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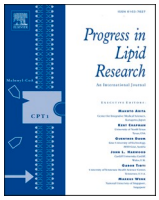

Increasing oil content in Brassica oilseed species

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ABSTRACT

Brassica oilseed species are the third most important in the world, providing approximately 15 % of the total vegetable oils. Three species (*Brassica rapa, B. juncea, B. napus*) dominate with *B. napus* being the most common in Canada, China and Europe. Originally, *B. napus* was a crop producing seed with high erucic acid content, which still persists today, to some extent, and is used for industrial purposes. In contrast, cultivars which produce seed used for food and feed are low erucic acid cultivars which also have reduced glucosinolate content. Because of the limit to agricultural land, recent efforts have been made to increase productivity of oil crops, including Brassica oilseed species. In this article, we have detailed research in this regard. We have covered modern genetic, genomic and metabolic control analysis approaches to identifying potential targets for the manipulation of seed oil content. Details of work on the use of quantitative trait loci, genome-wide association and comparative functional genomics to highlight factors influencing seed oil accumulation are given and functional proteins which can affect this process are discussed. In summary, a wide variety of inputs are proving useful for the improvement of Brassica oilseed species, as major sources of global vegetable oil.

1. Introduction

Brassica oilseed species (BOS) have been cultivated for thousands of years with detailed records of the crops in India from 1500 years ago. Demand increased rapidly in the 20th century due to improvements in seed varieties and agricultural and processing methods [1]. Rapeseed is now the third most important oil crop (after palm and soybean) and produces about 15 % of the total vegetable oils. It is a major oil crop for the European Community, Canada, China and India (Table 1) [2].

Three BOS, or more specifically rapeseed species, dominate crop

Abbreviations: ABI, abscisic acid insensitive; ACCase, acetyl-CoA carboxylase; ACP, acyl-carrier protein; AGPase, ADP-glucose pyrophosphorylase; AGR, associated genomic region; BAAC, biotin attachment domain-containing; BC, backcross; BOS, Brassica oilseed species; BSA, bulk-segregant analysis; CCT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-1, 2-diacyl-*sn*-glycerol cholinephosphotransferase; DAG, 1, 2-diacyl-*sn*-glycerol; DAP, days after pollination; DArT, diversity array technology; DCR, defective cuticular ridges; DEG, differentially expressed gene; DGAT, diacylglycerol acyltransferase; DH, doubled haploid; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; FA, fatty acid; FAD, fatty acid desaturase; FAS, fatty acid synthase; FAT, fatty acyl-acyl carrier protein thioesterase; GRF2, growth-regulating factor 2-like; GPAT, *sn*-glycerol-3-phosphate acyltransferase; GWA, genome-wide association; G3P, *sn*-3 glycerol 3-phosphate; G3PDH, *sn*-3 glycerol-3-phosphate dehydrogenase; HEAR, high erucic acid rapeseed; LACS, long chain acyl-CoA synthetase; LEA, late embryogenesis abundant protein; LEAR, low erucic acid rapeseed; LEC, leafy cotyledon; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; MCA, metabolic control analysis; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; MGDG, monogalactosyl diacylglycerol; MGD, monogalactosyldiacylglycerol synthase; NIL, near-isogenic line; NTD, N-terminal domain; OB, oil body; OPPP, oxidative pentose phosphate pathway; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCK, pyruvate dehydrogenase complex kinase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; PLA, phospholipase A; PUFA, polyunsaturated fatty acid; QTG, quantitative trait gene; QTL, quantitative trait locus; QTV, quantitative trait variant; RIL, recombinant inbred line; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SBE, starch branching enzyme; SDP1, sugar-dependent 1; SNP, single nucleotide polymorphism; SOC, seed oil content; TAG, triacylglycerol; TDCA, top-down control analysis; TF, transcription factors; TMD, transmembrane domain; TT, transparent testa; TWA, transcriptome-wide association; WRI1, wrinkled 1; WSD, wax synthase-diacylglycerol acyltransferase; WT, wild type.

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Table 1
Rapeseed by country (million metric tons).

	1985	1995	2009	2018
EU	5.8 (30 %)	10.7 (31 %)	20.9 (34 %)	19.5 (26 %)
Canada	3.5 (18 %)	6.4 (19 %)	11.8 (19 %)	20.3 (27 %)
China	5.6 (29 %)	9.8 (29 %)	13.5 (22 %)	13.3 (18 %)
India	3.1 (16 %)	5.8 (17 %)	7.2 (12 %)	8.4 (11 %)
Total	19.2	34.2	61.6	75.2

Data taken from data in Wikipedia (https://en.wikipedia.org/wiki/Rapeseed——accessed 5th Sept. 2023).

production in oil producing countries. *Brassica rapa* is the most cold-hardy and, therefore, is grown to some extent in Western Canada. On the other hand, *B. juncea* is better adapted to dryer climates and is common in China and Northern India. *B. napus* is the most common species grown in Europe, Canada and China and can be obtained in spring and winter varieties. The latter is favored because of its good yields provided climate conditions are suitable [1]. *B. napus* is an allotetrapoid crop, resulting from interspecies breeding of varieties of *B. oleracea* and *B. rapa* [3,4].

The earlier varieties of rapeseed usually contained high amounts of erucic acid (22:1Δ^{13cis}, hereafter 22:1), reported to damage animal cardiac muscle, which precluded its widespread use in food or feeds [5]. They also contained glucosinolates (3-butenyl-, 4-pentenyl-, 2-hydroxy-3-butenyl- and 3-hydroxy-4-pentenyl glucosinolate) which causes limited palatability and nutritional content for livestock and poultry.

Although high erucic acid rapeseed (HEAR) oil has industrial uses as a lubricant, nutritional requirements led to the breeding of *B. napus* (and other) varieties which were low in both erucic acid and glucosinolates, first in Canada in 1974 [6,7] and later in Europe. These were known as 'double low' varieties and later given the trade name 'canola' by which they are called in North America. The term 'canola' is used to describe varieties with <2 % erucic acid in their oil and < 30μmoles glucosinolates per g of meal. In European and other countries canola falls into the low erucic acid rapeseed (LEAR) category whereas HEAR produces oil with high erucic acid content. Both Europe and the U.S.A. stipulate that edible oils must have <2 % erucic acid.

Typical LEAR (including canola) varieties contain palmitic (16:0; 4 %), stearic (18:0; 2 %), oleic (18:1Δ^{9cis}, hereafter 18:1; 62 %), linoleic (18:2^{9cis,12cis}, hereafter 18:2; 22 %) and α-linolenic (18:3^{9cis,12cis,15cis}, hereafter α-18:3; 10 %) acids (Table 2) [1]. On the other hand, HEAR varieties contain only about 15 % oleic acid which had been elongated to eicosenoic (10 %) and erucic (45 %) acids (Table 2). As in most other oil crops, the accumulating lipid is almost exclusively triacylglycerol (TAG) formed by the four reaction steps of the Kennedy pathway [8,9]. The major TAG molecular species found in LEAR oils are OOO (22 %), LOO (22 %), LnOO (10 %), LLO (9 %), LnLO (8 %), LOP (6 %) and POO (5 %), where P, L, Ln and O refer to 16:0, 18:2, α-18:3 and 18:1, respectively. The distribution of TAG molecular species in different varieties of rapeseed are given in [1] and their spatial distribution within LEAR seeds, as revealed by MALDI-MS (matrix-assisted laser/desorption ionisation-mass spectrometry imaging), are shown in [10]. The distribution of fatty acids (FAs) on the glycerol backbone of TAG is similar to other oil crops with saturated FAs enriched at the *sn*-1 and *sn*-3 positions while the polyunsaturated fatty acids (PUFAs, including 18:2 and α-18:3) are concentrated at the *sn*-2 position [11]. For LEAR varieties, typical lipid contents are 45 % dry weight [10] of which TAG represents about 95 % of the total acyl lipids [12]. Typical crop yields are given in [1].

Table 2
Fatty acid composition of different *B. napus* oils.

Fatty acid	LEAR	HEAR	LLCAN	HOCAN	LTCAN
16:0	3.6	4.0	3.9	3.4	2.7
18:0	1.5	1.0	1.3	2.5	1.6
18:1	61.6	14.8	61.4	77.8	32.8
18:2	21.7	14.1	28.1	9.8	0.8
18:3	9.6	9.1	2.1	2.6	0.5
20:1	1.4	10.0	1.5	1.6	0.8
22:1	0.2	45.1	0.1	0.1	0.5

LLCAN = low linolenic rapeseed oil; HOCAN = high oleic rapeseed oil; LTCAN = rapeseed oil enriched in medium chain fatty acids (38.8 % lauric, 4.1 % myristic). Adapted from [11].

Depending on the country concerned, the use of rapeseed oil varies somewhat, although the LEAR varieties are used mainly for food. Substantial amounts have been used as biofuel in Europe [2], although this has declined recently since it is determined by government policy. In addition, genetically-engineered lines are available and these have been grown extensively in North America [2]. The production and utilization of such lines has been summarized in Weselake et al. [13]. These include transgenics with increased levels of 18:0 [14] or total saturates [15,16], increased medium-chain acids [17–19] or reduced saturated FAs [20]. Although LEAR/Canola varieties of rapeseed contain large amounts (about 60 %) of oleic acid, this can be increased further at the expense of PUFAs [21,22] (Table 2). Further manipulations, including methods to increase seed oil content (SOC) in *B. napus* and comparisons with other crops are covered in Weselake et al. [13].

