Comparative Transcriptomics Analysis of *Brassica napus* L. during Seed Maturation Reveals Dynamic Changes in Gene Expression between Embryos and Seed Coats and Distinct Expression Profiles of Acyl-CoA-Binding Proteins for Lipid Accumulation

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Production of vegetable oils is a vital agricultural resource and oilseed rape (*Brassica napus*) is the third most important oil crop globally. Although the regulation of lipid biosynthesis in oilseeds is not fully defined, the acyl-CoA-binding proteins (ACBPs) have been reported to be involved in such metabolism, including oil accumulation, in several plant species. In this study, progressive changes in gene expression in embryos and seed coats at different stages of seed development were comprehensively investigated by transcriptomic analyses in *B. napus*, revealing dynamic changes in the expression of genes involved in lipid biosynthesis. We show that genes encoding BnACBP proteins show distinct changes in expression at different developmental stages of seed development and show markedly different expression between embryos and seed coats. Both isoforms of the ankyrin-repeat BnACBP2 increased during the oil accumulation period of embryo development. By contrast, the expression of the three most abundant isoforms of the small molecular mass BnACBP6 in embryos showed progressive reduction, despite having the highest overall expression level. In seed coats, BnACBP3 and BnACBP4 and BnACBP5 expressions remained constant during development, whereas the two major isoforms of BnACBP6 increased, contrasting with the data from embryos. We conclude that genes related to fatty acid and triacylglycerol biosynthesis showing dynamic expression changes may regulate the lipid distribution in embryos and seed coats of *B. napus* and that BnACBP2 and BnACBP6 are potentially important for oil accumulation.

**Keywords:** ACBP • *Brassica napus* • Embryos • Fatty acid • Oilseed rape • Seed coats.

**Accession numbers:** The transcriptome raw data are deposited at the Sequence Read Archive (SRA) database of GenBank under the accession number PRJNA510462. Sequence data for BnACBPs used in this study were retrieved from the CNS-Genoscope (http://www.genoscope.cns.fr/brassicanapus/; Chalhoub et al. 2014): BnACBP1 (BnaA02g10270D and BnaC02g44810D), BnACBP2 (BnaA01g16660D and BnaC01g20440D), BnACBP3 (BnaA01g13710D, BnaA03g46540D, BnaC01g16110D and BnaC07g38820D), BnACBP4 (AIS76194, AIS76195, AIS76196, AIS76199, AIS76200 and AIS76201), BnACBP5 (AIS76197 and AIS76199) and BnACBP6 (BnaA0ng25690D, BnaA05g36060D, BnaCnng15340D and BnaA08g07670D). BnTIP41 (EV222761) was retrieved from the GenBank data library

**Introduction**

Oil crops are a very important agricultural commodity contributing about 155 million tonnes of oil per annum (Gunstone et al. 2007). Moreover, the demand for vegetable oils has been rising at 5% per year for the last 50 years (Gunstone et al. 2007). With increased interest in plant oils as renewable chemicals and, to a lesser extent, as biofuels this demand is only likely to increase (Harwood et al. 2017). Four major crops account for over 80% of total production and are, in order of importance, oil palm, soybean, rapeseed and sunflower (Weselake et al. 2017).

Oilseed rape, mainly *Brassica napus* L., is the main oil crop in Europe (Harwood et al. 2017). Together oilseed rape accounts for around 16% of total vegetable oil production (Gunstone et al. 2007, Taylor et al. 2011). Oilseed rape is also the main source of biodiesel in Canada and Europe (Harwood et al. 2017).

Triacylglycerol (TAG) is the dominant oil component and is produced in two overall steps. First, fatty acid (FA) biosynthesis in plastids is catalyzed by the multienzyme complexes of acetyl-CoA carboxylase and FA synthase. In most plants (and oilseed rape is typical), the main products of FA synthase, palmitate and stearate, are produced in a ratio of about 1:6. Stearate (as its acyl carrier protein ester) is a substrate for a very active Δ-9

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desaturase in the chloroplast stroma to yield oleate. Apart from a few plants like cocoa (Theobroma cacao) or shea (Butyrospermum parkii), stearate rarely accumulates (Harwood et al. 2017). The palmitate and oleate products of de novo FA formation are hydrolyzed by thioesterase enzymes (FATA and FATB; Salas and Ohlrogge 2002) and then re-esterified to coenzyme A by long-chain acyl-CoA synthetases (LACs; Weselake et al. 2009, Lu et al. 2011, Bates et al. 2013, Bates 2016). They then join the acyl-CoA pool in the cytosol. Acyl-CoA esters participate in various acyltransferase reactions for acyl lipid formation in the endoplasmic reticulum (ER). In addition, the production of phosphatidylcholine is important (Chen et al. 2015) because this phosphoglyceride is a substrate for the ER FA desaturases (FAD2 and FAD3) that give rise to linoleate and α-linolenate, respectively (Wallis and Browse 2002).

It is clear that there is substantial movement of FAs from plastids to the ER during TAG accumulation in oil crops (Wallis and Browse 2002, Bates et al. 2013). As lipids (even as thioesters) are poorly soluble in aqueous solutions, it is more efficient to bind them to proteins for transport. Moreover, acyl-CoAs are toxic to many enzymes through detergent-like effects and even at concentrations as low as 10⁻⁷ M can regulate metabolism (Faergeman and Knudsen 1997). Thus, binding (and transport) proteins have very important functions in vivo. Various proteins have been identified which can bind lipids (including acyl-CoAs; Du et al. 2016) and these include acyl-CoA-binding proteins (ACBPs) of which there are six well-characterized forms in Arabidopsis and in rice (Oryza sativa; Du et al. 2016).

