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Step changes in leaf oil accumulation via iterative metabolic engineering

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ABSTRACT

Synthesis and accumulation of plant oils in the entire vegetative biomass offers the potential to deliver yields surpassing those of oilseed crops. However, current levels still fall well short of those typically found in oilseeds. Here we show how transcriptome and biochemical analyses pointed to a futile cycle in a previously established *Nicotiana tabacum* line, accumulating up to 15% (dry weight) of the storage lipid triacylglycerol in leaf tissue. To overcome this metabolic bottleneck, we either silenced the *SDP1* lipase or overexpressed the *Arabidopsis thaliana LEC2* transcription factor in this transgenic background. Both strategies independently resulted in the accumulation of 30–33% triacylglycerol in leaf tissues. Our results demonstrate that the combined optimization of *de novo* fatty acid biosynthesis, storage lipid assembly and lipid turnover in leaf tissue results in a major overhaul of the plant central carbon allocation and lipid metabolism. The resulting further step changes in oil accumulation in the entire plant biomass offers the possibility of delivering yields that outperform current oilseed crops.

1. Introduction

Petroleum has been a cornerstone of our society since the industrial revolution. For over a century, it has provided a cheap means of energy and a wide suite of tailored oleochemicals. Recent price volatility and growing environmental concerns have fuelled an interest in plant-derived storage lipids such as triacylglycerol (TAG) as a sustainable, carbon-neutral alternative (Carlsson et al., 2011). TAG, one of the most energy-dense forms of carbon found in nature, consists of long acyl chains that bear structural similarity to aliphatic hydrocarbon compounds. Currently, plant oils are predominantly used for nutritional purposes. Increased demand for food, energy and chemical feedstock applications have sparked the food *versus* fuel debate and imposed a significant pressure on the future supply of this major commodity. Consequently, alternative production platforms such as algal- and biomass-derived lipids have attracted considerable attention in recent years.

Vegetable oils are largely sourced from seed or fruit tissues. The biosynthesis and accumulation of TAG in vegetative tissues has the potential to outyield current oil crops, especially when engineered in high-biomass crops (Ohlrogge and Chapman, 2011; Weselake, 2016). Vegetative organs including leaf and stem contain only minor amounts of TAG, where it is believed to serve as an intermediate storage pool for toxic free fatty acids released during membrane recycling or damage (Fan et al., 2013a, 2014; Troncoso-Ponce et al., 2013). Typically TAG accounts for less than 1.5% of the total leaf fatty acids and takes up less than 3% of *de* novo synthesized fatty acids (Fan et al., 2013a; Yang and Ohlrogge, 2009). Although this demonstrates an inherent metabolic capability of non-seed plant tissues to synthesize TAG, achieving high levels such as those typically found in oilseeds is a daunting metabolic engineering task due to the wide range and complexity of enzymatic pathways involved. Plant acyl-lipid metabolism is composed of at least 120 enzymatic steps and over 600 genes (Li-Beisson et al., 2013). In addition, our knowledge of the complex regulatory network controlling

Abbreviations: DAG, diacylglycerol; DAS, days after sowing; DGDG, digalactosyldiacylglycerol; DW, dry weight; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; SDP1, Sugar-dependent lipase1; TAG, triacylglycerol; VLCFA, very long chain fatty acids

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the flux of carbon into storage and membrane lipid biosynthesis is still fragmented (Marchive et al., 2014).

Early metabolic engineering attempts to accumulate storage lipids in vegetative plant tissues focused on overexpression or downregulation of single genes involved in fatty acid or TAG metabolism (Vanhercke et al., 2014a). Such strategies typically resulted in small TAG yield increases, highlighting the need for a simultaneous optimization of multiple critical enzymatic steps. As a result, several groups have recently explored a variety of combinatorial metabolic engineering approaches to increase fluxes through the *de novo* fatty acid ('Push') and TAG biosynthesis ('Pull') pathways while stabilizing the cytosolic lipid droplets ('Package') and minimizing lipid turnover ('Protect') (Vanhercke et al., 2014a; Weselake, 2016; Xu and Shanklin, 2016). Examples in potential crop species include the combined overexpression of the genes coding for WRI1, a regulator of the late glycolysis and *de novo* fatty acid biosynthesis pathways, the DGAT1 acyl-CoA: diacylglycerol acyltransferase and the OLEOSIN oil body protein in *Nicotiana tabacum* and sugarcane, yielding more than 15% and close to 2% oil in transgenic leaf tissue on a dry weight (DW) basis, respectively (Vanhercke et al., 2014b; Zale et al., 2016).

Here we show how differential gene expression analysis and pulse-chase labelling pointed to a futile cycle of TAG synthesis and degradation as a major factor limiting further increases in leaf oil content in the previously established TAG accumulating *N. tabacum* transgenic line (Vanhercke et al., 2014b). To further elevate TAG levels in this transgenic high oil background, we explored two independent metabolic engineering strategies. Firstly, we silenced the SUGAR-DEPENDENT LIPASE1 (SDP1) to interrupt the first step of TAG turnover. The *A. thaliana SDP1* gene product was originally characterized as a major TAG lipase during germination and initial seedling establishment (Eastmond, 2006; Kelly et al., 2011). Subsequent studies showed that this particular lipase is also expressed in vegetative tissues and involved in TAG turnover in leaf tissue (Fan et al., 2014; Kelly et al., 2013; Thazar-Poulot et al., 2015). Because SDP1 is epistatic to enzymatic steps catalyzing downstream lipid turnover processes such as β-oxidation, we explored an alternative and independent strategy to minimize TAG turnover by attempting to improve the stability of the oil droplets in transgenic leaf tissues. LEC2 is a master regulator of embryogenesis and oilseed maturation developmental programs, during which TAG accumulates as the major storage lipid (Angeles-Nunez and Tiessen, 2011; Stone et al., 2001). Amongst its transcriptional targets are *WRI1* and oil droplet proteins including oleosin and steroleosin (Braybrook et al., 2006; Che et al., 2009; Kim et al., 2014). Ectopic expression of *LEC2* in vegetative tissues leads to elevated levels of TAG in the form of cytosolic oil bodies containing oleosin (Feeney et al., 2013; Kim et al., 2014; Santos Mendoza et al., 2005). Overexpression of *LEC2* could therefore assist indirectly in further stabilizing TAG-containing lipid droplets via upregulation of endogenous oil body proteins. To minimize negative pleiotropic, we opted for a senescence-specific promoter to drive *LEC2* overexpression. We demonstrate how the independent stacking of either construct dramatically increased TAG in leaf tissue to around 30% (DW) while transitory starch levels were reduced. Transmission electron microscopy (TEM) analysis revealed an abundance of very large lipid droplets occupying the majority of the cytosol in transgenic leaf mesophyll cells. Our results demonstrate that, despite the complexity of lipid and carbon metabolism, modulating the expression of four genes is sufficient to achieve oilseed-like levels of storage lipids in leaf tissue.

2. Material and methods

2.1. Plant material and growth conditions

A *N. tabacum* line (T_3) overexpressing *A. thaliana WRI*, *A. thaliana DGAT1* and the *S. indicum OLEOSIN* has been described (Vanhercke et al., 2014b). Wildtype (Wisconsin 38) and transgenic plants were grown either in the glasshouse during summer conditions without artificial light (T_0-T_1) or in a growth cabinet (T_2) under 16 h/8 h light conditions (750 μmol m^{-2} s^{-1}). Growth temperatures in glasshouse and growth cabinets were set at 24 °C/18 °C. Unless otherwise stated, analyses were conducted on a minimum of three individual plants for each genotype. Samples from each plant were analyzed in triplicate.

