in Gly3P level in seeds at 30 and 40 DAF, respectively. Similar changes in Gly3P content were observed in a further trial using the T3 generation (Figure S1c, see 'Supplementary material').

GPD1 expression leads to an increase in total fatty acid content of developing and mature seeds

To investigate the influence of increased Gly3PDH activity and Gly3P content on the amount of oil accumulated, transgenic lines were further analysed for total fatty acid content in developing and mature seeds using gas chromatography (GC) analysis of fatty acid methyl esters. In the T4 generation, *gpd*1 expression led to a significant increase of up to twofold in the fatty acid content of developing seeds at 30 DAF (Table 1), and to a significant increase of 40% in the fatty

Table 1 Expression of *gpd*1 leads to a specific increase in lipid content in developing seeds. Storage product content was determined in developing seeds [30 days after flowering (DAF)] of wild-type, transgenic control (line 3) and three *gpd*1-expressing lines 6, 8 and 9 in the T4 generation (see Figure 1). Values (mg/g fresh weight) are the means ± standard error (n = 4). Values determined by Student's t-test to be significantly different ($P < 0.05$) from the controls (wild-type and line 3) are marked in bold

Storage product	Wild-type	Line 3	Line 6	Line 8	Line 9
Total fatty acids	43.6 ± 1.6	41.5 ± 1.2	**72.7 ± 4.6**	**81.4 ± 10**	**57.4 ± 1.2**
Proteins	39.4 ± 1.6	42.9 ± 1.8	41.6 ± 5.2	36 ± 0.6	38.9 ± 2.7
Starch	5.76 ± 0.15	6.2 ± 0.69	5.98 ± 0.39	5.35 ± 0.29	7.57 ± 0.38

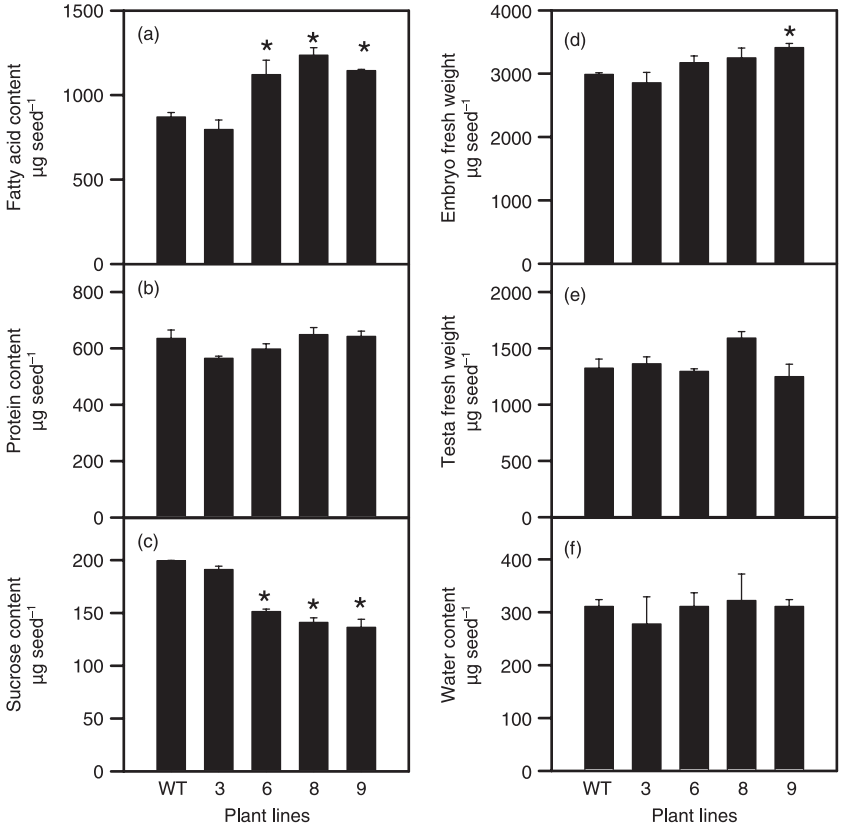

Figure 2 GPD1 expression leads to an increase in the final lipid content of seeds. Storage product content was analysed in mature seeds [55 days after flowering (DAF)] of wild-type, transgenic control (line 3) and three *gpd*1-expressing lines 6, 8 and 9 in the T4 generation (for expression data, see Figure 1). (a) Total fatty acid content measured by gas chromatography of fatty acid methyl esters; (b) protein content; (c) sucrose content; (d) embryo fresh weight; (e) testa fresh weight; and (f) water content of seeds. Values are means ± standard error ($n = 5$). Values significantly different from the controls (wild-type and line 3) based on Student's *t*-test (with $P < 0.05$) are indicated with an asterisk. Control plants were planted randomly intermixed with the transgenics.

acid content of mature seeds (Figure 2a), which correlated with the increase in Gly3PDH activity (see Figure 1b) and Gly3P content (see Figure 1c). The increase in seed oil content was also observed in a further trial using the T3 generation (Figure S1d, see 'Supplementary material'). The lipid content of the wild-type seeds used in this study (approximately 800 µg per seed) was similar to the values reported by Turnham and Northcote (1983) and Vigeolas *et al*. (2004) for glasshouse-grown plants (500 and 800 µg per seed, respectively).

