Running head:

Oil accumulation in sugar-dependent1

Correspondence:

Peter J. Eastmond
Rothamsted Research
Harpenden
Hertfordshire
AL5 2JQ, UK

E-mail: peter.eastmond@rothamsted.ac.uk
Tel: +44 (0) 1582 763133
Fax: +44 (0) 1582 763010

One-sentence summary: Knocking out a triacylglycerol lipase boosts the oil content of vegetative tissues in wild type plants and also in transgenic plants, which have been genetically engineered to make more oil.
Title of article:

The SUGAR-DEPENDENT1 lipase limits triacylglycerol accumulation in vegetative tissues of Arabidopsis

Authors’ names:

Amélie A. Kelly¹, Harrie van Erp², Anne-Laure Quettier¹, Eve Shaw¹, Guillaume Menard², Smita Kurup² and Peter J. Eastmond²

Addresses:

¹School of Life Sciences, University of Warwick, Coventry, Warwickshire, CV4 7AL, UK
²Department of Plant Biology and Crop Science, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK
Footnotes:

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Current address for AAK is: Life Sciences Institute, National University of Singapore, Singapore 117456.
ABSTRACT

There has been considerable interest recently in the prospect of engineering crops to produce triacylglycerol (TAG) in their vegetative tissues as a means to achieve a step-change in oil yield. Here we show that disruption of TAG hydrolysis in the *Arabidopsis thaliana* lipase mutant *sugar-dependent1* (*sdp1*) leads to a substantial accumulation of TAG in roots and stems, but comparatively much lower TAG accumulation in leaves. TAG content in *sdp1* roots increases with the age of the plant and can reach more than 1% of dry weight (DW) at maturity; a 50-fold increase over wild type. TAG accumulation in *sdp1* roots requires both ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1) and PHOSPHATIDYLCHOLINE:DIACYLGLYCEROL ACYLTRANSFERASE1 and can also be strongly stimulated by the provision of exogenous sugar. In transgenic plants constitutively co-expressing WRINKLED1 and DGAT1, *sdp1* also doubles the accumulation of TAG in roots, stems and leaves, with levels ranging from 5 to 8% of DW. Finally provision of 3% (w/v) exogenous sucrose can further boost root TAG content in these transgenic plants to 17% of DW. This level of TAG is similar to seed tissues in many plant species and establishes the efficacy of an engineering strategy to produce oil in vegetative tissues that involves simultaneous manipulation of carbohydrate supply, fatty acid synthesis, TAG synthesis and also TAG breakdown.
INTRODUCTION

Vegetable oils (triacylglycerols) are a major global commodity. They contribute significantly to human and livestock nutrition, provide versatile feedstock for the chemical industry and also serve as a renewable energy source in the form of biodiesel (Durrett et al., 2008; Dyer et al., 2008). World production has increased dramatically in the last decade and now exceeds 150 million metric tons per year (http://www.fas.usda.gov/oilseeds/). There is a pressing need to improve the yield of oil crops to meet societies growing demand for renewable oils (Lu et al., 2011). It has recently been proposed that a step-change in oil yield may be achievable in terrestrial crops if they can be engineered to produce oil in vegetative tissues rather than in seeds (Durrett et al., 2008; Ohlrogge et al., 2009). It has been estimated that, if the perennial biomass crop Miscanthus giganteus produced 20% of its harvestable dry mass as oil, the total oil yield per hectare would be three times that of a conventional oilseed crop such as Brassica napus (Durrett et al., 2008).

Lipid bodies have been observed in the leaves of many plants (Lersten et al., 2006) and oil in vegetative tissues has previously been proposed to play a role in carbon storage and membrane lipid remodelling (Murphy, 2001; James et al., 2010). Nevertheless the oil content of leaves, stems and roots is extremely low in all but a very few plant species (Durrett et al., 2008). For example, oil accounts for much less than 0.1% of dry weight (DW) in Arabidopsis thaliana leaves (Yang and Ohlrogge, 2009). However, a number of studies have established that the oil content can be boosted by the overexpression of individual oil biosynthetic enzymes such as ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1) (Bouvier-Nave et al., 2000), or transcriptional ‘master’ regulators that govern the expression of multiple enzymes in the pathway such as WRINKLED1 (WRI1), LEAFY COTYLDON1 (LEC1) and LEC2 (Cernac and Benning, 2004; Sanjaya et al., 2011; Mu et al., 2008; Andrianov et al., 2010). In addition, several mutants have been identified that exhibit ectopic oil accumulation (Ogas et al., 1997; Xu et al., 2005; Slocombe et al., 2009; Kunz et al., 2009; James et al., 2010). Among these are pxa1 (cts or ped3) and cgi58, which are associated with lipid catabolism. PXA1 is a peroxisomal ABC transporter that is required for fatty acid import for β-oxidation (Zolman et al., 2001) and
CGI58 is a protein that has intrinsic lipase, phospholipase and lysophosphatidic acid acyltransferase activities (Ghosh et al., 2009).

