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RESEARCH PAPER

Mobilization of lipid reserves during germination of oat (*Avena sativa* L.), a cereal rich in endosperm oil

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Abstract

Since the cereal endosperm is a dead tissue in the mature grain, β-oxidation is not possible there. This raises the question about the use of the endosperm oil in cereal grains during germination. In this study, mobilization of lipids in different tissues of germinating oat grains was analysed using thin-layer and gas chromatography. The data imply that the oat endosperm oil [triacylglycerol (TAG)] is not a dead-end product as it was absorbed by the scutellum, either as free fatty acids (FFAs) released from TAG or as intact TAG immediately degraded to FFAs. These data were supported by light and transmission electron microscopy (LM and TEM) studies where close contact between endosperm lipid droplets and the scutellum was observed. The appearance of the fused oil in the oat endosperm changed into oil droplets during germination in areas close to the aleurone and the scutellar epithelium. However, according to the data obtained by TEM these oil droplets are unlikely to be oil bodies surrounded by oleosins. Accumulation of FFA pools in the embryo suggested further transport of FFAs from the scutellum. Noticeably high levels of TAG were also accumulated in the embryo but were not synthesized by re-esterification from imported FFAs. Comparison between two oat cultivars with different amounts of oil and starch in the endosperm suggests that an increased oil to starch ratio in oat grains does not significantly impact the germination process.

Key words: *Avena sativa*, cereal, endosperm, germination, lipid mobilization, oat, oil bodies, scutellum, starch, triacylgycerol.

Introduction

The supply of vegetable oils today relies upon only a few crops: palm oil (*Elaeis guineensis*), soybean (*Glycine max*), oil-seed rape (*Brassica napus*), and sunflower (*Helianthus annuus*), which in 2007 accounted for 83% of total world production (FAOSTAT, 2008). Increased knowledge of oil metabolism in plants is therefore of crucial importance for the development of novel oil crops required to meet the future demands for a sustainable and increased plant oil production.

The endosperm of cereal grains is one of the most important biological structures used for food and feed. Oat (*Avena sativa* L.) is unique among the cereals due to its high content of oil in relation to starch and protein (Price and Parsons, 1975; Åman and Hesselman, 1984). Oat varieties differ in oil content between 2% and 18% (Brown *et al.*, 1966; Peterson and Wood, 1997; Frey and Holland, 1999; Zhou *et al.*, 1999; Leonova *et al.*, 2008), whereas, in other cereals (i.e. wheat; *Triticum aestivum*, barley; *Hordeum vulgare*) this range is limited to 2–3% (Price and Parsons, 1975; Peterson and Wood, 1997). Although the highest oil concentrations in the oat grain are found in the aleurone layer and embryonic axis, these tissues represent a small proportion of the whole grain, and the main part of the oil reserve is found in the endosperm cells (Price and Parsons,

Abbreviations: DAG, diacylglycerol; DAI, days after imbibition; DW, dry weight; FA, fatty acid; FFA, free fatty acid; LM, light microscopy; PL, polar lipid; TAG, triacylglycerol; TEM, transmission electron microscopy.

1979; Fulcher, 1986; Peterson and Wood, 1997; Zhou *et al.*, 1999; Banas *et al.*, 2007). Maize, the only cereal which has a similar high grain oil content to oat, accumulates the oil mostly in the embryo, not in the endosperm (Leng, 1961). Thus, oat is a unique model plant to study different aspects of oil synthesis, storage, and degradation in the endosperm of cereals.

During germination of cereals, nutrients stored in the endosperm are degraded by enzymes synthesized and secreted from the aleurone and scutellum tissues, resulting in transportable nutrient molecules that are transferred through the absorptive scutellum to nourish the growing embryo (Fincher, 1989). Due to its importance to the malting and food industry, both starch degradation into sugars and protein degradation into amino acids in the cereal endosperm during germination have been extensively studied (Beck and Ziegler, 1989; Fincher, 1989; Ziegler, 1995). However, there have been few studies on oil degradation during germination of cereals, and in oat they have only concerned how oil and its degradation products cause processing difficulties in the food industry (for a review, see Kaukovirta-Norja *et al.*, 2004). Therefore, it is still not known how, and if, the energy trapped as oil in the endosperm of cereals is supplied to the growing embryo during germination. Starch is normally considered to be the major energy source for the germinating cereal grain. Increased oil content in oat grains has been shown to be negatively correlated to starch (Frey and Holland, 1999). Therefore, knowledge about the capacity of the cereal grain also to utilize the oil reserves efficiently during germination is of importance for the breeding of high-oil cultivars of cereals.

To mobilize energy stored as seed oil [triacylglycerol (TAG)], the action of lipases must first release the esterified fatty acids (FAs) from TAG. Free fatty acids (FFAs) can then be degraded through the β-oxidation and glyoxylate cycles and subsequently converted into sugars (Clarke *et al.*, 1983; Graham, 2008). In dicot seeds, the oil reserve is mainly stored in the embryo (as in oil-seed rape) or in the endosperm (as in castor bean; *Communis ricinis*), and FFA degradation in both of these tissues occurs in the specialized peroxisomes called glyoxysomes (Beevers, 1979; Huang *et al.*, 1983). However, since the endosperm of monocot cereal grains goes through programmed cell death upon maturation (Young and Gallie, 2000), this tissue lacks peroxisomes and can therefore not serve as a site for FFA degradation during germination. Moreover, previous studies have shown that the oil in the endosperm of oat fuses upon maturation of the grain whereas, in the other tissues, it exists as discrete oil bodies (Banas *et al.*, 2007; Heneen *et al.*, 2008). Oil bodies are thought to be a form of oil storage that enhance oil degradation due to the larger surface area available for enzymatic attack (Hsieh and Huang, 2004; Siloto *et al.*, 2006). The fused oil bodies in the endosperm of mature oat grains could therefore indicate that this oil might be a dead-end product which is not available as an energy source during germination. It has been reported in previous studies of barley that lipases secreted from the scutellum into the endosperm (Jensen and Heltved, 1982) can explain how TAG could be converted into FFAs in the cereal endosperm. However, this still leaves questions about the fate of the FFAs in the oat endosperm during germination.

The aim of this study, was to add understanding of how oil and other lipid classes in different parts of the cereal grain are mobilized during germination. Lipid mobilization was compared with changes in starch and sugar content. Similarities to and differences from other plant species with oil reserves in the endosperm are discussed.