The first ACBP reported in plants was a 10-kDa protein which was strongly expressed in seeds, flowers and cotyledons of oilseed rape (B. napus L.; Hills et al. 1994). Further studies, especially in Arabidopsis and rice identified four different classes of ACBPs. Class I proteins are small molecular mass proteins (around 10 kDa) which are soluble and located in the cytosol, whereas Class II proteins have a transmembrane domain and ankyrin repeats. Class III ACBPs also have a transmembrane domain, whereas Class IV ACBPs have Kelch motifs but no transmembrane motif. All the ACBPs show a conserved acyl-CoA-binding domain (see Xiao and Chye 2011, Du et al. 2016).

Apart from Arabidopsis and rice, ACBPs have been reported (and characterized) from a number of plants (Du et al. 2016). Six ACBPs representing four classes are found in oilseed rape (Raboanatahiry et al. 2015a, Raboanatahiry et al. 2015b, Raboanatahiry et al. 2018). The 10-kDa ACBP first reported by Hills et al. (1994) was shown to bind various long-chain acyl-CoAs (Brown et al. 1998, Yurchenko et al. 2009). Notably, it also regulated the activities of important enzymes involved in oil synthesis such as glycerol 3-phosphate acyltransferase (GPAT; Brown et al. 1998), lysophosphatidic acid acyltransferase (LPAAT; Brown et al. 2002) and lysophosphatidylcholine acyltransferase (LPCAT; Yurchenko et al. 2009). It was also shown to participate in acyl-CoA transport (Johnson et al. 2002) and to promote exchange between the acyl-CoA and phosphoglyceride pools (Yurchenko et al. 2009, Yurchenko et al. 2014). The function of ACBPs in FA biosynthesis has also been reported previously in Arabidopsis (Yurchenko et al. 2009, Yurchenko et al. 2014, Lung et al. 2017, Lung et al. 2018). The overexpression of B. napus 10-kDa ACBP in developing Arabidopsis seeds resulted in increased polyunsaturated FAs (18:2-FA and 18:3-FA), at the expense of saturated and very long monounsaturated FAs (20:1; Yurchenko et al. 2009, Yurchenko et al. 2014). Furthermore, Arabidopsis ACBP1 overexpressors or an acbp1 mutant showed changed FA composition in siliques and phloem, whereas an AtACBP3-RNAi line displayed a reduced FA content (Hu et al. 2018).

Although the embryo is the major site for seed oil accumulation, the seed coat also plays an important role for lipid biosynthesis (Shi et al. 2012, Woodfield et al. 2017). The Arabidopsis transcription factor GLABRA2 (GL2) regulates the MUCILAGE MODIFIED4 (MUM4) gene in the seed coat which affects seed oil biosynthesis (Shi et al. 2012). Moreover, loss of GL2 or MUM4 function in the seed coat resulted in increased seed oil, at the expense of seed coat mucilage biosynthesis (Shi et al. 2012). The distinct lipid metabolism within seed coats compared with embryos (Woodfield et al. 2017) allows a comparison in order to delineate the roles of individual proteins, such as ACBPs, in B. napus. Furthermore, although Arabidopsis ACBPs have been extensively studied regarding their function during stress (Xiao and Chye 2011), rather less is known of their role in development and oil accumulation in any plant species (Du et al. 2016).

To understand the molecular basis of the different distribution of TAGs and phosphatidylcholines (PCs) between embryos and seed coats and to reveal putative ACBP functions for TAG biosynthesis during seed development, we conducted a comparative transcriptomics analysis and detailed study of ACBP expression during seed development in a low erucic acid (LEAR:Canola) variety of B. napus. The results show that genes related to FA biosynthesis in plastids may regulate the lipid distribution between embryos and seed coats. Also, the results reveal some very specific changes in expression patterns for different BnACBPs and their individual isoforms that may indicate not only distinct roles during oil accumulation and seed development but also tissue-specific differences in their functions.

**Results**

**Characterization of B. napus seed morphology and oil content during development**

When the morphology of B. napus embryos at different developmental stages was analyzed, embryos at 27, 38 and 45 days after flowering (DAF; Fig. 1A) were identified to coincide with three important developmental stages that represent the early, rapid and late stages of oil accumulation in oilseed rape, respectively (Borisjuk et al. 2013, Woodfield et al. 2017, Woodfield et al. 2018). Fresh seed weight gradually increased throughout the period (Fig. 1B). FA content on a fresh weight basis increased from approximately 21 µg/mg at the early stage of oil accumulation (27 DAF) to approximately 100 µg/mg at 38 DAF. The latter time is toward the end of the rapid phase of oil accumulation (Fig. 1C) in agreement with the seed morphology
By 45 DAF, oil accumulation appeared to be complete (Fig. 1C), again in agreement with morphology (Fig. 1A). Values for total FAs per seed increased from 116 to 658 µg and then 806 µg/seed during the developmental period (Fig. 1D).