Oil content is controlled by the balance between synthesis and breakdown in many eukaryotes and a deficiency in triacylglycerol hydrolysis has been shown to result in greater oil deposition (Grönke et al., 2005; Kurat et al., 2006; Zimmermann et al., 2004). We previously identified a small family of triacylglycerol lipase genes in Arabidopsis, consisting of SUGAR-DEPENDENT1 (SDP1) and SDP1-LIKE (SDP1L), which appear to be directly responsible for initiating oil breakdown in the seeds following germination (Eastmond, 2006; Kelly et al., 2011). SDP1 and SDP1L are members of an unorthodox group of lipases that are related to patatin from potato (Solanum tuberosum), but contain a divergent active site (Scherer et al., 2010). Well-characterized examples include Homo sapiens adipose triglyceride lipase (Zimmermann et al., 2004), Drosophila melanogaster Brummer (Grönke et al., 2005) and Saccharomyces cerevisiae triacylglycerol lipase 3, 4 and 5 (Athenstaedt and Daum, 2005; Kurat et al., 2006).

Interestingly although SDP1 is most strongly expressed in seeds, transcripts can also be detected in all vegetative tissues (Eastmond, 2006; Kelly et al., 2011). Likewise, genes encoding enzymes that catalyse the committed step for oil synthesis such as DGAT1, DGAT3, and PHOSPHATIDYLCHOLINE:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1) are also expressed in vegetative tissues (Zhang et al., 2009; Hernández et al., 2012). Given evidence that key enzymes for both oil synthesis and breakdown are expressed in vegetative tissues, the aim of this study was to investigate whether SDP1-mediated oil turnover might limit oil accumulation in leaves, stems and roots of wild type (WT) Arabidopsis and also transgenic lines engineered to synthesise more oil. Our data suggest that this is the case particularly in heterotrophic tissues such as roots where oil can accumulate to more than 1% of DW in sdp1, while levels as high as 17% of DW are achievable in this sdp1 roots by providing an exogenous sugar supply and enhancing fatty acid synthesis and TAG synthesis by over-expression of WRI1 and DGAT1.
RESULTS

SDP1 is expressed in vegetative tissues of Arabidopsis

To confirm that the SDP1 promoter is active in the vegetative tissues of Arabidopsis, and to investigate the spatial distribution in more detail, we created β-glucuronidase (GUS) reporter lines containing GUS driven by 1.5 kb of the SDP1 promoter. Quantitative assays performed on two independent transgenic lines showed that GUS is expressed in leaves, stems and roots of four week old plants, as well as in 3 d old seedlings (Fig. 1A) where seed storage oil mobilisation is occurring (Eastmond, 2006). Histochemical staining for GUS also suggested that expression was in all cells, but that it was strongest in the veins of the leaf and stele of the roots, and also in the tips of main and lateral roots (Supplemental Figure S1). To confirm that SDP1 protein is also present in these tissues Western blots were performed on material from a complemented sdp1-5 mutant line expressing hemagglutinin (HA)-tagged SDP1 under the same SDP1 promoter (Kelly et al., 2011). SDP1-HA could clearly be detected in leaves, stems and roots, as well as in seedlings (Fig. 1B).

Disruption of SDP1 leads to TAG accumulation in vegetative tissues

To determine whether SDP1 plays a limiting role in TAG turnover in vegetative tissues of Arabidopsis, the TAG content of leaves, stems and roots of four week old WT and sdp1-5 mutant plants was measured (Fig. 2). TAG was separated from total lipids by thin layer chromatography (TLC), trans-methylated and quantified by gas chromatography of fatty acid methyl esters (Xu et al., 2005). The data showed that TAG levels are extremely low (around 0.02% of DW) in all three WT tissues, consistent with previous studies (Yang and Ohlrogge, 2009). However, in the stems and roots of sdp1-5 substantially more TAG accumulated, while the effect was much less pronounced in leaves (Fig. 2A). The highest level was found in roots where around 20% of total fatty acids were in TAG (Fig. 2B). We decided to focus further work on roots as a convenient model heterotrophic tissue. The identity of the TAG in sdp1-5 roots was confirmed using electrospray ionisation tandem mass spectroscopy (ESI-MS/MS) (Supplemental...
Figure S2A). The TAG is largely made up of C54 molecular species that are enriched in α-linolenic acid (Supplemental Figure S2B). The accumulation of cytosolic lipid droplets in the root cells of sdp1-5 plants could also be visualised by laser scanning confocal microscopy using Nile red staining (Fig. 3).