Materials and methods

Plant material, germination conditions, and sampling

Field-grown material from a high-oil oat cv. Matilda (10% oil) and a medium-oil cv. Freja (6% oil) that both share the same parental lines (Svalöf Weibull AB, Svalöv, Sweden) were dehulled manually before analysis. Grains with weights of 31–37 mg were included in the analyses. To provide germination conditions, the grains were placed on moistened layers of paper tissues in Petri dishes, wrapped in aluminium foil, and kept in darkness at room temperature. Sampling was done after 2 h (0 days or 'non-germinated'), and at 1, 2, 4, 7, and 10 days after imbibition (DAI). Each grain was dissected using a scalpel to separate embryo+scutellum from the endosperm. Since it was not possible to separate the embryo+scutellum properly from the endosperm from non-germinated grains (without including endosperm tissue), only the endosperm tissue was analysed for the amount of starch at this time point. However, lipid analyses were also carried out on embryo+scutellum samples at this time point since the dense oil concentration in this part of the grain was regarded not to be significantly affected by the oil from the contamination of endosperm (estimated to be at most 10%). Shoots and roots were considered as a product of the developing embryo during germination and therefore were included in the 'embryo+scutellum' sample. However, at the last time point (10 DAI) no embryo+scutellum samples were taken because shoots and roots were too difficult to handle. To estimate the contribution of lipids and carbohydrates from the scutellum in the embryo+scutellum, scutellum samples were dissected without the embryo in parallel on a separate set of grains at 0, 1, 2, 4, 7, and 10 DAI. Samples were either frozen in liquid N_2 and stored at –80 °C prior to lipid analysis (six grains per replicate) or dried at 80 °C for dry weight (DW), starch, and sugar determination (three grains per replicate). Three replicates were sampled for both lipid and carbohydrate analyses. For microscopy, samples were collected at 1 and 4 DAI. Data from the high-oil cv. Matilda are presented here, while data from medium-oil cv. Freja can be found as Supplementary data at *JXB* online.

Lipid analyses

Total lipids were extracted according to Bligh and Dyer (1959) with the difference that 1 mM ethylenediaminetetraacetic acid (EDTA) in 0.15 M acetic acid (HAc) was added to prevent lipase activity. To determine lipid class content and FA composition, total lipids were separated and analysed using thin-layer and gas–liquid chromatography (GC) as described previously (Leonova *et al.*, 2008). Lipid amounts in grains are given as nmol FA per grain determined by using methyl-heptadecanoate as an internal standard in GC analysis of the methyl esters (FAME) for all FAs. FA profiles of the different lipid classes are given as mol% of the total amount of FAs in the lipid class.

Carbohydrate analyses

Starch and sugar (sucrose, D-glucose, and D-fructose) determinations were conducted enzymatically using two kits: Megazyme K-TSTA 01/05 (amyloglucosidase/α-amylase method for starch determination) and Megazyme K-SURFG 12/05 (for the measurement of D-glucose, D-fructose, and sucrose) (Megazyme, Wicklow, Ireland). Sugars were extracted from dried samples by homogenizing and boiling in 80% ethanol. After centrifugation, the pellet was washed in ethanol and then immediately used for starch analysis according to the supplier's manual. The ethanol extract containing free sugars was kept at –20 °C until sugar analysis was performed according to the manual.

Light and transmission electron microscopy

Grains at two time points (1 and 4 DAI) of cv. Matilda were used for light microscopy (LM) analyses. Samples of the proximal part of the grain, including the embryo and scutellum, and whole grains were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) at room temperature with rotation at 100 rpm for 4–12 h depending on the sample size. After washing with buffer (3× 15 min) the samples were post-fixed in buffered 2% OsO4, rinsed with buffer (3× 10 min) and dH_2O (3× 20 min), dehydrated in acidified 2,2-dimethoxypropane, followed by three acetone–Spurr epoxy series before being embedded in fresh Spurr epoxy resin (Spurr, 1969) and polymerized at 70 °C for 20 h (low viscosity kit, Ted Pella Inc., Redding, CA, USA). Semi-thin sections of 2 μm were cut and placed on object slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany) and stained with methylene blue–azur A–safranin O (MAS; Warmke and Lee, 1976) or Sudan Black (SB; O'Brien and McCully, 1981) as described previously (Heneen *et al.*, 2008). Structural studies with TEM using the same material as for LM were performed as in Heneen *et al.* (2008).

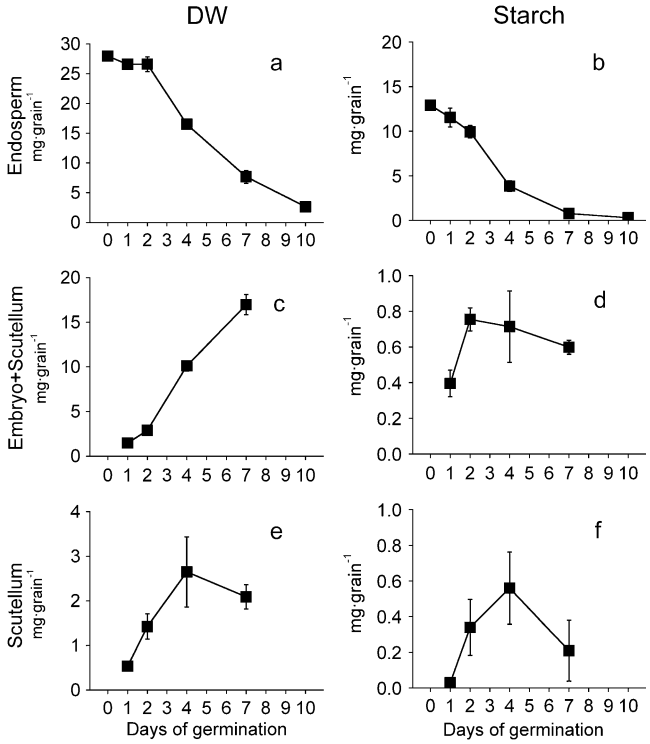

Fig. 1. Dry weight (DW, a, c, e) and starch amount (b, d, f) in the endosperm, embryo+scutellum, and scutellum of germinating oat grains of high-oil cv. Matilda. Results are mean values ± standard deviation of three samples.

Results

Changes in dry weight, starch, and sugar content during germination

The nutrients stored in the endosperm of oat grains were depleted after 10 d of germination with the most drastic decrease of endosperm DW and starch between 2 and 4 DAI (Fig. 1a, b). The DW of growing tissues (i.e. embryo and scutellum) increased more or less steadily during germination (Fig. 1c, e) while the starch content of these tissues first increased (up to 4 DAI) after which it decreased in the scutellum (Fig. 1d, f). The pattern of starch accumulation in the scutellum of oat grains found in this study with a sharp peak at 4 DAI is in line with previous studies where the pattern of accumulation and consequent degradation of starch granules in the scutellum of germinating seeds from several grasses was observed (Smart and O'Brien, 1979; Matsukura *et al.*, 2000).

Sugars (fructose, glucose, and sucrose) were non-detectable or present at very low levels in non-germinated oat grains (Fig. 2). Glucose and sucrose started to accumulate in all tissues after a few days of germination, whereas fructose mostly accumulated in the embryo (Fig. 2). Glucose (which together with maltose is the major product of starch degradation in cereal grains; Bewley and Black, 1994; Aoki *et al.*, 2006) accumulated in the endosperm and peaked at 4 DAI when it constituted 3% of total endosperm DW (Fig. 2d). This peak in glucose content correlated well with the time point when the major part of the starch reserve in the endosperm was depleted (Fig. 1b). Between 4 DAI and 7 DAI, the amount of glucose in the endosperm dropped significantly and was depleted after 10 d of germination (Fig. 2d). Since it is unlikely that sucrose and fructose could be synthesized in the endosperm of germinating oat grain, the most probable explanation of their presence in the endosperm is leakage of these sugars from surrounding living cells of the scutellum and aleurone layer.