The major breeding targets for oilseed rape are yield, quality and resistance. Increasing SOC in rapeseed is a component of yield and quality and is a major goal of breeding and biotechnological programs [7,23–25]. This review focuses on the use of biotechnological strategies for increasing SOC. It begins by presenting information on oil biosynthesis in rapeseed in order to provide a metabolic context for the various genetic interventions that will be discussed later. Great improvements in increasing SOC and yield have come through conventional breeding, with more recent molecular approaches being introduced. Therefore, a section dealing with genetic and omics approaches to identifying potential targets for manipulation of SOC is included. Thereafter, an overview of Metabolic Control Analysis (MCA) as a tool to help identify metabolic engineering strategies is covered. This is followed by specific attention to diacylglycerol acyltransferase (DGAT) and further enzymes in TAG assembly. Other possible targets in carbon metabolism and lipid transport are then surveyed. Overall, we believe that the review provides an informative survey of how BOS have been manipulated and suggest further ways for the future.

2. Triacylglycerol biosynthesis

The majority of TAG biosynthesis in developing seeds of rapeseed occurs in the zygotic embryo during seed development and involves several enzymes and other functional proteins operating in the plastid and endoplasmic reticulum (ER). Several review articles have described the metabolic pathways involved (e.g. [26–32]).

FAs, which are eventually incorporated into various membranes and TAG, are synthesized through the sequential action of acetyl-CoA carboxylase (ACCase) and the fatty acid synthase (FAS) complex of the plastid to the level of 16:0 and 18:0 while attached to the acyl-carrier protein (ACP) component of the FAS complex. A soluble stearoyl-ACP desaturase catalyses the conversion of 18:0 to 18:1 while the fatty acyl chain is still attached to ACP. In an investigation using microspore-derived cultures of *B. napus* L. cv Jet Neuf, Andre et al. [33] have demonstrated that a reduced requirement for de novo FAs leads to accumulation of 18:1-ACP, which directly inhibits plastidial ACCase, thereby resulting in a reduction in FA biosynthesis. Thioesterases catalyse the release of 16:0 and 18:1 which can then move across the inner plastidial membrane through the involvement of fatty acid transporter 1 [34,35]. The released FAs are then esterified to CoA to form their respective acyl-CoAs on the outside of the plastid. A recent study has suggested that long-chain acyl-CoA synthetase (LACS) 2 is involved in catalysing the production of acyl-CoA [36]. The glycerol backbone for TAG assembly in the ER is provided by *sn*-3 glycerol 3-phosphate (G3P) [8,9]. G3P is produced from dihydroxyacetone phosphate (DHAP) via

the catalytic action of *sn*-3 glycerol 3-phosphate dehydrogenase (G3PDH) [37]. DHAP is produced via glycolysis.

A generalized view of TAG assembly in the ER of developing seeds of oleaginous plants producing TAG containing PUFAs is depicted in Fig. 1. The main pathway for TAG biosynthesis is historically referred to as the G3P pathway or Kennedy pathway [8,9]. This process involves a sequential acylation of the glycerol backbone, starting with G3P, catalysed by three membrane-bound acyl-CoA-dependent acyltransferases. In addition, phosphatidic acid phosphatase (PAP) catalyses the liberation of inorganic phosphate from phosphatidic acid (PA) to form 1, 2-diacyl-*sn*-glycerol (DAG) prior to the final acylation. The acylation of G3P to form lysophosphatidic acid (LPA) is catalysed by *sn*-glycerol-3-phosphate acyltransferase (GPAT) whereas the further acylation of LPA to form PA is catalysed by lysophosphatidic acid acyltransferase (LPAAT). In developing seeds of *Arabidopsis thaliana* (hereafter Arabidopsis), type-9 GPAT has been shown to be active within the Kennedy pathway [38,39]. The acylation of DAG to form TAG is catalysed by DGAT, which in rapeseed, is a key enzyme involved in controlling the flow of carbon into seed oil [25,40–44]. Analysis of the lipidome conducted during seed development in *B. napus* has suggested that the selectivity properties of the DGAT isoforms involved may influence the fatty acyl composition of TAG [45].

Although LEAR is enriched in oleic acid, substantial levels of PUFAs are also present in the seed oil [7]. Production of the PUFA component of TAG involves a complex metabolic interplay between the Kennedy pathway and further membrane metabolism (Fig. 1). PA and DAG, produced in the Kennedy pathway, are involved in the formation of anionic and zwitterionic phospholipids, respectively [32,46]. The production of PUFAs, which may eventually end up in TAG, are formed at the level of phosphatidylcholine (PC) via the catalytic actions of membrane-bound fatty acid desaturase (FAD) 2 and FAD3. These desaturases sequentially catalyse the production of PC enriched in 18:2 and α-18:3. PC with 18:1 at the middle position typically serving as the substrate of FAD2.

DAG synthesized in the Kennedy pathway can be converted to PC by the catalytic action of CDP-:1, 2-diacyl-*sn*-glycerol cholinephosphotransferase (CPT) [32]. Phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) can also utilise DAG produced in the Kennedy pathway by converting it to PC by catalytic transfer of a phosphocholine headgroup from PC enriched in PUFAs [47]. The resulting PUFA-enriched DAG can thus be used by the Kennedy pathway in the formation of PUFA-enriched TAG. Recently, PDCT isoforms in canola-type *B. napus* have been shown to be responsible for less than 20 % of the PUFAs found in seed TAG, compared to 40 % in Arabidopsis [48]. Bai et al. [48] and Li et al. [49] have suggested that care must be taken in extrapolating results from the model plant Arabidopsis to the oilseed crop, *B. napus*.

In the forward reaction, lysophosphatidylcholine acyltransferase (LPCAT) catalyses the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to form PC [28]. In Arabidopsis, LPCAT1 and LPCAT2 have been shown to be involved in the remodeling of phospholipids derived from the Kennedy pathway [50]. Based on studies with various oleaginous plants, the combined forward and reverse reactions of LPCAT, however, have been shown to release PUFAs from PC into the acyl-CoA pool thereby providing additional opportunities for the incorporation of PUFAs into PC [44,51–53]. A 10-kDa soluble acyl-CoA-binding protein, present in developing seeds of *B. napus*, has been shown to enhance acyl-exchange between acyl-CoA and PC [53].

Another enzyme, known as phospholipid:diacylglycerol acyltransferase (PDAT), catalyses the non-acyl-CoA-dependent acylation of DAG to also produce TAG [54,55]. In this case, nitrogenous phospholipids (PC and phosphatidylethanolamine) serve as acyl donors instead of acyl-CoA. DGAT1 and PDAT have been shown to overlap in function during seed development in Arabidopsis [56]. Arabidopsis DGAT1, however, has been shown to be more important in seed oil accumulation [57]. Moreover, in vitro enzyme measurements implied that DGAT was

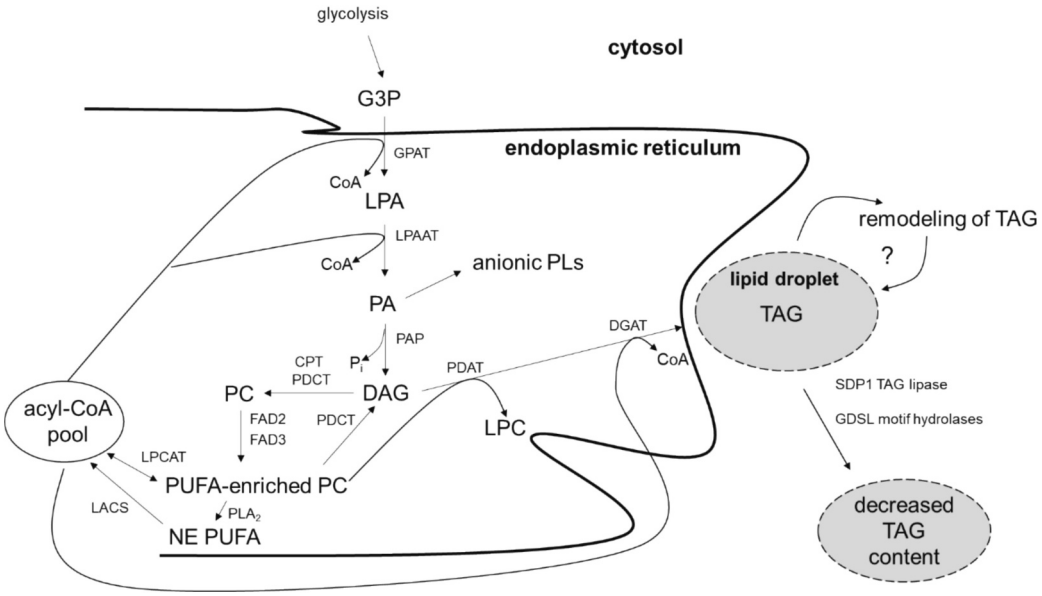

Fig. 1. Generalized overview of the triacylglycerol (TAG) biosynthesis in developing seeds of oleaginous plants producing TAG containing polyunsaturated fatty acids (PUFAs).