2.2. RNA isolation and transcriptome sequencing

Total RNA was extracted as three biological replicates from leaves sampled from three wild type *N. tabacum* plants and three transgenic plants (Vanhercke et al., 2014b). Sampling stages corresponded to vegetative growth (69 days after sowing, DAS), flowering (104 DAS) and seed setting (139 DAS) as described previously (Vanhereke et al., 2014b). A modified version of rapid CTAB-based method was used for RNA extraction followed by purification using MagJET Plant RNA Kit (Thermo Fisher Scientific, Australia). Quantity and quality of total RNA was determined by NanoDrop spectrophotometer and Bioanalyzer. High quality RNA was submitted to ACRF Biomolecular Resource Facility (Canberra) for subsequent cDNA library preparation and sequencing by Illumina HiSeq™ 2500 system. Raw sequencing reads (100 bp, paired end) were subjected to stringent quality control to remove adaptor sequences, ambiguous reads (5%) and low quality sequences (Q20) using FastQC (version 0.10.1; Babraham Bioinformatics) and an in-house toolkit, Biokanga (version 3.4.17). Raw reads from individual samples were deposited to the National Center for Biotechnology Information (NCBI) Short Read Archive under accession number SRP081111.

2.3. De novo *transcriptome assembly, annotation and differential expression analysis*

High quality reads were assembled into contigs using Trinity (version r20140413p1) (Grabherr et al., 2011) using a *k*-mer length of 25 to yield a total of 350615 contigs with a N50 of 1508. The assembled transcripts were mapped against sequence databases from different plants using BLASTX ($1e^{-5}$) in Biokanga. The databases used for annotation included the Arabidopsis TAIR database (http://www.arabidopsis.org), *N. benthamiana* (http://sydney.edu.au/science/molecular_bioscience/sites/benthamiana/) and *N. sylvestris* (http://www.ncbi.nlm.nih.gov). The fully annotated *N. tabacum* leaf transcriptome was made available via CSIRO data access portal (http://doi.org/10.4225/08/5763594279730). To measure the expression of each transcript, high-quality reads from each sample were mapped to the final transcriptome assembly using Biokanga. The parameters used include a maximum number of two mismatches and a minimum read count of 10 in at least two samples. A count matrix was generated and loaded into R (version 3.0.1; https://www.r-project.org/) for differential expression analysis using the edgeR package (version 3.2.4; https://bioconductor.org/packages/release/bioc/html/edgeR.html). Read counts were normalised using Trimmed Mean of M-values (TMM) method (Robinson and Oshlack, 2010) and Hochberg's FDR adjustment approach (Benjamini and Hochberg, 1995) was employed to estimate fold changes. A *P*-value cut-off ≤0.05 was used to identify significant fold changes.

After filtering for the presence of a minimum of 10 reads in at least two libraries, 79,054 contigs were subjected to differential expression analysis. A total of 16278 genes were differentially expressed at the vegetative stage, out of which 8601 were upregulated and 7677 were downregulated. At the flowering stage, 4870 genes displayed increased expression whilst 3814 were downregulated. A total of 90 genes showed a differential expression profile between the wild type and transgenic plants at seed setting. In this group, 70 were found to be upregulated whilst expression levels of 20 genes were reduced. Differentially expressed genes at the first two time points were

subjected to KEGG (http://www.genome.jp/kegg/) pathway enrichment analysis. Both stages exhibited differential gene expression changes in a total of 25 pathways, 15 of which were affected at both time points. To validate the expression patterns, expression levels of six transcripts were determined by Digital Droplet PCR. The log2-fold change values were in agreement with those obtained by RNA-seq (Supplementary Fig. 10). Contigs encoding different subunits of pyruvate dehydrogenase and acetyl-CoA carboxylase complexes were identified by individually blasting against the Arabidopsis TAIR 10 database (http://www.arabidopsis.org).

2.4. Generation of SDP1 knockdown and LEC2 overexpressing lines

A 3.9 kb synthetic fragment was synthesized containing the *N. tabacum* enTCUP2 constitutive promoter, a hairpin composed of a 713 bp fragment of the *N. benthamiana SDP1* gene (Nbv5.1tr6385200; http://benthgenome.qut.edu.au/) flanked by attB1 and attB2 sites and interspersed by *Pdk* and *cat* intron sequences, and followed by the *Rhizobium radiobacter* OCS polyadenylation site. Subsequent subcloning of this fragment into the *Sma*I and *Kas*I restriction sites of a pORE04-based binary expression vector backbone containing the hygromycin resistance selectable marker gene yielded pOIL051. The *A. thaliana LEC2* gene was synthesized as a 3.6 kb DNA fragment, flanked by the *A. thaliana* SAG12 senescence-specific promoter and the *Glycine max* Lectin polyadenylation region. This fragment was subsequently inserted between the *Sac*I and *Not*I restriction sites of a pORE04-based binary expression vector backbone containing the hygromycin resistance selectable marker gene, resulting in pOIL049. Gene synthesis was done by GeneArt (Thermo Fisher Scientific, Germany). Both constructs were transformed independently into a homozygous high oil transgenic *N. tabacum* line, as previously described (Vanhercke et al., 2014b).

2.5. Digital Droplet PCR

Copy number was determined by Digital Droplet PCR (Glowacka et al., 2016) using primer and probes specific for the *HPH* selectable marker and *N. tabacum NFL2* reference gene (Kelly et al., 1995) (Accession #AH006599). *BamH*I and *Hind*III digested genomic DNA was added to 2x ddPCR mastermix at concentrations between 20 and 120 ng DNA per 20 µL PCR reaction. Final concentrations of TaqMan assay primers and probes in the reaction were 900 nM and 250 nM, respectively. Droplets were generated using a Droplet Generator QX100 (Bio-Rad, Australia) following the manufacturer's instructions. Next, 40 µL of oil droplets were transferred to a 96 well plate and loaded in a C1000 Thermal Cycler (Bio-Rad, Australia). The PCR program consisted of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 59 °C for 1 min, followed by 98 °C for 10 min, with a 2.5 °C/s ramping at each step. After amplification, plates were loaded into the QX100 Droplet Reader (Bio-Rad, Australia) for the detection of amplicons in individual droplets. Data analysis was performed using the Quanta soft software (version 1.7.4.0917; Bio-Rad, Australia).

For gene expression analysis by Digital Droplet PCR, total RNA from leaf tissue was extracted and purified from three biological replicates as described above. Next, 2 µg total RNA was reverse transcribed using SuperScript VILO Master Mix (ThermoFisher Scientific, Australia). Each 20 µL reaction contained 10 µL of 2x ddPCR master mix, 500 nM of each primer and 250 nM of the TaqMan probe for target and reference genes. Oil droplet generation and PCR amplification were done as described above. The plates were then transferred into QX200 Droplet Reader and data were analyzed as described above. The *N. tabacum L25* gene (Accession #L18908) was used as reference (Schmidt and Delaney, 2010). Primer and probe sequences are detailed in Supplementary Table 9.

2.6. Germination assay

Wildtype and transgenic *N. tabacum* seeds were sterilized using chlorine gas for 75 min as described by Kereszt et al. (2007). A total of 3×21 seeds were inoculated either onto MS media containing 1.5% sucrose or moistened Whatman filter paper. Seed viability was scored following incubation in the dark for 12 days at 24 °C.

2.7. Lipid analyses

Initial screening of primary transformants was done visually following separation of total lipids by thin layer chromatography (TLC). Extraction and quantification of TAG from leaf, stem and root tissues by TLC and gas chromatography (GC) were performed as previously described (Vanhercke et al., 2013).