Because the development of oilseed rape was slower in Hong Kong than for other studies in Canada or Europe, we checked that the total FA composition of seeds was not affected significantly. During seed development from 27 to 45 DAF, there were notable decreases in the percentage of saturated (palmitate and stearate) and polyunsaturated FAs (linoleate and linolenate; Fig. 1E; Supplementary Table S1). These were compensated by the relative increase in oleate, as expected from the dominance of this acid in rapeseed oil. There was also a small increase in the percentage of eicosenoate and a decrease in that of n-7 octenoate (the latter noted to be concentrated in the seed coat of B. napus; Woodfield et al. 2017). These data agreed with typical values for different low erucate cultivars of developing oilseed rape (Turnham and Northcote 1983, Gunstone et al. 2007, Harwood et al. 2017, Woodfield et al. 2017). So, although the development of seeds was slower under our growth conditions, their overall characteristics (Fig. 1; Supplementary Table S1) were as expected.

Differential gene expression in embryos and seed coats during seed development

To understand the gene expression characteristics of each stage of seed development, we performed Next-Generation RNA-Seq (RNA-Seq) on B. napus embryos and seed coats at 27, 38 and 45 DAF, and subsequently performed a comparative transcriptomic analysis to identify genes that show
differential expression throughout the different developmental
stages. When comparative RNA-Seq analysis was performed on
embryos and seed coats during seed development, the results
indicated that 32,719, 31,608 and 12,919 differentially expressed
genes (DEGs) were identified in embryos in 27 vs. 38 DAF, 27 vs.
45 DAF and 38 vs. 45 DAF comparisons, respectively (Fig. 2A),
whereas 12,163, 22,429 and 16,189 DEGs were identified in the
equivalent comparisons for seed coat samples (Fig. 2A). It was
noted that the vast majority of DEGs identified in embryos (88–
95%) was downregulated, in contrast to DEGs identified in seed
coats (37–62%; Fig. 2A–D). We identified a core set of 5,673
genes that showed differential expression in all three time-point
comparisons in embryos, and a set of 3,197 DEGs for equivalent
comparisons in seed coat samples (Fig. 2B; Supplementary
Table S2). In embryos, most DEGs in the core set showed
downregulation throughout seed development (Fig. 2C),
whereas in seed coats, the numbers of upregulated and down-
regulated DEGs were more equivalent (Fig. 2D).

Gene ontology (GO) category analysis showed that DEGs
identified in both embryos and seed coats in the 27 vs. 45
DAF (i.e. earliest to latest developmental stage) comparison
showed similar profiles for cellular component, with a high
proportion of DEGs associated with ‘cell’, ‘cell part’, ‘organelle’,
membrane’ and ‘membrane part’ (Fig. 2E, F). For the biological
process category, DEGs in both embryos and seed coats were
mostly associated with ‘cellular process’, ‘metabolic process’,
‘biological regulation’, ‘regulation of biological process’ and ‘re-
response to stimulus’ (Fig. 2E, F), whereas for the molecular
function, ‘binding’ and ‘catalytic activity’ were the most
highly represented categories (Fig. 2E, F). GO analysis of 27
vs. 45 DAF and 38 vs. 45 DAF yielded similar results
(Supplementary Figs. S1, S2).

Pathway enrichment analysis was conducted by identifying the
top 20 most enriched Kyoto Encyclopedia of Genes and
Genomes (KEGG) pathways for DEGs from each comparison in
embryo and seed coat tissue (Supplementary Figs. S3, S4). DEGs
from both the embryo and seed coat samples for all three de-
velopmental stage comparisons were enriched for metabolic
pathways, including enrichment of pathways associated with
carbon fixation and metabolism, amino sugar and nucleotide
sugar metabolism, photosynthesis, porphyrin and chlorophyll
metabolism, and glyoxylate/2-carboxylate metabolism. FA bio-
synthesis/metabolism was also enriched in all comparisons and
tissues except 27 vs. 38 DAF in seed coats, where enrichment of
glycerolipid metabolism and biosynthesis of cutin, suberin and
wax pathways was evident instead. Also in the 27 vs. 38 DAF
seed coat comparison, we noted greater enrichment for path-
ways related to nucleic acid metabolism (including DNA repli-
cation and repair), whereas the equivalent embryo comparison
showed greater enrichment of protein and amino acid metab-
olism pathways. It was also evident that the embryo samples
showed greater enrichment of protein and amino acid metab-
olism pathways in the 27 vs. 45 DAF comparison, whereas the
seed coat samples were more enriched for carbohydrate me-
tabolism and the biosynthesis of secondary metabolites. In the
38 vs. 45 DAF comparisons, both embryos and seed coat sam-
ple showed enrichment of unsaturated FA biosynthesis and
secondary metabolites. Similarly, KEGG second pathway term
classification revealed that most DEGs mapped to translation,
amino acid metabolism, carbohydrate metabolism, energy me-
tabolism, lipid metabolism, transport and catabolism and fold-
ing, sorting and degradation (Supplementary Fig. S5).