The Kennedy pathway [8,9] is shown in relation to some possible acyl-trafficking reactions along with phospholipid:diacylglycerol acyltransferase (PDAT) action. Possible specialized pools of 1, 2-diacyl-sn-glycerol (DAG), including DAG synthesized de novo in the Kennedy pathway and PC-modified DAG [286], are not specifically depicted. Phosphatidylethanolamine can also serve as an acyl donor for the PDAT-catalysed reaction. Other abbreviations: CPT, CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase; FA, fatty acid; FAD, fatty acid desaturase; GPAT, acyl-CoA:sn-glycerol-3-phosphate acyltransferase; G3P, sn-glycerol-3-phosphate; LACS, long-chain acyl-CoA synthetase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltansferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; NE, non-esterified; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDCT, phosphatidylcholine:diacylglycerol; cholinephosphotransferase; Pi, inorganic phosphate; PL, phospholipid; PLA2, phospholipase A2; SDP1, sucrose-dependent 1. This figure is a slightly modified version of Fig. 9 from [40] and is based on information from: [28–30,40,46,61–63,65,66].

more important than PDAT in *B. napus* [58] which was confirmed by a detailed analysis of the molecular species of lipid classes during oil accumulation [45]. Recently, over-expression of *PDAT* in *B. napus* was shown to alter the cellular distribution of molecular species of PC and TAG along with decreasing the level of unsaturation of these lipid classes [59,60]. The increase in PDAT activity during seed development may have allowed reduced time for desaturation of PC catalysed by FAD2 and FAD3.

In addition to the substrate selectivities of the Kennedy pathway enzymes, combined with PDCT, LCAT and PDAT action, the acyl composition of seed TAG may be further influenced by other enzyme activities such as those of phospholipases and LACS [26,28,29]. In particular, the process of 'acyl editing' (or re-modeling) should be mentioned. The metabolism involved in plant acyl editing has been described well [26] and is particularly important for plants accumulating polyunsaturated or modified (e.g. hydroxylated) fatty acids. Although oilseed rape accumulates an oil enriched in oleate, the related model plant Arabidopsis has been studied extensively with regard to acyl editing [26] and the process is discussed further in Section 5.

Recent evidence has been presented in support of the remodeling of TAG in developing seeds of *Physaria fendleri* [61,62]. Although there is no evidence for this type of remodeling in *B. napus*, the oilseed has been shown to lose some of its accumulated TAG due to sucrose-dependent 1 (SDP1) TAG lipase action occurring during the desiccation phase of seed development [63,64]. Recently, hydrolases containing a distinct GDSL motif have also been shown to contribute to the reduction in SOC of *B. napus* observed at later stages of seed maturation [65,66]. Other catabolic processes may also contribute to the decrease in SOC at later stages of development [67].

During seed maturation in oleaginous plants, TAG accumulates in the form of cytoplasmic oil bodies (OBs) which are formed by pinching off from the ER where TAG biosynthesis occurs [68–70]. The OBs are encapsulated by a monolayer of phospholipid with proteins embedded in the surface, serving to stabilize the supramolecular structures in the mature seed. Oleosins are the most abundant of the OB proteins. Jolivet et al. [69] have analysed temporal changes in OB size, lipid composition changes and OB-associated proteins during seed development in *B. napus* L. cv Jet Neuf. Up to 50 days after pollination (DAP), OBs were $>$ 4 μm in diameter but were smaller in mature seed (about 1.2 μm). OBs at earlier stages of seed development also had a different FA and oleosin composition compared to OBs appearing at later stages.

Omics-based investigations, involving analysis of the transcriptome, proteome and metabolome (and more specifically the lipidome) during seed development in *B. napus* have also contributed to our knowledge of enzymes and functional proteins involved in TAG accumulation *in B. napus* [45,71–78]. Some omics-based investigations have involved comparisons of global gene expression between *B. napus* and those of other oilseed crops revealing some commonalties in the expression patterns of certain genes [79–81]. Schwender et al. [76,82] have indicated, however, that analysis of only transcript abundance is not reliable for inferring metabolic fluxes in developing embryos of *B. napus*. Interestingly, Tan et al. [77] have reported that metabolite and gene expression profiles in developing seeds of *B. napus* are closely correlated, especially for metabolites and expressed genes associated with FA and TAG biosynthesis. Lu et al. [75] have conducted a spatial analysis of the lipidome and transcriptome in developing seeds of high- and low-oil *B. napus* which revealed a tissue-specific heterogeneity of lipid metabolism. Later in this review, the role of comparative functional omics will be discussed in relation to identifying functional proteins influencing TAG accumulation in *B. napus*.

Omics-based research has also shown the involvement of a complex interplay of transcription factors (TFs) affecting the expression of genes encoding lipid biosynthetic enzymes in in the seeds of developing oleaginous plants, including *B. napus* [25,40,83–87]. For example, Zhang et al. [88] recently demonstrated that the TF *BnaZFP1* interacts with the promoter region of the gene encoding *B. napus* type-1 DGAT to positively control 18:1 levels. Isoforms of the DGAT1 and DGAT2 families involved in TAG biosynthesis in *B. napus* will be discussed later. Regulatory single nucleotide polymorphisms (SNPs) occurring between high- and low-oil content cultivars of *B. napus* have been shown to have the potential to alter phenotype by affecting the DNA-binding properties of TFs by altering the sequence of TF binding sites in promoter regions of target genes [74].

Non-coding RNAs are also known to be regulatory elements involved in plant growth and development. MicroRNAs are short non-coding RNAs which range in length between 20 and 24 nucleotides [89] whereas long non-coding RNAs are larger than 200 nucleotides [90]. Recent studies have suggested that both microRNAs and long non-coding RNAs participate in the regulatory network of lipid accumulation during seed development in *B. napus* [91,92]. For example, microRNA-mediated regulation of TFs may be involved in transitioning developing seeds from embryogenesis to maturation [72]. In addition, Li et al. [93] recently identified two long non-coding RNAs, designated *MSTRG.22563* and *MSTRG.86004*, which may affect SOC in *B. napus*. *MSTRG.22563* might affect respiration and the TCA cycle, whereas *MSTRG.86004* had a role in prolonging seed development.

Feeding studies with radiolabeled metabolite precursors have been used to probe storage lipid biosynthesis in developing seeds of *B. napus* (e.g. [43,94,95]). In addition, stable isotope experiments with ^{13}C have been used to study metabolic fluxes in developing zygotic embryos of *B. napus* [96–98]. In developing seeds of *B. napus*, starch accumulation occurs early in zygotic embryo development and eventually gives way to increased TAG accumulation, essentially indicating a developmental dependent shift in carbon partitioning [99]. Using nine selected germplasm accessions, Schwender et al. [76] have examined genotypic differences in carbon partitioning in in vitro cultured developing embryos of *B. napus*. Biomass composition along with numerous fluxes, metabolites and enzyme activities were assessed. Enzyme activity/flux and metabolite/flux correlations suggested that plastidic pyruvate kinase exerts flux control and that the metabolic switch from starch to lipid accumulation observed during seed development probably involves allosteric feedback regulation of phosphofructokinase and ADP-glucose pyrophosphorylase. Additional implications of the study of Schwender et al. [76] are presented in Section 4. In bottom-up approaches involving the assessment of a plethora of individual biochemical reactions, computational methods have been used to integrate data from stable isotope experiments with omics data to generate biochemical networks for developing embryos of *B. napus* with assigned fluxes [100–106]. Under in vivo conditions, NMR-based analysis has shown that metabolic fluxes are locally regulated and linked to the architecture of the seed [107].

Metabolic control analysis (MCA), using a top-down approach to analyse metabolic control, has been particularly useful in the estimation of flux control coefficients for various steps leading to TAG in developing seeds of *B. napus* [43,58,108,109]. In top-down control analysis (TDCA), flux control coefficients are usually assigned to blocks of reactions rather than individual biochemical reactions (see Section 4).

Photosynthesis in developing leaves, siliques and seeds has been shown to provide precursors for FA biosynthesis and TAG accumulation in the zygotic embryos of maturing seeds of *B. napus* [110–114]. Although ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) has been shown to be active in fixing carbon dioxide by the usual carboxylation reaction in developing zygotic embryos of *B. napus*, the enzyme did not operate as part of the Calvin cycle, but instead was part of a previously undescribed metabolic route [115]. This newly identified metabolic process involving Rubisco action provides 20 % more acetyl-CoA for FA biosynthesis, with a 40 % reduction in the loss of carbon as carbon dioxide. Overall, this metabolic route occurring in the zygotic embryo resulted in enhanced efficiency of carbon use during TAG accumulation. In addition, increases in light intensity have been shown to result in increased growth, efficiency of carbon storage and TAG biosynthesis in developing *B. napus* embryos due to increased provision

of reductant and/or ATP [116]. The oxidative pentose phosphate pathway (OPPP) is one of the sources of reductant support for FA biosynthesis [117]. Within Section 7.2, a molecular genetic strategy for increasing SOC in Arabidopsis is discussed, which is based on increasing the production of NADPH. Furthermore, numerous studies have indicated that silique wall photosynthesis provides for a substantial maternal effect on TAG accumulation in the zygotic embryo [111,118–123]. Liu et al. [120] have demonstrated that the developing silique wall, seed coat (ovule) and embryo all contribute positively to increased seed oil accumulation.

It is important to note that the liquid endosperm represents the main component of a maturing seed of *B. napus* seed at earlier stages of development and a substantial portion of carbon resources fueling zygotic embryo development arrive via the liquid endosperm [124,125]. The liquid endosperm is eventually utilised by the zygotic embryo, with the embryo taking over the space formerly occupied by the liquid endosperm [107,125]. In the Brassicaceae family, including *B. napus*, at maturation, the endosperm is reduced to a single layer of cells surrounding the embryo which contains lipid enriched in ω7 FAs [10,126]. During seed development, the concentration of hexose sugars in the liquid endosperm has been shown to decrease while sucrose concentration increases [124]. Both the endosperm transcriptome and proteome have been examined in *B. napus* during seed development [125,127]. Increasing our knowledge of the biochemical interplay between the liquid endosperm and zygotic embryo could lead to innovative strategies for enhancing embryo development and associated lipid accumulation [127]. Indeed, as indicated above, the liquid endosperm plays an important role in its biochemical interplay with the developing zygotic embryo. Thus, metabolic engineering interventions involving the liquid endosperm may affect SOC.

