Synergistic effect of WRI1 and DGAT1 coexpression on triacylglycerol biosynthesis in plants

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ABSTRACT

Metabolic engineering approaches to increase plant oil levels can generally be divided into categories which increase fatty acid biosynthesis (‘Push’), are involved in TAG assembly (‘Pull’) or increase TAG storage/decrease breakdown (‘Accumulation’). In this study, we describe the surprising synergy when Push (WRI1) and Pull (DGAT1) approaches are combined. Co-expression of these genes in the Nicotiana benthamiana transient leaf expression system resulted in TAG levels exceeding those expected from an additive effect and biochemical tracer studies confirmed increased flux of carbon through fatty acid and TAG synthesis pathways. Leaf fatty acid profile also synergistically shifts from polyunsaturated to monounsaturated fatty acids.

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1. Introduction

Biosynthesis of triacylglycerols (TAG) mainly occurs via the Kennedy or glycerol-3-phosphate (G3P) pathway in which three acyl chains are esterified to a glycerol backbone [1]. The first acylation step is catalyzed by the glycerol-3-phosphate acyltransferase (GPAT) using acyl-CoA esters as acyl-donor and G3P as acyl-acceptor. Lyso phosphatidic acid is subsequently acylated by the lysophosphatidic acid acyltransferase (LPAAT). The product, phosphatic acid, is further dephosphorylated to diacylglycerol (DAG) by the phosphatidic acid phosphatase (PAP). Finally, diacylglycerol acyltransferase (DGAT) esterifies a third acyl chain to DAG to form TAG. Since DAG can also serve as a precursor for membrane phospholipids, the reaction catalysed by DGAT is the only step within the Kennedy pathway that is solely committed to the biosynthesis of storage lipids. A second enzymatic reaction catalyzed by the phosphatidyl glycerol acyltransferase (PDAT) also results in the conversion of DAG to TAG but uses phospholipids instead of acyl-CoA as acyl-donor. A number of recent studies in different plants, however, have uncovered an intricate flux network between neutral lipid intermediates of the Kennedy pathway and membrane phospholipids thus complicating metabolic engineering efforts for increased storage lipid accumulation [2–4].

Current metabolic engineering strategies for increasing plant lipids can be broadly categorized as either upregulation of fatty acid biosynthesis (a ‘Push’ approach), increasing TAG assembly (a ‘Pull’ approach) and optimizing TAG storage/preventing TAG breakdown (the ‘Accumulation’ approach). Examples of these approaches include the overexpression of DGAT1 in leaf [5,8] and seed tissue [7,8], use of monoacylglycerol as a substrate for TAG biosynthesis in Nicotiana benthamiana leaves by heterologous expression of a mammalian MGAT acyltransferase [5], overexpression of acetyl-CoA carboxylase (ACCase) in potato tubers and tobacco chloroplasts [10,11], blocking lipid breakdown by targeting TAG lipases and β-oxidation [12,13], mutation of the TGD1 chloroplast lipid transporter [14], heterologous expression of transcription factors (LEC1, LEC2, WRI1) that drive seed development and maturation [15–21] as well as diverting the flow of carbon from starch biosynthesis to lipids by silencing of the ADP-glucose-pyrophosphorylase (AGPase) [17].

Top-down metabolic control analyses have revealed that both the Push and Pull side can control the overall flux of carbon into lipids with the relative importance of each depending on the oilseed species and tissue [7,22,23].

Few reports describe combinations of various oil increase technologies, especially those comprising the simultaneous optimization of both Push and Pull aspects of TAG metabolism. One
notable exception is a study which reports the overexpression of WRI1 and silencing of AGPase in Arabidopsis thaliana [17]. It is likely that TAG increases significantly greater than those achieved in the above studies will require a metabolic engineering approach which simultaneously addresses limitations on both push and pull sides of storage oil biosynthesis. In this study we use the transient N. benthamiana leaf assay system to show that combining Push and Pull approaches can yield surprisingly synergistic relationships and result in a larger than expected increase in TAG levels in plant tissues. We also describe the large shifts in fatty acid profile that occur under such a synergistic relationship.