The expression of *gpd*1 did not lead to significant changes in the protein content (Table 1; Figure 2b), but resulted in a decrease in the final sucrose content in mature seeds (Figure 2c). This shows that the increase in final oil content was partially compensated for by a decrease in the sucrose content, probably as a result of an increased conversion of sucrose to fatty acids in mature seeds (cf. Figure 2a and 2c). Starch levels were analysed in developing seeds only and showed no significant changes in response to *gpd*1 expression (Table 1). In seeds of oil-seed rape, starch accumulates transiently during seed development, and decreases to very low levels in mature seeds (DaSilva *et al*., 1997; Vigeolas *et al*., 2004).

In mature seeds, the expression of *gpd*1 led to an increase in embryo weight, which was statistically significant in line 9 (Figure 2d). The embryo weight increased by approximately 0.4 g, which corresponds to the increase in lipid content (see above). The fresh weight of the testa (Figure 2e) was not significantly changed (wild-type values similar to those reported by Perry and Harwood, 1993). The seed water content (approximately 0.3 mg/seed; Figure 2f) was similar to the values published by Shahidi *et al*. (1990), and did not show any significant changes across the lines. There were also no visible changes in plant growth, flowering characteristics or silique number per plant.

As increased Gly3P levels may affect which types of fatty acid are esterified on to the glycerol backbone by GPAT, we analysed the acyl composition of the seed oil. There were no significant changes in fatty acid composition between mature wild-type and transgenic seeds in the T3 (Figure S2, see 'Supplementary material') and T4 (Table 2) generations. The fatty acid composition in mature seeds was similar to the values reported by Zhang *et al*. (2001) for oil-seed rape.

Effect of GPD1 expression on metabolite levels, adenylate energy state and enzyme activities in developing seeds

To investigate whether the increased rate of lipid synthesis may be attributable to a general increase in metabolite levels,

Table 2 Fatty acid composition of seed oil from wild-type and *gpd*1-expressing plants in the T4 generation. The fatty acid composition was determined by gas chromatography of fatty acid methyl esters using oil from mature seeds [55 days after flowering (DAF)]. Values are the means ± standard error (n = 5)

Fatty acid (mol.%)	Wild-type	3	6	8	9
16:0	5 ± 0.1	5.6 ± 0.3	5.3 ± 0.3	5.3 ± 0.1	5.1 ± 0.1
18:0	1.2 ± 0.13	1.5 ± 0.15	1.4 ± 0.3	1.1 ± 0.04	1.1 ± 0.02
16:3	0.5 ± 0.08	1.6 ± 0.01	0.47 ± 0.1	0.5 ± 0.02	0.6 ± 0.2
18:1	60.6 ± 2.2	56.6 ± 2.5	58 ± 7	56.9 ± 1.6	61.1 ± 1.6
18:2	24.4 ± 1.5	24.9 ± 1.5	27.8 ± 3.3	25.3 ± 1.4	23.7 ± 1
20:0	0.3 ± 0.05	0.6 ± 0.05	0.45 ± 0.09	0.4 ± 0.01	0.4 ± 0.01
18:3	7.2 ± 0.7	9.5 ± 1	12.1 ± 4.5	9.4 ± 0.4	7.1 ± 0.5

rather than to a specific increase in Gly3P, we measured the *in vivo* levels of sugars and various metabolites in developing seeds (Table 3). Increased expression of *gpd*1 did not lead to significant changes in the levels of major sugars, such as sucrose, glucose or fructose. There were also no significant changes in the levels of glycolytic intermediates and precursors of fatty acid synthesis, such as uridine diphosphoglucose, glucose-6-phosphate and 3-phosphoglycerate, or intermediates of fatty acid synthesis, such as acetyl-CoA. Crucially, the stimulation of lipid synthesis was accompanied by a decrease in the level of DHAP and an increase in the level of Gly3P, which are the immediate substrate and product of Gly3PDH, respectively. It should be noted, however, that the reported metabolite levels are overall values and may not represent the concentration in the cytosol or plastid. Direct measurements of subcellular metabolite levels will be needed to confirm our interpretation. This requires the establishment of techniques to resolve oil-seed metabolism at a subcellular level.

The lack of changes in glycolytic metabolite levels was also reflected by a lack of changes in the overall activities of glycolytic enzymes. The expression of *gpd*1 did not lead to changes in the activities of sucrose synthase, invertase, hexokinase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase (Table 4). There were also no

Table 3 Metabolite levels in seeds of *gpd*1-expressing lines. Metabolite levels were determined in developing seeds [30 days after flowering (DAF)] of wild-type, transgenic control and three *gpd*1-expressing lines 6, 8 and 9 in the T4 generation (see Figure 1). The data presented (nmol/g fresh weight) are the means ± standard error (n = 5–6). Values determined by Student's t-test to be significantly different ($P < 0.05$) from the controls (wild-type and line 3) are marked in bold