3. Genetic- and omics-based approaches for identifying functional proteins associated with increased seed oil content

3.1. Genetic-based approaches

Creating novel varieties with higher SOC using hybrid breeding approaches have achieved significant successes in the past few decades; however, this process takes a huge amount of labour and time. In addition, when selecting hybrid offspring with high SOC, the desired agronomic traits are often associated with negative traits leading to decreased agronomic performances [128,129], and are susceptible to environmental factors [130,131]. To prevent adverse effects, accelerate progress and gain in-depth knowledge of germplasm at genetic levels, it is critical to isolate individual quantitative trait loci (QTL) to dissect the mechanism behind the complex traits.

A QTL is a region of DNA located on a chromosome that contributes to quantitative traits [132]. In a natural population, differences in complex traits are usually controlled by multiple QTL. These QTL can be enriched in different kinds of metabolic pathways which are directly and indirectly associated with the specific trait. As a quantitative trait, SOC has been proven to negatively correlate with seed protein content [72,133,134] and seed coat content [135–137], which suggests that numerous enzymes and/or transcriptional factors regulate it. Therefore, it is essential to examine key QTL and explore the mechanisms that lead to SOC variation among populations in order to guide crop breeding.

With ever-advancing high-throughput sequencing and bioinformatics, plenty of QTL affecting SOC in Brassica species have been identified

Table 3
Some representative QTL/QTGs associated with seed oil content (SOC) in *Brassica* oilseed species identified in mapping studies.

Species	Method(s)	Population	Population size	Major QTL/QTGs	Comment	Ref
B. napus	Linkage mapping	DH	151	*qOC.A10*	• A total of 30 QTL were identified • Compared with low-SOC parent, a near-isogenic line targeting *qOC.A10* increased in SOC by 1.3 %–6.9 % without adverse effects on other agronomic traits	[144]
B. napus	GWA mapping, metabolomics and transcriptomics	Natural population	388	*BnA04.SHP1, BnaA05.CPC, BnaA03.TT4, BnaC02.TT4 and BnaAC05.UK*	• 240 candidates were selected based on multi-omics data • All these major QTGs are negatively correlated to SOC • The SOC of the two *BnaTT4* knockout lines increased by 4.8 % and 5.6 %, respectively, and showed a yellow-seeded phenotype • The SOC of two *BnaC05.UK* knockout lines increased by 2.6 % and 3.1 %, respectively	[174]
B. napus	GWA mapping, linkage mapping and transcriptomics	Natural population and RIL	158 and 204	*BnaA07.STR18, BnA07. NRT1 and BnaA07g12880D*	• Nine QTL were identified • The *qA07.SOC* was the major QTL related to SOC • Three important QTGs were predicted within this interval	[214]
B. napus	GWA mapping and transcriptomics	Natural population	382	*BnA09.TT8, BnaC09.TT8 and BnaC07.CCRL*	• Three major QTL related to seed coat content were identified • Disruption of *BnaTT8s* and *BnaCCRLs* resulting in increased in SOC by 4.9–6.0 % and 3.0–4.2 % with lower seed coat and lignin content	[137]
B. napus	Linkage mapping and transcriptomics	RIL	186	*N/A*	• 26 QTL were identified in multi-environments, including 13 novel QTL • 21 differentially expressed genes were recognized and 11 were associated with high SOC	[397]
B. napus	Linkage mapping and BSA	DH	300	*uqA9–12*	• 77 unique QTL were identified by colocalisation of seed lignin, cellulose, hemicellulose and oil content • Eight unique SOC-QTL were detected by BSA, seven of these QTL were also found in the linkage mapping	[156]
B. juncea	Linkage mapping	DH	112	*O-B3*	• Six and five QTL were significant for SOC and seed protein content • Three of them are tightly linked to each other • The *O—B3* were considered to unlink with other protein-related QTL	[133]

Abbreviations: BSA, bulk-segregant analysis; CCRL, cinnamoyl-CoA reductase-like; CPC, CAPRICE; DH, doubled haploid; GWS, genome-wide association; NRT1, nitrate transporters 1; QTGs, quantitative trait genes; QTL, quantitative trait locus; RIL, recombinant inbred line; SHP1, SHATTERPROOF 1; STR18, SULFURTRANSFERASE 18; TT, transparenta testa.

throughout the genome (Table 3). Various types of molecular markers were used for genetic mapping and combined with omics data to narrow down QTL to quantitative trait genes (QTGs) and/or quantitative trait variants (QTVs) [138]. However, due to the high diversity of plant genomes, loci with minor contributions to SOC are difficult to localise. Moreover, identified QTL are rarely validated using experimental approaches [139].

The analysis of QTL can be generally divided into three approaches: linkage mapping, association mapping and bulk-segregant analysis (BSA) [140]. They vary in terms of mapping populations, sample size, population structure and statistical models [141,142]. Although BSA is considered to reduce scale and expense, and is a powerful tool, linkage mapping is the most popular one for identifying QTL. This method involves family-based analysis and can be used to confirm the linkage between particular traits and QTL. Through linkage mapping, the position and effect of each QTL can be determined. In other words, when genetic markers are linked to specific traits, significant variations in phenotypes can be observed among individuals with different genotypes [143]. In linkage mapping, genetic populations are needed to collect data, which may come from biparental, three-way or even multi-parental cross and/or backcross. The commonly used sample populations are F_2, F_2-derived F_3 ($F_{2:3}$; the population derived from self-crossing of the F_2 individuals with the aim to retain the individual's genetic constitution), backcross (BC), recombinant inbred lines (RILs), near-isogenic lines (NILs) and doubled haploids (DHs); among them RILs, NILs and DHs are immortal mapping populations.

Based on previous studies, QTL identification using linkage mapping alone can select many candidate loci (Table 3). However, only a few researchers selected one or two major loci to verify their performance in plants. Zhang et al. [144] constructed a DH population based on two backbone parent cultivars *B. napus* ZY50 (with high-SOC) and *B. napus* 19514 A (with low-SOC), and evaluated their SOC and other agronomic traits under eight environments. Of the 30 QTL identified in this study, *qOC.A10* could be detected in six environments. To validate the effect of this locus on SOC in *B. napus*, a series of NILs were generated via backcross with *B. napus* 19514 A. The results based on the NIL populations showed that *qOC.A10* explained 22.8 % of the genotypic variations and could be used to increase the SOC in *B. napus* 19514 A without adverse effect on other agronomic traits. Because of the low cost of next-generation sequencing technology, it has become possible to establish commercial genotyping platforms for *B. napus* that can be used for linkage mapping and/or association mapping, such as high-density SNP Illumina Infinium arrays [145,146] and target capture sequencing SNP genotyping platform [147]. These platforms have been widely used to investigate complex quantitative traits in BOS including SOC [148–151]. In many linkage mapping studies, researchers frequently use SNP arrays to exploit novel QTL, because they are cost-effective, highly accurate, reproducible and convenient for data analysis [152,153]. A recent example of using SNP arrays to detect SOC-related QTL is based on DH population generated by crossing N53–2 (yellow-seeded with high oil content) and Ken-C8 (black-seeded with relatively low oil content). Through the multi-environment trials, a total of ten QTL were identified including one major QTL *cqSC-A09*. Combined with the bulked segregant RNA-Seq results, this QTL can explain over 41 % of the phenotypic variance. The candidate genes localised in the A09 interval can be enriched in lipid metabolism, flavonoid biosynthesis and phenylpropanoid biosynthesis pathways [136]. The detailed characteristics of yellow-seeded versus black-seeded lines are discussed in Section 3.2.

Compared with conventional linkage mapping, which requires collection of data from all individuals of the segregated population, BSA is a powerful tool to screen for QTL of individuals with extreme opposite/representative phenotypes (for a review, see [154]). This approach involves constructing two pools containing individuals with extreme phenotypes, and genotyping of the parents and the pools, through screening for molecular markers between parents and the pools to accomplish the QTL mapping for target traits (for a review, see [155]). In this way, it has effectively overcome the limitation that many crops do not have NILs or they are difficult to create. In *B. napus*, this method was usually used to confirm and narrow down the interval of SOC-QTL selected from linkage mapping based on the overlap of these two approaches. Chao et al. [134] found 67 QTL for SOC in N53–2×Ken-C8 populations, and 38 associated genomic regions (AGRs) overlapped with or narrowed down the SOC-QTL identified in the linkage mapping based on BSA data. In another study that utilised the same population, BSA results confirmed that seven out of the eight novel QTL found regulated SOC [156].

Although the linkage mapping and BSA have provided a significant boost to QTL identification in BOS, the discovery of QTGs and/or QTVs in the last decade has owed greatly to the innovation and application of genome-wide association (GWA) mapping. This mapping strategy is based on linkage disequilibrium, usually using SNPs as molecular markers, and aims to analyse the entire target genome to identify causative loci/genes associated with the phenotypes [141]. Similar to linkage mapping, many bi-parental and multi-parental populations can be used for GWA mapping and they are considered to address some drawbacks of wild populations such as undesirable traits or genetic incompatibility. However, previous studies have relied mostly on natural populations because of the abundance of trait variation and genetic diversity (for a review, see [157]). In addition to the benefits mentioned above, the advantages of GWA mapping include: saving time and effort as there is no need to generate populations by crossing, a high-density map can be constructed, and allowing for validation by focusing only on marker positions (for a review, see [158]).