2. Materials and methods

2.1. Genes and expression vectors

The construction of a binary vector containing the A. thaliana DGAT1 gene expressed by the constitutive CaMV-35S promoter with duplication enhancer region is described elsewhere in detail [24,25]. A second binary vector containing the A. thaliana WRI1 gene expressed by the CaMV-35S promoter was constructed by cloning a codon optimised version of the gene as an EcoRI fragment into the EcoRI site of a binary vector already containing a 35S promoter with duplicated enhancer region and Agrobacterium tumefaciens NOS terminator. Both constructs were transformed in A. tumefaciens AGL1. A chimeric construct containing the tomato bushy stunt virus p19 viral silencing suppressor gene under the control of the 35S promoter and transformed into A. tumefaciens GV3101 was obtained from Dr. Peter Waterhouse (CSIRO Plant Industry).

2.2. Transient expression in N. benthamiana

Transient expression in N. benthamiana leaves was performed as described [24,25] with some minor modifications. A. tumefaciens cultures containing the gene coding for the p19 viral suppressor protein and the chimeric gene(s) of interest were mixed such that the final OD900 of each culture was equal to 0.125 prior to infiltration. For lipid analyses, a total of 18 leaves from 6 plants were infiltrated with the different gene combinations. Samples being compared were randomly located on the same leaf. After infiltration, N. benthamiana plants were grown for a further five days before leaf discs were harvested, pooled across the three leaves from the same plant, freeze-dried, weighed and stored at −80°C.

2.3. 14C acetate radiolabelling

A radiolabel biochemical tracer study was performed with two leaves infiltrated on the same plant. Three leaf discs of each infiltration were pooled randomly across the two leaves for each feeding time point. The assay was performed using [14C] acetate (56 mCi/mmol from American Radiolabeled Chemicals) as a substrate at a concentration of 0.4 mM in potassium phosphate buffer (0.1 M pH 7.2). After incubation, total lipids were extracted from each time point as described by [9]. Lipid samples were loaded on a thin layer chromatography (TLC) plate (20 × 20 cm, Silica gel 60, Merck) and developed in two-step solvent system. Samples were first run to 12 cm in chloroform:methanol:acetic acid:water (68:22:6:4 v/v/v/v), followed by a second separation in hexane:diethyl ether:acetic acid (70:30:1 v/v/v). The TLC plate was exposed to phosphor imaging screens overnight and analysed by a Fujifilm FLA-5000 phosphorimager. Radiolabelled lipid spots were measured using a Beckman-Coulter Ready Safe liquid scintillation cocktail and Beckman-Coulter LS 6500 Multipurpose Scintillation Counter.

2.4. Neutral and polar lipid quantification and fatty acid profiling by TLC and gas chromatography (GC)

Total lipids were extracted from leaf tissues using chloroform:methanol:0.1 M KCl (2:1:1 v/v/v). Freeze dried leaf tissues were first homogenized in chloroform:methanol in a microcentrifuge tube containing a metallic ball using Reicht tissue lyser (Qiagen) for 3 min at 20 frequency/s. After mixing homogenate at 2000 rpm (Vibramax 10, Heidolph) for 10 min, KCl was added and mixed for a further 5 min. Finally, the mixture was centrifuged for 5 min at 10 000×g and the lower lipid phase collected. The remaining phase was washed once with CHCl3 and lipid phase pooled to the earlier extract. Solvent of lipid phase was evaporated completely using N2 flow and a known volume of CHCl3 was added for per mg leaf dry weight.