Mobilization of lipids during germination

Mobilization of endosperm TAG reserves (~8000 nmol FA $grain^{-1}$) during germination of oat grains started 2 DAI, and at 10 DAI there was 20% of the initial amount left (Fig. 3a). Compared with TAG, other complex lipids [polar lipids (PLs) and diacylglycerol (DAG)] were present at much lower levels in the endosperm in non-germinated grains and were more or less degraded during germination (Fig. 3a). However, the amount of FFA in the endosperm increased at 2 DAI and reached levels of 1300 nmol FA $grain^{-1}$ at 10 DAI (Fig. 3a). Thus the loss of lipids (mainly TAG) in the endosperm was much larger than the amount of FFAs that accumulated in this tissue. This indicates that the FAs from lipid degradation in the endosperm either were transported from or degraded within this tissue. Another explanation could be direct uptake of endosperm TAG by the scutellum.

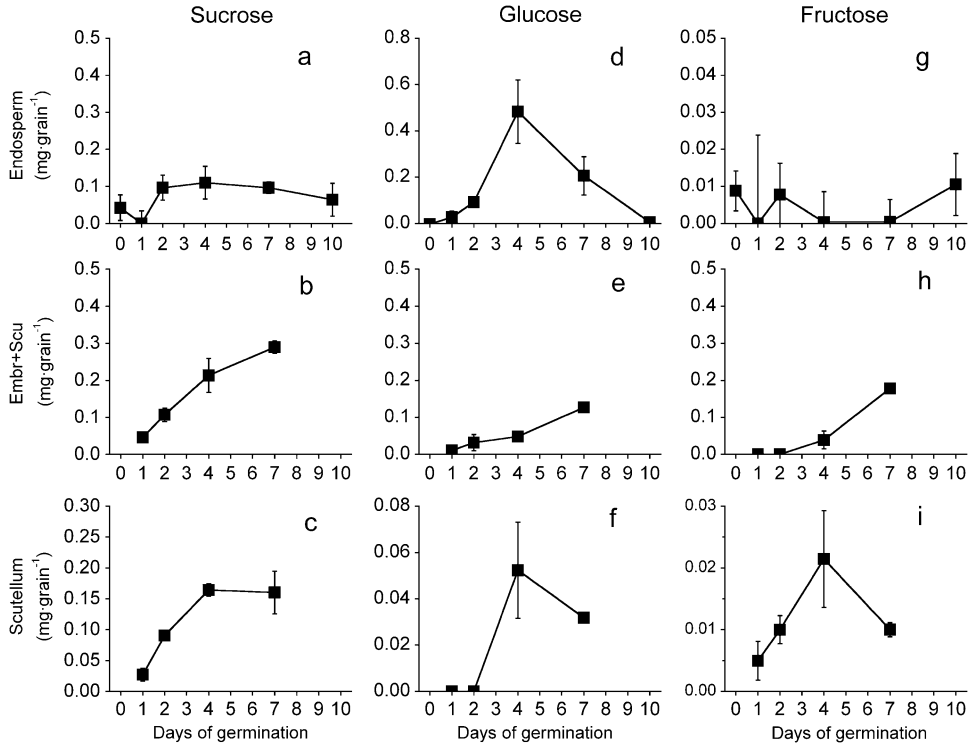

Fig. 2. Sugar content in endosperm, embryo+scutellum, and scutellum of germinating oat seeds of high-oil cv. Matilda. Sucrose (a, b, c), D-glucose (d, e, f), and D-fructose (g, h, i). Results are mean values ±SD of three samples.

The onset of lipid degradation at 2 DAI in the endosperm coincided with the accumulation of lipids in the embryo+scutellum; not only of PLs which is expected in growing tissues but also a high level of accumulation of TAG and FFAs (Fig. 3b). The fact that accumulation of FFAs in the scutellum started at 2 DAI whereas TAG degradation in this tissue did not start until 4 DAI indicates that FFAs in the scutellum most probably originated from FAs from lipids in the endosperm (Fig. 3c). The high levels of FFA in living cells as seen in the embryo+scutellum at 4 d and 7 d were unexpected since FFAs are potential detergent molecules. We therefore carried out investigations to determine if the extraction method could give rise to these FFAs by action of esterases on complex lipids and acyl-CoAs. However, no significant production of FFA could be seen from radioactive lipids or acyl-CoAs added to the frozen grain tissues just prior to extraction (results not shown).

The accumulation of PLs in the embryo+scutellum during germination was confined to the embryo since the scutellum alone did not contain any high levels of PLs during germination (Fig. 3b, c). Due to the high accumulation of lipids in the embryo+scutellum, the net loss of lipids from the whole grain (either in respiration or by conversion to other metabolites) during the first 7 d of germination was only 23%.

During the first day of germination, the TAG in the embryo+scutellum was degraded with no concomitant accumulation of FFA anywhere in the grain. The decrease of TAG reserves in the scutellum did not start until 4 DAI (assuming that TAG was not degraded in the scutellum during the first day of germination as reported for wheat; Tavener and Laidman, 1972) and at 10 DAI there was still 70% of the initial amounts left (Fig. 3c). The TAG left in the embryo+scutellum tissue at 1 DAI was mainly located in the scutellum (Fig. 3b, c) meaning that the major part of the TAG in the embryo was degraded early during germination of the oat grain.

Changes in FA profiles of lipids during germination

The FA composition of total lipid extracts from oat grains consists of three major FAs, palmitic (16:0), oleic (18:1), and linoleic acids (18:2), which altogether make up >95% of the total FA content, and several minor FAs [i.e. stearic; 18:0, linolenic; 18:3, avenoleic; Δ15-hydroxy-18:2$^{\Delta 9,12}$, and some other hydroxy and epoxy FAs (Banas *et al.*, 2007; Leonova *et al.*, 2008)]. In this study the FA profiles of the three most abundant lipid classes (TAG, FFAs, and PLs) were investigated in different tissues of oat grains during germination (Fig. 4).

The FA profile of TAG reserves differed between the endosperm and the embryo+scutellum, but there was no or very small changes in the FA profile of TAG during germination (Fig. 4a, d, g), which is in agreement with a previous study of malted oat grains (Peterson, 1999). However, the composition of the FFAs accumulating in all tissues of the oat grains during germination changed to a pattern almost identical to that of the TAG in the endosperm (Fig. 4b, e, h). The TAG in the scutellum started to be degraded 4 DAI but the FA profile of FFAs accumulating in this tissue continued to be much more