**TAG accumulation in sdp1 roots is greater than in several other lipid catabolism mutants**

Previous studies have shown that disruption of several other genes associated with lipid catabolism can also lead to TAG accumulation in vegetative tissues, although heterotrophic tissues have not generally been investigated (Slocombe et al., 2009; Kunz et al., 2009; James et al., 2010). PXA1 is a peroxisomal ABC transporter that is required for fatty acid import for β-oxidation (Zolman et al., 2001) and CGI58 is an enzyme that has been reported to have lipase, phospholipase and lysophosphatidic acid acyltransferase activities (Ghosh et al., 2009). Analysis of TAG content in pxa1-1 and/cgi58-1 roots showed that both accumulated TAG, but not as much as sdp1-5 (Fig. 4A). Furthermore, analysis of sdp1-5 pxa1-1 and sdp1-5/cgi58-1 double mutants showed no additive effect on root TAG content (Fig. 4A). SDP1 also has a single homologue in Arabidopsis called SDP1-LIKE (Kelly et al., 2011) and analysis of sdp1L-2 and sdp1-5 sdp1L-2 roots suggested that SDP1L has a comparatively minor role in TAG turnover in roots but that disruption of both genes does lead to a marginally greater accumulation of TAG than sdp1-5 alone (Fig. 4A). This is consistent with gene expression data which show that SDP1 transcripts are greater than 10-fold more abundant in vegetative tissues than SDP1L (Kelly et al., 2011).

**TAG accumulation in sdp1 roots is partially dependent on DGAT1 and PDAT1**

TAG synthesis in Arabidopsis seeds relies jointly on the activities of DGAT1 (Katavic et al., 1995) and PDAT1 (Zhang et al., 2009). However, analysis of dga1 and pdat1 mutants suggests that DGAT1 is quantitatively more important (Zhang et al., 2009). To determine whether these genes are required for TAG synthesis in sdp1-5 roots, the accumulation of TAG was measured in sdp1-5 dga1-1 and sdp1-5 pdat1-1 double mutants. Disruption of DGAT1 resulted in a greater
than 60% reduction in the accumulation of TAG in the sdp1-5 background while disruption of PDAT1 also had a small, but statistically significant ($P < 0.05$), negative effect (Fig. 4B). We did not attempt to make a triple mutant because dgat1 pdat1 has been shown to be lethal (Zhang et al., 2009).

**TAG accumulates in sdp1 roots as the plant ages and is enhanced by exogenous sugar**

To investigate what conditions maximise TAG accumulation in sdp1-5 roots, plants were grown for increasing lengths of time and also in the presence of increasing concentrations of exogenous sucrose. Both plant age and sugar supply enhanced total root TAG content (Fig. 5). TAG content increased by about 0.1% of dry weight (DW) per week when plants were grown in the absence of exogenous sucrose (Fig. 5A). After four weeks grown in the presence of increasing levels of sucrose up to 5% (w/v), TAG content was also enhanced by up to four-fold (Fig. 5B). Because sugar provision strongly stimulated TAG accumulation in sdp1-5 roots, we also chose to investigate whether it affected leaves where comparatively less TAG accumulation was found under normal growth conditions (Fig. 2A). Analysis of leaves from four week old sdp1-5 plants grown on media containing 3% (w/v) sucrose suggested that there is a positive effect. Many more lipid bodies were observed in leaves using Nile red staining and more TAG was also detected by TLC (Supplemental Figure S3).

**Disruption of SDP1 in lines expressing WRI1 and DGAT1 leads to an additive effect on TAG accumulation in vegetative tissues**

Having obtained evidence that SDP1 function limits TAG accumulation in vegetative tissues of WT plants we chose to test whether this is also true of lines genetically engineered to have higher oil biosynthetic capacity. Previous studies have shown that over-expression of DGAT1 and WRI1 individually lead to enhanced oil accumulation in vegetative tissues (Bouvier-Nave et al., 2000; Cernac and Benning, 2004) and also that a synergistic effect is achievable when they are expressed in combination (Vanhercke et al., 2013). WRI1, is a transcription factor that activates the expression of genes encoding multiple enzymes primarily of lower glycolysis and
fatty acid synthesis (Cernac and Benning, 2004; Baud et al., 2007) and DGAT1 is the major enzyme that catalyses the final committed step in TAG synthesis (Katavic et al., 1995; Zhang et al., 2009).