In BOS, GWA mapping helped the discovery of many QTGs which positively or negatively affect SOC [149,159,160]. In a study of dissecting seed-quality traits of *B. napus*, whole-genome resequencing data obtained from 238 cultivars and inbred lines under multiple environments were used for GWA mapping. A total of 17 loci were identified and 37 genes involved in acyl lipid metabolism were annotated for SOC. Eight of these were identified in previous studies, encoding a dienoyl-CoA reductase, a Sac domain containing phosphoinositide phosphatase [161], CTP: phosphoethanolamine cytidylyltransferase, ketoacyl-CoA synthase [162], phosphatidate phosphatase [163], a MYB transcription factor, glycerophosphoryl diester phosphodiesterase and the CER1 aldehyde decarboxylase [149]. Moreover, four novel candidates, *BnaA10g23290D* (BnaA10.GDPD6, which encodes the glycerophosphodiester phosphodiesterase), *BnaCO4g45690D* (BnaC4. GPAT), *BnaCO4g45790D* (BnaC4.LTP2) and *BnaCO445800D* (Bna.LTP1) were found to be highly correlated with variations in SOC [164]. For SOC-related QTGs identification in other BOS, such as *B. rapa*, GWA mapping is also a powerful tool. Kaur et al. [165] utilised diversity array technology for genotyping 195 inbred lines and their derivatives. Through association mapping five genes, *GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID TRANSFER PROTEIN 5*, *GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID TRANSFER PROTEIN 33*, *FRUCTOKINASE 7*, *SHRUNKEN SEED 1*, and *LACS8*, which directly or indirectly regulated SOC, were detected. Although genes within the interval of identified SOC-related QTL are attracting a great part of the interest, there are a number of SNPs localised in the non-coding region that are also associated with oil content. A GWA study on SOC collected data from 290 core germplasm and identified *BnaC07g30920D*, *a PATATIN-LIKE LIPASE* gene, and also found that any one of six SNPs upstream of this gene could cause a 4.7–6.2 % reduction in oil content. Through in silico analysis, it was determined that these SNPs were localised in the CAAT-box [166] within the promoter region and may, therefore, affect the expression of this gene. The CAAT-box is a common *cis*-element, usually localised 60-100 bp upstream of the transcription start site, which acts as a recognition site for various transcription factors, such as CBF (CAAT-binding factor) [167]. A mutation within the CAAT-box can lead to significant changes in the strength of the promoter, which can affect the expression levels of the downstream gene. One representative example which may provide some

clues to explain the correlation between the CAAT-box and SOC is *LEAFY COTYLEDON1* (LEC1). LEC1, a key regulator of embryonic development and FA biosynthesis [168], encodes a protein with a HAP3 subunit of CBF which can interact with the CAAT motif to precisely regulate the gene expression involved in lipid metabolism [169,170].

In order to overcome the issues caused by using a single mapping approach, some studies have combined linkage mapping with GWA mapping to detect SOC-related QTL [171,172]. Sun et al. [171] constructed two F_2 populations by using 6F313 and 61,616 (with high SOC) with 51,070 (with low SOC) for both linkage and GWA mapping. Based on the data from 227 accessions in multiple environments, 40 and 29 QTL were identified by linkage and association mapping, respectively. Seven QTL were in common between the two methods, and they explained 10 to 25 % phenotypic variation of SOC.

Besides genomic information, other molecules such as transcripts and metabolites can also be used to link SOC to QTGs/QTVs in *Brassica* species. Traditional GWA studies involve analysis of common variants to identify phenotype-associated loci, but most of the identified loci are located in non-coding regions, and it is difficult to elucidate how they affect the phenotype. Therefore, more biological datasets from multi-omics studies are needed to understand complex quantitative traits [138]. There are a great number of studies combining transcriptomics and metabolomics with GWA mapping to uncover SOC-related QTGs (Table 3). One representative example for using GWA and transcriptome-wide association (TWA) on SOC is based on 505 *B. napus* inbred lines in eight environments. Overall, 692 genes and four gene modules that significantly associated with SOC were identified. These genes are mainly related to lipid metabolism, flavonoid biosynthesis, transcript factors and transporters. Among them, *PUTATIVE METHYL-TRANFERASE6,* which has two homologs, has been verified via genetic manipulation. When knocked out by the CRISPR-Cas technique, SOC showed 1.1–1.2 % and 2.6–3.0 % absolute increase in the single and double knockout lines, respectively. Moreover, there are many genes down-regulated in *BnaPMT6* over-expression lines that are positively correlated with SOC [173]. Li et al. [174] examined metabolites via GWA and TWA studies to study SOC in 388 *B. napus* accessions. They found 131 marker metabolites associated with SOC and constructed a triple relationship network to investigate the relationship between SOC and these compounds. Through this network analysis, 240 genes were selected and considered to be involved in regulation of SOC. Furthermore, three genes *BnaA03.TT4*, *BnaC02.TT4*, and *BnaC05.UK* (a *PHENYLALANINE AMMONIA-LYASE-LIKE* gene), were validated to negatively affect SOC by generating loss-of-function lines. Due to the complexity of the *Brassicaceae* species genomes and the challenges associated with constructing populations, utilising the established associative transcriptomics platform for SOC related traits study is regarded as a highly feasible approach as well [175].

3.2. Omics-based approaches

The analysis of global gene expression in *B. napus* lines differing in SOC has proven to be a useful approach for identifying functional proteins which might be associated with seed oil biosynthesis. [75,176–179]. The general idea here is that transcripts and/or proteins exhibiting substantially altered levels in high SOC lines, when compared to low SOC lines, may represent potential gene targets for manipulation to enhance SOC in various cultivars of BOS. In an investigation involving two NILs of *B. napus* differing in SOC by 10 %, Li et al. [177] used subtractive suppression hybridization to identify 30 up-regulated genes in the high oil line. Among the highly expressed genes in the high SOC line were those related to chloroplast function, suggesting that photosynthesis was enhanced in this line. In addition, sucrose synthase production was increased in the high SOC line: this enzyme plays a role in supplying carbon for TAG biosynthesis in developing seeds of *B. napus* [112]. The gene encoding plastidial pyruvate kinase also showed enhanced expression in the high SOC line [177]. Plastidial pyruvate kinase has a role in supporting FA biosynthesis in developing seeds [180]. This latter observation on enhanced expression was corroborated further via enzyme activity measurements on the high versus low oil lines [177]. The expression of the gene encoding 6-phosphogluconate dehydrogenase was also increased in the high SOC line. This enzyme operates within the OPPP which, as previously mentioned in Section 2, provides reductant to support FA biosynthesis [117]. In contrast, among the differentially expressed genes (DEGs) identified in [177], the gene encoding mitochondrial pyruvate dehydrogenase complex kinase (PDCK) showed decreased expression. PDCK catalyses the phosphorylation of the mitochondrial pyruvate dehydrogenase complex which controls entry of carbohydrates into the TCA cycle. Marillia et al. first demonstrated in Arabidopsis that both constitutive and seed-specific partial silencing led to increased SOC [181]. Several other genes identified in this study encode products related to lipid metabolism, including a lipid transfer protein, fatty acid elongase, oleosin, phospholipase, acyl CoA oxidase, thioesterase and an ABC transporter [177].

In another study, differential gene expression analysis comparing two *B. napus* lines (zy036 and 51,070), differing in SOC, resulted in the identification of the *B. napus GROWTH-REGULATING FACTOR 2-LIKE* gene (*BnaGRF2*) in the high SOC line (zy036) [178]. *BnaGRF2* encodes a protein that is thought to function as a TF, and the genes encoding this protein, *BnaGRF2a* and *BnaGRF2b*, were identified in the A and C genomes, respectively. Constitutive and seed-specific over-expression of cDNA encoding BnaGRF2a in Arabidopsis resulted in relative SOC increases of >40 %, when compared to the wild type (WT), and was associated with upregulation of genes involved in cell proliferation, photosynthesis and oil synthesis, thus potentially influencing seed weight and overall oil content [178]. Furthermore, the genes, *BnaGRF2a* and *BnaGRF2b* were identified in the A and C genomes, respectively. Constitutive and seed-specific over-expression of cDNA encoding BnaGRF2a in Arabidopsis resulted in relative SOC increases of >40 %, when compared to the wild type (WT) and was associated with upregulation of genes involved in cell proliferation, photosynthesis and oil production. Comparative gene expression analysis of the *B. napus* NILs, YC13–559 and YC3–554, representing high- and low-SOC lines respectively, has revealed the up-regulation of several lipid biosynthesis-related genes encoding enzymes including ACCase, DGAT and LACS [179].

Comparative proteomics-based approaches have also been applied to high- versus low-SOC lines of *B. napus* [78,182]. Gan et al. [182] performed ultrastructural analysis combined with proteomic and comparative genomic analyses to investigate differences between a high- (55 % SOC) and low-SOC line (37 % SOC). OBs were much closer to each other in the high-SOC line. Proteins representing OB proteins, other oil formation-related proteins and proteins involved in dehydration were among 119 differentially accumulated proteins. Zhou et al. [202]. used a quantitative proteomic approach involving two *B. napus* lines differing in SOC which showed the differential accumulation of 342 proteins. Late embryogenesis abundant protein 57 (BnaLEA57) increased in abundance in the high-SOC line when compared to the low-SOC line. Over-expression of *BnaLEA57* in Arabidopsis resulted in increased SOC [78].

