Journal of Cereal Science

Accumulation and deposition of triacylglycerols in the starchy endosperm of wheat grain

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	wheat; starchy endosperm; triacylglycerol; oleosin
Corresponding Author:	Peter Shewry Rothamsted Research Herts, United Kingdom
First Author:	Irene Gonzalez-Thuillier
Order of Authors:	Irene Gonzalez-Thuillier
	Till K. Pellny
	Paola Tosi
	Rowan A. C. Mitchell
	Richard Haslam
	Peter Shewry
Abstract:	A combination of lipidomics, transctiptomics and bioimaging has been used to study triacylglycerol synthesis and deposition in the developing starchy endosperm of wheat. The content of TAG increased between 14 and 34 days after anthesis, from 50 to 115 mg/g dry wt and from about 35 to 175 mg/g dry wt in two experiments. The major fatty acids were C16 (palmitic C 16:0 and palmitoleic C16:1) and C18 (stearic C18:0, oleic C18:1, linoleic C18:2 and linolenic C18:3), with unsaturated fatty acids accounting for about 75-80% of the total throughout development. Linoleic acid (C18:2) was the major component at all stages and the proportion increased during development. Transcript profiling indicated that predominant route to TAG synthesis and oil accumulation is via the Kennedy pathway and diacylglycerol acyltransferase (DGAT) activity. Confocal microscopy of stained tissue sections showed that TAG accumulated in droplets concentrated in the cells below the sub-aleurone cells which are associated with protein. Transcripts encoding 16kd oleosins were also expressed, indicating that the oil droplets are stabilised by oleosin proteins.
Suggested Reviewers:	Sean Finney sfinney@bsm.com Expert on wheat lipids
	Bran Pareyt Puratos BPareyt@puratos.com Expert on wheat lipids
	Christophe Courtin KUL, Leuven christophe.courtin@kuleuven.be Expert on wheat chemistry

Dear Colleagues,

I am pleased to submit a manuscript entitled “Accumulation and deposition of triacylglycerols in the starchy endosperm of wheat grain”. This provides the first detailed integrated study of the synthesis and deposition of triacylglycerols (storage lipids) in the developing starchy endosperm of wheat. It therefore has significance for the quality and utilisation of white flour and should be on interest to the wider readership of JCS.

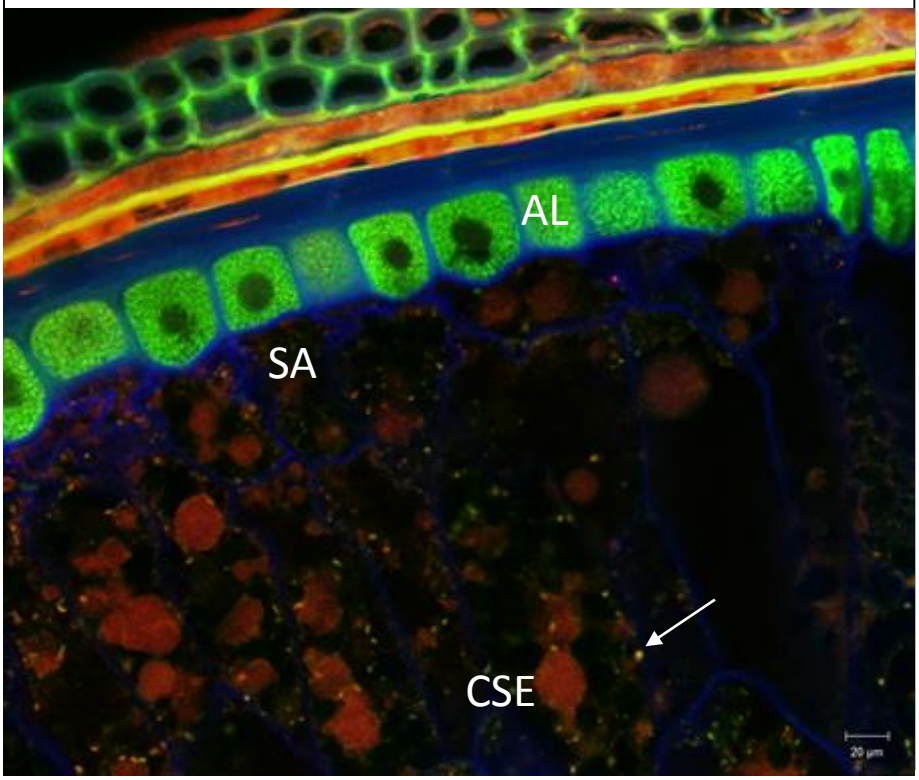
Thank you,

Peter Shewry.

Highlights

- Integrated study of triacylglycerol synthesis and deposition in wheat endosperm
- First lipidomic analysis of triacylglycerol composition in wheat starchy endosperm
- Bioimaging of lipid deposition in starchy endosperm cells
- Transcriptome analysis shows operation of Kennedy pathway for lipid synthesis

Lipids (tricylglycerols) are deposited in discrete bodies in the developing starchy endosperm cells of bread wheat



Accumulation and deposition of triacylglycerols in the starchy endosperm of wheat grain

Irene Gonzalez-Thuillier[a,1], Till K. Pellny[a], Paola Tosi[b], Rowan A. C. Mitchell[a], Richard Haslam[a*] and Peter R. Shewry[a*]

[a]Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK.

[b]School of Agriculture, Policy and Development, University of Reading, Whiteknights Campus, Early gate RG6 6AR, Reading UK

[1]Current address: Biosciences, Jeallots Hill Research Station, Syngenta, Warfield, Bracknell, RG42 6EY, UK

*Corresponding authors

Peter Shewry peter.shewry@rothamsted.ac.uk

Richard Haslam richard.haslam@rothamsted.ac.uk

Abstract

A combination of lipidomics, transctiptomics and bioimaging has been used to study triacylglycerol synthesis and deposition in the developing starchy endosperm of wheat. The content of TAG increased between 14 and 34 days after anthesis, from 50 to 115 mg/g dry wt and from about 35 to 175 mg/g dry wt in two experiments. The major fatty acids were C16 (palmitic C 16:0 and palmitoleic C16:1) and C18 (stearic C18:0, oleic C18:1, linoleic C18:2 and linolenic C18:3), with unsaturated fatty acids accounting for about 75-80% of the total throughout development. Linoleic acid (C18:2) was the major component at all stages and the proportion increased during development. Transcript profiling indicated that predominant route to TAG synthesis and oil accumulation is via the Kennedy pathway and diacylglycerol acyltransferase (DGAT) activity. Confocal microscopy of stained tissue sections showed that TAG accumulated in droplets concentrated in the cells below the sub-aleurone cells which are associated with protein. Transcripts encoding 16kd oleosins were also expressed, indicating that the oil droplets are stabilised by oleosin proteins.

Key words: wheat, starchy endosperm, white flour, oil, triacylglycerol, oleosin

1. Introduction

Wheat is the dominant crop and major staple food in Europe, North Africa, West and Central Asia and North and South America, where it contributes between 20% and 50% of the total calories in the human diet. Furthermore, the consumption of wheat is also increasing in countries where it is not readily grown, particularly Sub-Saharan Africa. The global success of wheat is due to its wide adaptability and to the grain processing properties, in particular the ability of wheat flour to be processed into bread, other baked products, pasta and noodles.

The processing properties of wheat are largely determined by the gluten proteins which interact to form a continuous viscoelastic network in dough: this provides the cohesion required for making pasta and noodles, and enables the entrapment of carbon dioxide released during proofing to give the light porous crumb structure of leavened bread. Consequently, wheat proteins have been widely studied (Shewry et al., 2009a). However, gluten proteins are not the sole determinant of processing quality and other grain components also contribute, including starch, cell wall polysaccharides and lipids.