Lipidomic analysis of leaf samples was done by liquid chromatography-mass spectrometry (LC-MS) on an Agilent 6550 QQQ mass spectrometer with Jetstream and ifunnel technology. Briefly, total lipid extracts were diluted to 1 mg/mL DW and 1 µL was injected. Lipid species were separated on an Agilent 1290 LC system as previously described (Reynolds et al., 2015). The mass spectrometer conditions were based on Sandra et al. (2010) and different lipid species were identified by isolation of the parent ion mass in Q1 and the resulting daughter ions were monitored after CID induced neutral loss of one acyl chain. Approximately 400 Multiple Reaction Monitoring experiments were conducted to quantify lipid species and acyl chain composition. Data were integrated using Mass Hunter Quantitative software (version B.07.07 SP1/Build 7.1.524.1; Agilent Technologies). All further data analyses were conducted using R-studio (version 3.3.0; https://www.rstudio.com/). As no internal standards were included in the samples, LC-MS results only indicate qualitative differences within each lipid class.

2.8. Starch, soluble sugar and total carbon quantification

Ground, freeze-dried leaf tissue (~15 mg) was boiled four times in 80% (v/v) ethanol. The supernatants were pooled and soluble sugars were measured in the ethanol extract while the pellet was retained for starch determination. Aliquots of ethanol extract were boiled in anthrone reagent (0.2% (w/v) anthrone in 70% (v/v) concentrated H_2SO_4) for 10 min and the absorbance at 630 nm was measured (Yemm and Willis, 1954). For starch determination, the pellet was resuspended in 350 µL 0.2 M KOH, boiled for 30 min and neutralised using 3.5 µL glacial acetic acid. The starch content of duplicate 100 µL aliquots was determined relative to a control/blank aliquot using a Megazyme Total Starch Kit (Megazyme International, Ireland). Spectrophotometric measurements were performed using a Thermo Multiscan Spectrum plate reader. Total carbon content of 2–2.5 mg freeze-dried leaf tissue was determined using a Europa 20-20 isotope ratio mass spectrometer with an ANCA preparation system, comprising a combustion and reduction tube operating at 1000 °C and 600 °C, respectively.

2.9. Acetate pulse-chase experiment

In vivo labelling experiments with [1-^{14}C]acetate were carried out according to the method described by Koo et al. (2004) with the following modifications. Individual disks (5 mm diameter) were cut from mature leaves of four flowering-stage tobacco plants and incubated in continuous light (100 µmol m^{-2} s^{-1}) at 22 °C with shaking in 10 mL of medium consisting of 1 mM acetate, 20 mM MES buffer (pH 5.5), 1/10-strength MS salts and 0.01% (v/v) Tween 20. The assay was started by the addition of 0.1 mCi of [^{14}C]acetate (57 mCi $mmol^{-1}$; PerkinElmer, UK). At the end of the incubation, leaf disks were washed three times with 0.01% (v/v) Tween 20 and blotted onto filter paper. For the chase period, leaf disks were then incubated in the same

medium lacking [^{14}C]acetate. Tissue was frozen in liquid nitrogen and total lipids were extracted by homogenization in chloroform/methanol/formic acid (1/1/0.1, v/v/v) and 1 M potassium chloride-0.2 M phosphoric acid and TAG was purified by TLC. Radioactivity associated with total lipids was determined by liquid scintillation counting.

2.10. Microscopy analyses

Fully expanded leaves from flowering plants were sampled early afternoon. For confocal miscroscopy, cross sections of leaf tissues were hand cut into thin slices (40 μm thickness) and stained with a 4 μg/mL solution of BODIPY 493–503 in 50 mM PIPES buffer. Imaging was done using a Leica SP8 confocal scanning microscope with excitation and emission wavelengths of 493 nm and 520 nm, respectively. Lipid droplets were imaged at 40x magnification and analyzed using the Leica LAS AF Lite software package (www.leica-microsystems.com).

Samples for TEM imaging were cut into approximately 2×2 mm squares and directly fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 under vacuum for 24 h. Samples were subsequently washed three times by incubating with 0.1 M phosphate buffer for 10 min each. Secondary fixation was carried out with 1% osmium tetroxide at room temperature for 4 h. The fixed samples were rinsed with distilled water, dehydrated using acetone and embedded in Spurr's resin overnight at 70 °C (Spurr, 1969). Following sectioning (70–90 nm thickness) with a Leica EM UC7 Ultra microtome, sections were stained with uranyl acetate and lead citrate for 10 min each. Images were observed and recorded using the Hitachi 7100 TEM at an accelerating voltage of 100 kv.

3. Results

3.1. Differential expression of carbon and lipid metabolic pathways in oil accumulating tobacco leaf tissue

Previously we reported how overexpression of *WRI1*, *DGAT1* and *OLEOSIN* in *N. tabacum* resulted in the accumulation of TAG levels up to 15% (leaf DW) (Vanhercke et al., 201b). As a first step towards gaining a better understanding of the underlying metabolic changes, we studied the differential gene expression patterns in wildtype and TAG accumulating transgenic leaf tissues overexpressing *WRI1*, *DGAT1* and *OLEOSIN* at different stages during plant development. The time points chosen corresponded to vegetative, flowering and seed setting stages (Vanhercke et al., 2014b). Since very few genes (90) showed a differential expression profile at seed setting, we focussed on the first two stages for the remainder of this study. Differentially expressed genes at vegetative and flowering stages were subjected to KEGG pathway enrichment analysis to identify metabolic pathways affected by overexpression of the three lipid-related transgenes. The majority of the pathways exhibiting differential gene expression changes at the vegetative and flowering stages were related to carbon assimilation and partitioning, including photosynthesis, the pentose phosphate pathway, glycolysis and the citric acid cycle alongside fatty acid biosynthesis and metabolism.

In the transgenic leaf tissue, genes involved in the photosynthetic electron transport chain and photorespiration generally showed increased expression levels (Supplementary Tables 1 and 2). Within the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was upregulated in high oil tobacco at both time points (Supplementary Fig. 1). Transcription of the plastidial isoforms of phosphoglycerate mutase, enolase and pyruvate kinase was highly elevated whilst those encoding cytosolic isoforms were either down- or only slightly upregulated. Both sucrose-phosphate synthase and sucrose-phosphate phosphatase genes were downregulated. The glucose (GLT1), triose phosphate/phosphate (TPT) and phosphoenolpyruvate/phosphate (PPT1) transporters, implicated in the transfer of glycolytic metabolites across the inner plastidial membrane, also exhibited increased expression.

Genes related to *de novo* fatty acid synthesis in the chloroplast were highly expressed as a result of *WRI1* transgene overexpression (Supplementary Fig. 2). Examples included different subunits of the pyruvate dehydrogenase and acetyl-CoA carboxylase that catalyses the conversion of pyruvate to malonyl-CoA (Supplementary Table 3), malonyl-CoA: ACP malonyltransferase, the different components of the fatty acid synthase complex and the long chain acyl-CoA synthetase 9 (LACS9). Transcripts of the FATA thioesterase and the soluble plastidial Δ9 desaturase (SAD) were also increased. This is in line with higher levels of oleic acid (C18:1$^{\Delta 9}$) observed in TAG accumulating tobacco leaf tissue (Vanhercke et al., 2014b). Ectopic overexpression of *WRI1* did not have a dramatic effect on the expression levels of the various acyltransferases that mediate synthesis of membrane and storage lipids (Supplementary Fig. 2 and Supplementary Table 4). Two phospholipase D isoforms which convert phosphatidylcholine into the Kennedy intermediate phosphatidic acid were upregulated. Other genes exhibiting increased expression included the membrane bound Δ12-desaturase (FAD2). The latter is consistent with the increase in linoleic acid (C18:2$^{\Delta 9,12}$) as previously observed in transgenic high oil tobacco leaves (Vanhercke et al., 2014b).