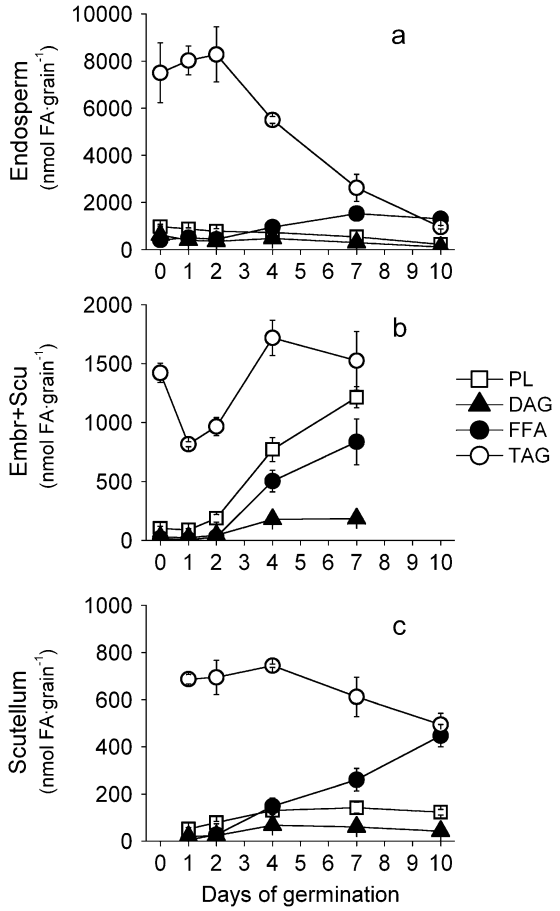

Fig. 3. Lipid content in endosperm (a), embryo+scutellum (b), and scutellum (c) of germinating oat seeds of the high-oil cv. Matilda. Polar lipids (PL), free fatty acids (FFA), and triacylglcyerol (TAG). Results are mean values ±SD of three samples.

similar to that of endosperm TAG and not to that of the TAG in the scutellum itself. This is another clear indication of endosperm TAG transfer to the scutellum, either as FFAs that are released by lipases from TAG in the endosperm or as intact TAG that is immediately degraded to FFAs by lipases in the scutellum. The possibility of direct uptake of TAG into the scutellum requires immediate degradation into FFAs for two reasons: no net increase in TAG amount in the scutellum was observed (Fig. 3c); and the FA profile of this TAG did not change into a profile more similar to that of the endosperm TAG during germination (Fig. 4a, g). The large pools of FFAs in the embryo+scutellum with a FA profile similar to that of FFAs in the scutellum suggested that there was further transport of FFA from the scutellum to the embryo during germination.

Interestingly, at 4 DAI when a significant proportion of endosperm TAG had been mobilized and FFAs had started to accumulate in all tissues (Fig. 3), the FA profile of different FFA pools in different tissues of the oat grains was not the same. The FFAs in the embryo and scutellum showed an FA profile at 4 DAI very similar to that of the endosperm TAG (see boxes in Fig. 4a, e, h), whereas the FA profile of the FFA in the endosperm itself at this time was different, with a much lower 18:1/18:2 ratio (see circle in Fig. 4b). It was estimated that the FA profile of the FFA pool present in the endosperm at 4 DAI would be likely to show the combined profile of FAs released from PLs and TAG (in amounts of 30% and 70%, respectively) between 0 DAI and 4 DAI. A prerequisite for this conclusion would be that the larger amount of TAG lost during this time is transferred to the scutellum as discussed above. Therefore, even though the amount of FAs released from PLs during this time was relatively small, it still had a high impact on the FA profile of the FFA pool in the endosperm at 4 DAI. As the accumulating FFA pool in the endosperm during germination was gradually reaching a level where the contribution from PLs can be neglected, the endosperm FFAs showed a profile identical to that of the endosperm TAG at 10 DAI.

The PL fractions from different tissues of the oat grain were distinguishable from each other by having a very different FA profile especially in 18:1/18:2 ratios (Fig. 4c, f, i). The difference in FA profile of PLs in the embryo and the scutellum at later stages of germination can be explained by the fact that these tissues have different functions; the embryo develops into shoots and roots during germination, whereas the scutellum is an absorptive structure inside the endosperm. Since PLs form the cellular membranes, it is therefore not unexpected that tissues with different functions show different FA profiles of PLs.

Structural study of germinating oat grains

LM was conducted to visualize changes that occurred in the oat grain at 1 DAI and 4 DAI (Fig. 5). At later stages of germination, fixation and sectioning became almost impossible due to the soft and rather liquid texture of the oat grain interior. Two staining methods, MAS and SB were used. MAS staining shows lipids as white, proteins as dark blue/green, and starch as pink; SB staining shows lipids as black, proteins as light brown, and starch as white. The different tissues of the oat grain were identified according to Fulcher (1986). The contours of the scutellum, a shield-shaped absorptive organ that penetrates the oat grain along the dorsal surface (Smart and O'Brien, 1979), can be seen through the pericarp of the grain in Supplememtary Fig. S1b, and in the longitudinal cut of the grain in Supplementary Fig. S1e available at *JXB* online.

At 1 DAI the oil in the subaleurone and starchy endosperm cells was present as confluent masses spread out between protein and intact large starch granules (Fig. 5a), whereas the aleurone and scutellum contained distinct oil droplets as previously shown (Banas *et al.*, 2007; Heneen *et al.*, 2008). At this time point, the cell walls of the endosperm cells were still intact (Fig. 5a, c), and the epithelium cells of the scutellum were tightly pressed against each other (Fig. 5c). The parenchyma of the scutellum was filled with dense material including oil droplets. No vacuoles or starch granules were visible in the scutellum at this stage (Fig. 5c).

At 4 DAI the most apparent change was the degradation of cell walls in the starchy endosperm, whereas in the