Wheat lipids are typically minor components of grain, accounting for only 2.5 to 3.3% of whole grain and 2.6 to 2.7% of white flour (Chung et al., 2009). Nevertheless, they affect the volume and texture of loaves and other baked products (Pycarelle et al., 2019), probably by a combination of effects including binding to and plasticising the gluten network and stabilizing the gas cells which are formed during dough mixing and expanded during fermentation (Köhler, 2001; Chung et al., 1978; Salt et al., 2018).

Wheat grain lipids display wide structural diversity, with over 70 molecular species being identified, and comprise neutral (acylglycerols and free fatty acids) and polar (glycolipids and phospholipids) components. Polar lipids are structural components of membranes, with the galactolipids being characteristic of the membranes of the amyloplasts (modified plastids), which contain the starch granules (Haschke et al., 1990). Recent studies have focused on surface-active galactolipids which are present in the air-water interface surrounding the gas bubbles in dough and may contribute to their stability (Schaffarczyk et al., 2014; Salt et al., 2018; Melis et al., 2020; Min et al., 2020).

Triacylglycerols (TAGs) are the major storage lipids in seeds but, with the exception of oats, are minor components in cultivated cereals. They are concentrated in the aleurone layer and scutellum of the embryo, where they account for 60 to 80% of the total lipids in these tissues (Chung et al., 2009) and are located in discrete oil bodies. By contrast, although TAGs account for about a third of the total lipids in the starchy endosperm tissue from which white flour is produced (Chung al., 2009; Gonzalez-Thuillier et al., 2015), nothing in known about their synthesis and deposition and is has been suggested that some transfer of lipids (including TAGs) from the aleurone and embryo to the flour occurs during milling (Morrison, 1994).

The production of fatty acids and the synthesis of TAGs in plants is a complex process that involves multiple cellular organelles. Fatty acids are synthesized in the plastid by a Type II fatty acid synthase complex. A repeated series of condensation, reduction and dehydration reactions then adds two carbon units to the extending fatty acid chain. The final products of these reactions are fatty acids typically 16 or 18 carbons (C16 and C18) long and attached to an acyl-carrier protein (ACP). While in the plastid a double bond can be introduced through the action of a fatty acid Δ9-desaturase. The ACP moeity is removed by thioesterases and the fatty acids produced in the plastid are then exported to the cytosol, converted to CoA forms and rapidly incorporated into phosphatidylcholine (PC). Further modification by additional desaturation (via fatty acid desaturases, FADs) (see Hajiahmadi et al. 2020 for a detailed description of wheat FADs) or incorporation of functional groups can then occur. The lipids of wheat grains typically contain C16 and C18 fatty acids, notably palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). A process of acyl editing exchanges fatty acids between PC and the acyl-CoA pool. Once located in the endoplasmic reticulum (ER), fatty acids are assembled into TAG by a combination of two pathways (Supplementary Figure S1). The acyl-CoA-dependent Kennedy pathway begins with the sequential acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferases (GPATs) and lysophosphatidic acid acyltransferases (LPAATs) using acyl-CoA to produce phosphatidic acid (PA). This PA can then be dephosphorylated by PA phosphatases to create de novo diacylglycerol (DAG). The DAG is then available for two different acyltransferase reactions: diacylglycerol acyltransferases (DGAT) transfer acyl-CoAs to the sn-3 position of DAG to produce TAG; alternatively phospholipid:diacylglycerol acyltransferases (PDAT) transfers the sn-2 acyl group of from phospholipids to DAG, forming

TAG (see Li-Beisson et al. 2013 for a detailed description). The contribution of DGAT and PDAT to TAG synthesis is known to vary between species.

We report here the first detailed study of TAG accumulation and deposition in the wheat starchy endosperm during the major grain filling period. This is based on the analysis of hand-dissected tissues to avoid lipid transfer between tissues with the lipid profiles being combined with transcript analysis and confocal microscope imaging of tissue sections. This study therefore add to our currently limited knowledge of the synthesis and deposition of TAG in the starchy endosperm during grain maturation and provides a basis for determining the contributions of TAGs to flour processing and breadmaking.

2. Experimental

2.1. Plant material

Wheat cv Hereward was grown in field trials with three replicate blocks in 2016 (year 1) and 2017 (year 2) at Rothamsted Research (Harpenden, UK) for lipidomics and microscopy. For transcript analysis cv Yumai 34 was grown in a glass house with 20ºC/15ºC day/night cycles and a photoperiod of 16 hours, supplementary lighting being provided when ambient levels fell below 400 µmol m^{-2} s^{-1}. Heads were tagged at anthesis and caryopses harvested from the middle thirds of ears for each developmental stage (14, 21, 28 and 34/5 days post-anthesis (dpa)). Starchy endosperm tissue was dissected by hand and frozen in liquid nitrogen for lipidomics or transcript analysis. Lipid analysis was carried out on on. Lipid analysis was carried out on three replicate samples of twelve caryopses each, representing the outer fully developing ones of the central six florets of one ear. For transcriptomics caryopses from two or three individual ears were pooled for each sample with two replicates per time point. Whole caryopses were used directly for microscopy.

2.2. Lipid extraction

Samples were transferred to a glass tube with 1 mL of propan-2-ol. Samples were crushed with a glass rod, vortexed and then heated at 75ºC for 20min. Chloroform, methanol and H_2O (1:1:0.7) were then added and the mixture vortex followed by 1mL chloroform and 1 mL water and the mixture vortexed again. Two phases were separated by centrifugation and the lower phase, containing the lipids, was removed to a new tube. The extraction was repeated with an additional millilitre of chloroform and, after mixing and centrifugation, the lower phase

was removed to the same tube. The samples were dried with a current of nitrogen and re-suspended in 200µl chloroform and stored at -80ºC.(Bligh and Dyer 1959; Kates et al. 1986)

2.3. TAG analysis

Triacylglycerols were identified and quantify by ESI-MS/MS as described by Li et al. (2014) with modifications (Gonzalez-Thuillier et al., 2015). A portion of lipid extract (10µL) and 0.857nmol tri15:0-TAG (Nu-Chek Prep, Minnesota, USA) were mixed with chloroform:methanol:300 mM ammonium acetate (24:24:1.75: v/v) to a final volume of 1 ml for direct infusion into the mass spectrometer. TAG was detected as [M+NH4]+ ions by a series of different neutral loss scans, targeting losses of fatty acids. The data were processed using the program Lipid View Software (AB-Sciex, Massachusetts, USA) where isotope corrections are applied. The peak area of each lipid was normalized to the internal standard and further normalized to the weight of the initial sample.

2.4. Sample preparation and staining for imaging

Developing caryopses were hand-dissected and immediately fixed in 4 % (w/v) paraformaldehyde in 1x phosphate buffer solution (PBS) for 4 or 5 hours after removal of the two ends to facilitate penetration of the fixative in the tissue. The samples were then washed x 3 with PBS and cut into 150 µm transverse sections using a Vibratome (Leica VT1000S, Germany). Sections were collected with a fine brush and washed briefly in PBS, before being subjected to two additional hours fixation in fresh 4 % (w/v) paraformaldehyde in PBS, following which they were washed three times with PBS for 5 to 10 minutes. For confocal microscopy the sections were sequentially stained with BODIPY 493/503 (Thermo Fisher Scientific) for neutral lipids, calcofluor white for cell walls and rhodamine for proteins. The sections were submerged for 2 minutes in BODIPY solution (1µg BODIPY per mL of PBS) then washed for 5 min with PBS, 1 min in distilled water and 1 min in PBS. They were then stained with 0.05% calcofluor white for 30 seconds and washed as above followed by 0.5 µg rhodamine in 1ml of PBS and washed again. The stained sections were mounted on a slide in fluorescence mounting medium (CITIFLUOR AF1, England) and observed by confocal microscopy (Zeiss LSM780, Germany). Images were acquired with Zen 2011 software.