TAG and polar lipids were fractionated by TLC (Silica gel 60, MERCK) in hexane:diethyl ether:acetic acid (70:30:1 v/v/v) and visualized by spraying Primuline (Sigma, 5 mg/100 ml acetone:water (80:20 v/v)) and exposing plate under UV. Fatty acid methyl esters (FAME) of TAG and PL (polar lipids) were produced by incubating corresponding bands in 1 N methanolic–HCl (Supeco, Bellefonte, PA) at 80°C for 2 h together with known amount of heptadecanoic acid (Nu-Chek PREP, Inc. USA) as internal standard to quantify TAG. To quantify total fatty acids in total lipids, known amount of heptadecanoic acid (Nu-Chek PREP, Inc. USA) was used as internal standard. FAME were analyzed by GC-FID (7890A GC, Agilent Technologies, Palo Alto, CA) equipped with a 30 m BPX70 column (0.25 mm inner diameter, 0.25 mm film thickness, SGE, Austin, Tx) as described previously [9]. Peaks were integrated with Agilent Technologies ChemStation software (Rev B.04.03 (16)).

TAG content in leaf was calculated as the sum of glycerol and fatty acyl moieties using the relation: % TAG by weight = 100 × [(total mol FAME/3) + (total g FAME-(15 × total mol FAME))]/g leaf dry weight, where 41 and 15 are molecular weights of glycerol moiety and methyl group, respectively.

2.5. Statistical analysis

TAG levels were transformed using the cube-root equation \( X = Y^{1/3} \) to achieve approximate variance homogeneity on the transformed scale. The transformed data were analyzed as a randomize complete block design using the R statistical software (version 2.15.1). To investigate the significance of a synergistic effect on the original scale of the data, a 95% confidence interval for the interaction contrast (representing the size of the synergistic effect) was obtained on the original scale using a Bayesian bootstrap approach [26]. This interval (1.302–1.617) did not include zero, confirming a statistically significant synergistic effect on TAG levels at the 5% level of significance.

In the case of 18:1 TAG levels, data were first transformed (log10) to achieve approximate variance homogeneity. The transformed values were then analyzed and a 95% confidence interval for the synergistic effect was derived as described for the TAG quantification data. Similarly, this interval (8.336–14.197) did not include zero, confirming a significant synergistic effect on 18:1 TAG levels at the 5% level of significance.

3. Results

3.1. Synergistic effect of WRI and DGAT1 expression in N. benthamiana

During seed development, expression of several enzymes involved in the glycolysis and de novo fatty acid biosynthesis pathways is controlled by the WRI1 transcription factor [27,28]. We
therefore first tested the effect of expression of the *A. thaliana* WRI1 transcription factor on TAG levels and fatty acid profiles in *N. benthamiana* leaves. Under these assay conditions TAG levels were found to be increased 22-fold compared to the p19 negative control and corresponded to 0.5% on a dry weight basis (Fig. 1). Next we compared this with the effect of expressing the *A. thaliana* DGAT1 gene which had previously been shown to increase TAG levels in *N. benthamiana* leaf tissue [9]. Expression of this acyltransferase was found to yield similar TAG levels (0.45%, Fig. 1). When both the *A. thaliana* WRI1 and DGAT1 genes were co-infiltrated, a significant synergistic effect on leaf oil accumulation was observed with TAG levels increasing almost 100-fold and making up 2.48% of the leaf dry weight (Fig. 1). A mere additive rather than synergistic effect between the two genes would account for only 1.02% on a dry weight basis (Fig. 1). As a result, 59% of the total TAG yield obtained upon the coexpression of the WRI1 and DGAT1 genes can be attributed to a synergistic effect between the two genes. Interestingly, no significant differences were observed when polar lipids were quantified.

### 3.2. Changes to fatty acid profile

The fatty acid profile of the TAG fraction of each infiltration was determined by GC (Table 1). Analysis of the TAG profile revealed a similar significant synergistic effect on 18:1(^18) levels. This increase came mostly at the expense of 18:3(^12,14,16) levels. Expression of WRI1 seemed to result in decreased levels of 18:0 while expression of DGAT1 positively affected 18:2(^18) levels. Similar although less outspoken changes in both 18:1(^9) and 18:2(^11,13,15) were also detected in the polar lipid fraction (Table 1).