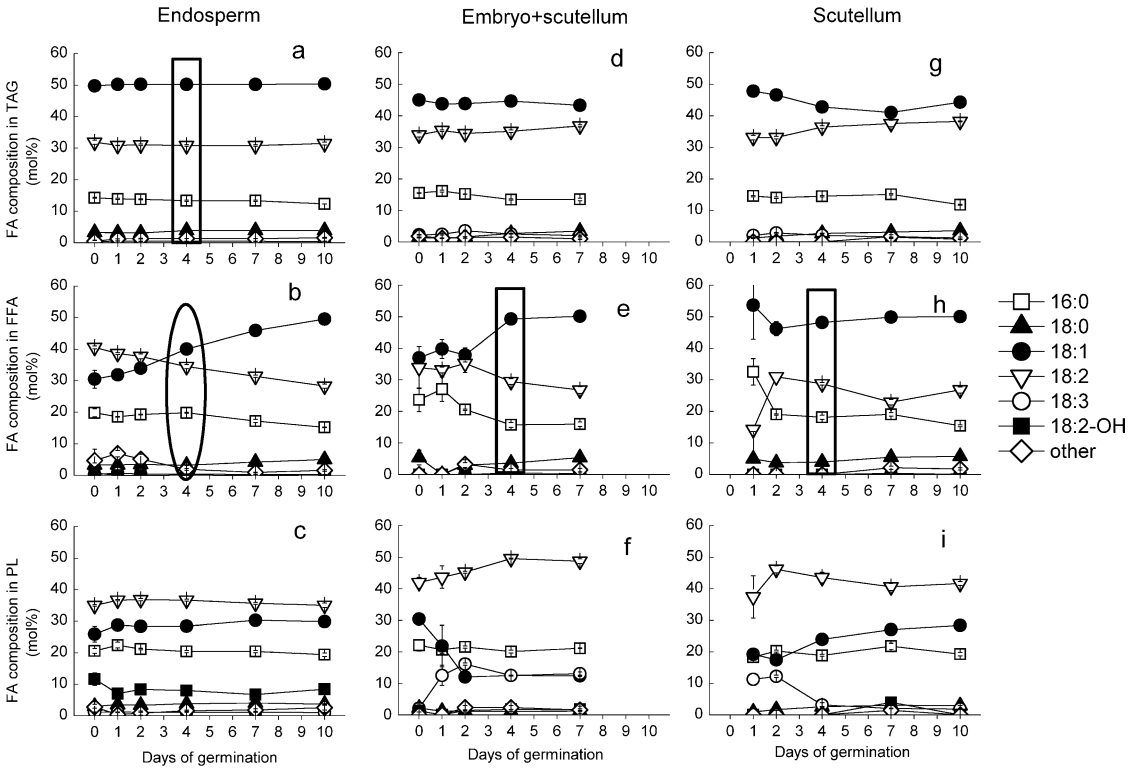

Fig. 4. Fatty acid profiles of the three main lipid classes present in the endosperm (a–c), embryo+scutellum (d–f), and scutellum (g–i) of germinating oat seeds of the high-oil cv. Matilda. Triacylglycerol (TAG; a, d, g), free fatty acids (FFA; b, e, h), and polar lipids (PL; c, f, i). Results are mean values ±SD of three samples. Boxes and circle highlight details discussed in the text.

aleurone layer the cell walls were still intact (Fig. 5b). In almost the entire endosperm, a major proportion of the previously large intact starch granules were now degraded into smaller granules (Fig. 5b, d, g). Moreover, the appearance of lipids in the endosperm cells in regions close to the aleurone differed from day 1. Lipids in the subaleurone cells and nearby endosperm cells seemed to have changed from the previous confluent oil masses to groups of oil droplets (Fig. 5b). At the same time, in the starchy endosperm near the scutellum, lipids could be seen as confluent masses as well as round droplets, either in close proximity to or sometimes in direct contact with the scutellar epithelium cells (Fig. 5d, e, g, h). This droplet-like lipid material could either be TAG, or a mixture of TAG breakdown products, including FFAs, released by lipases secreted from the scutellum. The fact that both staining methods (MAS and SB) stained the same particles with a dark colour could be an indication of co-localization of lipids and proteins. Moreover, accumulation of darkly stained material, most probably lipids, was observed during the germination process inside the epithelium cells (Fig. 5d). The remnants from cell walls seen at 1 DAI in the depleted layer next to the scutellar epithelium were no longer visible at 4 DAI.

At 4 DAI, the scutellar epithelium cells had become very elongated (Fig. 5d, g), with the lateral sides separated from each other, giving them a finger-like appearance as previously shown (Bewley and Black, 1994). This adds to the surface area capable of intake of nutrients from the starchy endosperm. At 4 DAI, starch granules were observed accumulating in the parenchyma cells of the scutellum (Fig. 5f). At the same time, the cell content in the scutellar epithelium had become less dense (seen on both MAS and SB staining) with a concomitant appearance of vacuoles (Fig. 5f, g). The development of vascular tissues in the scutellar parenchyma at 4 DAI was obvious (not shown).

The TEM study was undertaken to get a closer view of regions where close contact between scutellar epithelium and oil droplets was observed at 4 DAI (Fig. 6). The study revealed that these oil droplets do not have distinct borders but instead are rather diffuse. No organelles (putative peroxisomes or glyoxysomes) were seen around them. The oil droplets were positioned tightly along the cell walls of scutellum epithelium cells.

Comparison of nutrient reserve mobilization between two oat cultivars with different oil amounts

The comparison of two oat cultivars with different amounts of oil and starch in the endosperm reflects if and how an increased oil content of the cereal storage tissue affects the germination process. For this purpose, oat grains of high-oil cv. Matilda were analysed in parallel with the medium-oil cv. Freja (see Supplementary Figs S2–S4 at *JXB* online). The mature grains of cv. Matilda had 44% more oil and 7% less starch in the endosperm compared with the medium-oil cv. Freja (Figs 1b, 3a, Supplementary Figs S2b, S4a).

The onset of TAG mobilization in both the endosperm and the scutellum started 1 d earlier in the medium-oil cv.

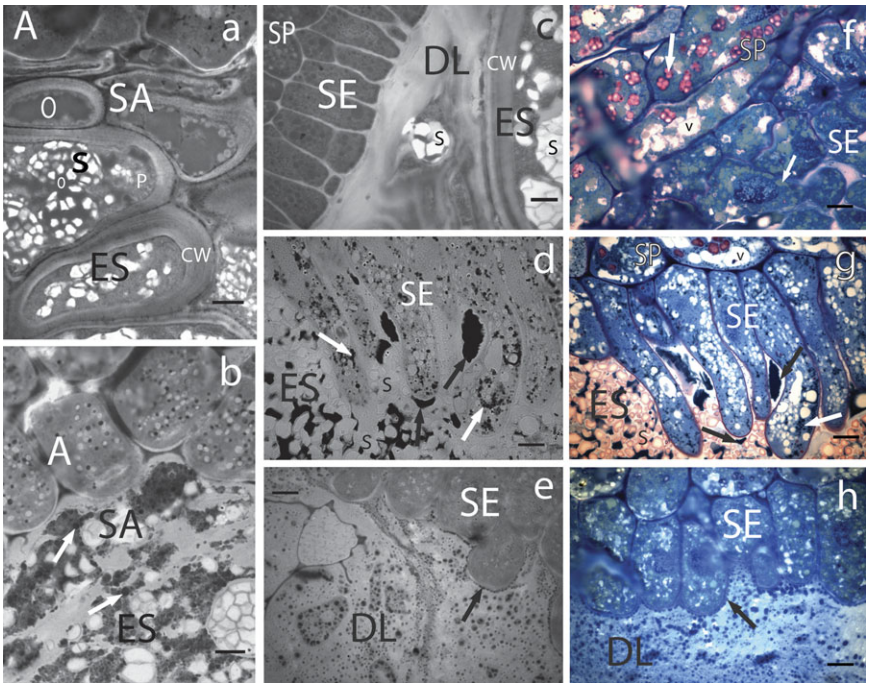

Fig. 5. Light microscopy of sectioned oat grains (cv. Matilda) 1 DAI (a, c) and 4 DAI (b, d–h) post-stained with Sudan Black (a–e) and methylene blue–azur A–safranin O (f–h). (a, b) An oat grain at 1 DAI (a) and 4 DAI (b) with the aleurone layer (A), subaleurone layer (SA), and inner endosperm cells (ES), depicting oil mass (O), proteins (P), starch (S), cell walls (CW), and oil droplets (arrow). (c) An oat grain at day 1 with endosperm, depleted layer (DL), and scutellar epithelium cells (SE) tightly pressed against each other and scutellar parenchyma cells (SP) filled with dense cell material with no vacuoles or starch granules visible. (d, g) Scutellum at 4 DAI with accumulation of darkly stained material, most probably lipids, inside the epithelium cells (white arrows) and oil masses in the endosperm outside the epithelium cells (black arrows), v, vacuoles. (e, h) Scutellum epithelium cells at day 4 surrounded by a chain of droplets. (f) Lipids (white arrow) in the scutellum epithelium and parenchyma cells, and starch granules (black arrow) in parenchyma cells. Scale bars are 10^μm. All sections were taken from the same part of the seed (the first third of the grain proximal to the embryo).