2.5. Transcript analysis

Transcript profiles were determined essentially as described by Pellny et al (2012) for cv Cadenza. In short, central samples of starchy endosperm were dissected by hand, snap frozen in liquid nitrogen and total RNA extracted using a CTAB method. Transcriptome analysis was performed at the University of Bristol Transcriptomics facilities using Illumina single reads.

3. **Results and Discussion**

3.1. TAG accumulation

Starchy endosperm tissue was prepared from developing caryopses of wheat cv Hereward between 14 and 34 days post-anthesis (dpa) which correspond to the most active phase of grain filling (Shewry et al., 2009b). Total TAGs and TAG molecular species were determined by lipidomic profiling as described by Min et al. (2020). The experiment was carried out in two years, 2016 and 2017 (called Years 1 and 2, respectively), and the results are summarised in Figure 1 and Supplementary Figure S1.

The content of TAG increased during development from about 50 to 115 mg/g dry wt in Year 1 and from about 35 to 175 mg/g dry wt in Year 2 (Figure 1A). The fatty acid profiles of the total TAG fractions were broadly similar over the two experiments, although the precise proportions of the fatty acids differed (Figure 1B, C): this almost certainly resulted from differences in the growth conditions, which are known to strongly affect grain lipid composition (Salt et al., 2018). The major fatty acids were C16 (palmitic C 16:0 and palmitoleic C16:1) and C18 (stearic C18:0, oleic C18:1, linoleic C18:2 and linolenic C18:3), with unsaturated fatty acids accounting for about 75-80% of the total throughout development. Linoleic acid (C18:2) was the major component at all stages and the proportion increased during development.

Twenty-four TAG species were determined (Figure 2), including two minor saturated species (C46:0, C48:0). The major species in both years was C52:4, but the proportions of all species varied with no consistent trends between stages and years. The accumulation of TAG during endosperm development aligns with its role as a storage reservoir of fatty acids. Furthermore, it is possible that TAG composition is dynamic during development, exchanging fatty acids with cell membranes to modulate membrane properties, including adaptation to environmental conditions (de Carvalho and Caramujo, 2018).

3.2. Transcript analysis of TAG synthesis

The profiles of transcripts encoding enzymes catalysing the synthesis and assembly of TAG were determined using transcript libraries for hand-dissected starchy endosperm samples from the cultivar Yumai 34, harvested at similar developmental stages to those analysed from cv Hereward (Figure 3).

The transcripts for enzymes catalysing fatty acid synthesis show an initial burst of activity which then continues through grain development (Figure 3, Panel A). Abundant transcripts include β-ketoacyl-acyl carrier protein (ACP) synthase II (KASII), which elongates 16:0-ACP to 18:0-ACP in the plastid at the first branch point of fatty acid synthesis. At the same time (10 to 15 DAP) transcripts for acyl-ACP desaturase (stearoyl-acyl carrier protein-desaturase; DES/SAD), which introduces the first double bond into the acyl chain of saturated fatty acid in plastids (C18:0 to C18:1), and FAD2 (which converts C18:1 to C18:2) are also abundant. Long-chain acyl-CoA synthetases (LACSs), typically esterify 16-carbon and mono- and polyunsaturated 18-carbon fatty acids to acyl-CoA and therefore play vital and diverse roles typically associated with cuticular wax synthesis. However, it is not uncommon for LACs mutants to produce less seed oil than wildtype. LACS transcript activity remains high through grain development which is consistent with its established role in supplying substrates for wax biosynthesis and its contribution to TAG assembly (Zhao et al. 2019). The levels of transcripts for FAD3, which converts C18:2 to C18:3, are consistently low, which is consistent with the low proportion of C18:3 in grain lipid profiles (~5%).

Triacylglycerol (TAG) is synthesised by two routes, either in a reaction that uses acyl-CoA as acyl donor and diacylglycerol (DAG) as acceptor (the Kennedy pathway) or from phosphatidyl choline in an acyl-CoA independent reaction (Supplementary Figure S1).

Analysis of the transcript profiles of three acyltransferases involved in the Kennedy pathway (Panel B) shows that the pathway is highly active during grain development, with consistently high levels of transcripts for sn-1 glycerol-3-phosphate acyltransferase (GPAT) which acylates glycerol-3-phosphate to form lysophosphatidic acid (LPA), lysophosphatidic acid acyltransferase (LPAAT) which acylates LPA to give phosphatidic acid and diacylglycerol acyltransferase (DGAT) which catalyses the third acylation, following dephosphorylation of PA to give diacylglycerol (DAG), to give TAG.

However, transcripts are also present for activities involved in the production of TAG from PC and DAG by the acyl-CoA independent pathway, namely phospholipid:diacylglycerol acyltransferases (PDAT), phosphatidylcholine:diacylglycerol choline phosphotransferase (PDCT) and diacylglycerol cholinephosphotransferase (DAG-CPT). Together with lysophospholipid acyltransferases (LPCAT), these enzymes are responsible for the exchange of DAG and PC head groups and the mixing of acyl-CoA species into the TAG biosynthetic pathway. DAG produced from these exchange activities is often referred to as PC-derived DAG. Hence, both *de novo* synthesised DAG and PC-derived DAG can be used as a substrate for DGAT TAG synthesis. However, the transcript levels for the acyl-CoA independent pathway are generally lower than those for the Kennedy pathway.

The transcript profiles therefore indicate that the predominant route to TAG synthesis and oil accumulation in the developing starchy endosperm of wheat is via the Kennedy pathway and DGAT activity. However, the acyl-CoA independent pathway clearly also operates and the precise contributions of *de novo* DAG (Kennedy) and PC-derived DAG remain to be determined. The important of the Kennedy pathway supported by the transcript analysis of wheat grains reported by Grimberg et al. (2020) which also showed low levels of PDAT activity early in grain development and higher levels of DGAT/TAG1 activity.

3.3. TAG Deposition

Oil bodies in seed tissues are usually stabilised by a surface layer of oleosin proteins associated with phospholipids, with smaller amounts of other proteins (notably, caleosins, LD associated protein and OB associated protein) (Huang, 2018). Oil body proteins have not been identified in white flour or starchy endosperm cells of wheat, although oleosins have been identified in bran, a fraction which contains aleurone cells (Tsen et al., 1990) and embryos (Lv et al., 2016) in addition to the outer tissues of the grain.

We therefore initially used confocal laser microscopy to study the accumulation of oil in the developing starchy endosperm cells of wheat and determine whether the oil deposits were associated with proteins. Staining with BODIPY showed clear droplets of neutral lipids in the starchy endosperm cells of grain sections at 14 and 28 days after anthesis (Figure 4D). In order to visually compare the distribution of lipid bodies, confocal images of grain sections at 14 and 28 days after anthesis are displayed as three-dimensional images in Figure 4A and 4C.