### 3.3. $[^{14}C]$ acetate labelling

In order to investigate the biochemical nature of the TAG accumulation response further we performed labelling studies with $[^{14}C]$ acetate substrate [2]. In particular, we wanted to determine the likely involvement of PC in TAG synthesis as a series of papers by Bates et al. [2–4,29] have shown that, depending on the plant species, DAG is formed either de novo from PA or derived from PC. However, no such biochemical data is available for the *N. benthamiana* model system. As shown in Fig. 2 and Supplementary Figure the label was readily incorporated into newly synthesised fatty acids within 10 min after feeding. The flux of radiolabelled carbon was observed to increase through both fatty acid synthesis and TAG assembly pathways in both the WRI1 and DGAT1 samples. Incorporation of the label into TAG in the leaf co-expressing WRI1 and DGAT1 was greatest (42 pmol) with less label incorporated in either the WRI1 (7 pmol) or DGAT1 (24 pmol) samples (Fig. 2). Conversely, incorporation of the label into phosphatidylcholine (PC) in DGAT1 infiltrated leaves increased sevenfold (16.5 pmol of PC) when compared to the control p19 sample (2.4 pmol). Unlike the TAG response, accumulation of the label in PC upon the co-expression of WRI1 and DGAT1 was only slightly higher compared to DGAT1 alone (Fig. 2). Interestingly, no further increase of the label in DAG was observed when both wri1 and dgat1 genes were co-expressed. In plants, DAG forms a minor and transient neutral lipid class while a possible signalling role has also been proposed [30,31]. As a result, accumulation of this neutral lipid might be tightly regulated and rapid conversion to PC and TAG might be two mechanisms to achieve this.

### 4. Discussion

We selected WRI1 and DGAT1 as representative Push and Pull genes. The effects of these genes on lipid synthesis in seed and vegetative tissues have been well documented. *A. thaliana* DGAT1 mutants accumulate less oil in leaves [12] and seeds [32]. Overexpression of DGAT1 increased both TAG and total fatty acid levels in tobacco leaves [5,6] and seed oil content in *A. thaliana* [7,8]. Similarly, constitutive overexpression of the WRI1 gene in *A. thaliana* and maize yielded higher TAG levels in vegetative tissues [17] and seed [19–21] while an *A. thaliana* wri1 knockout mutant exhibited reduced seed oil content [28,33]. In addition, this transcription factor has been suggested to play a major role in the accumulation of high levels of storage lipids in the oil palm mesocarp [34]. Unlike other master transcription factors (LEC1, LEC2) that regulate the synthesis of seed storage compounds, ectopic overexpression of WRI1 is not associated with negative phenotypic effects such as impaired growth, reduced germination, somatic embryogenesis or other abnormal morphologies [15,20,35,36]. Hence WRI1 has become a target of significant interest to increase oil content in both seeds and vegetative tissues.

Despite the central roles of WRI1 and DGAT1 in lipid biosynthesis the authors are not aware of any studies reporting the co-expression of both genes. We observed a significant synergistic effect on TAG accumulation when both DGAT1 and WRI1 were co-infiltrated in *N. benthamiana* leaves. This is likely due to the combined upregulation of both the late glycolysis and fatty acid biosynthesis pathways by WRI1 overexpression (Push) and an increased demand for the resulting acyl and G3P building blocks for TAG biosynthesis by DGAT1 overexpression (Pull). Co-expression of WRI1 and DGAT1 also resulted in a significant shift in fatty acid profile with 18:1(^9) increasing markedly at the expense of polyunsaturated fatty acids. A similar (but less outspoken) change in the fatty acid profile has been observed in tobacco leaves expressing the *A. thaliana* DGAT1 gene [6] and seed fatty acid profiles of *A. thaliana* wri1 mutants are typically enriched in the polyunsaturated 18:3(^9,12,15) whilst 18:1(^9) levels are reduced [28,33]. We observed an increase in radiolabel accumulation in PC under all treatments including DGAT1 [6]. Interestingly, the polar lipid level did not significantly change under any treatment in the transient expression system. It will be interesting to observe this further in stable transformants.