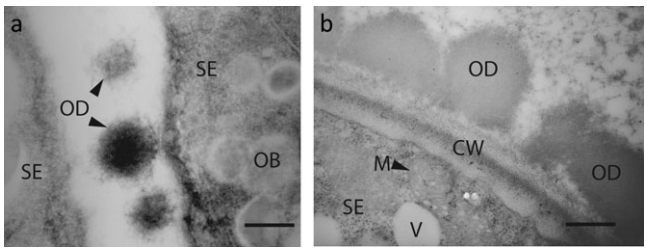

Fig. 6. TEM of germinating oat grains at 4 DAI. (a) The oil bodies (OB) inside the scutellum epithelium cells and the oil droplets (OD, arrowheads) between the two scutellum epithelium cells (SE) are seen. Note the rather diffuse boundaries of OD compared with OB. (b) Oil droplets (OD) are seen to be attached to the cell wall (CW) of a scutellum epithelium cell (SE). Mitochondria (M) and vacuoles (V) are visible. Scale bar 0.5^μm.

Freja compared with the high-oil cv. Matilda (Fig. 3, Supplementary Fig. S4 at *JXB* online). At 10 DAI all the TAG reserves in the endosperm of cv. Freja were depleted, whereas in cv. Matilda the endosperm still had 20% of the initial amounts left. The high-oil cultivar was also more efficient in accumulating lipids in the embryo+scutellum compared with cv. Freja, thus providing a much lower net loss of lipids from the whole grain during the first 7 d of germination (23% and 44% of initial amounts, in cv. Matilda and Freja, respectively, which corresponds to 2690 nmol FA grain^{-1} and 3839 nmol FA grain^{-1}, respectively; Fig. 3, Supplementary Fig. S4). The average rate of lipid transport from the endosperm between the onset of TAG degradation and 10 DAI was 46% higher in cv. Matilda compared with cv. Freja (9800 nmol FA grain^{-1} d^{-1} and 670 nmol FA grain^{-1} d^{-1}, respectively).

There was a remarkable peak in starch accumulation in the scutellum of the high-oil cv. Matilda on day 4 when the scutellum contained 20% starch by DW, after which it decreased dramatically (Fig. 1f). This was in sharp contrast to cv. Freja where starch levels of the scutellum were much lower all throughout germination (Supplementary Fig. S2f at *JXB* online). However, there was a tendency towards a higher sugar content in germinating tissues of cv. Freja compared with those of cv. Matilda, especially for glucose in the embryo (Fig. 2e, Supplementary S3e).

Discussion

Mobilization of lipid reserves in the cereal grain during germination

Very little is known about the mobilization of oil in the endosperm of cereals and in which form it is supplied as energy to the growing embryo during germination. There

are three alternatives by which the oil reserves of the endosperm could be supplied to the growing embryo during germination; either as intact TAG, as FFAs (released from TAG by lipases), or as sugars (through FFA degradation by β-oxidation with subsequent gluconeogenesis), or a combination of these alternatives. Evidence for uptake of TAG in plant cells is lacking but should not be excluded. Moreover, the cereal endosperm is a dead tissue and FA breakdown through peroxisomal β-oxidation and further conversion into sugars should therefore not be possible in this tissue (Young and Gallie, 2000). This is in contrast to both the aleurone layer and the scutellum of germinating cereal grains where enzymes for FFA degradation are present (Oaks and Beevers, 1964; Doig and Laidman, 1972; Jones, 1972; Doig *et al.*, 1975). Together with the fact that oil in the mature oat endosperm appears as large fused oil areas rather than oil bodies, this raises the question of whether the cereal oil reserve cannot be mobilized for use in the growing embryo during germination (Banas *et al.*, 2007; Heneen *et al.*, 2008). However, the present data on germinating oat grains clearly show that the oil reserve in the endosperm is mobilized during germination and transferred to the growing tissues of the grain.

It is generally believed that the oil stored in the embryo and aleurone of cereals is mobilized early during germination before the arrival of sugars from starch degradation in the endosperm fuels embryo growth (Clarke *et al.*, 1983). The present study showed that in oat, just like in wheat (Tavener and Laidman, 1972), the oil stored in the embryo is mobilized at an early phase of germination, whereas the oil reserves of the scutellum are mobilized at a later stage. Since FFAs did not accumulate in the embryo (or anywhere else in the grain) during the first day of germination, it is most likely that the oil degraded in the embryo during the first day of germination was either instantly respired or converted into sugars.

The fate of the TAG reserve in the endosperm during germination

Mobilization of TAG reserves in the endosperm of oat did not start during the first day of germination as reported for wheat (Tavener and Laidman, 1972); it started later at 1–2 DAI. This coincided with the accumulation of FFAs in this tissue with a FA profile that gradually turned identical to that of the disappearing endosperm TAG, which demonstrates the presence of lipase activity in the endosperm. In cereals, both aleurone and scutellum are thought to be possible sources of lipases (Jensen and Heltved, 1982; Urquhart *et al.*, 1983; Ekstrand *et al.*, 1992).

Taken together, the facts that, first, FFAs did not accumulate in the oat endosperm in stochiometric amounts corresponding to the quantity of TAG degradation in this tissue, and, secondly, FFAs with a profile similar to that of the endosperm TAG accumulated in both the scutellum and embryo, indicate that the oat scutellum is absorbing the FFAs released from the endosperm lipids (mainly TAG) during germination. This conclusion is supported by studies of germinating oil palm (*Elaeis guineensis*) kernel, a non-cereal monocotyledonous seed, where the release of FFAs from TAG occurs in the endosperm and the FFAs are then absorbed by the haustorium, a large absorptive structure characteristic of the palm tree family (Boatman and Crombie, 1958; Oo and Stumpf, 1983; Alang *et al.*, 1988). However, it should be mentioned that we could not exclude the possibility that endosperm TAG was instead taken up directly by the scutellum and immediately degraded into FFAs by lipases in this tissue even though evidence for such TAG transport is lacking.

The absorbed FFAs in the oat scutellum are most probably then degraded through β-oxidation and subsequently converted into sugars that can be further transported to the embryo through the scutellar vasculature as proposed for germinating wheat (Aoki *et al.*, 2006). This is supported by the presence of β-oxidation and glyoxylate cycle activities in the scutellum of germinating maize (Oaks and Beevers, 1964).

The observation of slower change from a PL-like to TAG-like FFA profile in oat endosperm compared with that in scutellum and embryo could be explained by two different sources of lipases in the oat grains during germination: one lipase from the aleurone cells that is mainly active towards endosperm PLs (but also to a lower extent to TAG) and one lipase from the scutellum that is mainly active towards endosperm TAG. At the onset of germination, TAG near the scutellum would then be degraded by its lipases and these FFAs are immediately absorbed, while FFAs from PLs that are degraded away from the absorptive scutellum are still present and thus influence the FA profile of the endosperm. Later, PLs are completely mobilized and only FFAs from TAG, the result of both types of lipases, are left. This hypothesis is in agreement with the suggested sources of lipases in the cereal grain during germination as discussed above (Jensen and Heltved, 1982; Urquhart *et al.*, 1983; Ekstrand *et al.*, 1992). The presence of acylhydrolases with substrate specificity towards different lipid classes including PLs, shown in algae and *Arabidopsis*, also supports this hypothesis (Terasaki and Itabashi, 2003; Illijas *et al.*, 2008; Seo *et al.*, 2009).