These show a clear increase in the number of oil deposits between 14 and 28 days. They also show that oil deposits are concentrated in the central endosperm cells (CES) at both stages, and absent from the sub-aleurone cells (SA). Both sections also show aleurone cells (AL), demonstrating the abundant lipid deposits in this tissue. To determine whether the oil deposits were associated with protein the sections were co-stained for neutral lipids (BIODIPY), protein (rhodamine) and cell wall glucans (β-glucan and cellulose, stained with calcofluor white) (Figure 4B). The distributions of these components were then determined across transects of the section, running from the outer cell wall of the aleurone layer to the central endosperm. These transects passed through oil deposits, showing that they were clearly associated with peaks in protein concentration (see arrows in Figure 4B and 4D).

Oleosins are present in cereal seeds in two isoforms with masses of about 16,000 and 18,000 (16kDa and 18 kDa oleosins, respectively) (Tsen et al., 1990) and Aalen (1995) reported that transcripts for both were present in the aleurone and starchy endosperm of the barley grain. The expression profiles of transcripts encoding these two forms were therefore determined using the starchy endosperm transcript libraries described above. No transcripts for the 18 kDa oleosins (*Ole-1*) were detected, but transcripts corresponding to the 16 kDa oleosin (*Ole-2*) genes on the B and D (but not A) genomes were detected (Figure 2D). The *Ole-2* transcipts from the B and D genomes show similar deceases in expression during caryopsis development. These data are therefore consistent with the oil deposits in the starchy endosperm cells being stabilised by 16 KDa oleosins.

3.4. General Discussion

We have demonstrated that the triacylglycerols deposited in the starchy endosperm cells of developing caryopses are rich in linoleic acid (C18:2), with the proportion of this fatty acid also increasing during caryopsis development. Furthermore, TAGs are deposited in discrete oil deposits which are associated with protein. The presence of *Ole-2* transcripts encoding the 16 kDa oleosin isoform suggests that these bodies are stabilised by oleosins as in most other oil-storing seed tissues. Although we have not directly demonstrated a role of oleosin in stabilising lipid bodies in this paper, recent studies carried out in transgenic wheat support this suggestion: overexpression of the AsWRI1 transcription factor from oat in the starchy endosperm of wheat resulted in increase in accumulation of TAGs by up to nine-fold and in up-regulation of oleosin-encoding genes (Grimberg et al., 2020). The fact that oleosins have

not been reported in published proteomic studies of wheat starchy endosperm or white flour probably reflects technical problems, as oleosins are highly hydrophobic and not readily extracted in buffers used for proteomic studies.

Oats differs from other cereal grain in containing 5-6% oil which is concentrated in the starchy endosperm as well as in the embryo and aleurone. Furthermore, oil is deposited in the sub-aleurone as well as the central starchy endosperm cells. Although an early study using fluorescence microscopy and Nile blue staining to visualise lipids reported the presence of oil bodies (termed spherosomes) in the subaleurone cells of wheat (Hargin et al 1980), this was not observed in our work, where BODIPY was used as a stain specific for neutral lipids.

Wheat also appears to differ from oats in that the oil deposits in the starchy endosperm remain discrete whereas they merge in the developing oat endosperm, resulting in an oily matrix surrounding the starch and protein in the mature grain (Heenan et al, 2008). This merging of the oil bodies in the starchy endosperm of oats may result from an imbalance between the synthesis of oil and oleosins as Heenen et al (2008) showed that oleosins are present in all oil-storing tissues of the oat grain, but the amount relative to oil content is much lower in the endosperm (aleurone and starchy endosperm) than in the embryo. In view of the low oil content it is unlikely that a similar merging of oil bodies occurs in the wheat starchy endosperm, but they will certainly become disrupted as the grain matures and the cell contents merge.

Funding

Rothamsted Research receives strategic funding from the Biotechnology and Biological Sciences Research Council (BBSRC) and the work forms part of the Designing Future Wheat strategic programme (BB/P016855/1). The work was also supported by the BBSRC Crop Improvement Research Club grant BB/J019526/1 “The role of lipids in determining gas bubble retention in wheat dough”

CRediTt authorship statement

Irene Gonzalez-Thuillier: Conceptualization, Methodology, Formal Analysis, Writing- review and editing. **Till K. Pellny:** Formal analysis, Investigation, Methodology, Validation. **Paola**

Tosi: Conceptualization, Methodology, Writing - review & editing, Visualization **Rowan Mitchell: Methodology,** Formal analysis, Investigation; Writing- review and editing. **Richard Haslam.** Conceptualization; Methodology, Formal Analysis**;** Supervision, Writing - original draft, Writing - review & editing **Peter R. Shewry:** Conceptualization; Project Administration, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper

Appendix A. Supplementary data Supplementary data to this article can be found online at:

References

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

Capuano, F., Beaudoin, F., Napier, JA and Shewry, PR. (2007). Properties and exploitation of oleosins. Bitechnology Adv., 25, 203-206.

Chung, O. K., Pomeranz, Y., & Finney, K. F. (1978). Wheat flour lipids in breadmaking. Cereal Chem., 55, 598-618.

Chung, O. K.; Ohm, J.-B.; Ram, M. S.; A., H. C., Wheat lipids. In Wheat Chemistry and Technology, 4th ed.; K. K.; Shewry, P. R., Eds. AACC St Paul, MN, USA, **2009**; pp. 363-399.

De Carvalho, C.C.D/R and Caramujo, M. J. (2018). The various roles of fatty acids. Molecules, 23, 2583.

Dong K, Ge P, Ma C, Wang K, Yan X, Gao L, Li X, Liu J, Ma W, Yan Y. (2012) Albumin and globulin dynamics during grain development of elite Chinese wheat cultivar Xiaoyan 6. J Cereal Sci. 56, 615–622..

Finnie, S.M., Jeannotte, R., & Faubion, J.M. (2009). Quantitative characterization of polar lipids from wheat whole meal, flour, and starch. Cereal Chem., 86, 637–645.

Gerits, L. R., Pareyt, B., & Delcour, J. A. (2014). A lipase-based approach for studying the role of wheat lipids in bread making. Food Chem., 156,190-196.

Gonzalez-Thuillier, I., Salt, L., Chope, C., Penson, S., Skeggs, P., Tosi, P., Powers, S. J., Ward, J. L., Wilde, P., Shewry, P. R. & Haslam, R. P., (2015). Distribution of lipids in the grain of wheat (cv. Hereward) determined by lipidomic analysis of milling and pearling fractions. J. Agric. Food Chem. 63, 10705-10716.

Grimberg, A., Wilkinson, M. D., Snell, P., De Vos, R. P., Gonzalez-Thuillier, I., Tawfike, A., Ward, J. L., Carllson, A. S., Shewry, P. R., Hofvander, P. (2020) Transitions in wheat endosperm metabolism upon transcriptional induction of oil accumulation by oat endosperm WRINKLED1. BMC Plant Biology, 20, 235.

Hajiahmadi Z, Abedi A, Wei H, Sun W, Ruan H, Zhuge Q, & Movahedi A. (2020) Identification, evolution, expression, and docking studies of fatty acid desaturase genes in wheat (Triticum aestivum L.) BMC Genomics 21(1):778. doi: 10.1186/s12864-020-07199-1.

Hargin, W. R. Morrison, and R. G. Fulcher (1980) Triglyceride Deposits in the Starchy Endosperm of Wheat Cereal Chem. 57, 320 - 325.

Haschke, H. P.; Kaiser, G.; Martinoia, E.; Hammer, U.; Teucher, T.; Dorne, A. J.; Heinz, E., Lipid profiles of leaf tonoplasts from plants with different CO_2-fixation mechanisms. Bot. Acta. 1990, 103, 32-38.