3.2. TAG accumulation in N. tabacum leaf tissue is limited by a futile cycle

Various lipid turnover related transcripts were increased in high oil tobacco, especially at the second time point. These included the SDP1 lipase as well as enzymes of the peroxisomal β-oxidation pathway (ACX, ECH, HAD and KAT) (Fig. 1). To obtain biochemical evidence for the upregulation of both lipid biosynthesis and TAG degradation pathways in metabolically engineered high oil tobacco leaves overexpressing *WRI1*, *DGAT1*, and *OLEOSIN*, we performed an *in vitro* pulse-chase experiment using isolated leaf discs and ^{14}C-labeled acetate as a substrate for lipid biosynthesis (Fig. 2a). *De novo* lipid biosynthesis was increased in transgenic leaves compared to the wildtype control. During the chase period, however, we observed considerable lipid turnover in the transgenic line which accounted for almost half of the label in total lipids disappearing after 3 days. As expected, leaves overexpressing *WRI1*, *DGAT1* and *OLEOSIN* accumulated the majority of the label at the end of the chase as TAG (Fig. 2b).

3.3. SDP1 silencing or LEC2 overexpression further increase TAG content in N. tabacum leaves

As a first strategy to overcome the futile TAG cycle, we silenced the *SDP1* lipase. Secondly, we decided to overexpress the embryogenic transcription factor *LEC2* to test the effect on both TAG biosynthesis and degradation. To reduce possible unwanted pleiotropic effects in developing leaf tissue as a result of *LEC2* overexpression (Feeney et al., 2013; Kim et al., 2014; Santos Mendoza et al., 2005; Stone et al., 2008), we used the *A. thaliana* SAG12 senescence-specific promoter for transgene expression (Noh and Amasino, 1999) (Supplementary Fig. 3). Both constructs were transformed independently into the previously established homozygous *N. tabacum* transgenic line that overexpresses the *WRI1*, *DGAT1* and *OLEOSIN* transgenes ('high oil parent') (Vanhercke et al., 2014b).

Following a preliminary rapid screening of 33 (*SDP1*) and 75 (*LEC2*) primary transformants prior to flowering, we subsequently re-sampled leaves of the best performing independent events for each construct at seed setting. Quantification by TLC-GC yielded up to 28.7% and 32.9% TAG in leaf tissue (DW) upon *LEC2* overexpression and *SDP1* silencing, respectively (Supplementary Table 5). Senescing leaves of several independent *LEC2* overexpressing lines at seed setting contained higher TAG levels compared to green leaf tissue. This is in agreement with the leaf senescence-specific expression pattern of the

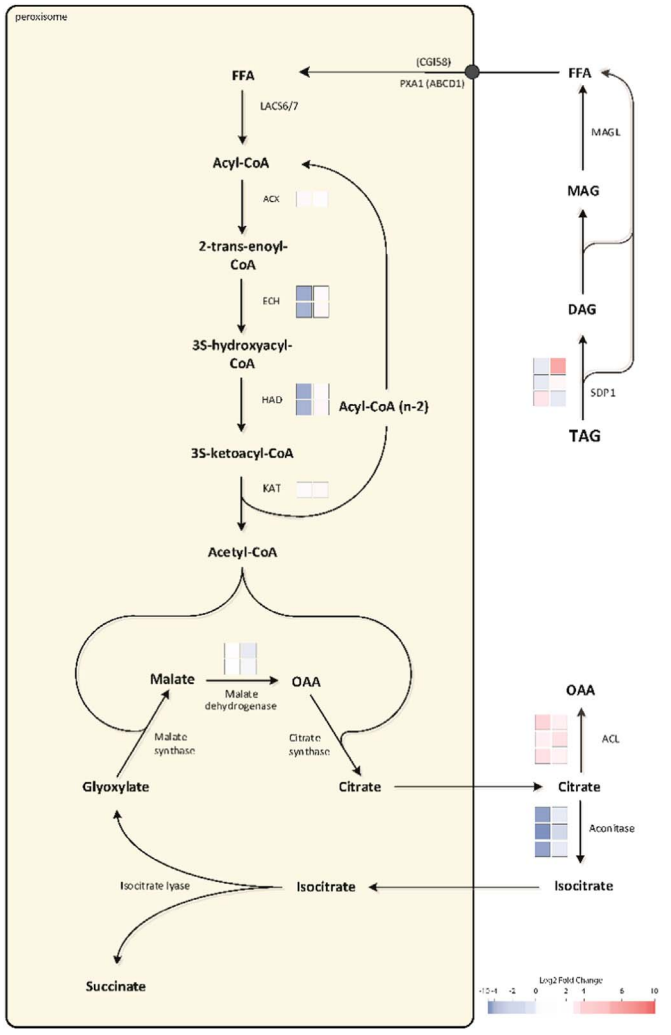

Fig. 1. Differential gene expression pattern in the lipid catabolic pathway in oil accumulating *N. tabacum* leaf tissue expressing three transgenes involved in lipid biosynthesis (*WRI1*, *DGAT1*, *OLEOSIN*). Boxes on the left and right represent differentially expressed genes at vegetative and flowering time points, respectively. Colour of the boxes correspond to fold change (Log2) values compared to wildtype. For enzymes with more than three contigs, only the three highest expressed contigs are included. Significant fold changes ($p \leq 0.05$) only are represented.

SAG12 promoter. We selected the following two lines for further progeny analysis: *SDP1* silenced line #69 contained three T-DNA insertions whilst *LEC2* #32 was a single copy event, as determined by Digital Droplet PCR (results not shown). The *LEC2* overexpressing line #23 did not develop elongated stems in the next generation.

T_1 progeny of the *LEC2* and *SDP1* selected lines were grown alongside wildtype and the high oil parent for comparison. Fully expanded yellow-green senescing leaves were sampled at 21 weeks after sowing when the plants were carrying dried seed pods. After an initial screening of the entire segregating T_1 population, the three best individual events for each construct were subjected to further detailed lipid analysis (Fig. 3a). Similar to our previous findings (Vanhercke et al., 2014b), TAG constituted a minor lipid species in wildtype leaves (0.1% DW) while levels in the high oil parent typically ranged between 13% and 17% (DW). Maximum TAG levels in leaves of *LEC2* and *SDP1* transgenic plants (T_1) averaged 29.8% and 33.3% (DW), respectively. As *SDP1* silencing has been reported to increase neutral lipid content in heterotrophic tissues (Kelly et al., 2013), we also quantified TAG in stems and roots of selected *LEC2* and *SDP1* transgenic plants (Fig. 3b). TAG levels in both stem and root tissues of the high oil parent plants were increased 8- and 3-fold compared to the wildtype controls, respectively. *LEC2* overexpression and *SDP1* silencing further elevated TAG content in stem tissues to 4.9% and 7.4% (DW), respectively. A similar trend was observed for transgenic *LEC2* and *SDP1* roots although the absolute TAG amounts were lower compared to stem tissue of the same plant.

To test the effect of *LEC2* overexpression and *SDP1* silencing on total lipid synthesis and turnover, we subjected isolated leaf disks to an *in vitro* pulse chase experiment in the presence of ^{14}C-labeled acetate. Following the chase period, both *SDP1* and *LEC2* leaves exhibited reduced lipid turnover compared to the high oil parent (Fig. 2).