Breeding of rapeseed in China has resulted in the genetic conversion of HEAR cultivars with relatively low SOC to LEAR cultivars with high SOC over a period of about two decades [183]. In this regard, Hu et al. [183] have compared the transcriptomes of cultivar Zhongyou 821, with high erucic acid content and low SOC, to that of its low-erucic acid descendant Zhongshuang 9. Selective pressure for zero 22:1, low glucosinolate content, high 18:1, high SOC and high yield resulted in increased expression of genes encoding FAD3, ACCase, fatty acid elongation 1, caleosin, glyceraldehyde-3-phosphate dehydrogenase and phosphoenolpyruvate carboxylase.

Other comparative studies have focused on analysing the expression of specific gene families in high- versus low-SOC lines of *B. napus* [184,185]. Xiao et al. [185] used genome-wide identification coupled with comparative expression analysis to examine *LACS* genes in a high-

(P1-HO) versus a low-SOC (P2-LO) line. BnaLACS proteins were arranged into four groups based on phylogenetic analysis. Eighteen *BnaLACS* genes were highly expressed during seed maturation. Comparative analysis of gene expression between the two lines revealed that two of the *BnaLACS* genes, *BnaLACS1–10* and *BnaLACS4–1*, may play a key role in lipid biosynthesis in *B. napus*. Recently, genome-wide identification of OB proteins has revealed up to 88 genes encoding OB proteins in *B. napus* [186]. Jia et al. [184] conducted a genome-wide analysis of *B. napus* to identify 53 *OLEOSIN* genes. Comparative expression analysis between two lines differing in SOC revealed that *BnaOLEOSIN* genes were directly linked to genes encoding lipid metabolic enzymes, TFs, lipid transport proteins and carbohydrate metabolic enzymes, all contributing to the molecular network underlying seed oil accumulation.

Yellow-seeded lines of *B. napus* have a thinner seed coat and lower husk content which is associated with increased SOC [187]. Yellow-seeded lines were briefly mentioned in the previous Section 3.1 in relation to genetic-based approaches for increasing SOC. When processed, yellow-seeded lines result in nutritionally improved meal [187–189]. Yellow-seeded lines of *B. napus* appear yellow because of mutations in *TRANSPARENT TESTA* (*TT*) genes which lead to a transparent seed coat (testa) revealing the yellow-colored zygotic embryo [190]. Yellow-seed lines have been associated with increased SOC and the processed seed results a nutritionally improved meal [188,189]. Several of the *TT* genes have been shown to be involved in the regulation of flavonoid biosynthesis leading to accumulation of proanthocyanidins (condensed tannins) in the seed coat thereby imparting a darker color to seeds of regular dark-seeded lines [190–193]. For example, BnaTT16 isoforms have been shown to be involved with the transcriptional regulation of *ANTHOCYANIDIN REDUCTASE* and *DIHYDROFLAVONOL REDUCTASE* along with five other genes involved in proanthocyanidin synthesis [192]. In most cases, defects in seed coat pigments do not result in detrimental physiological effects [93,190]. RNA interference-mediated down-regulation of *TT16* in canola-type *B. napus*, however, resulted in impaired embryo and seed development with alterations in the expression of genes related to lipid metabolism [194]. Hong et al. [195] have compared the transcriptomes between yellow- and brown-seeded NILs. In the yellow-seeded line, 3128 up-regulated and1,835 down-regulated genes were identified during seed maturation. Genes involved in encoding proteins associated with phenylpropanoid and flavonoid biosynthetic pathways were identified among the down-regulated genes. In another comparative study, Jiang et al. [188] also identified an enrichment in DEGs encoding proteins related to flavonoid and lignin synthesis in a yellow-seeded line compared to a black-seeded line. In yet another study, gene expression analysis between a yellow- and black seeded lines revealed 23 co-expression modules involving differentially spliced genes, which included genes encoding proteins associated with flavonoid biosynthesis such as *TT5*, *TT8* and *TT12* [189]. Recently, gene editing via CRISPR/Cas 9 was used to introduce targeted mutations into various *BnaTT* gene forms [196,197]. Zhai et al. [197] introduced targeted mutations into *BnaA09.TT8* and *BnaC09.TT8b* to produce the yellow-seeded phenotype. This phenotype could only be recovered when both gene forms were mutated indicating the redundant roles of the two gene forms. Interestingly, both SOC and protein content of edited seed were elevated. In another study, genome-wide association mapping of seed coat color in *B. napus* has shown that 22 single-nucleotide polymorphisms distributed on seven chromosomes are associated with seed color [198]. The underlying causes for enhanced SOC in yellow-seeded appears to be complicated [199]. Because of the reduced pigment accumulation in the seed coat of yellow-seeded lines, more carbon may be allocated to oil deposition in the zygotic embryo. Along with down-regulation of genes related to testa-based pigment accumulation in yellow-seeded lines, a large number of genes related to FA biosynthesis have been shown to be up-regulated: in addition, genes related to β-oxidation were down-regulated [199].

Given the maternal influence of the silique on SOC, comparative functional omics-based studies have also been conducted on the developing silique wall of high- versus low-SOC lines of *B. napus* [111,118,121]. Hua et al. [111] have performed comparative transcriptomics of the developing silique walls of line zy036 (high SOC line) and 51,070 (low SOC line), which were mentioned earlier in this section of the review. The results implied that genes associated with photosynthesis were highly expressed in both total silique wall expressed genes and genes that were differentially expressed between the two genotypes. Flux control coefficients are given for these processes in Section 4. Two other studies, using high- and low-SOC lines, have also revealed the up-regulation of genes associated with photosynthesis in the developing silique wall [118,121]. Developing silique walls from the high-SOC line L192 also exhibited increased expression of genes encoding sugar transporters when compared with the low-SOC line A260 [118].

Rapeseed SOC is also known to be influenced by environmental factors [23,25,200]. The effect of temperature on seed oil accumulation in *B. napus* has been known for a long time [201]. Zhu et al. [202] developed two NILs of *B. napus* (NIL-9 and NIL-1) differing in SOC, with NIL-9 exhibiting higher SOC. The difference in SOC between the two lines was mainly associated with a QTL region on chromosome C2 when seeds were allowed to mature at high temperature. NIL-9 exhibited a higher SOC at all temperatures tested, especially higher temperatures. Increasing temperature resulted in a decrease in SOC that also involved the down-regulation of several genes including genes associated with photosynthesis and lipid metabolism. Zhou et al. [203] have explored the effect of high night temperature on FA content, FA composition and transcriptome of a low- (Jiuer-13) versus high- (Zheyou-50) SOC lines of *B. napus*. Increased night temperatures in both the low- and high-SOC lines resulted in decreased SOC, but high night temperatures caused less of a relative decrease in SOC than for the low-SOC line. In addition, in both lines, high night temperatures resulted in a decrease in the proportions of 18:0 and 18:1 and increase in 18:2 and α-18:3. Transcriptomic analysis showed that genes encoding proteins involved in gibberellin signaling and FA catabolism were up-regulated at high night temperature. The results on the effects of high night temperature on FA composition observed by Zhou et al. [203] are in contrast to what was previously reported by Canvin [201] where increasing growth temperatures resulted in a decrease in PUFA content and an increase in 18:1 content. SOC was also decreased with growth at higher temperatures. In addition, Vuorinen et al. [204] have shown that low temperature (15 °C) and short days (12h) increased the proportion of α-18:3 in the seed oil of *B. rapa.* The study of Zhou et al. [203], however, focused on changes in night temperature.

A high-SOC *B. napus* line known as H105 was known to exhibit a SOC of about 46 %, 54 % and 53 % when grown in Nanjing (altitude 8.9 m), Xining (altitude 2261 m) and Lhasa (altitude 3658 m), respectively [205]. Transcriptomic analysis of H105 grown at different altitudes revealed that 363 genes and 421 genes were changed by two-fold or more when H105 was grown in Xining and Lhasa, respectively, compared to growth in Nanjing, the lowest altitude. Some genes encoding functional proteins involved in sugar metabolism were up-regulated. The results suggested possible gene targets for manipulation to increase SOC at lower elevations.

Numerous recent studies have combined QTL analysis with other omics-based analyses [134,137,173,202,206–214]. In a very recent example, Zhang et al. [213] have used high-throughput chromatin conformation capture technology to examine the differential three-dimensional landscape of genome architecture between two *B. napus* lines differing in SOC (high SOC N53–2 and low SOC Ken—C8). Multi-omics analysis showed that SOC-associated genes were strongly correlated with genome structural variations in the QTL/associated genomic region (AGR) which encodes the biotin carboxylase subunit of heteromeric acetyl-CoA carboxylase (ACCase). A candidate gene identified as *Bna09g48250D*, which encodes the biotin carboxylase subunit of heteromeric ACCase, exhibited structural variation in the QTL/AGR region

of chrA09. Heteromeric ACCase acts along with the FAS complex in the plastid to produce FAs de novo (see Section 2). Over-expression and knockout of this candidate gene resulted in significant increases and decreases in SOC, respectively, in transgenic lines. Taken together, comparative omics analysis of low and high SOC *B. napus* lines could reveal valuable information, but overexpression of the identified genes would not necessarily always lead to even higher SOC in the high SOC lines, given the constraints that will be presented in Section 4. In addition, the genes may be exploited for SOC improvement in other cultivars not displaying high expression levels of these genes or gene variants.

Omics-based approaches have identified a plethora of DEGs and gene products in high versus low oil lines of *B. napus*. In some cases, selected genes were further evaluated in metabolic engineering experiments to check if SOC could be increased in Arabidopsis and/or *B. napus* transgenic lines. The results of comparative omics, however, are based on a 'guilt-by-association' approach. Thus, it is possible that many of the DEGs identified in the various studies may not result in increases in SOC when evaluated further for functionality in transgenic plant systems.