Heenen, W., Karlsson, G., Brismar, K., Gummerson, P-O., Marttila, S., Leonova, S., Carlsson, A.S., Bafor, M., Banas, A., Mattsson, B. DFebski, H. and Stymne, S. (2008). Fusion of oil bodies in endosperm of oat grains. Planta 228, 589-599.

Heneen,K., Banas, A., Leonova, S., Carlsson, A. S., Marttila,S., Debski, H., Stymne, S. (2018). Plant lipid droplets and their associated proteins: potential for rapid advances. Plant Physiol. 178, 1894-1918.

Kates AL, Park IRA, Himmshagen J, Kopecky J (1986) Thyroxine 5'deiodinase Activity in Brown Adipose-Tissue of Rats and Mice - Lack of Effect of Diet. Fed. Proc. 45 :610-610

Köhler, P. (2001). Study of the effect of DATEM. 3: Synthesis and characterization of DATEM components. LWT-Food Sci. Technol., 34, 359-366.

Li-Beisson Y, Shorrosh B, Beisson F, Andersson M, Arondel V, Bates PD, Baud S, Bird D, DeBono A, Durrett TP, Franke RB, Graham IA, Katayama K, Kelly AA, Larson TR, Markham JE, Miquel M, Molina I, Nishida I, Rowland O, Samuels L, Schmid KM, Wada H, Welti R, Xu C, Zallot R, & Ohlrogge J (2013) Acyl-Lipid Metabolism. Arabidopsis Book 11: e0161.

doi: 10.1199/tab.0161

Li MY, Baughman E, Roth MR, Han XL, Welti R, Wang XM (2014) Quantitative profiling and pattern analysis of triacylglycerol species in Arabidopsis seeds by electrospray ionization mass spectrometry. Plant J. 77, 160-172

Lv Y., Zhang S., Wang J and Hu Y. 2016. Quantitative proteomic analysis of wheat seeds during artificial ageing and priming using isobaric tandem mass tag labelling. PlosONEDOI:10.1371/journal.pone.0162851

MacRitchie, F. & Gras, P. W. (1973). Role of flour lipids in baking. Cereal Chem., 50, 292-302.

Melis, S., Meza Morales, W.R. & Delcour J.A. (2020). Lipases in wheat flour bread making: importance of an appropriate balance between wheat endogenous lipids and their enzymatically released hydrolysis products. Food Chem. 289, 125002.

Min, B., González-Thuillier, I., Powers, S.J., Wilde P., Shewry, P.R., & Haslam, R.P. (2017). Effects of cultivar and nitrogen nutrition on the lipid composition of wheat flour. J. Agric. Food Chem. 65, 5427-5434.

Morrison, W.R., 1994. Wheat lipids: structure and functionality. In “Wheat: production properties and quality” Eds Bushuk W and Rasper V.F. pp128–142. Chapman and Hall, London.

Pellny, T.K., Lovegrove, A., Freeman, J., Tosi, P., Love, C.G., Knox, J.P., Shewry, P.R. and Mitchell, R.A.C. (2012) Cell walls of developing wheat starchy endosperm: comparison of composition and RNA-Seq transcriptome. Plant Physiol. 158, 612–627.

Pareyt, B., Finnie, S. M., Putseys, J. A., & Delcour, J. A. (2011). Lipids in bread making: sources, interactions and impact on bread quality. J. Cereal Sci., 54, 266-279.

Pycarelle, S.C., Winnen, K.L.J., Bosmans, G.M., Van Haesendonck, I. V., Pareyt, B., Brijs, K. & Delcour, J.A. (2019). Wheat (Triticum aestivum L.) flour free lipid fractions negatively impact the quality of sponge cake. Food Chem. 27, 401-409.

Salt, L.J., González-Thuillier, I., Chope, G., Penson, S., Tosi, P., Haslam, R.P., Skeggs P.K., Shewry, P.R., & Wilde, P.J. (2018). Intrinsic wheat lipid composition effects the interfacial and foaming properties of dough liquor. Food Hydrocolloids, 75, 211-222.

Schaffarczyk, M., Østdal, H., & Koehler, P. (2014). Lipases in wheat breadmaking: analysis and functional effects of lipid reaction products. J. Agric. Food Chem. 62, 8229-8237.

Shewry, P.R., D'Ovidio, R., Lafiandra, D., Jenkins, J.A., Mills, E.N.C. and Bekes, F. (2009a) Wheat grain proteins. In: Wheat: Chemistry and Technology 4th edition (eds. K. Khan and P.R. Shewry). AACC, St Paul, MN, USA. pp. 223-298.

Shewry, P.R., Underwood, C., Wan, Y., Lovegrove, A., Bhandari, D., Toole, G., Mills, E.N.C., Denyer, K. and Mitchell, R.A.C. (2009b) Storage product synthesis and accumulation in developing grains of wheat. J. Cereal Sci. 50, 106-112.

Tsen JTC., Lai Y-K, Chan K-L and Huang, AHC. 1990. Oleosin isoforms of high and low molecular weight are present in the oil bodies of diverse seed species. Plant Physiol. 94, 1282-1289.

Wan YF, Gritsch CS, Hawkesford MJ, Shewry PR (2014) Effects of nitrogen nutrition on the synthesis and deposition of the omega-gliadins of wheat. Ann. Bot. 113, 607-615.

Zhao L, Haslam TM, Sonntag A, Molina I, Kunst L (2019) Functional Overlap of Long-Chain Acyl-CoA Synthetases in Arabidopsis. Plant Cell Physiol. 2019 May 1;60(5):1041-1054.

Figure Captions:

Figure 1. Total triacylglycerols (TAG) (A) and percentage compositions of fatty acids in TAGs (B,C) in developing endosperm of wheat grown in Years 1 (A,C) and 2 (A,D).

Fatty acids are: C16:0, palmitic; C16:1, palmitoleic; C18:0, stearic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic.

406 Fig 2. Triacylglycerol (TAG) molecular species in developing starchy endosperm of wheat
407 grown in Year 1 (A) and 2 (B)

408 Molecular species are defined as the sums of carbon atoms (44 to 54) and double bonds (0 to
409 7) in the three fatty acid moieties.

410 Fig 3. Expression profiles of transcripts encoding enzymes involved in TAG synthesis and
411 oleosin in developing starchy endosperm of wheat (RNAseq data mapped to IWGSC refseq
412 1.1] log scale).

413 Panel A shows transcript profiles for enzymes associate with fatty acid synthesis:KASII, β-
414 ketoacyl-acyl carrier protein (ACP) synthase II; FAD2, fatty acid desaturase 2; FAD3, fatty acid
415 desaturase 3; LACS, long-chain acyl-CoA synthetase; long-chain acyl-CoA synthetases
416 DES/SAD, stearoyl-acyl carrier protein-desaturase

417 Panel B shows acyltransferases catalysing TAG synthesis via the Kennedy pathway: DGAT,
418 diacylglycerol acyltransferases; LPAAT, lysophosphatidic acid acyltransferase; GPAT, glycerol-
419 3-phosphate acyltransferases.

420 Panel C shows enzymes catalysing TAG synthesis via the CoA-independent pathway: DGAT-
421 CTP, diacylglycerol cholinephosphotransferase; PDCT/ROD1;
422 phosphatidylcholine:diacylglycerol cholinephosphotransferase; PDAT,
423 phospholipid:diacylglycerol acyltransferases

424 Panel D shows transcripts for 16 kDa oleosins (*Ole-1*) encoded by the A, B and D genomes.

425 Fig 4. Lipid deposition in the starchy endosperm cells of the developing wheat caryopsis.

426 Panels A and C show three-dimensional representations of the distribution of lipids
427 reconstructed from CLSM images of BODIPY-stained cross sections of developing caryopses
428 at 14 days post anthesis DPA (panel A) and 28 DPA (panel C). Panel B shows the intensity levels
429 of staining with Rhodamine (protein), BODIPY (lipids) and calcofluor (cell wall
430 polysaccharides), as detected along the transect showed in panel D. Panel D shows across
431 section of the developing caryopsis at 28 DPA wheat grain stained for protein, lipids and cell
432 wall polysaccharides.