Expression dynamics of FA and lipid biosynthesis
and storage genes during seed development
To further explore the possible mechanism that causes the
differential distribution of TAGs and PCs between embryos
and seed coats, expression analysis of genes related to TAG
storage, FA biosynthesis in plastids, TAG synthesis in the ER
and phospholipase C and D genes (Supplementary Fig. S6) was
performed by RNA-Seq. Results indicated that the TAG storage-
related genes were significantly increased during development
(Fig. 3A), coinciding with the increased of lipid content during
seed development (Fig. 1C, D). Most FA biosynthesis-related
genes associated with plastids, including PYRUVATE
DEHYDROGENASE (PDH), ACETYL-COA CARBOXYLASE
(ACCase), MALONYL-ACOA: ACP MALONYLTRANSFERASE
(MCMT), KETOACYL-ACP SYNTHASEI (KASI), KASII,
KETOACYL-ACP REDUCTASE (KAR), β-HYDROXYACYL-ACP
DEHYDRATASE (HAD), ENOYL-ACP REDUCTASE (ENR), ACYL
CARRIER PROTEIN (ACP), STEAROYL-ACP DESATURASE (SAD),
FATA, GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPDH)
and LACS were significantly downregulated in embryos
during seed development (Fig. 3B). Similarly, most of these
genes showed downregulation in seed coats, though the mag-
nitude of downregulation was reduced compared with embryos
(Fig. 3B). It was noted that most FA biosynthesis-related genes
were more highly expressed in 27 DAF embryos than seed coats,
except MALIC ENZYME (ME) and FATB (Fig. 3B). ME catalyzes
the conversion of malate to pyruvate, which can be used as a
precursor for the biosynthesis of FAs in plastids. Consistently, ME
expression significantly increased in both embryos and seed coats
during seed development (Fig. 3B). For genes related to TAG
synthesis at ER, most were downregulated in embryos, including
GPAT, LPAAT, PHOSPHATIDIC ACID PHOSPHATASE (PAP),
DIACYLGLYCEROL ACYLTRANSFERASES (DGAT1&2), CDP-
CHOLINE: DIACYLGLYCEROL CHOLINEPHOSPHOTRANSFERASE
(CPT), PHOSPHOLIPID: DIACYLGLYCEROL ACYLTRANSFERASE
(PDAT), PHOSPHATIDYLCHOLINE: DIACYLGLYCEROL
CHOLINEPHOSPHOTRANSFERASE (PDCT), FAD2, FAD3 and
LCAT (Fig. 3C), while their expression levels were not not-
ably reduced in seed coats (Fig. 3C). For phospholipase D and
C genes, PLDα1 was found to be upregulated in late-stage
embryos and NPC-PLC showed a mild downregulation in 38
vs. 45 DAF, but no other significant differences were observed
in either embryos or seed coats (Fig. 3D).

Expression profiling of BnACBP5s in embryos and
seed coats during seed development
Given their important role in oil accumulation (Supplementary
Fig. S6), we analyzed the expression of genes encoding the
ACBP5s during seed development by RNA-Seq (Fig. 4) and
validated results by quantitative real-time reverse transcription
Identification of DEGs and cluster analysis of DEGs in *B. napus* embryos (EM) and seed coats (SC) during seed development. (A) Number of DEGs in embryos and seed coats at different developmental stages. DEGs were identified by the NoISeq method (see Materials and Methods section). FPKM was used for calculating expression levels. Definition of differential expression: \((\log_2 \text{ratio} \geq 1 \text{ and } \text{diverge probability} \geq 0.8)\). Red, upregulated genes; green, downregulated genes. (B) Venn diagram showing DEG overlaps among the 27 vs. 38 DAF, 27 vs. 45 DAF and 38 vs. 45 DAF pairwise comparisons in embryos (EM) and seed coats (SC). (C) Cluster analysis of DEGs in EM for each of the pairwise comparisons. Red, \(\log_2 \text{ratio} \geq 0\); green, \(\log_2 \text{ratio} \leq 0\). (D) Clustering as in (C) but for SC pairwise comparisons. (E) GO classification for DEGs identified in 27 vs. 38 DAF pairwise comparisons in embryos (EM) and seed coats (SC). (F) GO classification for DEGs identified in 27 vs. 45 DAF comparisons for EM. (G) GO classification for DEGs identified in 27 vs. 45 DAF comparisons for SC.
**Fig. 3** Differential expression of genes related to FA and lipid metabolism in *B. napus* embryos (EM) and seed coats (SC) during seed development. (A) Genes related to TAG storage, (B) FA biosynthesis in plastids, (C) TAG synthesis at the ER and (D) phospholipase C and D in *B. napus* embryos (EM) and seed coats (SC) during seed development. Average FPKM values from three biological repeats were used in calculating log2 ratio and diverge probability in each pairwise comparison. DEG: log2 ratio ≥ 1, diverge probability ≥ 0.8. Upregulated DEGs are marked in red and downregulated DEGs in blue. The color scale shows the magnitude of change in gene expression. Log 2 ratio of FPKM in each sample pair was used in generating a heat map from http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi. The *B. napus* genome reported by Chalhoub et al. (2014) was used as template for the identification of Gene ID in *B. napus*. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier

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**A** greater than zero
less than zero

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less than zero

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<th>DAF EM 27 vs 38 FPKM</th>
<th>DAF SC 27 vs 38 FPKM</th>
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<td>NPC-PLC</td>
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**D** greater than zero
less than zero

(continued)
To obtain comprehensive information about the expression of *BnACBPs* in both embryos and seed coats and at the three different developmental stages (27, 38 and 45 DAF), RNA-Seq data were displayed in three different ways. First, the expression level (fragments per kilobase of transcript per million fragments mapped, FPKM) of the different *BnACBPs* in each tissue and the developmental stage was quantified (Supplementary Fig. S8). Second, changes in expression of each *BnACBP* were analyzed across the three developmental stages (Fig. 4; Supplementary Fig. S7). Third, the expression of each *BnACBP* was compared between embryos and seed coats (Supplementary Fig. S9).

In embryos, *BnACBP6* (encoding a small molecular mass Class I protein) was the most highly expressed of the *BnACBPs* throughout seed development (Supplementary Fig. S8), with the highest expression at the earliest developmental stage (Fig. 4A). At 27 DAF, *BnACBP1* (encoding a class II membrane-localized ankyrin-repeat protein) was the next highest expressed *ACBP*, followed by *BnACBP4* and *BnACBP2*, while *BnACBP3* and *BnACBP5* showed the lowest expression levels. When the expression of each *BnACBP* was compared at three different developmental stages, the results indicated that all *BnACBPs*, except *BnACBP2*, showed a decline during the period of rapid oil accumulation in embryo development.