4. Metabolic control analysis of storage lipid biosynthesis

Having discussed genetic and omics approaches for identifying factors related to increased SOC in BOS, the review now moves onto MCA as a means of identifying points of control in biochemical pathways leading to seed TAG. A substantial component of this section, however, is devoted to explaining MCA theory.

Yield of a metabolic product, such as a seed component, in a plant is the integral of the rate (or flux) of the metabolic pathway over the period of biosynthesis into the site of deposition. Whereas the overall product yield from plants grown in field or greenhouse can be readily measured, it is less easy to determine the pathway flux and even more so to quantify the effects on it of specific modifications of the metabolic apparatus. This is further exacerbated where the product is a mixture of different but related compounds such as the TAGs of oilseeds.

As usual in agriculture, most of the past increases in the yield of seed oil and changes in composition have come from selective breeding, exploiting natural or induced genetic variation, but generally not targeting specific components of metabolism. More recently it has been possible to use techniques of genetic manipulation to introduce targeted changes in the metabolic pathways from photosynthate to TAG in seed oil. That raises the question of which of the many steps of metabolism to target. Many decades ago, the conventional response would have been to choose the rate-limiting step (RLS) of the target pathway and activate or amplify it. This concept still lives on in spite of many contrary examples, such as the failure of over-expression of the gene encoding phosphofructokinase in yeast, the widely cited RLS of glycolysis, to have any effect on glycolytic flux [215]. There are, of course, two potential modes of failure of such attempts: choosing a step that is not a major limitation on the pathway flux, and not succeeding in sufficiently increasing the quantity of target enzyme and/or sufficiently activating the enzyme in the event that it has allosteric properties.

Deeper understanding of the limitations on a metabolic flux and the effects of changing the activity of a component enzyme has come from MCA, devised independently but contemporaneously by Kacser and Burns [216] and Heinrich and Rapoport [217–219] as a critique of the RLS concept and the criteria that were supposed to identify it. The starting point for MCA was that the relationship between the pathway flux at metabolic steady state and the activity of any one enzyme (at constant levels of the others), is typically concave (Fig. 2), even approximately rectangular hyperbolic like the Michaelis-Menten equation, based on evidence from experimental observation, computer simulation and mathematical analysis of the kinetics of multistep enzyme pathways. In other words, an enzyme's influence on metabolic flux is continuously variable and diminishes as the enzyme activity increases. The original specific examples, and many subsequent ones, are reviewed in Fell [220]. (See Fig. 3.)

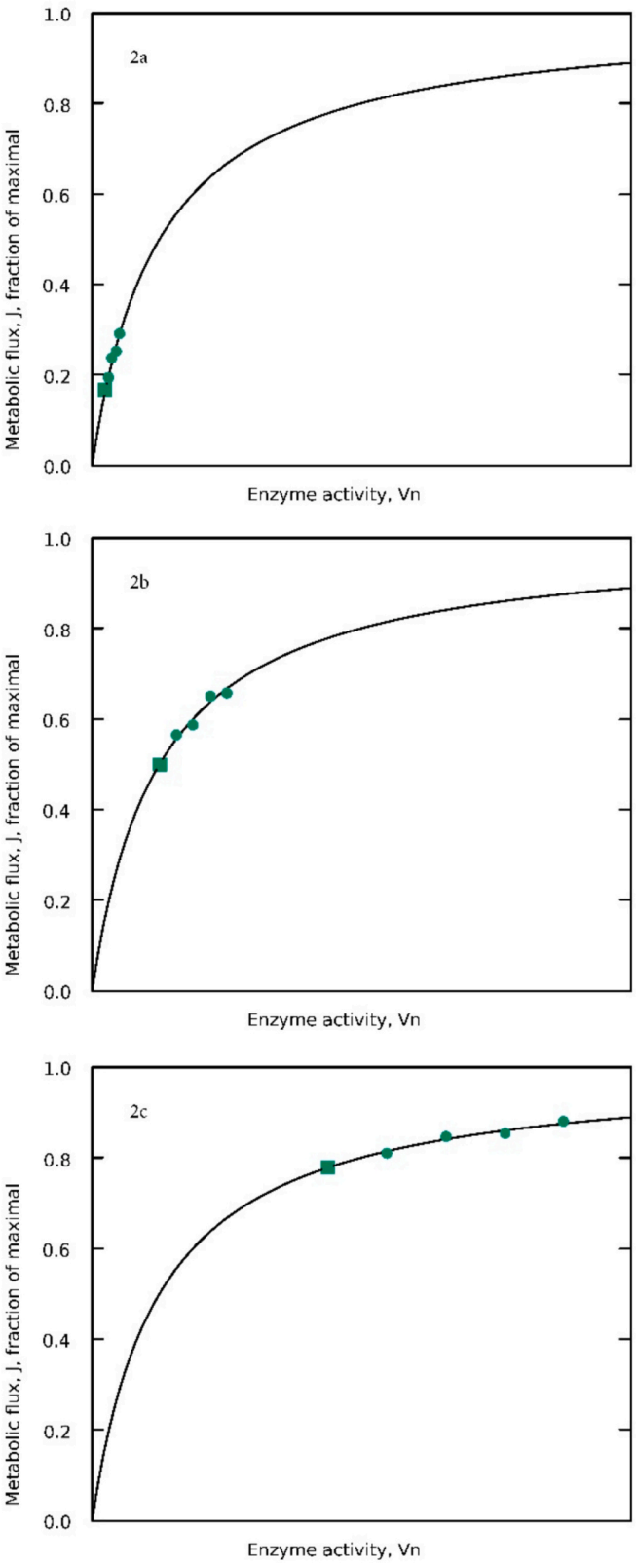

Fig. 2. The concave dependence of metabolic flux on the activity of one enzyme, 'n', at constant activities of the other enzymes of the pathway. Overlaid on the same flux-enzyme curve are the outcomes of hypothetical over-expression experiments starting from three different wild type (WT) activities, marked by squares, in increasing order 2a to 2c. In each panel, a series of steps are shown up to a two-fold amplification over the respective initial WT activities.

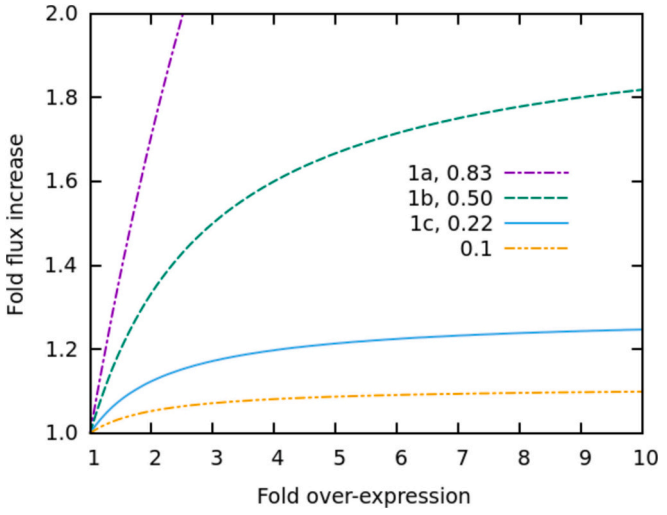

Fig. 3. Dependence of the response to enzyme over-expression on the enzyme's flux control coefficient.
The three cases of Fig. 2a-c are shown, plus a fourth case for a flux control coefficient of 0.1, in the higher range of values measured to date for individual enzymes of triacylglycerol accumulation. In this figure, over-expression refers to an increase in enzyme abundance.

The implications of this are illustrated in Fig. 2 for three hypothetical cases of experiments to modify the flux of a pathway by doubling the WT activity of a single enzyme in a sequence of steps at constant activities of the remainder of the enzymes. In each case, the flux correlates with the enzyme activity, but the outcomes are significantly different. In the example of Fig. 2a, the initial activity is relatively low, and the flux almost doubles, in direct proportion to the change in activity. Furthermore, the flux is still only about 30 % of the maximum obtainable so there is scope for further increase. In the case of Fig. 2b, flux is no longer directly proportional to the enzyme activity and its relative increase compared to WT is only about 30 %. In addition, the scope for a further increase is much reduced. Finally, as shown in Fig. 2c, doubling of enzyme activity changes the flux from about 75 % of maximum to 88 %, but it will now be difficult to achieve much further improvement. Hence it is apparent that qualitative statements about the limitation of the metabolic flux by Enzyme n, though not wrong, do not convey the information needed to discriminate between three such scenarios, nor to predict the response to increased production of enzyme.

To provide a measure that would allow characterisation of these different behaviours, both the Kacser and Heinrich groups turned to the concepts of sensitivity analysis to provide a numerical measure of the enzyme's influence at any point on the curve. This coefficient was later renamed as the *flux control coefficient*, and can be approximately regarded as the percentage change in metabolic flux that results from a 1 % change in enzyme activity. (The technical definitions and earlier terminology are reviewed by Mazat [221] Its value at the WT levels shown in Fig. 2 are: in 1a, 0.83; in 1b, 0.50, and in 1c, 0.22. Although flux control coefficients can be measured experimentally by specific up- or down-modulation of an enzyme's activity, a 1 % modulation would not permit accurate evaluation, so larger modulations need to be used, and appropriate methods have to be used to compute the result to take into account that the flux control coefficient itself will change over the measurement interval [222,223].