433

434

435

436

437

438

439

Figure 1

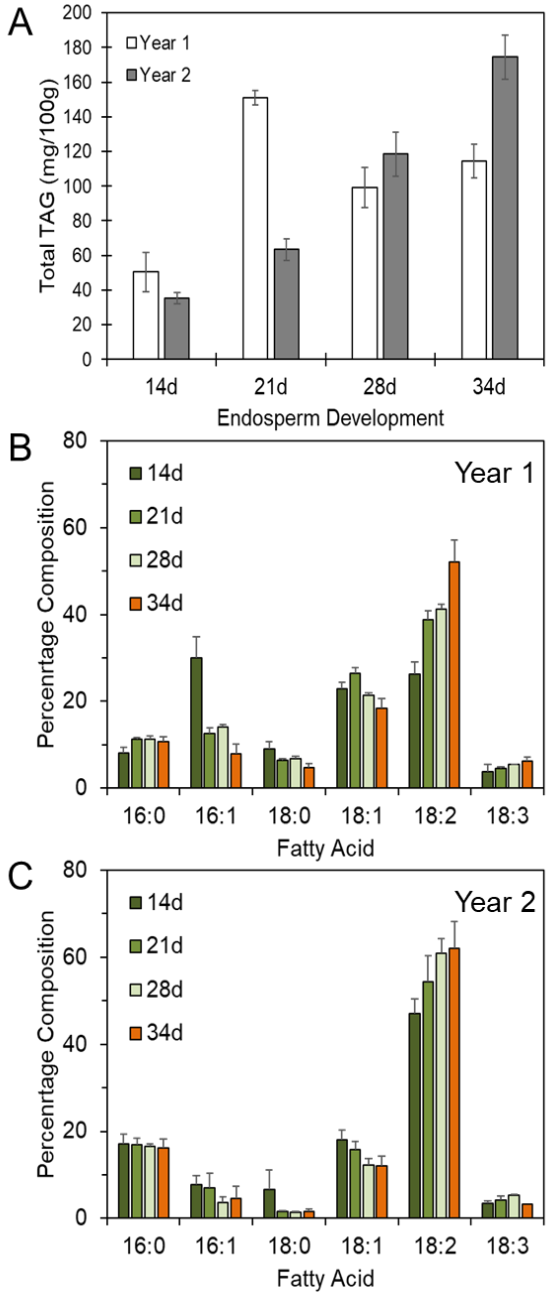
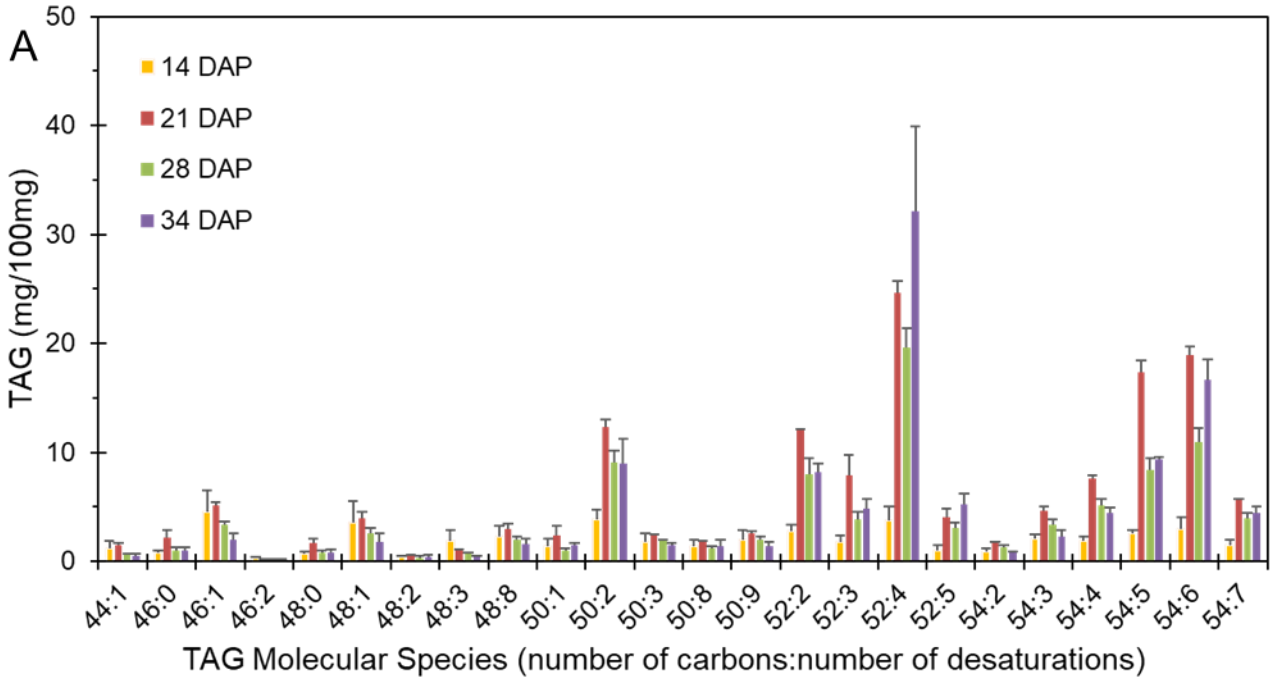