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**Fig. 4** Expression profiling of *B. napus* ACBPs in embryos (A) and seed coats (B) during seed development by RNA-Seq. Total RNA was extracted from dissected embryos at 27, 38 and 45 DAF. Expression levels are represented by FPKM values. Data are means ± SD of three independent replicates; a, significant difference (*P* < 0.05 by Student’s t-test) when 38 or 45 DAF compared with 27 DAF; b, significant difference (*P* < 0.05 by Student’s t-test) between 38 and 45 DAF.

---

**Fig. 3** Continued

protein; CALO, caloelin; CPT, CDP-choline: diacylglycerol cholinephosphotransferase; DAF, days after flowering; DGAT, diacylglycerol acyltransferase; EM, embryo; ENR, enoyl-ACP reductase; FAD, fatty acid desaturase; FATA and FATB, fatty acyl-ACP thioesterases; FPKM, fragments per kilobase of transcript per million fragments mapped; GPAT, glycerol-3-phosphate acyltransferase; GPDH, glycerol-3-phosphate dehydrogenase; HAD, C-3-hydroxyacyl-ACP dehydratase; KAR, ketoacyl-ACP reductase; KAS, ketoacyl-ACP synthase; LACS, long-chain acyl-CoA synthetase; LCAT, lysocephatidyicholine acyltransferase; ME, malic enzyme; MCMT, malonyl-CoA:ACP malonyltransferase; PLC, phospholipase C; OBO, oil body oleosin; PAP, phosphatidic acid phosphatase; PDH, pyruvate dehydrogenase; PDAT, phospholipid:diacylglycerol acyltransferase; PC, phosphatidylcholine:diacylglycerol cholinephosphotransferase; PLD, phospholipase D; SC, seed coat; SAD, stearoyl-ACP desaturases; SEIPINS, lipodystrophy proteins; STERO, steroleosins. For simplicity, expression levels of isoforms from a gene family have been combined where appropriate.
especially BnACBP6 which showed the greatest relative reduction in expression level (Fig. 4A). Except for BnACBP3, this reduction was maintained or increased at maturity (45 DAF). By contrast, the expression of BnACBP2 increased during seed development, demonstrating that this gene displays different expression dynamics to the other members of the BnACBP family. The expression profiles of BnACBPs in embryos during seed development were verified by qRT-PCR in independent experiments and the results were consistent with the RNA-Seq data: all BnACBP genes, except BnACBP2, showed declining expression during seed development, while BnACBP2 expression increased (Supplementary Fig. S7A). These results independently confirm the RNA-Seq data.

In 27 DAF seed coats, BnACBP6 was also the most highly expressed ACBP, while BnACBP1, BnACBP2, BnACBP3 and BnACBP4 showed similar, lower levels of expression and BnACBP5 displaying the lowest expression level of all BnACBPs in this tissue (Supplementary Fig. S8). Changes in ACBP expression during seed development tended to be less dramatic in seed coats than in embryos. In contrast to embryos, BnACBP6 expression in seed coats increased slightly throughout development (Fig. 4B) suggesting distinct roles for BnACBP6 in the two tissues. BnACBP2 expression increased substantially during development in seed coats, just as in embryos, whereas BnACBP3 showed a transient increase at 38 DAF, suggesting that BnACBP3 could play a role in seed coats during active oil accumulation. BnACBP1 expression showed a mild decrease during seed development, as did BnACBP4 and BnACBP5 to a lesser degree. The expression profiles of BnACBPs in seed coats during seed development were validated by qRT-PCR (Supplementary Fig. S7B), and showed an increase in BnACBP2 and BnACBP6 expressions during seed development, with a transient peak of BnACBP3 expression at the 38 DAF time point, whereas BnACBP4 and BnACBP5 showed a slight decrease in expression, consistent with the RNA-Seq data.

Direct comparison of the expression of BnACBPs in embryos and seed coats is shown in Supplementary Fig. S9. The data highlight the reciprocal expression dynamics of BnACBP6, with expression decreasing markedly in the embryo but increasing in the seed coat as seed development progressed. Conversely, BnACBP2 showed increasing expression in both tissues during seed development. These differences in expression profile may reflect distinct roles of these ACBPs in embryos and seed coats. Furthermore, the differential expression of the Class 1 (ankyrin repeat) ACBPs (BnACBP1 and BnACBP2) during seed development imply that, of these two genes, BnACBP2 may have the predominant role in embryonic lipid accumulation, as it is most abundant ACBP of this class in the late-stage embryo (Fig. 4A; Supplementary Fig. S8).

### Expression profiling of BnACBP isoforms in embryos and seed coats during seed development

*Brassica napus* ACBPs exist in a number of isoforms (Raboanatairy et al. 2015a) that arise from alternative splicing of pre-mRNAs transcribed from the six BnACBP loci. Two isoforms have been reported for BnACBP1, BnACBP2 and BnACBP5 and four isoforms for BnACBP3, BnACBP4 and BnACBP6 (Raboanatairy et al. 2015a, Raboanatairy et al. 2015b).