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**Fig. 1.** Polar lipid and TAG levels in transiently-transformed *N. benthamiana* leaf tissue as a percentage of dry tissue weight. All genes were constitutively expressed by the CaMV 35S promoter with gene identities being tomato bushy stunt virus p19 viral silencing suppressor gene (negative control, common to all samples), *A. thaliana* WRI1, *A. thaliana* DGAT1 and a combination (Comb.) of *A. thaliana* WRI1 and DGAT1. Error bars denote standard deviation with n = 6 for all samples.
More work is required to understand the synergistic relation-
ship between WRI1 and DGAT1. It will be interesting to determine
how feedback inhibition of the plastidial ACCase [37] is being af-
fected by this gene combination and whether the route taken by
18:1 D9-CoA exported from the plastid to TAG is altered under these
high fatty acid and TAG synthesis conditions. Once exported from
the plastids, the majority of acyl chains are usually incorporated
into membrane phospholipids of the endoplasmic reticulum mem-
brane first where they can undergo further desaturation prior to
entering or re-entering the Kennedy pathway [2,3]. Upregulation
of both fatty acid and TAG biosynthesis pathways may result in
more 18:1 D9-CoA entering the Kennedy pathway directly and, as
a result, being largely inaccessible for further modification such
as desaturation by the endogenous FAD2/FAD3 Δ12- and Δ15-
desaturases. Alternatively, there could be limitations on desatura-
tion activity under these conditions. The combined expression of
WRI1 and DGAT1 might also lead to changes in other enzymatic
activities involved in acyl transfer between different lipid pools
which influence the availability of acyl substrates to PC-type desat-
urases: examples of this could include upregulation of phospholi-
pase D which releases DAG from PC, downregulation of PDCT
which converts DAG back to PC or reduced LPCAT activity involved
in acyl editing.

Table 1
Fatty acid profile of both the TAG and polar lipid fractions of leaf discs expressing the tomato bushy stunt virus p19 viral silencing suppressor gene (negative control, common to all samples), A. thaliana WRI1, A. thaliana DGAT1 and a combination (Comb.) of A. thaliana WRI1 and DGAT1. Errors denote standard deviation with n = 6 for all samples.