Figure 2



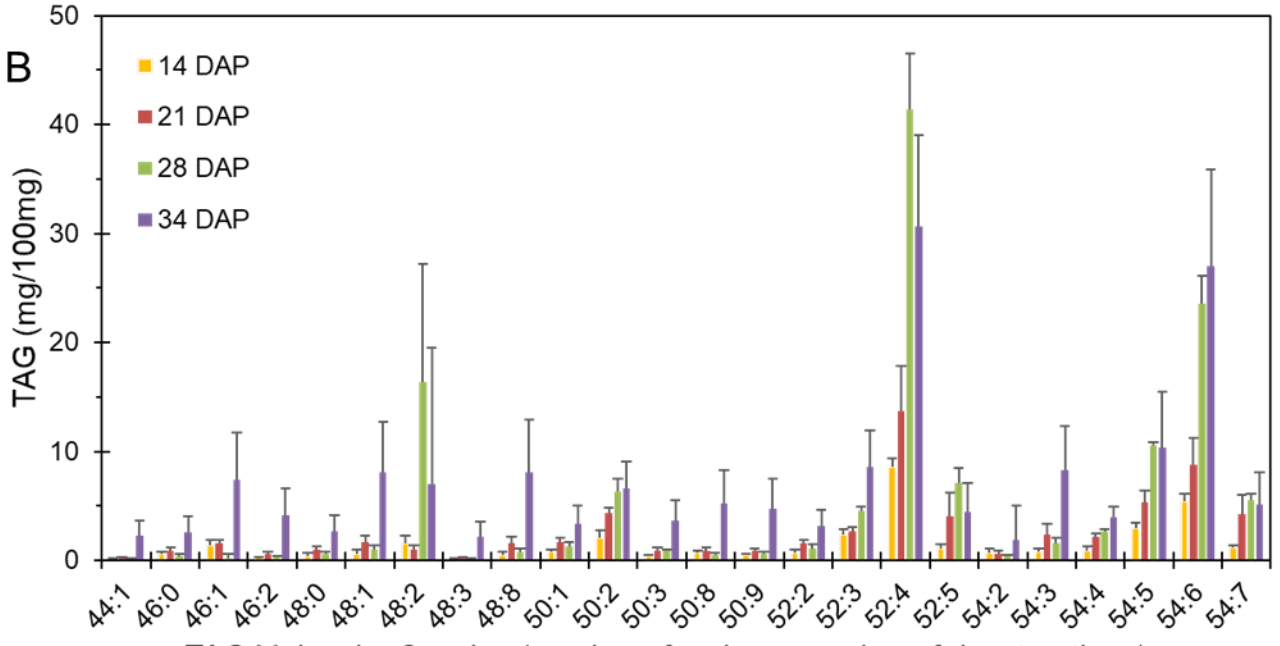


Figure 3

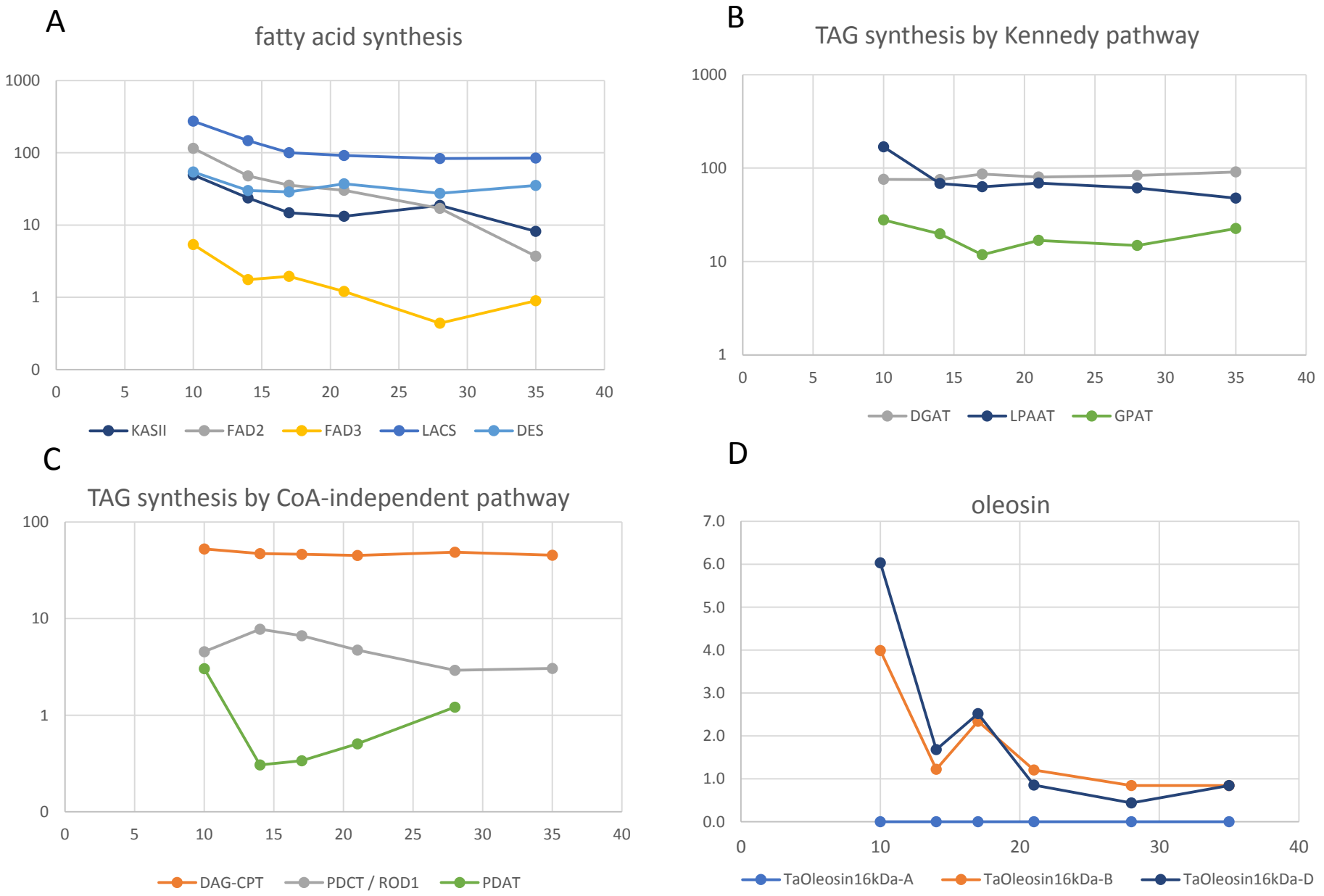
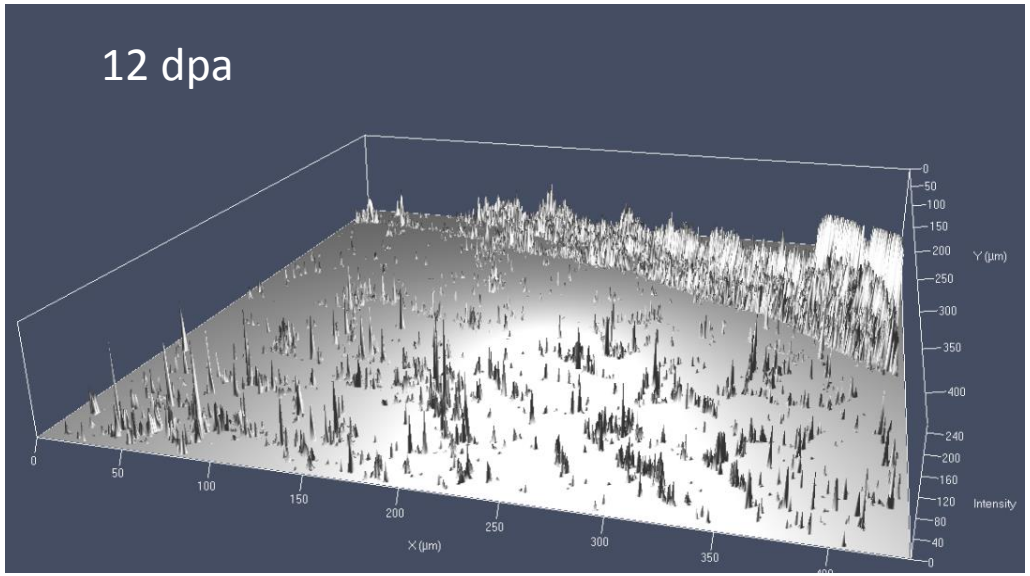
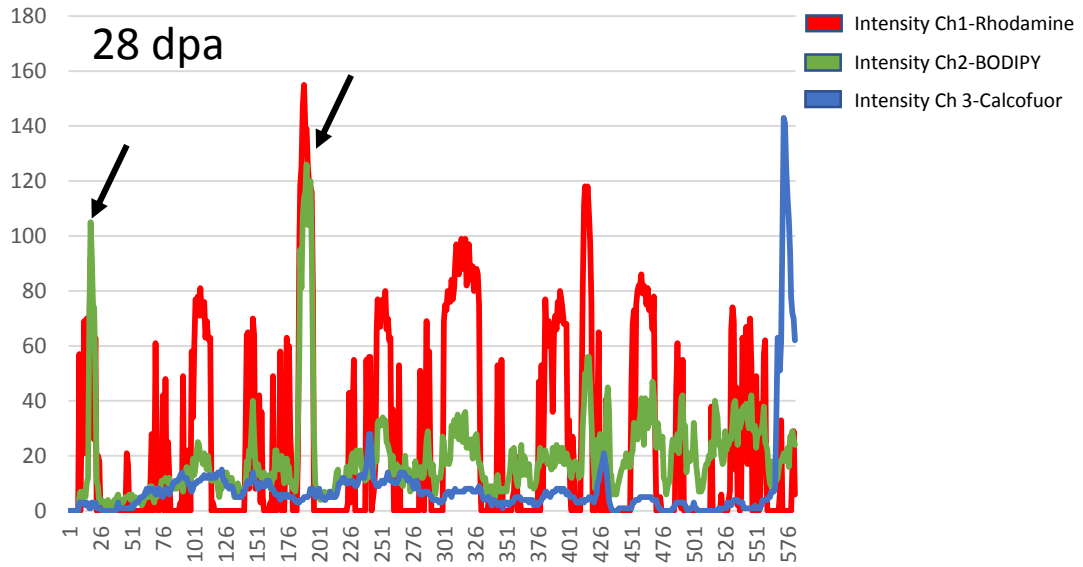