Metabolite	Wild-type	3	6	8	9
Sucrose (×10^3)	57 ± 1.2	67 ± 4.6	64 ± 2	62 ± 1.4	61 ± 1
Uridine diphosphoglucose	117 ± 8	125 ± 7	135 ± 4	143 ± 7	133 ± 3
Glucose-6-phosphate	191 ± 38	217 ± 29	223 ± 40	243 ± 14	188 ± 20
3-Phosphoglycerate	156 ± 14	117 ± 16	152 ± 8	178 ± 5	145 ± 14
Dihydroxyacetone phosphate (DHAP)	222 ± 25	209 ± 11	**130 ± 11**	**128 ± 16**	**160 ± 14**
Giycerol-3-phosphate (Gly3P)	87.5 ± 2.7	99.6 ± 6	**308 ± 17**	**270.4 ± 21**	**247.4 ± 22**
Acetyl-coenzyme A	3.4 ± 0.7	4.2 ± 0.6	3.6 ± 0.3	3.9 ± 0.6	4.6 ± 0.47
ATP/ADP ratio	2 ± 0.1	2.6 ± 0.2	2.1 ± 0.3	2.6 ± 0.2	1.9 ± 0.1
Gly3P/DHAP ratio	0.4 ± 0.004	0.5 ± 0.003	**2.4 ± 0.02**	**2.1 ± 0.03**	**1.55 ± 0.02**

Table 4 Enzyme activities in seeds of *gpd*1-expressing lines. Enzyme activities were determined in developing seeds [30 days after flowering (DAF)] of wild-type, transgenic control (line 3) and three *gpd*1-expressing lines 6, 8 and 9 (see Figure 1) in the T4 generation. The data presented (nmol/g fresh weight/min) are the means ± standard error (n = 5–6). Values determined by Student's t-test to be significantly different ($P < 0.05$) from the control (wild-type and line 3) are marked in bold

Enzyme	Wild-type	3	6	8	9
Sucrose synthase	1.4 ± 0.07	1.6 ± 0.15	1.7 ± 0.11	1.3 ± 0.05	1.5 ± 0.16
Invertase	0.27 ± 0.009	0.34 ± 0.01	0.35 ± 0.03	0.37 ± 0.05	0.24 ± 0.007
Hexokinase	0.28 ± 0.028	0.32 ± 0.05	0.35 ± 0.05	0.3 ± 0.065	0.31 ± 0.021
Aldolase	2.1 ± 0.09	2.2 ± 0.3	2.25 ± 0.17	1.9 ± 0.004	2.3 ± 0.2
Pyrophosphate-dependent phosphofructokinase	2.1 ± 0.6	2.4 ± 0.3	2.9 ± 0.12	2.9 ± 0.6	3.1 ± 0.4
Glyceraldehyde-3-phosphate dehydrogenase	0.3 ± 0.019	0.4 ± 0.04	0.5 ± 0.02	0.36 ± 0.04	0.44 ± 0.01
Acetyl-CoA carboxylase	0.28 ± 0.016	0.27 ± 0.04	**0.46 ± 0.06**	**0.40 ± 0.015**	**0.42 ± 0.04**
Diacylglycerol acyltransferase (×10^3)	2.22 ± 0.22	2.15 ± 0.34	1.98 ± 0.36	2.25 ± 0.26	2.77 ± 0.17
Glycerol-3-phosphate acyltransferase (×10^3)	8.5 ± 2.4	6.4 ± 1.7	6.25 ± 2.7	**2.85 ± 0.63**	7.6 ± 3.7

changes in the ATP/ADP ratio indicative of the energy state of the seeds (Table 3). This shows that the increased rates of lipid synthesis were not a result of the stimulation of energy-generating pathways in the seeds.

Interestingly, lines with increased expression of *gpd*1 showed a 1.6-fold increase in the overall activity of the key enzyme of fatty acid synthesis, ACCase, whereas the activities of enzymes involved in TAG assembly, such as GPAT and DAGAT, were not significantly altered (Table 4).

Discussion

Previous attempts to manipulate oil quantity in plants have mainly concentrated on the genes involved in the biosynthesis and use of fatty acids, neglecting the possible role of Gly3P supply for the rate of TAG synthesis. Gly3P is synthesized from the glycolytic intermediate DHAP via Gly3PDH, or from glycerol via GlyK, but studies investigating the *in vivo* importance of these enzymes to provide Gly3P for TAG assembly are lacking.

In this study, cytosolic Gly3PDH from yeast was expressed in transgenic oil-seed rape under the control of the napin promoter, which drives expression specifically in the middle and later stages of embryo development where rapid lipid synthesis occurs (Josefsson *et al*., 1987). A twofold increase in Gly3PDH activity led to a three- to fourfold increase in the level of Gly3P in developing seeds, resulting in a 40% increase in the lipid content of seed, whereas the accumulation of protein and starch remained unchanged and the final sucrose content decreased. This was accompanied by a decrease in the glycolytic intermediate DHAP, the direct precursor of Gly3PDH, whereas the levels of various metabolites involved in the pathway from sucrose to fatty acids remained unaltered. These results provide evidence that Gly3PDH is an important factor in the synthesis of storage lipids in developing seeds, with an increase in its activity being of considerable biotechnological importance to increase the overall level of oil in commercial oil-seed crops.

The results of this study are consistent with those of previous approaches used to increase the seed oil content, which have shown that engineering the TAG assembly in the cytosol is a more efficient strategy than engineering fatty acid synthesis in the plastid (see Thelen and Ohlrogge, 2002a). Our results show that *gpd*1 expression leads to an increase of up to 40% in the final seed oil content in two subsequent generations (Figures 2a and S1d), which is comparable with the relative increase in final seed oil content obtained by over-expressing DAGAT in *Arabidopsis* or a heterologous LPAT in rapeseed (see Zou *et al*., 1997; Jako *et al*., 2001). However, it is important to note that the results presented have been obtained with plants growing in a glasshouse, with light intensities of 300 μmol photons/m^2/s, and it remains to be established whether a similar increase in oil content can be achieved in the field. Previous studies with rapeseed and *Arabidopsis* have shown that external conditions, such as light, can have a considerable impact on oil accumulation in seeds (Ruuska *et al*., 2004; Goffman *et al*., 2005; Li *et al*., 2006).