While silencing of the SDP1 lipase affected TAG levels in vegetative tissues, germination was comparable to the wildtype control and high oil parent and close to 100% (Supplementary Table 6). Overexpression of *LEC2* resulted in a lower germination rate (74.3 - 86.8% depending on the line and condition). Whilst T_1 plants of the original high oil parent did not display major detrimental phenotypic effects compared to the wildtype (Vanhercke et al., 2014b), we did observe a reduction in final plant height in subsequent generations (Supplementary Figs. 4–5). In the T_2 generation, *SDP1* sister lines varied in plant height, overall development and leaf TAG content whilst the *LEC2* progeny behaved stably. We therefore did not further characterize the *SDP1* transgenic line. Homozygous *LEC2* overexpressing plants (T_2) at seed setting were comparable in height to the wild type control whilst both leaf and total plant DW biomass were reduced compared to the wildtype and high oil parent (Supplementary Figs. 4–5).

3.4. Profile changes in DAG and TAG neutral lipids

Compared to the high oil parent, leaves of *SDP1* silenced and *LEC2* overexpressing T_1 plants at the onset of senescence showed a further depletion of α-linolenic acid (C18:3$^{\Delta 9,12,15}$) in TAG to less than 4% of the fatty acid composition (Supplementary Table 7). In the *LEC2* leaves, increased linoleic acid levels largely came at the expense of oleic acid, being the dominant fatty acid in both the high oil parent and *SDP1* silenced lines. Similar fatty acid profile changes were detected in corresponding stem tissues with the exception of a general reduction in linoleic acid levels compared to the wildtype control. Wildtype roots displayed considerable variation in their TAG profile, likely a result of heterogeneity in root developmental stage during sampling. Unlike leaf and stem tissue, transgenic *LEC2* and *SDP1* transgenic roots exhibited decreased levels of palmitic acid (C16:0).

In addition, we performed a detailed compositional analysis by LC-MS of TAG and diacylglycerol (DAG). TAG species containing poly-unsaturated fatty acids were reduced in the high oil parent compared to the wildtype control (Supplementary Fig. 6). A similar shift was also observed in the distribution of DAG species composed of two C18 acyl chains (DAG 36:1-6) (Supplementary Fig. 7). The TAG profile in *SDP1* silenced leaves was similar to that of the high oil parent. In *LEC2* overexpressing plants, on the other hand, TAG 52:1-2 and TAG 54:1-2 molecular species were reduced while TAG 54:4-6 was increased. These findings are consistent with the GC results showing a decrease in oleic acid and an increase in linoleic acid containing TAG species. This change was also reflected in increased DAG 36:4 which predominantly consisted of linoleic acid. Other TAG species also exhibited altered fatty acid compositions. As an example, TAG 52:5 predominantly consisted of palmitic, linoleic and α-linolenic fatty acids in the high oil parental line while its composition in *LEC2* overexpressing plants mostly consisted of palmitoleic (C16:1$^{\Delta 9}$) and linoleic fatty acids.

3.5. SDP1 *and* LEC2 *transgenic tobacco mesophyll cells contain large cytosolic oil droplets*

Microscopic analysis confirmed the accumulation of neutral lipids in *LEC2* and *SDP1* transgenic leaves. Confocal microscopy revealed an abundance of lipid droplets measuring between 10 and 15 μm in diameter within the mesophyll cells (Fig. 4a). A high number of large

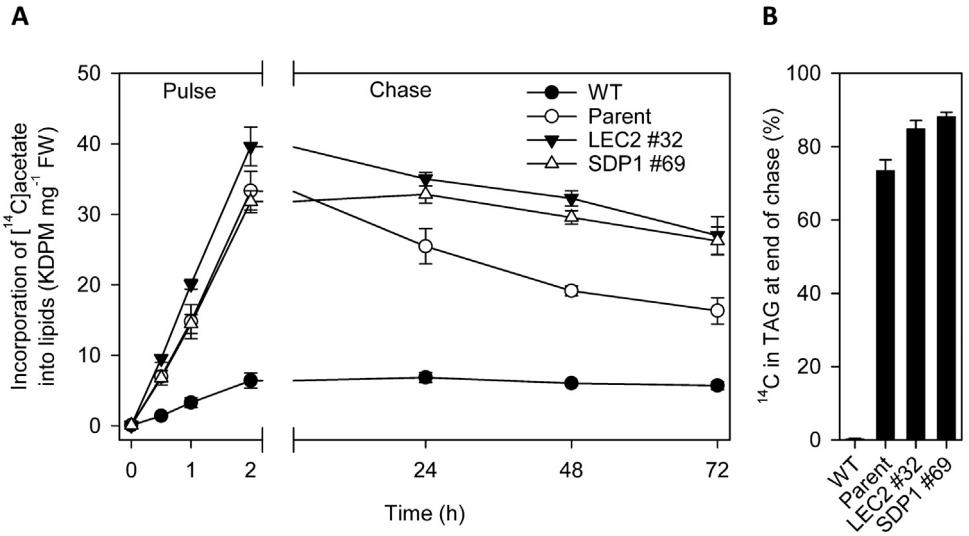

Fig. 2. [^{14}C]Acetate pulse-chase labelling of wildtype and transgenic *N. tabacum* leaves. (A) Label incorporation in total leaf lipids. (B) Percentage of label in triacylglycerol at the end of the chase. Leaf disks were sampled from mature leaves of flowering-stage *N. tabacum* plants. Wt, wildtype *N. tabacum*; Parent, high oil *N. tabacum* line expressing three transgenes involved in lipid biosynthesis (*WRI1*, *DGAT1*, *OLEOSIN*); SDP1, silencing of the SDP1 TAG lipase in the high oil background; LEC2, overexpression of the *A. thaliana LEC2* gene in the high oil background. Data are mean ± SE of measurements on leaf disks from four separate plants of each genotype.

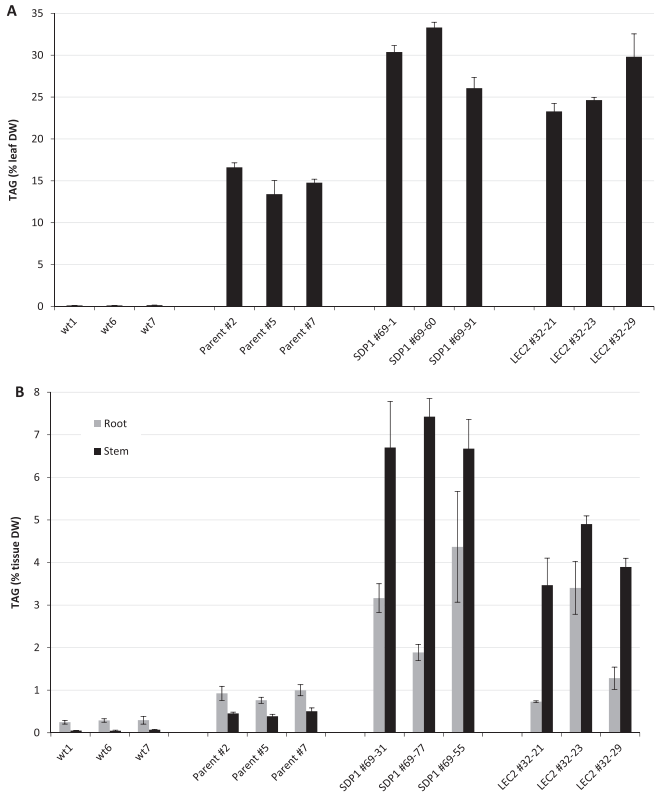

Fig. 3. Triacylglycerol (TAG) content on a dry weight (DW) basis in senescing leaves (A), stems and roots (B) of wildtype and transgenic (T_1) *N. tabacum* plants sampled at seed setting. Wt, wildtype *N. tabacum*; Parent, high oil *N. tabacum* line expressing 3 transgenes involved in lipid biosynthesis (*WRI1*, *DGAT1*, *OLEOSIN*); SDP1, silencing of the SDP1 TAG lipase in the high oil background; LEC2, overexpression of the *A. thaliana LEC2* gene in the high oil background. Error bars represent standard deviations of triplicate analyses on three individual plants for each genotype.

oil bodies were found to be densely packed in the cytosol of mesophyll cells in transmission electron micrographs of *SDP1* transgenic leaf tissue (Fig. 4b). Starch granules were typically smaller in size compared to those found in wildtype leaf tissue. Very few and small lipid droplets were observed in wildtype mesophyll cells.