Firstly T-DNA constructs containing DGAT1 or WRI1, under the control of the constitutive 35S promoter, were transformed into wild type and approximately 20 independent transformants were screened for elevated TAG content in the T2 generation using TLC. For each construct, three lines with the highest apparent TAG levels were then taken to the T3 generation and homozygous plants were identified by segregation analysis. Enhanced expression of the transgenes was confirmed in the roots of these homozygous lines by Q-PCR (Supplemental Table SI). The 35S:DGAT1 and 35S:WRI1 lines with the highest TAG content (D1 and W1; Supplemental Table SI) were then crossed together and homozygous plants carrying both constructs were recovered (D1/W1). Finally, sdp1-5 was also crossed into D1 and D1/W1 and homozygous plants containing the transgenes were obtained.

In order to compare the effects of all genotypic combinations and provision of exogenous sugar, the TAG content was measured in roots from four week old plants grown on agar medium either with or without 3 % (w/v) sucrose. The level of TAG increased progressively with the combination of each gene manipulation and exogenous sugar (Fig. 6A). The sum effect of DGAT1 overexpression, WRI overexpression and SDP1 deficiency in the sdp1/D1/W1 line was approximately 8% TAG content (as a percentage of DW) without sugar supplementation and around 17% with sucrose (Fig. 6A). Root biomass (total DW) was reduced by 20 to 30% in both cases (Fig. 6B). Regardless of exogenous sugar, disruption of SDP1 resulted in an approximate doubling in TAG content verses overexpression of DGAT1 and WRI1 alone (Fig. 6A). The fatty acid composition of TAG from D1/W1 and sdp1/D1/W1 roots was different from either WT or sdp1-5, with levels of oleic acid elevated at the expense of α-linolenic acid (Supplemental Table SII). Co-expression of WRI1 and DGAT1 in Nicotiana benthamiana leaves has previously been shown to have a similar effect on the level of oleic acid in TAG (Vanhercke et al., 2013).

Finally the TAG content of roots, stems and leaves of four week old soil-grown plants was also examined in D1/W1 and sdp1/D1/W1. In all three tissues the sdp1 genetic background had a strong additive effect, resulting in TAG content increasing from 2-3% of DW in D1/W1 to 5 to 8% of DW in sdp1/D1/W1 (Fig. 7A). Soil-grown D1/W1 and sdp1/D1/W1 plants also exhibited
significant reductions in both leaf and root biomass (measured as total DW) \( (P < 0.05) \) (Fig. 7B). On soil the \( sdp1/D1/W1 \) plants also had visibly smaller rosettes, but otherwise appeared to have no gross abnormalities (Supplemental Figure S4).

**DISCUSSION**

In this study we show that disruption of the TAG lipase SDP1 (Eastmond, 2006) leads to an accumulation of oil in vegetative tissues of Arabidopsis, suggesting that TAG turnover occurs in these tissues despite their very low steady-state TAG content (Yang and Ohlrogge, 2009). Although we show that \( SDP1 \) is expressed (and the protein is present) in all tissues of a rosette plant, the accumulation of TAG is far more pronounced in heterotrophic tissues (i.e. roots and stems) and much less substantial in leaves. TAG accumulation in whole root tissue appears to be gradual, progressing with the age of the plant, and at maturity TAG can account for more than 1% of DW. This is a 50-fold increase over WT and boosts the total fatty acid content of the tissue by more than 50%. Heterotrophic vegetative tissues can make up a significant proportion of the harvestable biomass of many crop plants and Arabidopsis has a comparatively short life cycle, so TAG accumulation might conceivably be even greater in these plants if \( SDP1 \) genes were disrupted. It has been suggested that even modest increases in total lipid content of crops may be commercially useful. Firstly, for biomass crops the increased energy density makes electricity generation via combustion more efficient (Ohlrogge et al., 2009). Secondly, in forage and in fodder crops the higher calorie content is advantageous for livestock nutrition and therefore meat and dairy production (Hegarty et al., 2013). Disruption of SDP1 genes can be achieved by conventional mutation breeding methods, as well as by transgenic approaches. In Arabidopsis, \( sdp1 \) has impaired seedling establishment (Eastmond, 2006). However, mutation of SDP1 genes might have less impact in non-oilseed species and/or species with vegetative propagation (e.g. many grasses and legumes).