Although the expression of yeast LPAT led to a change in seed oil composition (Zou *et al*., 1997), the expression of yeast GPD1 did not alter the fatty acid composition of the seed oil significantly (see Table 2). Obviously, increased levels of Gly3P do not lead to the preferred incorporation of specific fatty acids into TAG. Nevertheless, increasing Gly3P may provide an interesting strategy to increase the incorporation of unusual fatty acids into oils to yield oils with useful industrial properties. Previous attempts to synthesize industrial oils in plants were characterized by severe yield penalties (Jaworski and Cahoon, 2003).

The results presented here provide evidence that the stimulation of oil synthesis is attributable to an increase in the supply of Gly3P as a precursor for TAG assembly. First, the increase in fatty acid content correlated strongly with the increase in Gly3P levels (cf. Figure 1c, Figure 2a and Table 1; also see Figure S1). As the expression of *gpd*1 in transgenic plants was targeted to the cytosol, this should lead to an increase in cytosolic rather than plastid levels of Gly3P. Secondly, there were no significant changes in the levels of sucrose and other major metabolites of the pathway from sucrose to fatty acids, making it unlikely that the increase in TAG synthesis was driven by an increased supply of precursors for fatty acid synthesis (see Table 3). Thirdly, there was a specific decrease in the level of DHAP (see Table 3), indicating increased conversion of DHAP to Gly3P by Gly3PDH as a primary metabolic alteration, correlating with the stimulation of TAG synthesis. Moreover, the results are consistent with previous studies, which showed that increased Gly3P levels after feeding glycerol led to a stimulation of the rate of TAG synthesis in seeds of oil-seed rape (Vigeolas and Geigenberger, 2004).

The reaction catalysed by Gly3PDH also involves the oxidation of the co-substrate NADH to NAD^+. However, it is unlikely that this could be a factor leading to increased oil synthesis, as a decrease in the availability of redox equivalents would inhibit rather than stimulate fatty acid synthesis. Increased regeneration of NAD^+ from NADH by operation of Gly3PDH in the cytosol may become important under anoxic conditions, allowing increased rates of glycolysis by relieving

the inhibition of glyceraldehyde dehydrogenase. This may be relevant in developing seeds, where internal oxygen concentrations have been reported to be very low, restricting respiration and ATP production, as well as carbon fluxes to TAG synthesis (Vigeolas *et al*., 2003). However, inspection of Table 3 shows that *gpd*1 expression did not affect the levels of glycolytic metabolites that are indicative of an altered cytosolic redox state, or the ATP/ADP ratio in the seeds, making it unlikely that redox homeostasis or respiratory ATP production was largely improved.

It is tempting to speculate by what mechanism increased provision of Gly3P leads to an increase in the amount of fatty acid accumulated. GPD1 expression leads to a significant increase in overall ACCase activity, whereas other enzymes involved in glycolysis and lipid synthesis remain unaltered (see Table 4). According to previous studies, increased ACCase expression on its own is unlikely to increase lipid synthesis in plants (Thelen and Ohlrogge, 2002b). However, an increase in ACCase activity in combination with an increase in Gly3P supply may be more effective in stimulating TAG biosynthesis. Increased Gly3P may lead to a decrease in the size of the acyl-CoA pool, thereby signalling the need for enhanced fatty acid synthesis. The activity of ACCase is known to be feedback inhibited by acyl-CoA (Thelen and Ohlrogge, 2002b). Alternatively, Gly3P may act as a signal on its own, leading to the up-regulation of the pathway of fatty acid synthesis. Clearly, fatty acid synthesis is stimulated without major changes in metabolite levels or in the adenylate energy state, providing evidence for a very efficient regulation of the whole pathway. More studies are needed to investigate this regulatory network.

Although this study shows the importance of Gly3P supply for TAG synthesis by ectopic expression of cytosolic *gpd*1 in developing seeds, information is largely lacking concerning the role of Gly3PDH isoforms and the encoding genes in plants. Mutant studies on *sfd*1 and *gly*1, encoding plastidial Gly3PDH, provide evidence for a role of these genes in membrane lipid synthesis and the signalling of defence gene expression, rather than TAG synthesis (Kachroo *et al*., 2004; Nandi *et al*., 2004). In a recent study by Shen *et al*. (2006), *Arabidopsis* mutants defective in GPDHc1, encoding a cytosolic Gly3PDH, were investigated. When germinating seedlings were compared, the mutants exhibited strongly increased $NADH/NAD^+$ ratios, accompanied by a marked decrease in the level of pyruvate and decreased respiratory capacity, indicating a role of GPDHc1 in redox transport to mitochondria, analogous to the proposed role of cytosolic forms of Gly3PDH in other systems (Larsson *et al*., 1998; Rigoulet *et al*., 2004).