3.6. Further increases in leaf TAG content are correlated with a reduction in transitory starch

The elevated TAG levels in leaves of both *SDP1* and *LEC2 N. tabacum* plants were accompanied by a further reduction in starch content compared to the high oil parent (Fig. 5 and Supplementary Fig. 8). Levels of soluble sugars, potentially serving as metabolic precursors for *de novo* fatty acid and lipid biosynthesis, were similar between the different genotypes. Total leaf carbon content was positively correlated with TAG levels (Supplementary Fig. 9).

3.7. TAG accumulates to high levels in leaves of different ages

Since the *A. thaliana* SAG12 promoter has been reported to be active during leaf senescence, we decided to compare TAG levels between different aged leaves and determine the extent of a possible gradient of TAG accumulation across the entire plant. We therefore measured the total lipid and TAG contents in all leaves sampled from single wildtype, high oil parent and *LEC2* overexpressing plant (T_2) at seed setting stage (Fig. 6). In both transgenic plants, the lipid content in young leaves was generally higher compared to bottom senescing leaves. Maximum TAG levels detected in leaves of the high oil parent and *LEC2* plants grown under controlled cabinet conditions equalled 13.5% and 32.1% (DW), respectively. These levels are in good agreement with previous results obtained in the glasshouse (Fig. 3). The accumulation of over 30% TAG in younger leaves of the *LEC2* overexpressing line suggested leaky expression of the SAG12 promoter in non-senescing tissue.

4. Discussion

Although the direct biochemical function of genes such as *WRI1*, *DGAT1* and *OLEOSIN* in lipid metabolism have been extensively studied in an oilseed context, the effect on lipid and carbon metabolism upon overexpression in photosynthetic non-seed tissues such as leaves is still poorly understood. Differential expression analysis on the previously established high oil *N. tabacum* transgenic line suggests a direct flow of photosynthetic carbon in the plastids from 3-phosphoglycerate to pyruvate, the precursor for *de novo* fatty acid biosynthesis (Fig. 7). Many genes involved in subsequent *de novo* fatty acid biosynthesis were also upregulated as a result of *WRI1* overexpression. As fatty acid synthesis is a demanding process in terms of energy,

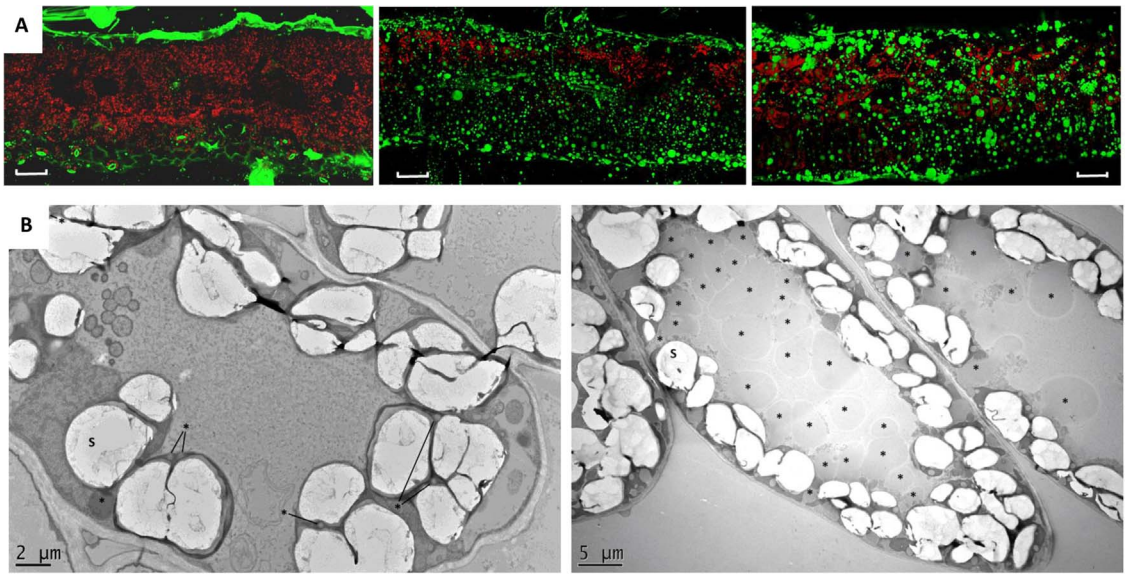

Fig. 4. Micrographs of wildtype and transgenic *N. tabacum* leaf tissues. (A) Accumulation and distribution of lipid droplets in leaves of wildtype (left), *LEC2* overexpressing (middle) and *SDP1* silenced (right) *N. tabacum* lines as detected by confocal microscopy (40 µm scale bars) (B) Transmission electron microscopy images of wildtype (left) and *SDP1* silenced (right) leaf mesophyll cells. Dominant oil bodies are marked with asterisks; S denotes a starch granule.

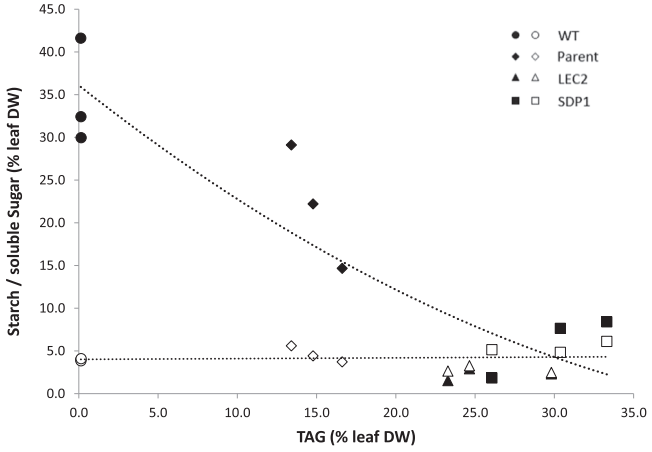

Fig. 5. Starch (black symbols) and soluble sugar (open symbols) contents on a dry weight (DW) basis in senescing leaves of wildtype and transgenic (T_1) *N. tabacum* plants sampled at seed setting. Wt, wildtype *N. tabacum*; Parent, high oil *N. tabacum* line expressing 3 transgenes involved in lipid biosynthesis (*WRI1*, *DGAT1*, *OLEOSIN*); SDP1, silencing of the SDP1 TAG lipase in the high oil background; LEC2, overexpression of the *A. thaliana LEC2* gene in the high oil background. Data points are based on triplicate analyses of three individual plants for each genotype.

additional ATP could be supplied via upregulation of photophosphorylation in the high oil tobacco. In line with our results, *WRI1* overexpression in *B. napus* induces expression of genes involved in photosynthesis in developing green seeds (Wu et al., 2014). However, an opposite effect on the expression of photosynthetic genes has been observed in *N. benthamiana* leaves (Grimberg et al., 2015), highlighting the metabolic complexity between different tissues. Increased transcription of several plastidial transporters suggests a model for carbon fluxes at different metabolic nodes from the cytosolic into the plastidial glycolysis pathways. Downregulation of biosynthetic steps involved in sucrose and starch biosynthesis indicates a reduced mobilization of photosynthetic carbon into both major sinks in leaf mesophyll cells. This is in agreement with the lower levels of transitory starch detected in oil accumulating transgenic *N. tabacum* leaves.