We also show that TAG accumulation in all vegetative tissues of Arabidopsis plants is limited by SDP1 function when they have been genetically engineered to synthesise more oil. Co-expression of DGAT1 and WRI1 in an \( sdp1 \) background results in roots, stems and leaves
with approximately double the TAG content of WT plants expressing DGAT1 and WRI1. Levels of TAG range from 5 to 8% in four week old soil-grown plants, and when cultured in the presence of 3 % (w/v) sucrose more than 17% TAG can be made to accumulate in roots. This TAG content, as a percentage of DW, is very nearly equivalent to that of soybean (*Glycine max*) seeds. Even higher levels of TAG may be possible with further optimisation of transgene expression. However, growth is also impaired as a result of these manipulations. In four week old plants grown on soil, leaf and root biomass (measured as DW) are reduced by more than 20%. There could be many reasons for growth retardation given the extent of the genetic manipulations. However, the shift in carbon partitioning alone might reasonably be expected to have some negative impact on plant growth. TAG has a two-fold higher energy density than carbohydrate and carbon conversion to fatty acids is also comparatively inefficient; although there is potential in photosynthetic tissues for re-fixation of the carbon released as CO₂ (Durrett et al., 2008).

The fact that exogenous sucrose boosts root TAG content so strongly indicates that substrate availability remains an important limiting factor for TAG accumulation in our transgenic lines. Sanjaya et al., (2011) recently showed that blocking transient starch accumulation in Arabidopsis enhances sugar levels and TAG accumulation in leaves expressing WRI1. This approach might also be effective in *sdp1* plants expressing WRI1 and DGAT1. Disruption of starch synthesis has also been shown to elevate sugar levels in roots, as well as leaves, at certain points during the diurnal cycle (Bläsing et al., 2005). Numerous plant species are known to preferentially allocate carbon to stems or roots for storage (Durrett et al., 2008) and these may present more appropriate hosts than Arabidopsis for engineering TAG accumulation in heterotrophic tissues. Sugars may also exert an additional effect over substrate provision by enhancing biosynthetic capacity. Increased sugar concentration triggers wide-scale changes in gene expression and enzyme activities in Arabidopsis, many of which are associated with primary metabolism (Bläsing et al., 2005; Osuna et al., 2007). For example, there is evidence that both *WRI1* and *DGAT1* are induced by sugar (Masaki et al., 2005; Lu et al., 2003).

It’s not clear why *sdp1* leaves fail to accumulate as much TAG as stems and roots. One explanation could be that additional lipases are present in photosynthetic tissues, which allow
TAG turnover to continue to occur in *sdp1*. Arabidopsis contains many genes that could potentially have this function (Li-Beisson et al., 2013). CGI58 is one candidate since the protein has lipase activity (Ghosh et al., 2009) and the mutant accumulates TAG in its leaves to around 0.2% of DW (James et al., 2010), which is higher than we detected in *sdp1*. The disruption of fatty acid β-oxidation also leads to TAG accumulation in leaves (Slocombe et al., 2009; James et al., 2010), but the impact on total fatty acid content appears to be small (Yang and Ohlrogge, 2009), unless the tissue is subjected to carbohydrate starvation (Slocombe et al., 2009; Kunz et al., 2009). Therefore, TAG turnover in the cytosol may simply be less rapid in leaves, than in roots and stems. Importantly, we observed that SDP1 disruption does boost TAG accumulation in leaves when exogenous sugar is applied or when DGAT1 and WRI1 are overexpressed. In both cases TAG synthesis is artificially stimulated (Masaki et al., 2005; Lu et al., 2003; Bouverie-Nave et al., 2000; Cernac and Benning, 2004) and SDP1-mediated TAG turnover must therefore become more active under these non-physiological conditions.

Lipid metabolism in Arabidopsis roots and stems has received rather less attention than in leaves (Li-Beisson et al., 2013). Our analysis of the *pxa1* mutant that is severely deficient in fatty acid β-oxidation (Zolman et al., 2001) showed that it also accumulates TAG in its roots. This suggests that β-oxidation does make a detectable contribution to the bulk turnover of fatty acid from membrane lipids in this tissue under normal growth conditions. Interestingly, *sdp1* roots accumulate more TAG than *pxa1* indicating that the rate of TAG turnover in roots might be greater than the rate of fatty acid breakdown, presuming that the rate of fatty acid synthesis is equivalent in these mutants. Analysis of a *sdp1 pxa1* double mutant also shows that PXA1 is epistatic to SDP1, which implies that SDP1 and PXA1 function in the same pathway. Finally, analysis of *sdp1 dgat1* and *sdp1 pdat1* double mutants showed that TAG accumulation in *sdp1* roots is largely dependent on DGAT1 function, but that PDAT1 is also required. It has already been established that DGAT1 is necessary for normal TAG synthesis in seeds, seedlings and leaves of Arabidopsis (Katavic et al., 1995; Lu et al., 2003; Slocombe et al., 2009), but *pdat1* has not previously been shown to have a TAG phenotype. Recently, a third acyltransferase (DGAT3), with specificity toward polyunsaturated fatty acids (PUFAs), has also been shown to play a role in TAG synthesis in Arabidopsis seedlings (Hernández et al., 2012). Currently, we
cannot discount a significant role for this gene in roots. The TAG that accumulates in \( sdp1 \) roots is enriched in PUFAs; a feature that has also been reported in \( cgi58 \) leaves (James et al., 2010).