### Table 1

<table>
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<tr>
<th>ACBP Family</th>
<th>Isoform 1</th>
<th>Isoform 2</th>
<th>Isoform 3</th>
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<tr>
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<tr>
<td>BnACBP2</td>
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### Discussion

#### Dynamic changes in gene expression in embryos and seed coats during seed development

In this study, a comparative transcriptomics analysis was performed to understand the changes in gene expression that occur during the critical phase for oil accumulation in *B. napus* seed development, and to reveal important differences in gene expression between the embryo (in which most oil
Table 1  Expression levels of *B. napus* ACBP isoforms in embryos and seed coats at three developmental stages by RNA-seq analysis

<table>
<thead>
<tr>
<th></th>
<th>27 DAF EM</th>
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<td>25.3 ± 3.6</td>
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Seeds were collected at 27, 38, and 45 days after flowering (DAF). Total RNA was extracted from dissected embryos at 27, 38, and 45 DAF. The expression levels of *B. napus* ACBP isoforms were represented by fragments per kilobase of transcript per million fragments mapped (FPKM) values generated from RNA-seq data. Data are means ± SD of three independent replicates. Values displayed significant changes (*P* < 0.05 by Student’s *t*-test) are shown in bold. Values increased in comparison to control (27 DAF EM for embryos or 27 DAF SC for seed coats) are marked in red, values decreased in comparison to control (27 DAF EM for embryos or 27 DAF SC for seed coats) are marked in blue.

accumulates) and the seed coat. When comparing the gene expression profiles among different stages of seed development (27, 38 and 45 DAF), more DEGs were identified in embryos than seed coats. Although most DEGs were progressively downregulated in embryos during seed development, a smaller proportion of DEGs were downregulated in seed coats, indicating different gene expression dynamics in these two tissues during seed development.

During seed development, oil is synthesized and accumulates mainly in the embryo. Consistent with this, we observed larger expression changes in DEGs associated with FA and lipid biosynthesis and storage in embryos than in seed coats. Most DEGs in the embryo were downregulated during development, especially those related to FA and TAG biosynthesis, consistent with a previous study (Troncoso-Ponce et al. 2011). However, genes related to oil storage increased in expression level during seed development, consistent with the progressive increase in oil content. Although most genes involved in FA biosynthesis in plasids were downregulated in both embryos and seed coats, some ER-associated TAG synthesis-related genes were reciprocally expressed between embryos and seed coats, suggesting that these differences may cause the distinct distribution of TAGs and PCs between these different tissues as previously observed (Woodfield et al. 2017).

**ACBP expression and function during embryo development**

*BnACBP6*, the only member of the small molecular mass Class-1 ACBPs, was the most highly expressed ACBP at all developmental stages, although its expression declined steadily during seed development. At 27 DAF, *BnACBP1* showed the next highest expression level, followed by *BnACBP4* and *BnACBP2*. Of these, the steady increase in expression of *BnACBP2*, which encodes a Class-2 ankyrin-repeat protein, is notable and may suggest its involvement in oil accumulation and, later, in seed maturation. *BnACBP3* and *BnACBP5* are expressed at very low levels and are, therefore, unlikely to be important for lipid formation in embryos. Therefore, the rise in *BnACBP2* expression during seed development and the overall high level of *BnACBP6* expression throughout this period mean that the two *BnACBPs* encoded by these genes are together likely involved in oil accumulation.

Hills et al. (1994) first reported that *BnACBP6* was more highly expressed in *B. napus* developing embryos and cotyledons than in leaves and flowers. The RNA-Seq results reported here, as well as other studies on embryos/seeds of major oil accumulating plants, indicate that *BnACBP6* is the most highly expressed of all the *BnACBPs* (Troncoso-Ponce et al. 2011, Chen et al. 2015). Furthermore, *BnACBP6* expression gradually decreased during seed development, consistent with the microarray data reported by Troncoso-Ponce et al. (2011). A similar trend for *ACBP6* expression has also been reported for *Ricinus communis* endosperm, *Euonymus alatus* endosperm and Arabidopsis seeds (Troncoso-Ponce et al. 2011). *BnACBP6* protein declined to low levels by the later stages of seed development (Brown et al. 1998) in agreement with our data showing that *BnACBP6* expression significantly decreased from early to later stages of development.
AtACBP6 from Arabidopsis was shown to bind long-chain acyl-CoA (C16 to C18-CoA) esters at least as efficiently as other Arabidopsis ACBPs such as ACBP4 and ACBP5 (Hsiao et al. 2015a). In addition, recombinant AtACBP6 (rAtACBP6) binds to long-chain acyl-CoA esters with \( K_d \) values ranging from 36 to 84 nM, in comparison to rAtACBP4 with ranges of 3–190 \( \mu \)M and rAtACBP5 with ranges of 35–92 \( \mu \)M (Hsiao et al. 2015a). Furthermore, Arabidopsis acbp6 mutant embryos accumulated more 18:1-CoA (Hsiao et al. 2015a). Transgenic Arabidopsis developing seeds overexpressing BnACBP6 showed reduced 18:1-CoA because the overexpressed BnACBP6 could bind more 18:1-CoA (Yurchenko et al. 2014). These results confirm a role for ACBP6 in acyl-CoA transport in seeds. Furthermore, seed weight was reduced in Arabidopsis double (acbp4, acbp6) and triple (acbp4, acbp5, acbp6) mutants, further implying a significant role for ACBP6 in seed development (Hsiao et al. 2015a).