Both transcriptome and biochemical analyses pointed to a futile cycle of lipid synthesis and degradation, likely limiting further TAG accumulation in the *N. tabacum* transgenic leaves. Of note, Grimberg et al. (2015) reported elevated transcript levels corresponding to putative lipases as well as upregulation of genes involved in the β-oxidation and glyoxylate pathways. Overexpression of the *Brachypodium distachyon WRI1* in the same species also resulted in the upregulation of *SDP1* and β-oxidation genes (Yang et al., 2015). Considerable TAG turnover rates have also been reported in *A. thaliana* leaves when compared to membrane phospholipids (Tjellstrom et al., 2015). The combined biochemical and transcriptome findings therefore indicate that TAG accumulation in metabolically engineered, oil accumulating leaves of *N. tabacum* and likely other species is limited by a futile cycle operating in mesophyll cells (Fig. 7). The loss of photosynthetic carbon during *de novo* fatty acid synthesis and TAG turnover might be salvaged subsequently due to the upregulation of RuBisCo in the metabolically engineered *N. tabacum* leaf tissue, much like the RuBisCo shunt pathway operating in green oilseed tissues (Schwender et al., 2004) (Fig. 7).

Silencing of the *SDP1* lipase or overexpression of *LEC2* in the high oil transgenic *N. tabacum* background reduced total lipid turnover and further elevated TAG levels in leaf tissues. Knock-out of *sdp1* in *A. thaliana* also resulted in a doubling of the TAG content in vegetative tissues when combined with *WRI1* and *DGAT1* transgene overexpression, though absolute levels achieved were much lower (Kelly et al., 2013). To the best of our knowledge, a direct or indirect effect of *LEC2* overexpression on lipid catabolism has not been reported. Recently, Kim et al. (2015) undertook an extensive gene expression study in *A. thaliana* using an alternative senescence-specific promoter to drive *LEC2* overexpression. Genes exhibiting upregulated expression included oleosin, various acyltransferases and several embryogenic transcription factors. However, the authors did not report on possible transcriptional changes of genes involved in β-oxidation and the glyoxylate cycle. One possible explanation for the observed reduction in lipid turnover in the *LEC2 N. tabacum* leaves could be the upregulation of endogenous oleosin-encoding transcripts which further stabilize cytosolic lipid droplets (Braybrook et al., 2006; Kim et al., 2015). Alternatively, *LEC2* overexpression could directly or indirectly down-regulate expression of *SDP1* or other downstream genes involved in lipid degradation. Further gene expression analyses and *in vitro* oil droplet stability assays are required to confirm these hypotheses. The characteristic increase in linoleic acid levels in *LEC2* overexpressing leaves is in agreement with previous *A. thaliana* results and might be explained by increased expression of the FAD2 fatty acid desaturase (Slocombe et al., 2009). Contrary to previous reports (Kim et al., 2015;

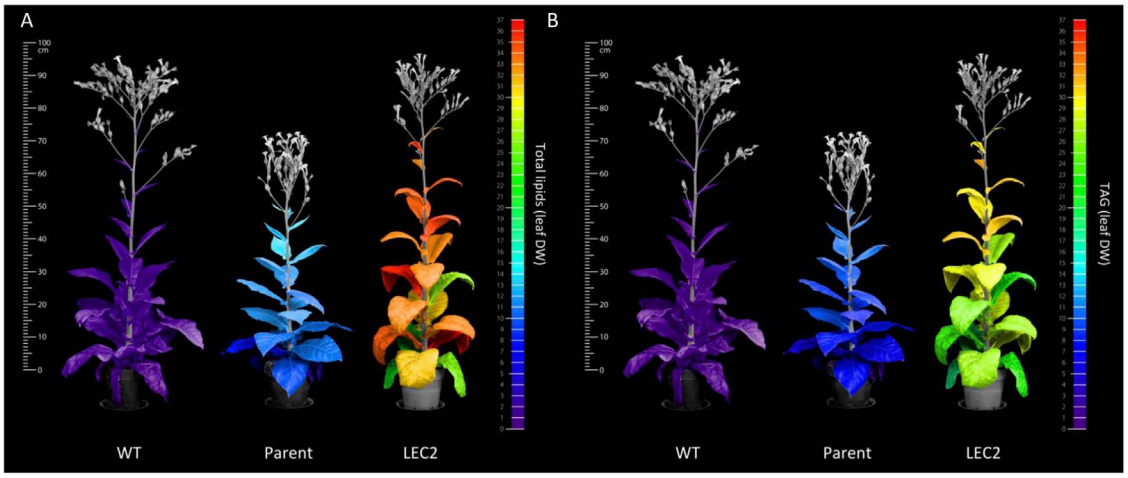

Fig. 6. Total lipid (A) and TAG (B) levels in individual leaves of a wildtype and transgenic *N. tabacum* plants (T_2) at seed setting. Total lipid and TAG contents, as determined by TLC-GC, are represented by the colour scale on the right. Wt, wildtype *N. tabacum*; Parent, high oil *N. tabacum* line expressing three transgenes involved in lipid biosynthesis (*WRI1*, *DGAT1*, *OLEOSIN*); LEC2, overexpression of the *A. thaliana LEC2* gene in the high oil background. Plants were grown in a growth cabinet (16 h/8 h; 650 µmol m^{-2} s^{-1}) (1 m scale bar).

Santos Mendoza et al., 2005; Slocombe et al., 2009), we only detected trace amounts of very long chain fatty acid species (VLCFA; ≥C20). In the *LEC2* overexpressing *N. tabacum* plants, a highly efficient incorporation of acyl chains into TAG might limit the time they reside in the cytosolic acyl-CoA pool where fatty acid elongation to VLCFA occurs.

In developing *A. thaliana* leaves, PDAT1 has been shown to be involved in TAG synthesis (Fan et al., 2013b). Active LPCAT-mediated acyl editing via the Lands Cycle has been demonstrated in *A. thaliana* and pea leaves (Bates et al., 2007; Wang et al., 2012). Recent *in vitro* labelling experiments have indicated the existence of multiple subcellular metabolic DAG pools in *A. thaliana* leaf tissue (Tjellstrom et al., 2015). Taken together, these findings support a leaf metabolic model in which *de novo* synthesized fatty acids are shunted into PC via acyl editing before incorporation into TAG, a situation very much resembling oilseed tissues and possible occurring via upregulation of phospholipases D.