In conclusion our data show that combined manipulation of carbohydrate supply, fatty acid synthesis, TAG synthesis and TAG breakdown can drive substantial TAG accumulation in vegetative tissues of Arabidopsis. Further work will clearly be required to establish whether this approach is also applicable to various tissues of crop species and to optimise the technology. The accumulation of TAG in \( sdp1 \) also raises a number of questions concerning the physiological role of TAG in plant vegetative tissues. Oil bodies have been found in the leaves of many plant species (Lersten et al., 2006) and roles for cytosolic TAG in carbon storage and membrane lipid remodelling have previously been proposed in a number of studies (Murphy, 2001; James et al., 2010). However, more evidence is required to support or refute these theories. Further investigation of the role of SDP1 in roots may help to address this question.

ACKNOWLEDGMENTS

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MATERIALS AND METHODS

Plant material and growth conditions

The Arabidopsis thaliana mutants; sdp1-4, sdp1-5, sdp1L-2, cgi58-1 and pxa-1 are described in Kelly et al., (2011). The dgat1-1 (tag1-1) and pdat1-1 mutants are described in Katavic et al., (1995) and Zhang et al., (2009), respectively. For experiments performed on media, seeds were surface sterilized, plated on agar plates containing one-half strength Murashige and Skoog salts (Sigma-Aldrich), with or without the addition of sucrose, and imbibed in the dark for 4 d at 4°C. The plates were then placed vertically in a growth chamber set to 16-h light (23°C) / 8-h dark (18°C); photosynthetic photon flux density = 250 μmol m⁻² s⁻¹. For some experiments plants were also transferred to soil after 5 d on media with 1% (w/v) sucrose to rescue sdp1 (Eastmond, 2006) and grown to maturity in the same conditions as described above.

Histochemical staining and Western blotting

Assays for β-glucuronidase (GUS) were performed as described by Jefferson et al., (1987). Lipid droplets were imaged in situ by laser scanning confocal microscopy using Nile red staining (Greenspan et al., 1985). Nile red stock was made to a concentration of 1mg ml⁻¹ in acetone and diluted to 1μg ml⁻¹ in water for a working concentration. Roots were stained for 1min and washed in deionised water. The material was mounted on a slide in water and imaged with a Zeiss LSM 780 using a 40x or 63x objective and an excitation wavelength of 514 nm, and detected using an emission band of 539-648 nm. Protein extraction, quantification, SDS-PAGE, and Western blotting were performed as described previously (Eastmond, 2004) except that anti-HA and anti-IgG-HRP (Invitrogen Ltd.) were used as primary and secondary antibodies at 1 in 1000 and 1 in 10,000 dilutions, respectively and HRP was detected using an Enhanced Chemiluminescence kit (PerkinElmer).

Lipid analysis
Total lipids were extracted from homogenised freeze dried tissue using the method of Dormann et al., (1995) except that tripentadecanoic acid (15:0 TAG) was added to the homogenized tissue to act as an internal standard. A proportion of the total lipid extract was subjected directly to trans-methylation according to the method of Browse et al., (1986) and the fatty acid methyl esters (FAMEs) quantified by GC-FID with reference to the standard (Kelly et al., 2011). The remaining lipid extract was applied to silica TLC plates and neutral lipids were separated using a hexane-diethyether-acetic acid (70:30:1 v/v) solvent system. The lipids were visualised under UV light by staining with 0.05 % (w/v) primuline in 80% (v/v) acetone and the TAG band scraped from the plate, trans-methylated and the FAMEs quantified as above. ESI-MS/MS analyses were performed on a 4000 QTRAP® LC/MS/MS (ABSiex, Framingham, USA) coupled with a TriVersa NanoMate mounted with an ESI-chip (Advion, Ithaca, USA). For all analysis the NanoMate was operated in positive ion mode with a spray voltage of 2 kV, vented headspace, and pressure of 0.4 psi. A 50μl aliquot of total lipid plus 15:0 TAG standard was mixed in 1ml of 1:1 (v/v) chloroform:methanol with 10 mM ammonium acetate (James et al, 2010). A 10μl aliquot of this mix was then loaded on the ESI-chip to perform the analyses. Full scans were performed using the Q1/MS mode to acquire the spectra. Molecular species of TAG were profiled using the method described by Krank et al., (2007) except that 12 periods of 2 min were used for neutral loss scans. The data were then analysed in Lipidview (V1.1). The amount of fatty acid was normalised by GC-FID.