The present structural studies on oat grains also indicated an important role for the scutellum in endosperm oil utilization during germination, i.e. the uptake of lipid reserves. Specifically, it showed that the oil in the endosperm came in close contact with the epithelium cells of the scutellum during germination. Regarding the lipid compounds inside the scutellum, it cannot be concluded from this study whether they are imported FFAs or other neutral lipids such as TAG. However, according to the present quantitative analytical data they are most probably FFAs since these are accumulating while the amount of TAG is either constant or decreasing in this tissue. The spatial division of TAG catabolism between the endosperm and other parts in the monocot seed contrasts with the situation in castor bean (*Ricinus communis*), an endospermic dicotyledon, where the complete conversion of TAG to sugars

takes place in the endosperm (Hutton and Stumpf, 1969; Huang and Beevers, 1974; Nishimura and Beevers, 1979).

Further transport of FFAs in the scutellum to the embryo

The accumulation of FFAs with a FA profile similar to that of the endosperm TAG, not only in the scutellum, but also in the embryo, suggested that there was a direct transport of oat endosperm FFAs via the scutellum into the embryo. The intra- and intermembrane mechanisms for cellular lipid transport in plants are not fully understood, but both vesicular and non-vesicular transport by specific proteins exist (Voelker, 2009). Lipid transport between cells is a less studied phenomenon. Interestingly, the fact that phloem sap of oil-seed rape was shown to contain lipids of which more than half was in the form of FFAs but also some TAG (Madey *et al.*, 2002) suggests that cell–cell transport of both FFAs and TAG actually occurs in living plant tissues.

In the present study, the FA profile of accumulating TAG in the embryo during germination was different from that of both accumulating FFAs in the embryo and endosperm and TAG in the endosperm. Therefore, this accumulating TAG in the embryo is most probably neither the result of long-distance transport of TAG from the ndosperm to the embryo via the scutellum nor the result of esterification of transported FFAs from the scutellum. Rather, these FFAs in the embryo are degraded to sugars, and the accumulating TAG is the result of *de novo* synthesized FAs in the embryo.

The physical appearance of oil reserves in the endosperm changes during germination

In some endosperm regions, especially close to the scutellum, the subaleurone layer, and nearby endosperm cells, there was a shift in the appearance of oil, from confluent oil masses at 1 DAI as previously described to be present in mature oat grains (Heneen *et al.*, 2008), to droplets at 4 DAI. As can be seen from Fig. 6, these oil droplets are unlikely to be true oil bodies surrounded by oleosins. No discrete boundaries were observed, but instead they had a rather diffuse character. Oleosins—proteins associated with oil bodies and believed to influence the size and stability of the oil bodies (Murphy, 1993; Hsieh and Huang, 2004; Siloto *et al.*, 2006; White *et al.*, 2008)—are known to be present at a high level in living cells of the embryo, scutellum, and the aleurone layer, but at a much lower level in the endosperm of mature oat grains (White *et al.*, 2005; Heneen *et al.*, 2008). The histological staining suggested that endospermal oil droplets in germinating oat grains contain proteins, but it is unlikely that oleosins are included among them. The presence of oleosins in endosperm tissues would have required these proteins to be synthesized in living tissues and transported to the endosperm during germination, which is rather unrealistic. Therefore, the proteins associated with the oil droplets in the LM study are most probably lipases hydrolysing the lipid molecules of the oil droplets.

Influence of endosperm oil amount on germination

The comparison between two oat cultivars with different amounts of oil in the endosperm showed that germination was not negatively affected by a higher oil content under the conditions used in the present study (the biomass increase of the growing grain tissues was the same for both cultivars). The fact that the high-oil cultivar showed a higher rate of lipid transfer from the endosperm during germination compared with the medium-oil cultivar suggests that lipid transport is not limiting the transfer of energy that is trapped in lipids in the endosperm cereals during germination, at least within the range of these oil contents.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Photos of oat grains of cv. Matilda showing growth of the embryo and scutellum after 0 (a), 1 (b), 2 (c), 4 (d), 7 (e), and 10 d (f) of germination. The contour of the scutellum can be seen under the pericarp (see arrow in b) and in the longitudinal cut of the grain (see arrows in e).

Figure S2. Dry weight and amount of starch in the endosperm, embryo+scutellum, and scutellum of germinating oat grains of medium-oil cv. Freja.

Figure S3. Amounts of sucrose, D-glucose, and D-fructose in the endosperm, embryo+scutellum, and scutellum of germinating oat grains of medium-oil cv. Freja.

Figure S4. Amounts of different lipid classes in the endosperm, embryo+scutellum, and scutellum of germinating oat grains of medium-oil cv. Freja.

Figure S5. Fatty acid profiles of the three main lipid classes present in the endosperm, embryo+scutellum, and scutellum of germinating oat grains of medium-oil cv. Freja.

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References

Alang ZC, Moir GFJ, Jones LH. 1988. Composition, degradation and utilization of endosperm during germination in the oil palm (*Elaeis guineensis* Jacq.). *Annals of Botany* **61,** 261–268.

Åman P, Hesselman K. 1984. Analysis of starch and other main consituents of cereal grains. *Swedish Journal of Agricultural Research* **14,** 135–139.

Aoki N, Scofield GN, Wang X-D, Offler CE, Patrick JW, Furbank RT. 2006. Pathway of sugar transport in germinating wheat seeds. *Plant Physiology* **141,** 1255–1263.

Banas A, Debski H, Banas W, *et al*. 2007. Lipids in grain tissues of oat (*Avena sativa*): differences in content, time of deposition, and fatty acid composition. *Journal of Experimental Botany* **58,** 2463–2470.

Beck E, Ziegler P. 1989. Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40,** 95–117.

Beevers H. 1979. Microbodies in higher plants. *Annual Review of Plant Physiology* **30,** 159–193.

Bewley JD, Black M. 1994. *Seeds. Physiology of development and germination*. New York: Plenum Press.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37,** 911–917.

Boatman SG, Crombie M. 1958. Fat metabolism in the West African oil palm (*Elaeis guineensis*). *Journal of Experimental Botany* **9,** 52–74.

Brown CM, Alexander DE, Carmer SG. 1966. Variation in oil content and its relation to other characters in oats (*Avena sativa* L.). *Crop Science* **6,** 190–191.

Clarke NA, Wilkinson MC, Laidman DL. 1983. Lipid metabolism in germinating cereals. In: Barnes PJ, ed. *Lipids in cereal technology*. London: Academic Press, 57–92.

Doig RI, Colborne AJ, Morris G, Laidman DL. 1975. Induction of glyoxysomal enzyme-activities in aleurone cells of germinating wheat. *Journal of Experimental Botany* **26,** 387–398.