An extra dimension to the data is provided by our analysis of BnACBP isoforms that arise via alternative pre-mRNA splicing. BnACBP6 has four isoforms, the majority (BnACBP6-1,
BnACBP6-2 and BnACBP6-3) declined significantly in the period 27–38 DAF and then decreased further toward the end of the seed maturation (45 DAF). By contrast, BnACBP6-4 expression increased at the end of the development. Even though the major BnACBP6 isoforms declined during development, they were still the most abundant ACBP transcripts at 38 DAF which is near the end of the rapid phase of oil accumulation, and at 45 DAF. The increase in both BnACBP2 isoform transcripts during development suggests that both play a similar role in lipid biosynthesis. Indeed, it can be noted that although AtACBP6 is a small molecular mass protein confined to the cytosol (Chen et al. 2008), AtACBP2, an ankyrin-repeat protein, is associated with the ER (Li and Chye 2003). Thus, we would suggest that these two ACBPs play complementary roles during oil accumulation. BnACBP6 (and its homologs) would be mainly involved in cytosolic acyl-CoA binding and inter-organellar transport, whereas BnACBP2 (and its homologs) would be important for enzyme interactions within the Kennedy pathway for TAG biosynthesis. The significance of ACBPs in the transport of acyl-CoA esters during embryo development has also been reported in mice where the depletion of the ACBP6 homolog as well as the phosphotyrosine-binding-domain-containing ACBD3 led to embryonic lethality (Zhou et al. 2007, Landrock et al. 2010).

**ACBP expression in the seed coat contrasts with that in the embryo**

Several BnACBP genes were significantly expressed in seed coats at 27 DAF. BnACBP1, BnACBP2, BnACBP3 and BnACBP4 all
showed moderate expression levels that were higher than in embryos except for BnACBP1, whereas BnACBP5 had the lowest expression level. Nevertheless, as in embryos, BnACBP6 was the most highly expressed of all BnACBP genes. However, in contrast to the embryos, the level of BnACBP6 increased in the seed coat during seed development. As in embryos, BnACBP2 increased steadily so that, by 45 DAF, its level was about three times that at 27 DAF. Although both BnACBP1 and BnACBP4 showed small decreases during seed development, BnACBP3 gave a peak of expression in the rapid oil accumulation phase at 38 DAF. Thus, most BnACBPs showed significant expression during this period pointing to complementary roles within the seed coat. In a sense, this is to be expected, because the embryo’s main function is to produce and store oil, while the seed coat has to ensure germination and seedling establishment. The myriad of BnACBPs available for these purposes includes significant amounts of five of the six classes which have membrane-localized or soluble features (Du et al. 2016). It has been reported previously that AtACBP1 and AtACBP2 have been immunolocalized to the developing embryo using antibodies specific to AtACBP1 and AtACBP2, respectively, and both displayed expression in developing seeds coinciding with lipid deposition (Chye et al. 1999, Chen et al. 2010). Furthermore, AtACBP1 has been detected to the plasma membrane of heart-, torpedo- and cotyledonary-staged embryos as well as in the seed coat as revealed by immunoelectron microscopy (Chye et al. 1999). Several of the ACBPs, such as AtACBP1 (Du et al. 2013, Lung et al. 2017, Chen et al. 2018, Lung et al. 2018) and AtACBP2 (Gao et al. 2009, Gao et al. 2010), have been proven to possess the potential to interact with other proteins based on the presence of the characteristic Class II ankyrin-repeat domain or the Class IV Kelch domain (Du et al. 2016). Within the BnACBP classes in seed coats, most isoforms behaved similarly in terms of their expression. The exception was BnACBP6 where BnACBP6-1 declined while BnACBP6-2 and BnACBP6-3 increased and BnACBP6-4 remained constant during development. Thus, comparative data for embryos and seed coat emphasize the subtle differences between spatially adjacent tissues where their physiology is distinct.

We used a combination of RNA-Seq and qRT-PCR for measurement of gene expression levels and found that gene expression values were generally in very good agreement, particularly for the more highly expressed BnACBPs. However, sometimes while the trend in expression level change was similar, the magnitude of differential expression varied between the two methods, which may be attributable to their different sensitivities. For the lower expressed BnACBPs, such as BnACBP3 and BnACBP5 and some of the low abundance isoforms, there were small deviations between RNA-Seq and qRT-PCR data but this likely reflects the limitations in quantifying very low gene expression levels. We realize that changes in expression levels of ACBP genes may not necessarily translate directly to alterations in metabolism (Voelckel et al. 2017). Moreover, in our past research on oil accumulation in oilseed rape, we have identified several factors other than ACBP levels that influence the process (e.g. Perry et al. 1999, Weselake et al. 2008, Tang et al. 2012, Woodfield et al. 2018). Nevertheless, the data reported here identify which ACBPs may be important for oil accumulation in embryos or lipid metabolism in seed coats.

In conclusion, this study has provided comprehensive information on differentially expressed lipid-related genes between embryos and seed coats and ACBP (isoform) expression in an important oil crop, B. napus. The apparent importance for BnACBP2 and BnACBP6 and their isoforms during oil accumulation offers possibilities for genetic manipulation which may significantly enhance TAG formation. In a world with limited agricultural land but increasing demand for vegetable oils, this is a very important potential application.

Materials and Methods

Plant materials

Wild-type B. napus cv. DH12075 (LEAR) seeds were germinated in pots containing soil mix (Tref Substrates, Jiffy). Ten-day-old seedlings were transplanted individually into 8.7 cm pots. Plants were grown in a greenhouse with a temperature of approximately 23° C and with a natural light period (11–13 h) at the School of Biological Sciences, the University of Hong Kong. Flowers were pollinated manually and tagged on the first day when flowers open. Siliques were collected at 27, 38 and 45 DAF, representing early, rapid and late stages of lipid accumulation in the oilseed rape plants.