Creation of DNA Constructs and Arabidopsis Transformation

**DGAT1**, **WRI1** and 1.5 kb of the **SDP1** promoter were amplified by PCR from either Arabidopsis cDNA or gDNA using the following primer pairs: **DGAT1** (5′-CACCATGGCGATTTTGGATTTCTGCTGGCG-3′ and 5′-TCATGACATCGATCCTTTTCGGTTCATCAGG-3′), **WRI1** (5′-CACCATGAAGAAGCGCTTAACCACTTCC-3′ and 5′-TTATTCAGAACCAACGAACAAGCCC-3′) and **SDP1p** (5′-CACCTTCGAGTTTTATTTTCGTTACTTCCA-3′ and 5′-TATTGATTTCGAAGATGAATTTGGGTGTGT-3′). The products were cloned into the
pENTR/D-TOPO vector and then (using the Gateway LR clonase enzyme mix) were transferred to the appropriate destination vector, according to the manufacturer’s instructions (Invitrogen Ltd.). SDP1p was transferred to pBGWFS7, DGAT1 to pB2GW7 and WRII to pK2GW7 (Karimi et al., 2002). The constructs were transformed into Agrobacterium tumefaciens strain GV3101 by heat shock and into Arabidopsis by the floral-dip method (Clough and Bent, 1998). Transformants containing T-DNA insertions were selected via antibiotic or herbicide resistance.

**Transcript Analysis**

DNAse-treated total RNA was isolated from Arabidopsis roots using the RNeasy kit from Qiagen Ltd. The synthesis of single-stranded cDNA was carried out using SuperScript™II RNase H- reverse transcriptase from Invitrogen Ltd. Quantitative real-time PCR was performed as described in Rajangam et al., (2013). The primer pairs used for real-time PCR were QDGAT1 (5'-TGGATTCTGCTGGCGTTACTAC-3' plus 5'-AGCCTATCAAGATCGACGAACACTCTC-3'), QWRII (5'-AAACGAGCCAAAAGGGCTAAAG-3' plus 5'-GGGCTTGTCGGGTTATGAGA-3') and QACT2 (5'-TGTGACAATGGTACCGGTATGG-3' plus 5'-GCCCTGGGAGCATCATCTC-3').

**Statistical Analyses**

Total lipid content, TAG content, and dry weight were compared among genotypes using paired t tests assuming unequal variance.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_120486 (SDP1), NM_202720 (SDP1L), AF378120 (PXA1), NM_202876 (CGI58), AJ238008 (DGAT1) and AY254038 (WRII).

**Supplemental Data**
Supplemental Figure S1. GUS staining of various tissues from a SDP1p::GUS reporter line.
Supplemental Figure S2. ESI-MS/MS analysis of total lipids from sdp1-5 roots.
Supplemental Figure S3. TAG accumulation in sdp1-5 leaves in the presence of sucrose.
Supplemental Figure S4. Images of wild type and sdp1/D1/W1 plants grown on soil.
Supplemental Table SI. Root TAG content and DGAT1 and WRI1 expression in transgenic lines.
Supplemental Table SII. Fatty acid composition of TAG from sdp1/D1/W1 roots.
LITERATURE CITED


Baud S, Mendoza MS, To A, Harscoët E, Lepiniec L, Dubreucq B (2007) WRINKLED1 specifies the regulatory action of LEAFY COTYLEDON2 towards fatty acid metabolism during seed maturation in Arabidopsis. Plant J 50: 825-838


Browse J, McCourt J, Sommerville CR (1986) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem 152: 141–145


Kelly AA, Quettier A-L, Shaw E, Eastmond PJ (2011) Seed storage oil mobilization is important but not essential for germination or seedling establishment in Arabidopsis. Plant Physiol 157: 866-875


Murphy DJ (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog Lipid Res 40: 325–438


Zhang M, Fan J, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell 21: 3885-3901


**FIGURE LEGENDS**

**Figure 1.** Expression of SDP1 in vegetative tissues of Arabidopsis.

(A) *SDP1* promoter activity in leaf, stem and root of four week old plants monitored using a transgenic line carrying an *SDP1p:GUS* construct.

(B) SDP1-HA protein content in leaf, stem and root of four week old plants monitored by Western blot using a transgenic *sdp1-5* line carrying an *SDP1p:SDP1-HA* fusion construct.

**Figure 2.** Triacylglycerol accumulation in *sdp1-5* mutant plants.

Triacylglycerol content (A) and total fatty acid content (B) of *sdp1-5* roots of leaf, stem and root of four week old wild type and *sdp1-5* plants grown on agar plates. Values are the mean ± SE of values from four separate batches of ten plants.