Figure 4

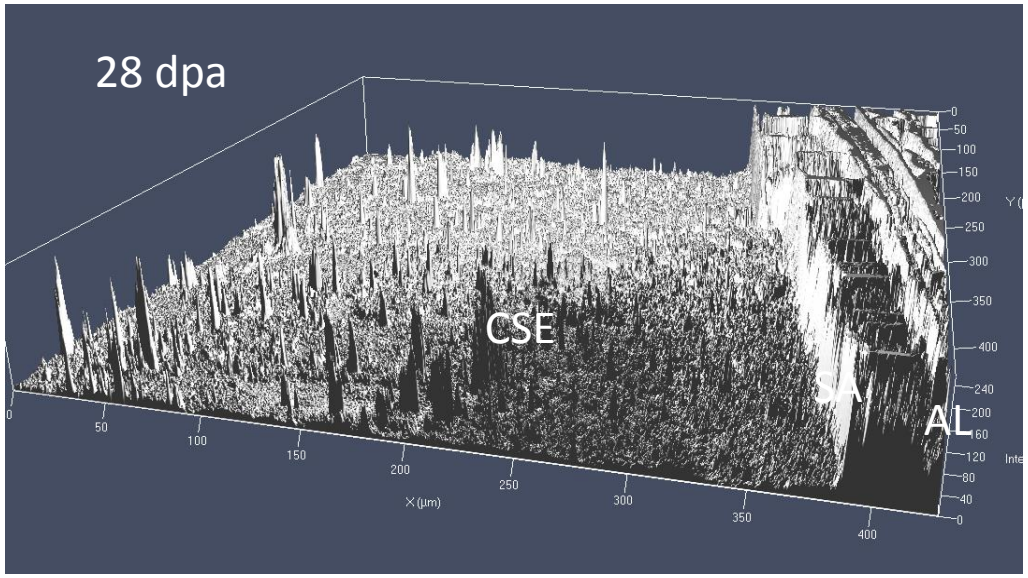
A



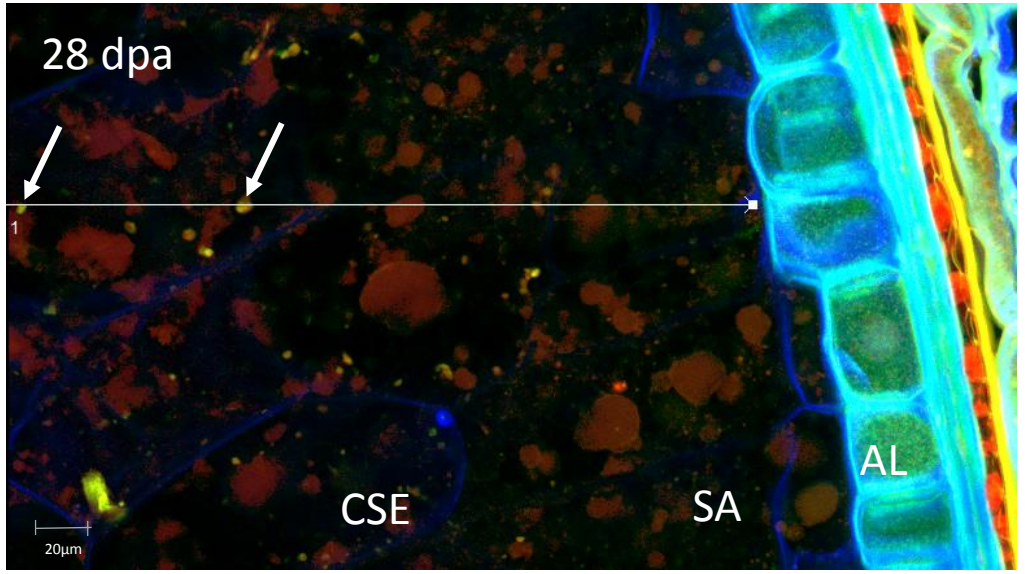
B



C

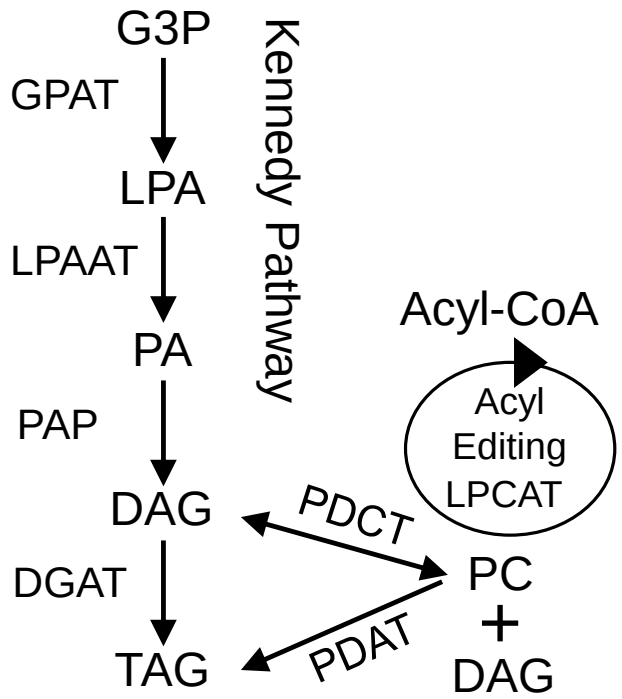


D



Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper



Supplementary Figure S1. Summary of the pathways of triacylglycerol biosynthesis.
Fatty acids are assembled into TAG by a combination of two pathways.
In the acyl-CoA-dependent Kennedy pathway glycerol-3-phosphate (G3P) is converted by glycerol-3-phosphate acyltransferases (GPATs) and lysophosphatidic acid acyltransferases (LPAATs) using acyl-CoA to produce phosphatidic acid (PA). PA is then dephosphorylated by PA phosphatases (PAP) creating de novo diacylglycerol (DAG). DAG and acyl-CoA are then available to diacylglycerol acyltransferases (DGAT) to produce TAG.
In the second acyl-CoA independent pathway phospholipid:diacylglycerol acyltransferases (PDAT) utilizes the sn-2 acyl group of phospholipids with DAG, forming TAG. Phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) exchanges head groups between PC and DAG, whilst LPCAT (lysophospholipid acyltransferase) is responsible for acyl editing, incorporating acyl-CoA species into PC.