Morphological analysis of Hong Kong-grown Brassica embryos

Due to the differences in temperature, light intensity and daylight length between the greenhouses in Hong Kong (11–13 h of natural light) and Cardiff (16 h with a light intensity of 250 μmol·m⁻²·s⁻¹; Woodfield et al. 2017, Woodfield et al. 2018), wild-type B. napus grew slower in Hong Kong than Cardiff. For Cardiff-grown wild-type B. napus, siliques were collected at 20, 27 and 35 DAF, representing early, rapid and late stages of lipid accumulation (Turnham and Northcote 1983, Woodfield et al. 2017, Woodfield et al. 2018). To match these with those of Hong Kong-grown B. napus, the morphology of embryos at different developmental stages from Hong Kong-grown wild-type B. napus was compared with the embryo architecture described by Borisjuk et al. (2013). Results were confirmed by lipid analysis. Subsequently, 27, 38 and 45 DAF were selected to represent early, rapid and late stages of lipid accumulation in siliques collected in Hong Kong. Embryos were dissected manually by a razor blade and photographed (Woodfield et al. 2017). Seeds from 10 siliques from each of the six plants were harvested for measurements of fresh weight.

FA profiling

Twelve seeds from different siliques were harvested at 27, 38 and 45 DAF for each biological repeat. In total, six biological repeats were used. FA extraction was performed as previously described (Woodfield et al. 2017, Woodfield et al. 2018). Seed samples were incubated in 1.2 ml of isopropanol at 70 °C for 30 min to inactivate any endogenous (phospho-) lipases. Nonadecanoic acid (19:0; Sigma, St. Louis, Missouri, USA) was used as an internal standard. Fatty acid methyl esters (FAMES) were analyzed by an Agilent GC-MS device (5,973 inert mass spectrometer combined with 6,890 N gas chromatograph) equipped with an Agilent J&W DA-WAX capillary column (30 m × 0.25 mm × 0.25 μm; Lung et al. 2017). The oven temperature was set to 170 °C for 3 min, increased to 220 °C at 4°C·min⁻¹, and held at 220 °C for 15 min (Woodfield et al. 2017). FAMES were routinely identified by comparing the retention time of peaks with the Superko 37 Component FAME MIX standard (Sigma) but had been identified fully in previous work (Woodfield et al. 2017).

RNA extraction

Siliques were harvested at 27, 38 and 45 DAF. Embryo and seed coat samples were dissected manually by a razor blade and stored immediately in liquid nitrogen before RNA extraction. Total RNA from embryos was extracted using an RNeasy
Plant Mini kit (Qiagen, Hilden, Germany). Total RNA from seed coats which contain procyanidins was extracted according to Wang and Vodkin (1994) with some modifications. Seed coat samples were first ground to a fine powder in liquid nitrogen, an equal volume of special RNA extraction buffer (450 µl) [100 mM Tris-HCl pH 8.0, 20 mM EDTA-Na, 1.5% SDS (w/v), 200 mM NaCl, 5% bovine serum albumin (BSA; w/v), 4% polyvinylpyrrolidone (w/v)] and the lysis buffer (RLC; 450 µl) from RNAeasy Plant Mini Kit with 1% 2-mercaptoethanol (v/v) were added and mixed by vortex. Then 212 µl of proteinase K (10 mg/ml) was added and incubated at 37°C with gentle shaking (80 rpm) for 20 min to digest the remaining BSA. Subsequently, seed coat RNA was extracted following the instructions of the RNAeasy Plant Mini Kit (Qiagen). On-column DNase digestion (Qiagen) was performed on RNA samples to remove potential DNA contamination.

**RNA-Seq analysis**

Embryo and seed coat RNA samples from each of the three developmental stages, with three biological replicates, were sent to Beijing Genomics Institute (BGI, Hong Kong, China) for RNA-Seq analysis (BGISEQ-500; Mak et al. 2017). The *B. napus* genome from ‘Darmor-bzh’ was used as a reference genome (Chalhoub et al. 2014). The gene expression level was quantified using a software package (RSEM) (Li and Dewey 2011). The FPKM values which represent expression levels for each transcript were generated (Li and Dewey 2011). The RNoSeq method (Tarazona et al. 2015) was used to screen for DEGs with the following criteria: fold change $\geq 2$ (log2 ratio $\geq 1$) and diverge probability $\geq 0.8$. GO enrichment analysis was performed by using the Cluster and Java Treeview software. GO classification was conducted using the WEGO software (Ye et al. 2006). Subsequently, pathway enrichment analysis of DEGs was carried out using the KEGG database (Kanehisa et al. 2008).

**Quantitative RT-PCR**

Total RNA (2.5 µg) was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer’s instructions. Subsequently, qRT-PCR was performed with the FastStart Universal SYBR Green Master (Roche) on the StepOnePlus Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Three independent biological repeats were performed for each reaction, and three technical repeats were conducted in each PCR reaction. *Brassica TIP41-LIKE PROTEIN* (TIP41) was used as internal control (see Chen et al. 2010). The data were analyzed using the $2^{-\Delta\Delta C_{T}}$ method (Schmittgen and Livak 2008). Each *BnACBP* primer was designed from sequences originating from the conserved region of *BnACBP* isoforms within the same class. Each *BnACBP* isoform-specific primer was designed from a specific region for each isoform. Details of primers for qRT-PCR are listed in Supplementary Table S3.

**Supplementary Data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