|          | TAG           | Polar          |
|----------|---------------|----------------|
|          | p19 WRI1 DGAT1 Comb. | p19 WRI1 DGAT1 Comb. |
| 14:0     | 0.8 ± 0.3 0.1 ± 0.0 0.1 ± 0.0 0.1 ± 0.0 | – – – – |
| 16:0     | 30.4 ± 1.7 33.9 ± 1.1 26.4 ± 1.3 27.8 ± 1.7 | 13.6 ± 0.3 17.3 ± 0.8 13.6 ± 0.3 16.4 ± 0.4 |
| 16:1Δ9   | – – – – | 3.1 ± 0.2 1.6 ± 0.2 3.1 ± 0.1 2.0 ± 0.1 |
| 16:1Δ11  | 0.4 ± 0.1 0.9 ± 0.1 0.1 ± 0.0 0.3 ± 0.0 | 0.2 ± 0.0 0.3 ± 0.0 0.1 ± 0.1 0.2 ± 0.0 |
| 16:3Δ7,10,13 | 1.1 ± 0.5 0.5 ± 0.0 0.5 ± 0.1 0.2 ± 0.0 | 8.9 ± 0.3 7.6 ± 0.3 8.8 ± 0.3 7.6 ± 0.1 |
| 18:0     | 10.4 ± 1.5 3.8 ± 0.2 9.7 ± 0.9 5.7 ± 0.1 | 2.4 ± 0.1 2.1 ± 0.1 2.3 ± 0.1 1.9 ± 0.0 |
| 18:1Δ9   | 3.9 ± 0.5 6.8 ± 1.1 6.3 ± 14 21.1 ± 2.5 | 1.9 ± 0.2 2.7 ± 0.4 2.8 ± 0.4 7.0 ± 0.8 |
| 18:1Δ11  | 0.3 ± 0.0 0.7 ± 0.1 0.2 ± 0.1 0.7 ± 0.0 | 0.3 ± 0.0 0.6 ± 0.1 0.4 ± 0.0 0.5 ± 0.0 |
| 18:2Δ9,12 | 20.4 ± 1.1 18.5 ± 0.7 27.1 ± 0.5 25.9 ± 0.6 | 11.5 ± 0.5 11.9 ± 0.5 14.0 ± 0.4 14.3 ± 0.5 |
| 18:3Δ9,12,15 | 29.5 ± 4.0 33.3 ± 1.4 21.7 ± 2.2 13.8 ± 0.3 | 59.7 ± 0.7 55.2 ± 1.4 53.8 ± 0.9 48.9 ± 0.7 |
| 20:0     | 1.4 ± 0.1 0.8 ± 0.1 3.7 ± 0.4 2.0 ± 0.2 | 0.3 ± 0.0 0.3 ± 0.0 0.3 ± 0.0 0.3 ± 0.0 |
| 20:1Δ11  | – 0.1 ± 0.0 0.3 ± 0.0 0.3 ± 0.0 | – – – – |
| 20:2Δ11,14 | – 0.1 ± 0.0 0.2 ± 0.0 0.1 ± 0.0 | – – – – |
| 20:3Δ11,14,17 | – 0.1 ± 0.0 0.1 ± 0.0 – | 0.1 ± 0.0 0.1 ± 0.0 0.1 ± 0.0 0.1 ± 0.0 |
| 22:0     | 0.6 ± 0.4 0.3 ± 0.0 2.1 ± 0.3 1.3 ± 0.1 | 0.2 ± 0.0 0.2 ± 0.0 0.2 ± 0.0 0.3 ± 0.0 |
| 24:0     | 0.8 ± 0.1 0.2 ± 0.0 1.5 ± 0.3 0.8 ± 0.1 | 0.2 ± 0.0 0.3 ± 0.0 0.3 ± 0.0 0.3 ± 0.0 |

Fig. 2. Amount of radiolabelled product in pmol (LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine) appearing 10 min after [14C] acetate feeding to leaf discs expressing the tomato bushy stunt virus p19 viral silencing suppressor gene (negative control, common to all samples), A. thaliana WRI1, A. thaliana DGAT1 and a combination (Comb.) of A. thaliana WRI1 and DGAT1. Error bars denote standard deviation with n = 3 for all samples.
practical difficulties associated with generating numerous transgenic events containing gene combinations. Assessing the combined effect of multiple genes on TAG accumulation requires a fast screening platform as a first selection tier that can replace the necessity of generating a large number of stable transformants. Recently, Tjellstrom et al. [38] proposed that A. thaliana cell suspension cultures as a model system for studying and engineering lipid synthesis in vegetative tissues. However, authors noted significant differences in lipid and fatty acid compositions between cell cultures and A. thaliana leaf tissue especially regarding TAG levels and fatty acid composition. We opted for the N. benthamiana transient leaf expression system as a rapid combinatorial gene screening platform due to the ease of the assay and the close relationship we have previously observed between transient and stable expression results [9,24,39–41].

In conclusion, we have described the significant synergistic effect on TAG biosynthesis and accumulation, as well as on 18:1Δ9 accumulation, when WRI1 and DGAT1 are co-expressed in plant tissue. This illustrates the great potential of simultaneously upregulating both fatty acid and TAG biosynthesis pathways. Further work is required to determine the extent of this synergy in plants stably-transformed with constitutive, tissue-specific and inducible expression systems. The authors expect that the longer expression window associated with stable transformation will result in TAG accumulation levels that significantly exceed currently-described levels and that these could be further enhanced by the addition or downregulation of other genes including oil body proteins (e.g. oleosin) and TAG lipases.

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Appendix A. Supplementary data

Supplementary data (TLC plate showing radiolabelled products described in Fig. 2; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; MAG, monoacylglycerol; FFA, free fatty acids) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febio.2012.01.018.

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