Interestingly, the level of DHAP and the lipid content remained unaltered in this mutant, which contrasts with the results of the present study. However, the study by Shen *et al*. (2006) was performed essentially with germinating seedlings, which are different to developing seeds. In seeds, there is a premium on the provision of Gly3P for oil synthesis, rather than on redox homeostasis, representing a different metabolic situation compared with seedlings or leaves. Moreover, other Gly3PDH isoforms may have compensated for a decrease in GPDHc1 in developing seeds. More studies are needed to further investigate the role of Gly3PDH isoforms and the encoding genes in plants.

In conclusion, the results of this work provide evidence that Gly3P supply co-limits oil synthesis in seeds. This has obvious implications for strategies that aim to increase the overall level of oil in commercial oil crops. This study shows that increased expression of Gly3PDH leads to an increase in seed oil content in glasshouse trials. Further studies are necessary to confirm these results in the field and to investigate the impact on yield. Furthermore, combined over-expression of Gly3PDH with the final enzymes of TAG assembly may lead to an additional increase in oil quantity, and combined expression with genes encoding unusual fatty acids may help to produce larger amounts of industrial oil in plants.

Experimental procedures

Plant material

Low erucic acid (LEA) *Brassica napus* cv. Westar plants were grown in a glasshouse (25 °C by day and 20 °C by night; 16-h day/8-h night photoperiod; light intensity, 300 μmol photons/m^2/s). Emerging flowers were tagged, and seed age was expressed in DAF. If no developmental stage is indicated in the text, experiments were performed with seeds at the age of 30 DAF. Seeds were harvested in the middle of the light period.

Generation of the transgenic lines

The cDNA encoding *gpd*1 from yeast (GenBank Acc. No. Z24454; Larsson *et al*., 1993) was subcloned into the plant expression vector pGPTV between the napin promoter and the ocs terminator. The construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 via electroporation. The helper plasmid in the *A. tumefaciens* strain was pAL4404 (Hoekema *et al*., 1983). Seedlings, 4–5 days old, from spring oil-seed rape cv. Westar were used as a source for transformation explants. According to the transformation method used (Moloney *et al*., 1989 with slight modifications), the petioles of the cotyledons were immersed for 15–20 min in a solution with *A. tumefaciens* containing the respective construct, and were thereafter transferred to a co-cultivation medium and cultured in the dark. After 2–3 days of co-cultivation, the explants were transferred to a

culture medium containing timentin and cultured in the light. After a 7-day rest period, the explants were then transferred to selection medium containing timentin and kanamycin for the selection of transformed cells. After 3–10 weeks, green shoots emerged from the cut end of the cotyledonary petioles. These were transferred to a shoot culture medium containing timentin and kanamycin for elongation and rooting. Transformed shoots were transplanted to soil and grown to maturity in the glasshouse.

RNA isolation and Northern blot analysis

Total RNA was extracted from frozen seeds using the RNA plant reagent of Invitrogen GmbH (Karlsruhe, Germany), and 25 μg of total RNA was used for Northern blot analysis (Kossmann *et al*., 1999). GPD1 mRNA levels were analysed using the yeast GPD1 full-length cDNA as hybridization probe.

Chemicals

Unless stated otherwise, chemicals were obtained from Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Metabolite and nucleotide analysis

In the middle of the day, siliques were rapidly frozen in liquid nitrogen, and the seeds were separated from the silique walls under liquid nitrogen. Frozen seeds were homogenized using a liquid nitrogen ball mill, and metabolites and nucleotides were analysed from trichloroacetic acid extracts as described in Jelitto *et al*. (1992). ATP, ADP, glucose-6-phosphate, uridine diphosphoglucose, Gly3P and acetyl-CoA were measured according to Gibon *et al*. (2002), and glucose, fructose and sucrose according to Geigenberger *et al*. (1998). DHAP was assayed as follows: 10 μL of extract or standard solution was added to 80 μL of 100 mM Tricine/KOH (pH 8), 5 mM $MgCl_2$ containing 0.4 U Gly3PDH (or water as the control assay) and 66 nmol NADH. After 1 h of incubation at room temperature, the tubes were incubated for 20 min at 95 °C. After cooling and centrifugation, the supernatants were transferred to a microplate and 10 μL of a solution containing 2 U glycerol-3-phosphate oxidase, 130 U catalase and 66 nmol NADH was added. The absorbance was monitored at 340 nm in an 'Anthos' reader (Anthos, Wals, Austria), and the rates of the reactions were calculated as the decrease in absorbance in mOD/min using 'BIOLISE' software (Biolise, Hombtechtikom, Switzerland).

Determination of total fatty acid content

Total fatty acids were extracted according to the method of Bligh and Dyer (1959), and the lipid content was measured by GC of fatty acid methyl esters using pentadecanoic acid as internal standard (Benning and Somerville, 1992).

Determination of protein and starch content

The protein content was measured according to Eastmond and Rawsthorne (2000). The protein concentration was determined using the dye-binding assay (Bradford, 1976) with bovine serum albumin as standard. The starch content was measured as described by Geigenberger *et al*. (1998).