Oilseed tissues typically accumulate TAG as discrete oil bodies with diameters ranging between 0.5 and 3 µm (Jolivet et al., 2004; Tzen et al., 1993). Their small and uniform size has been attributed to the presence of oil body proteins such as oleosin that coat the surface of the lipid droplets and prevent aggregation during seed desiccation (Shimada et al., 2008; Siloto et al., 2006; Wu et al., 2010). The relatively large size (10–15 µm) of the lipid droplets present within the transgenic *SDP1* and *LEC2 N. tabacum* leaf mesophyll cells is reminiscent of fruit tissues that accumulate oil in the absence of typical seed-specific oleosins (Chapman et al., 2012). Irregularly-shaped lipid droplets have also been reported in oat endosperm while *A. thaliana tgd5sdp1* seedling tissues accumulate droplets that measure up to 10 µm (Banas et al., 2007; Fan et al., 2015). In transgenic *N. tabacum* leaves, the occurrence of large lipid droplets could be caused by several

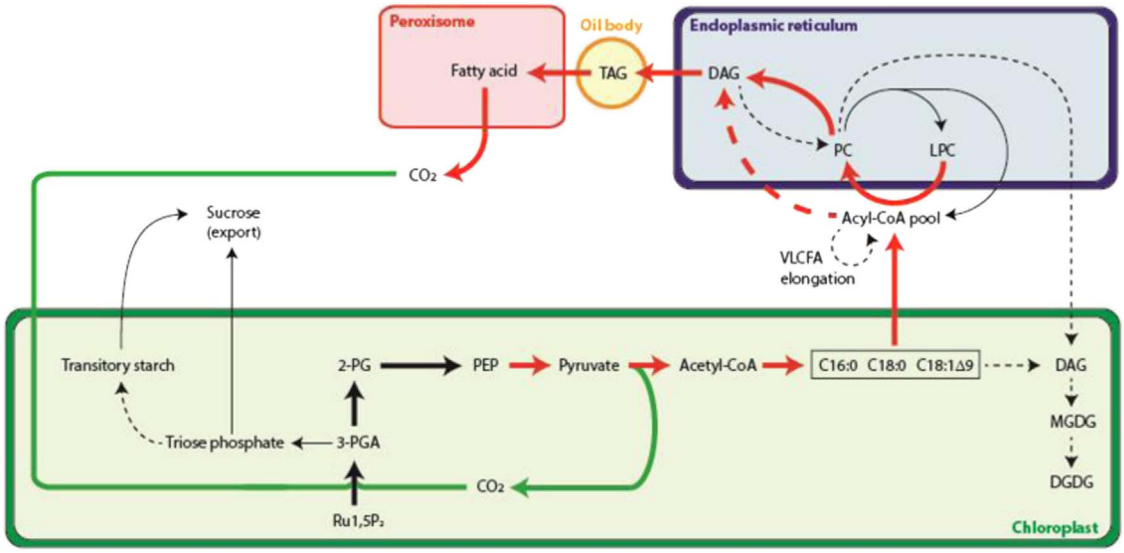

Fig. 7. Schematic representation of carbon and lipid fluxes in oil accumulating transgenic *N. tabacum* leaf tissue. Photosynthate in wildtype leaves is converted to sucrose for export to sink tissues while excess is stored as transitory starch to sustain growth and metabolism during the night. In *N. tabacum* leaves overexpressing *WRI1*, *DGAT1* and *OLEOSIN*, the central carbon flux is redirected into *de novo* fatty acid biosynthesis (thick black arrows) at the expense of transitory starch. Acyl chains are subsequently exported into the cytosol, followed by incorporation into PC via the Lands Cycle. PC serves as a substrate for DAG and TAG biosynthesis in the ER membrane while TAG accumulates as oil bodies in the cytosol. Alternatively, glycerol-3-phosphate can be sequentially acylated to TAG via the Kennedy pathway. Free fatty acids released by SDP1-mediated TAG hydrolysis enter the peroxisomes where they are subjected to stepwise chain shortening during β-oxidation. Acetyl-CoA is likely oxidized to CO_2 in the TCA cycle, completing the futile cycle (red arrows). Possible recycling of carbon released as CO_2 during fatty acid biosynthesis and respiration via a RUBISCO shunt pathway is indicated by green arrows. *N. tabacum SDP1* and *LEC2* transgenic leaves exhibit reduced lipid turnover, resulting in increased net TAG accumulation. Pathways competing for carbon and acyl chains are indicated by dashed black arrows. Ru1,5P_2, Ribulose 1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; DAG, diacylglycerol; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; LPC, pysophosphatidylcholine; TAG, triacylglycerol; VLCFA, very long chain fatty acid (≥C20).

factors. Firstly, an insufficient supply of ectopically expressed oleosin to match increased rates of fatty acid synthesis and TAG assembly might trigger the fusion of pre-existing lipid droplets. Secondly, changes in intracellular PC levels could have implications for the composition of the lipid droplet phospholipid monolayer. In *Drosophila melanogaster* S2 cells, PC is known to prevent fusion of lipid droplets while compromised PC biosynthesis results in increased lipid droplet size (Guo et al., 2008; Krahmer et al., 2011). While similar observations have yet to be made in plant cells, insufficient supply of PC in transgenic *N. tabacum* mesophyll cells might provide one plausible explanation for the occurrence of giant lipid droplets. Finally, active TAG biosynthesis might continue on the surface of small lipid droplets allowing for their continuous expansion within the cytosol, as observed in *D. melanogaster* and mammalian cells (Kuerschner et al., 2008; Wilfling et al., 2013). The exact mechanism of lipid droplet biogenesis and growth in transgenic oil accumulating *N. tabacum* leaf tissue, however, needs further detailed microscopy and biochemical investigation.

The synthesis and accumulation of high levels of storage lipids in non-seed tissues has important biotechnological applications. Using a combination of iterative gene stacking or silencing, we show that the central carbon flux in *N. tabacum* leaf tissues can be redirected from starch into oil, yielding more than 30% TAG on a DW basis. We estimate that in transgenic *SDP1* and *LEC2 N. tabacum* leaves TAG represents between 80% and 92% of the total carbon that is sequestered in starch, soluble sugars and TAG (Supplementary Table 8). This is in stark contrast with wildtype leaf tissue where more than 99% of the total carbon in starch, soluble sugars and TAG is present as starch. Due to the higher energy density of TAG compared to starch and soluble sugars, the calculated energy content of wildtype and transgenic *N. tabacum* leaf tissues also differs considerably. In wildtype *N. tabacum* leaves the energy contribution of TAG is estimated to be around 0.05 kJ (g^{-1} leaf DW). This value is increased more than 200-fold (13.2 kJ/g DW) in transgenic *SDP1* or *LEC2 N. tabacum* leaves. Although a significant amount of carbon appears to be redirected from transitory starch into storage lipids, other macromolecules such as cellulose, proteins and cell wall polymers could also be negatively impacted. Consequently, the need for additional carbon due to the increased TAG as a new sink is possibly met by a variety of carbon metabolic pools, as suggested by the observed trade-off in biomass. The implications of engineering an entirely new carbon sink in green tissues on photosynthetic efficiency, net carbon assimilation rates and carbon reallocation in wildtype and the different transgenic *N. tabacum* lines form the subject of current ongoing studies.

The accumulation of valuable storage lipids such as TAG in the entire biomass has the potential to deliver unprecedented yields. Assuming 20 ton dry biomass per hectare (Andrianov et al., 2010), a leaf-to-stem biomass ratio of 1.3 and a 35% observed biomass penalty (Supplementary Fig. 5), we estimate that the current transgenic *N. tabacum LEC2* plants have the potential to generate up to 2.4 metric ton oil per hectare, a yield that is more than two-fold higher than canola. The current step-change in absolute TAG levels is likely to be sufficient for biomass oil to become a novel high yielding and cost-effective production platform. Minimizing the effect on overall plant growth and biomass in the field will likely require fine-tuning of transgene expression using tissue- and developmental-specific promoters similar to the SAG12 promoter used in this study. Finally, deployment into high biomass crop species or a combination of the current high oil trait with metabolic engineering strategies aimed at increasing biomass, growth vigour, delaying senescence and improving photosynthetic capacity should allow for exploiting the full potential of the proposed biomass oil technology (Li et al., 2015; Roy Choudhury et al., 2014; Zhang et al., 2010).

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2016.12.007.

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