**Figure 3.** Lipid body accumulation in *sdp1-5* roots.

Laser scanning confocal images of Nile red stained roots of four week old wild type and *sdp1-5* plants grown on agar plates. Scale bar = 50 µm.

**Figure 4.** A comparison of TAG accumulation in roots of various mutants.

(A) TAG accumulation in lipid catabolism mutants.

(B) Effect of DGAT1 and PDAT1 deficiency on TAG accumulation in *sdp1-5*.

Values are the mean ± SE of values from four separate batches of ten plants grown for four weeks on agar plates. Asterisk denotes a statistically significant difference from WT (*P* < 0.05).

**Figure 5.** Effect of plant age and exogenous sucrose on total TAG content of wild type and *sdp1-5* roots.

(A) Effect of plant age on root TAG content when grown in the absence of sugar.
(B) Effect of exogenous sucrose on TAG content of roots from four week old plants.

Values are the mean ± SE of values from four separate batches of ten plants grown on agar plates.

**Figure 6.** Combinatorial effect of sucrose, DGAT1 and WRI1 over-expression and SDP1 deficiency on root TAG accumulation.

TAG content (A) and total root DW (B) of four weeks old plants grown on agar plates with, or without 3% (w/v) sucrose. Values are the mean ± SE of measurements on ten plants. D1 and W1 are independent 35S:DGAT1 and 35S:WRI1 lines, respectively. Asterisk denotes a statistically significant difference from WT \((P < 0.05)\).

**Figure 7.** Effect of SDP1 deficiency on TAG content of vegetative tissues of soil-grown plants over-expressing DGAT1 and WRI1.

TAG content (A) and total DW (B) of leaves, stems and roots of four weeks old plants grown on soil. Values are the mean ± SE of measurements on ten plants. The D1/W1 line is expressing DGAT1 and WRI1. Asterisk denotes a statistically significant difference from D1/W1 in (A) and from WT in (B) \((P < 0.05)\).
Figure 1. Expression of SDP1 in vegetative tissues of Arabidopsis.

(A) SDP1 promoter activity in 3 d old seedlings and leaf, stem and root of four week old plants of three independent lines carrying an SDP1p::GUS construct. Values are the mean ± SE of measurements on tissue from four separate plants.

(B) SDP1-HA protein content in 3 d old seedlings and leaf, stem and root of 4 week old plants monitored by Western blotting using a transgenic sdp1-5 line carrying an SDP1p:SDP1-HA fusion construct. The gel was loaded with an equal amount of total protein from each tissue.
Figure 2. Triacylglycerol accumulation in *sdp1-5* mutant plants.

Triacylglycerol content (A) and total fatty acid content (B) of leaf, stem and root of four week old wild type (WT) and *sdp1-5* plants grown on agar plates. Values are the mean ± SE of values from four separate batches of ten plants. Asterisk denotes a statistically significant difference from WT (*P* < 0.05).
**Figure 3.** Lipid body accumulation in *sdp1-5* roots.

Laser scanning confocal images of Nile red stained roots of four week old wild type and *sdp1-5* plants grown on agar plates. Scale bar = 50 µm.
**Figure 4.** A comparison of TAG accumulation in roots of various mutants.

TAG accumulation in lipid catabolism mutants (A) and effect of DGAT1 and PDAT1 deficiency on TAG accumulation in *sdp1-5* (B). Values are the mean ± SE of values from four separate batches of ten plants grown for four weeks on agar plates. Asterisk denotes a statistically significant difference from WT (*P* < 0.05).
Figure 5. Effect of plant age and exogenous sucrose on total TAG content of wild type and sdp1-5 roots.

(A) Effect of plant age on root TAG content when grown in the absence of sugar.

(B) Effect of exogenous sucrose on TAG content of roots from four week old plants.

Values are the mean ± SE of values from four separate batches of ten plants grown on agar plates.
Figure 6. Combinatorial effect of sucrose, DGAT1 and WRI1 over-expression and SDP1 deficiency on root TAG accumulation.

TAG content (A) and total root DW (B) of four weeks old plants grown on agar plates with, or without 3% (w/v) sucrose. Values are the mean ± SE of measurements on ten plants. D1 and W1 are independent 35S:DGAT1 and 35S:WRI1 lines, respectively. Asterisk denotes a statistically significant difference from WT ($P < 0.05$).
**Figure 7.** Effect of SDP1 deficiency on TAG content of vegetative tissues of soil-grown plants over-expressing DGAT1 and WRI1.

TAG content (A) and total DW (B) of leaves, stems and roots of four weeks old plants grown on soil. Values are the mean ± SE of measurements on ten plants. The D1/W1 line is expressing DGAT1 and WRI1. Asterisk denotes a statistically significant difference from D1/W1 in (A) and from WT in (B) ($P < 0.05$).