Analysis of enzyme activities

Enzyme extracts were prepared from frozen seed material according to Geigenberger and Stitt (1993). Gly3PDH activity was measured by monitoring the production of Gly3P from DHAP and NADH. Assays were incubated at room temperature. The reaction mixture contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffer, 4 mM DHAP, 0.2 mM NADH and an aliquot of protein extract (10 μL) in a final volume of 100 μL. After 30 min, the reaction was stopped by heating (20 min, 95 °C). In the control assay, the reaction mixture was immediately placed at 95 °C. Gly3P was measured according to Gibon *et al*. (2002). Sucrose synthase (EC 2.4.1.13) and invertase (EC 3.2.1.26) were measured according to Geigenberger *et al*. (1998), hexokinase (EC 2.7.1.1) as in Renz and Stitt (1993), UDP-glucose pyrophosphorylase (EC 2.7.7.9) and pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90) according to Merlo *et al*. (1993), and aldolase (EC 4.1.2.13) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) according to Trethewey *et al*. (1998). ACCase activity was determined by measuring the incorporation of ^{14}C from $NaH^{14}CO_3$ into malonyl-CoA; 10 μL of protein extract was pre-incubated with 10 μL of a solution containing 50 mM Tricine/KOH, pH 8.5, and 10 mM dithiothreitol (DTT) for 15 min at 30 °C. Then, 20 μL of the reaction mixture (50 mM Tricine/KOH, pH 8.5, 10 mM ATP, 10 mM $MgCl_2$, 2 mM acetyl-CoA, 20 mM $NaHCO_3$ and 2 mM $NaH^{14}CO_3$, 2.15 GBq/mmol) was added. After 20 min at 30 °C, the reaction was stopped by the addition of 40 μL of 6 M HCl. The total reaction mixture was then applied to Whatman paper discs and dried in a fume hood overnight. The incorporation of radioactive label into malonyl-CoA was measured in a scintillation counter. DAGAT (EC 2.3.1.20) and GPAT (EC 2.3.1.15) activities were determined according to Perry *et al.* (1999) and Bafor *et al.* (1990), respectively.

Acknowledgements

We are very grateful to Mark Stitt for his continuous support and interest in this work, to Peter Dörmann for kindly providing the GC facilities, to Alisdair Fernie for critical reading of the manuscript, and to Britta Hausmann for taking excellent care of the plants. This work was supported by grants from BASF Plant Science GmbH and the Max-Planck Society.

References

Albertyn, J., van Tonder, A. and Prior, B.A. (1992) Purification and characterization of glycerol-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *FEBS Lett*. **308**, 130–132.

Bafor, M., Jonsson, L., Stobart, A.K. and Stymne, S. (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea-lanceolata*. *Biochem. J*. **272**, 31–38.

Benning, C. and Somerville, C.R. (1992) Identification of an operon

involved in sulfolipid biosynthesis in *Rhodobacter-sphaeroides*. *J. Bacteriol*. **174**, 6479–6487.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys*. **37**, 911–917.

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

Broun, P., Gettner, S. and Somerville, C. (1999) Genetic engineering of plant lipids. *Annu. Rev. Nutr*. **19**, 197–216.

Cao, Y.Z. and Huang, A.H.C. (1986) Diacylglycerol acyltransferase in maturing oil seeds of maize and other species. *Plant Physiol*. **82**, 813–820.

Commodity Research Bureau (CRB) (2005) *CRB Commodity Yearbook*. Jersey City, NJ: Commodity Research Bureau.

Commodity Research Bureau (CRB) (2006) *CRB Commodity Yearbook*. Jersey City, NJ: Commodity Research Bureau.

DaSilva, P.M.F.R., Eastmond, P.J., Hill, L.M., Smith, A.M. and Rawsthorne, S. (1997) Starch metabolism in developing embryos of oilseed rape. *Planta*, **203**, 480–487.

Eastmond, P.J. (2004) Glycerol-insensitive *Arabidopsis* mutants: gli1 seedlings lack glycerol kinase, accumulate glycerol and are more resistant to abiotic stress. *Plant J*. **37**, 617–625.

Eastmond, P.J. and Rawsthorne, S. (2000) Coordinate changes in carbon partitioning and plastidial metabolism during the development of oilseed rape embryo. *Plant Physiol*. **122**, 767–774.

Finlayson, S. and Dennis, D. (1980) NAD+-specific glycerol-3-phosphate dehydrogenase from developing castor bean endosperm. *Arch. Biochem. Biophys*. **199**, 179–185.

Gee, R., Goyal, A., Gerber, D., Byerrum, R.U. and Tolbert, N.E. (1988) Isolation of dihydroxyacetone phosphate reductase from *Dunaliella* chloroplasts and comparison with isozymes from spinach leaves. *Plant Physiol*. **88**, 896–903.

Geigenberger, P. and Stitt, M. (1993) Sucrose synthase catalyzes a readily reversible-reaction in vivo in developing potato-tubers and other plant-tissues. *Planta*, **189**, 329–339.

Geigenberger, P., Hajirezaei, M., Geiger, M., Deiting, U., Sonnewald, U. and Stitt, M. (1998) Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose–starch interconversions, and increased levels of nucleotides in growing potato tubers. *Planta*, **205**, 428–437.

Gibon, Y., Vigeolas, H., Tiessen, A., Geigenberger, P. and Stitt, M. (2002) Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. *Plant J*. **30**, 221–235.

Goffman, F.D., Alonso, A.P., Schwender, J., Shachar-Hill, Y. and Ohlrogge, J.B. (2005) Light enables a very high efficiency of carbon storage in developing embryos of rapeseed. *Plant Physiol*. **138**, 2269–2279.

Gunstone, F.D. (2001) Soybeans pace boost in oilseed production. *Inform*. **11**, 1287–1289.