Doig RI, Laidman DL. 1972. The occurrence of an induced glyoxylate cycle in wheat aleurone tissue. *Biochemical Journal* **128,** 88.

Ekstrand B, Gangby I, Åkesson G. 1992. Lipase activity in oats—distribution, pH dependence, and heat inactivation. *Cereal Chemistry* **69,** 379–381.

FAOSTAT. 2008. *ProdSTAT, crops processed*. Rome: Food and Agriculture Organization of the United Nations.

Fincher GB. 1989. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annual Review of Plant Physiology and Plant Molecular Biology* **40,** 305–346.

Frey KJ, Holland JB. 1999. Nine cycles of recurrent selection for increased grain-oil content in oat. *Crop Science* **39,** 1636–1641.

Fulcher RG. 1986. Morphological and chemical organization of the oat kernel. In: Webster FH, ed. *Oats: chemistry and technology.* St Paul, MN: American Association of Cereal Chemists. 47–74.

Graham IA. 2008. Seed storage oil mobilization. *Annual Review of Plant Biology* **59,** 115–142.

Heneen WK, Karlsson G, Brismar K, *et al*. 2008. Fusion of oil bodies in endosperm of oat grains. *Planta* **228,** 589–599.

Hsieh K, Huang HC. 2004. Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. *Plant Physiology* **136,** 3427–3434.

Huang AHC, Beevers H. 1974. Developmental changes in endosperm of germinating castor bean independent of embryonic axis. *Plant Physiology* **54,** 277–279.

Huang AHC, Trelease R, Moore T. 1983. *Plant peroxisomes*. New York: Academic Press.

Hutton D, Stumpf PK. 1969. Characterization of the β-oxidation systems from maturing and germinating castor bean seeds. *Plant Physiology* **44,** 508–516.

Illijas MI, Terasaki M, Nakamura R, Iljima N, Hara A, Fusetani N, Itabashi Y. 2008. Purification and characterization of glycerolipid acylhydrolase from the red alga. *Gracilaria vermiculophylla. Fisheries Science* **74,** 670–676.

Jensen SA, Heltved F. 1982. Visualization of enzyme activity in germinating cereal seeds using a lipase sensitive fluorochrome. *Carlsberg Research Community* **47,** 297–303.

Jones RL. 1972. Fractionation of enzymes of barley aleurone layer—evidence for a soluble mode of enzyme release. *Planta* **103,** 95–109.

Kaukovirta-Norja A, Wilhelmson A, Poutanen K. 2004. Germination: a means to improve the functionality of oat. *Agricultural and Food Science* **13,** 100–112.

Leng ER. 1961. Predicted and actual responces during long-term selection for chemical composition in maize. *Euphytica* **10,** 368–378.

Leonova S, Shelenga T, Hamberg M, Konarev AV, Loskutov I, Carlsson AS. 2008. Analysis of oil composition in cultivars and wild species of oat (*Avena* sp.). *Journal of Agricultural and Food Chemistry* **56,** 7983–7991.

Madey E, Nowack LM, Thompson JE. 2002. Isolation and characterization of lipid in phloem sap of canola. *Planta* **214,** 625–634.

Matsukura C-a, Saitoh T, Hirose T, Ohsugi R, Perata P, Yamaguchi J. 2000. Sugar uptake and transport in rice embryo. Expression of companion cell-specific sucrose transporter (*OsSUT1*) induced by sugar and light. *Plant Physiology* **124,** 85–94.

Murphy DJ. 1993. Structure, functions and biogenesis of storage lipid bodies and oleosins in plants. *Progress in Lipid Research* **32,** 247–280.

Nishimura M, Beevers H. 1979. Subcellular distribution of gluconeogenetic enzymes in germinating castor bean endosperm. *Plant Physiology* **64,** 31–37.

O'Brien TP, McCully ME. 1981. *The study of plant structure: principles and selected methods*. Melbourne: Termarcarphi Pty. Ltd.

Oaks A, Beevers H. 1964. The glyoxylate cycle in maize scutellum. *Plant Physiology* **39,** 431–434.

Oo KC, Stumpf PK. 1983. Some enzymic activities in the germinating oil palm (*Elaeis guineensis*) seedling. *Plant Physiology* **73,** 1028–1032.

Peterson DM, Wood DF. 1997. Composition and structure of high-oil oat. *Journal of Cereal Science* **26,** 121–128.

Price PB, Parsons J. 1979. Distribution of lipids in embryonic axis, bran-endosperm, and hull fractions of hulless barley and hulless oat grain. *Journal of Agricultural and Food Chemistry* **27,** 813–815.

Price PB, Parsons JG. 1975. Lipids of seven cereal grains. *Journal of the American Oil Chemists Society* **52,** 490–493.

Seo YS, Kim EY, Kim JH, Kim WT. 2009. Ensymatic characterization of class I DAD1-like acylhydrolase members targeted to chloroplast in. *Arabidopsis. FEBS Letters* **583,** 2301–2307.

Siloto RMP, Findlay K, Lopez-Villalobos A, Yeung EC, Nykiforuk CL, Moloney MM. 2006. The accumulation of oleosins determines the size of seed oil bodies in Arabidopsis. *The Plant Cell* **18,** 1961–1974.

Smart MG, O'Brien TP. 1979. Observations on the scutellum I. Overall development during germinatiopn in four grasses. *Australian Journal of Botany* **27,** 391–401.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26,** 31–43.

Tavener RJA, Laidman DL. 1972. The induction of triglyceride metabolism in the germinating wheat grain. *Phytochemistry* **11,** 981–987.

Terasaki M, Itabashi Y. 2003. Glycerolipid acyl hydrolase activity in the brown alga *Cladosiphon okamuranus* Tokida. *Bioscience, Biotechnology, and Biochemistry* **67,** 1986–1989.

Urquhart AA, Altosaar I, Matlashewski GJ. 1983. Localization of lipase activity in oat grains and milled oat fractions. *Cereal Chemistry* **60,** 181–183.

Voelker DR. 2009. Genetic and biochemical analysis of non-vesicular lipid traffic. *Annual Review of Biochemistry* **78,** 827–856.

Warmke HE, Lee SLJ. 1976. Improved staining procedures for semithin epoxy sections of plant-tissues. *Stain Technology* **51,** 179–185.

White DA, Fisk ID, Gray DA. 2005. Characterisation of oat (*Avena sativa* L.) oil bodies and intrinsically associated E-vitamers. *Journal of Cereal Science* **43,** 244–249.

White DA, Fisk ID, Mitchell JR, Wolf B, Hill SE, Gray DA. 2008. Sunflower-seed oil body emulsions: rheology and stability assessment of a natural emulsion. *Food Hydrocolloids* **22,** 1224–1232.

Young TE, Gallie DR. 2000. Regulation of programmed cell death in maize endosperm by abscisic acid. *Plant Molecular Biology* **42,** 397–414.

Zhou M, Robards K, Glennie-Holmes M, Helliwell S. 1999. Oat lipids. *Journal of the American Oil Chemists Society* **76,** 159–169.

Ziegler P. 1995. Carbohydrate degradation during germination. In: Kigel J, Galili G, eds. *Seed development and germination*. New York: CRC Press, 447–474.