Hills, M.J. (2004) Control of storage-product synthesis in seeds. *Curr. Opin. Plant Biol*. **7**, 302–308.

Hippman, H. and Heinz, E. (1976) Glycerol kinase in leaves. *Z. Pflanzenphysiol*. **79**, 408–418.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of Vir-region and t-region of the *Agrobacterium-tumefaciens* Ti-plasmid. *Nature*, **303**, 179–180.

Huang, A.H.C. (1975) Enzymes of glycerol metabolism in storage tissues of fatty seedlings. *Plant Physiol*. **55**, 555–558.

Jako, C., Kumar, A., Wei, Y.D., Zou, J.T., Barton, D.L., Giblin, E.M., Covello, P.S. and Taylor, D.C. (2001) Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. *Plant Physiol*. **126**, 861–874.

Jaworski, J. and Cahoon, E.B. (2003) Industrial oils from transgenic plants. *Curr. Opin. Plant Biol*. **6**, 178–184.

Jelitto, T., Sonnewald, U., Willmitzer, L., Hajirezeai, M. and Stitt, M. (1992) Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing *Escherichia-coli* pyrophosphatase in their cytosol. *Planta*, **188**, 238–244.

Josefsson, L.G., Lenman, M., Ericson, M.L. and Rask, L. (1987) Structure of a gene encoding the 1.7 S storage protein, napin, from Brassica napus. *J. Biol. Chem*. **262**, 12 196–12 201.

Kachroo, A., Venugopal, S.C., Lapchyk, L., Falcone, D., Hildebrand, D. and Kachroo, P. (2004) Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101**, 5152–5157.

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

Kirsch, T., Gerber, D.W., Byerrum, R.U. and Tolbert, N.E. (1992) Plant dihydroxyacetone phosphate reductases – purification, characterization, and localization. *Plant Physiol*. **100**, 352–359.

Kossmann, J., Abel, G.J.W., Springer, F., Lloyd, J.R. and Willmitzer, L. (1999) Cloning and functional analysis of a cDNA encoding a starch synthase from potato (*Solanum tuberosum* L.) that is predominantly expressed in leaf tissue. *Planta*, **208**, 503–511.

Lacey, D.J. and Hills, M.J. (1996) Heterogeneity of the endoplasmic reticulum with respect to lipid synthesis in developing seeds of *Brassica napus* L. *Planta*, **199**, 545–551.

Lacey, D.J., Wellner, N., Beaudoin, F., Napier, J.A. and Shewry, P.R. (1998) Secondary structure of oleosins in oil bodies isolated from seeds of safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.). *Biochem. J*. **334**, 469–477.

Larsson, C., Pahlman, I.L., Ansell, R., Rigoulet, M., Adler, L. and Gustafsson, L. (1998) The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. *Yeast*, **14**, 347–357.

Larsson, K., Ansell, R., Eriksson, P. and Adler, L. (1993) A gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. *Mol. Microbiol*. **10**, 1101–1111.

Li, Y., Beisson, F., Pollard, M. and Ohlrogge, J. (2006) Oil content of *Arabidopsis* seeds: the influence of seed anatomy, light and plant-to-plant variation. *Phytochemistry*, **67**, 904–915.

Merlo, L., Geigenberger, P., Hajirezaei, M. and Stitt, M. (1993) Changes of carbohydrates, metabolites and enzyme-activities in potato-tubers during development, and within a single tuber along a stolon-apex gradient. *J. Plant. Physiol*. **142**, 392–402.

Miquel, M., Cassagne, C. and Browse, J. (1998) A new class of *Arabidopsis* mutants with reduced hexadecatrienoic acid fatty acid levels. *Plant Physiol*. **117**, 923–930.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989) High-efficiency transformation of *Brassica-napus* using *Agrobacterium* vectors. *Plant Cell. Rep*. **8**, 238–242.

Murphy, D.J. (1994) Biogenesis, function and biotechnology of plant-storage lipids. *Prog. Lipid Res*. **33**, 71–85.

Nandi, A., Welti, R. and Shah, J. (2004) The *Arabidopsis thaliana*

dihydroxyacetone phosphate reductase gene SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell*. **16**, 465–477.

Ohlrogge, J.B. and Jaworski, J.G. (1997) Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **48**, 109–136.

Ohlrogge, J.B., Kuhn, D.N. and Stumpf, P.K. (1979) Subcellular-localization of acyl carrier protein in leaf protoplasts of *Spinacia-oleracea*. *Proc. Natl. Acad. Sci. USA*, **76**, 1194–1198.

Perry, H. and Harwood, J.L. (1993) Changes in the lipid content of developing seeds of *Brassica napus*. *Phytochemistry*, **32**, 1411–1415.

Perry, H., 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.

Ramli, U.S., Baker, D.S., Quant, P.A. and Harwood, J.L. (2002) Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly. *Biochem. J*. **364**, 393–401.

Rawsthorne, S. (2002) Carbon flux and fatty acid synthesis in plants. *Prog. Lipid Res*. **41**, 182–196.

Renz, A. and Stitt, M. (1993) Substrate-specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato-tubers. *Planta*, **190**, 166–175.

Rigoulet, M., Aguilaniu, H., Averet, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X., Larrson, C., Pahlman, I.L., Manon, S. and Gustafsson, L. (2004) Organization and regulation of the cytosolic NADH metabolism in the yeast *Saccharomyces cerevisiae*. *Mol. Cell Biochem*. **256–257**, 73–81.

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

Ruuska, S.A., Schwender, J. and Ohlrogge, J.B. (2004) The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes. *Plant Physiol*. **136**, 2700–2709.

Sadava, D. and Moore, K. (1987) Glycerol metabolism in higher-plants – glycerol kinase. *Biochem. Biophys. Res. Commun*. **143**, 977–983.

Shahidi, F., Naczk, M. and Myhara, R.M. (1990) Effect of processing on the soluble sugars of Brassica seeds. *J. Food Sci*. **55**, 1470–1471.

Sharma, N., Phutela, A., Malhotra, S.P. and Singh, R. (2001) Purification and characterization of dihydroxyacetone phosphate reductase from immature seeds of *Brassica campestris* L. *Plant Sci*. **160**, 603–610.

Shen, W.Y., Wei, Y.D., Dark, M., Tan, Y.F., Taylor, D.C., Selvaraj, G. and Zou, J.T. (2006) Involvement of a glycerol-3-phosphate dehydrogenase in modulating the NADH/NAD(+) ratio provides evidence of a mitochondrial glycerol-3-phosphate shuttle in *Arabidopsis*. *Plant Cell*, **18**, 422–441.

Shintani, D., Roesler, K., Shorrosh, B., Savage, L. and Ohlrogge, J. (1997) Antisense expression and overexpression of biotin carboxylase in tobacco leaves. *Plant Physiol*. **114**, 881–886.

Singh, R. (1998) Carbon and energy sources for fatty acid biosynthesis in non photosynthetic plastids of higher plants. *Proc. Indian Natl. Sci. Acad*. **B64**, 335–354.

Stalberg, K., Ellerstom, M., Ezcurra, I., Ablov, S. and Rask, L. (1996) Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. *Planta*, **199**, 515–519.

Stymne, S. and Stobart, A.K. (1987) *Triacylglycerol Biosynthesis*. In *The Biochemistry of Plants: a Comprehensive Treatise*, Vol. 9. (Stumpf, P.K., ed), pp. 175–214. New York: Academic Press.

Taylor, D.C., Katavic, V., Zou, J.T., MacKenzie, S.L., Keller, W.A., An, J., Friesen, W., Barton, D.L., Pedersen, K.K., Giblin, E.M., Ge, Y., Dauk, M., Sonntag, C., Luciw, T. and Males, D. (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. Breed*. **8**, 317–322.

Thelen, J.J. and Ohlrogge, J.B. (2002a) Metabolic engineering of fatty acid biosynthesis in plants. *Metab. Eng*. **4**, 12–21.

Thelen, J.J. and Ohlrogge, J.B. (2002b) Both antisense and sense expression of biotin carboxyl carrier protein isoform 2 inactivates the plastid acetyl-coenzyme A carboxylase in *Arabidopsis thaliana*. *Plant J*. **32**, 419–431.

Trethewey, R.N., Geigenberger, P., Riedel, K., Hajirezaei, M.R., Sonnewald, U., Stitt, M., Riesmeier, J.W. and Willmitzer, L. (1998) Combined expression of glucokinase and invertase in potato tubers leads to a dramatic reduction in starch accumulation and a stimulation of glycolysis. *Plant J*. **15**, 109–118.

Turnham, E. and Northcote, D.H. (1983) Changes in the activity of acetyl-CoA carboxylase during rape-seed formation. *Biochem. J*. **15**, 223–229.

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., van Dongen, J.T., Waldeck, P., Hühn, D. and Geigenberger, P. (2003) Lipid storage metabolism is limited by the prevailing low oxygen concentrations within developing seeds of oilseed rape. *Plant Physiol*. **133**, 2048–2060.

Vigeolas, H., Möhlmann, T., Martini, N., Neuhaus, H.E. and Geigenberger, P. (2004) Embryo-specific reduction of ADP-glucose pyrophosphorylase leads to an inhibition of starch synthesis and a delay in oil accumulation in developing seeds of *Brassica napus* L. *Plant Physiol*. **136**, 2676–2678.

Zhang, H., Vasanthan, T. and Wettasinghe, M. (2001) Dry matter, lipids, and proteins of canola seeds as affected by germination and seedling growth under illuminated and dark environments. *J. Agric. Food Chem*. **52**, 8001–8005.

Zou, J.T., Katavic, V., Giblin, E.M., Barton, D.L., MacKenzie, S.L., Keller, W.A., Hu, X. 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.

Supplementary material

The following supplementary material is available for this article:

Figure S1 GPD1 expression leads to an increase in glycerol-3-phosphate (Gly3P) level and fatty acid content in seeds of transgenic oil-seed rape in the T3 generation. Developing seeds [40 days after flowering (DAF)] were analysed for steady-state mRNA levels of *gpd*1 (a), Gly3P dehydrogenase (Gly3PDH) activity (b) and Gly3P level (c). Total fatty acid content (d) was determined in mature seeds. Values are the

means ± standard error (n = 5). Values significantly different from the wild-type (based on Student's t-test with $P < 0.05$) are indicated with an asterisk.

Figure S2 Fatty acid composition of seed oil from mature wild-type and *gpd*1-expressing seeds in the T3 generation. Fatty acid composition was determined by gas chromatography of fatty acid methyl esters. Values are the means ± standard error (n = 5).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/pbi/10.1111/j.1467-7652.2007.00252.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