Starting from their mathematical definition of the flux control coefficient, the originators of MCA were able to explore its properties in relationship to the kinetics and control of metabolic pathways. One significant property is that the sum of the flux control coefficients of all the steps from the input substrate of a linear pathway to the output product is exactly 1. In the case of the example of enzyme *n* in Fig. 2, this means that the sum of the flux control coefficients of the other pathway enzymes is 1–0.83 = 0.17 in Fig. 2a, 0.50 in 1b and 0.78 in 1c, even though these enzymes did not have their activities modulated. Hence the flux control coefficient is not an intrinsic property of the enzyme itself but is a context-dependent system property (i.e., dependent on the specific environment). Furthermore, as the total available control is potentially distributed over the pathway, the more steps there are in the pathway, the less likely it is that any one step will have a large flux control coefficient. Experimental measurements over the years since have verified that this is generally the case (reviewed in [220]).

Further analysis showed that mathematical expressions for the value of a flux control coefficient depended on the kinetic attributes of the enzyme and reaction, such as maximal velocity, K_ms and degree of displacement of the reaction from equilibrium, but in combination with these same attributes for all the other steps [216,217]. As a result, no single measure or property could point to the most rate-limiting enzyme (i.e., the one with the largest flux control coefficient). A more recent analysis has shown that the degree of displacement of a reaction from equilibrium, in relation to the overall displacement of the pathway from equilibrium, sets a maximum attainable value for the flux control coefficient [224] but that this is not inevitably realised.

There are many ways to measure flux control coefficients. These have been reviewed by Fell [220,225] and they will not be detailed here. However, the underlying strategies can be divided into two broad areas. One, the bottom-up approach, corresponds to the description in Fig. 2., where the WT activity of an enzyme is selectively modulated up or down, or both, by techniques such as changing genetic expression or the action of a specific inhibitor. The change in pathway flux is measured when a new steady state is established, thus determining a segment of the flux-activity curve in the vicinity of the unperturbed state. Apart from the demands on selectivity and precision of measurement, this can be laborious for a long pathway.

The second approach is the top-down method [226,227]. Here, the pathway is divided into a small number of blocks (typically two or three) around a common metabolite whose concentration can be measured. The concept depends on the fact that the flux control coefficients of the enzymes within a block are additive so there will be an overall flux control coefficient for each block. If these are determined, this then gives a high-level view of the distribution of control. To determine the flux control coefficients of the blocks, the flux in one block is perturbed by some means that is selective to that block, such as an inhibitor or genetic modulation. Unlike in bottom-up analysis though, it is not necessary to know the magnitude of the perturbation. The system will come to a new steady state at a changed concentration of the common metabolite, and the fluxes through the other blocks are measured. This allows the kinetic response of these blocks to the change in concentration of the common metabolite to be determined in the form of a coefficient termed the *elasticity*, which like the flux control coefficients approximately represents the percentage change in the flux through the block for a 1 % change in the common metabolite. This is repeated with perturbations in the other blocks. Flux control coefficients and elasticities are linked via another theorem of MCA, the *connectivity theorem*, which, together with the flux summation theorem allows the flux control coefficients for the blocks to be calculated [228,229]. Choosing a different block structure with a different common metabolite can allow further dissection of how control is distributed. Top-down control analysis (TDCA) is not dissimilar to assigning source and sink strength in plant physiology.

Both bottom-up and top-down approaches have been applied in the determination of the control of flux to TAG, as will be detailed later in this review. It is also possible to combine results on a given metabolic system from both methods to obtain an enhanced view of the distribution of control (e.g. [108]).

Flux control coefficients are defined for small changes in activity that would be of little interest for metabolic engineering purposes, but it can be seen from Fig. 2 that they do indicate the scope for obtaining a significant increase of flux for a large change in an enzyme activity. The relationship between the fold-change in flux, *f*, obtained for an *r*-fold

change in an enzyme with a flux control coefficient, C_E^J is [222]:

$$f = \frac{1}{1 - \frac{r-1}{r} C_E^J} \quad (1)$$

Assuming a very large amplification of activity, where $r - 1 \approx r$, the maximum attainable fold change in flux is $f = \frac{1}{1 - C_E^J}$, which gives values of 5.9, 2.0 and 1.3-fold for the three examples of Fig. 2. The substantial differences in the response to enzyme activity over-expression are illustrated in Fig. 2 for these three examples, plus a fourth case for a coefficient of 0.1, which is closer to the range for some of the measured coefficients in TAG accumulation (see later). This illustrates the importance of the flux control coefficient in choosing targets for metabolic engineering.

These results may seem rather discouraging for the prospects of making substantial changes by metabolic engineering, but MCA theory does point to some potential improvements to the outcomes:

1. The finite change analysis of Small & Kacser [222] shows that if, in a linear sequence (or block) of enzymes, each is increased in activity *r*-fold, the gain in flux is governed by the sum of their flux control coefficients in Eq. 1, giving synergy in the effects of multiple enzyme amplifications. 'Gene-stacking' (as it is termed in plant engineering, [230,231]) is required to do this, but it is not simple, especially as approximately equal increases in enzyme activity are needed to avoid undue perturbations of metabolite concentrations.
2. Examination of large metabolic flux changes in living systems in the light of the issues pointed to by MCA shows that evolution has already discovered solutions. The most obvious examples are the operons in bacterial metabolism, which coordinately express adjacent enzymes. In eukaryotic metabolism, the mechanisms are not so overt, but without the distorted viewpoint of the RLS, it can be seen that very large flux changes with smaller changes in metabolite concentrations are obtained by 'multi-site modulation' [220,232]. This enrols various mechanisms, including signaling metabolites that are effectors of multiple enzymes, covalent modification cascades, signal transduction mechanisms, and transcription factors with multiple targets in a metabolic network. In the context of modifying TAG metabolism, examples of exploitation include the expression of the TF, WRINKLED1 (WRI1), which has extensive effects on central metabolism [233], and the antisense-based deactivation of mitochondrial pyruvate dehydrogenase complex kinase (PDCK) [231] to potentiate amplification of DGAT1.
3. In the specific context of TAG accumulation in *B. napus*, the growth of the zygotic embryo [234] compounds any stimulation of TAG synthesis so that typically the fractional increase in yield by the end of seed filling is about 3.5 times the fractional change in flux caused by an enzyme activation [234,235].

In addition to its explanatory power in metabolic engineering, MCA also has relevance to improvement of metabolic traits by artificial selection. The concave relationship between enzyme activities and metabolic flux underlies and can explain some examples of genetic phenomena such as dominance [236], heterosis and epistasis, as reviewed by de Vienne et al. [237]. It was also realised early in the development of MCA that, given a constraint on the total amount of enzyme protein available for a metabolic pathway, there would be an optimum distribution of that protein that would give the maximum flux. Although the flux control coefficients would not equalise, the variance of the flux control coefficients would be at a minimum, thus mitigating against a dominant control coefficient [238,239]. Correspondingly, if a pathway was not in the optimal state, but was then subject to natural or artificial selection, the largest increase in flux would be obtained by reassigning protein from enzymes with low flux control coefficients to the one with the highest, which is, therefore, under the greatest selection pressure. Continued selection will drive towards selective neutrality, and the maximum flux state with flux control distributed over the pathway [237,240]. Since the wild progenitors of an oilseed crop would have undergone selection for a range of attributes ensuring survival, it cannot be assumed that their pathway flux to TAG has been optimised. Once selection is instituted for SOC, the reassignment of enzyme protein will occur, along with reduction in the larger control coefficients so that the wild relatives (especially Arabidopsis!) will not be a guide to the distribution of control in the crop plant. A different distribution of enzyme activities between high- and low-yielding cultivars of *B. napus* has been observed in multi-omics studies previously mentioned in Section 3.2 [76,177] The enzymes with higher activities or abundances in the high SOC lines are likely to be among those with the higher flux control coefficients for TAG synthesis in the low-SOC lines. However, as shown in the previous paragraphs, their flux control coefficients are likely to be lower in the high-SOC lines, so it cannot be concluded from the data that they would be the best targets for achieving further improvements in SOC in these plants given that their reduced control would have led to increases in control by other enzymes.

5. Selected enzymes and functional proteins as targets for increasing seed oil content

The major strategies of engineering oil accumulation in BOS have focused on enzymes involved in lipid metabolic pathways. Many researchers have reviewed the genetic factors affecting oil content in different kinds of plant tissues which can be briefly divided into three categories: (1) increasing fatty acid supply, (2) enhancing the TAG assembly efficiency and (3) reducing TAG degradation (for reviews, [24,241–245]). However, regulating a single enzyme in lipid metabolism sometimes does not achieve expected results because its contribution to oil yield is relatively minor, as detailed in Section 4. It is worth noting that a multiple gene strategy is usually considered as a sustainable and stable way to promote oil accumulation (see Section 4 for details). With this approach, enzymes, transporters and TFs targeting different aspects of lipid metabolism can be manipulated simultaneously to enable oil accumulation [246–248]. Some representative studies are summarized in Table 4. Although it would have been valuable to calculate the flux control coefficients of each of the specific enzymes manipulated in order to rank their potential efficacy for increasing SOC, these could only be determined from the data provided in a few of these studies (Table 4). Nevertheless, the two enzymes with the biggest control coefficients (G3PDH and LPAAT) are linked with the largest changes in SOC. Other areas of metabolism that might accommodate enzymes with comparable control are indicated in Fig. 4.

Generally, oil biosynthesis in plants can be divided into three stages (see Section 2): the de novo synthesis of FAs, TAG assembly, and OB formation [40]. In oilseed crops, FAs are synthesized in the plastids with malonyl-ACP as the substrate and non-esterified (free) FAs as products. In this pathway, several enzymes and complexes are involved, which has attracted the interest of researchers to engineer seed oil accumulation in BOS by using them. Many attempts in increase SOC focused on the ACCase which is the initiating and a critical enzyme in FA synthesis. In plants, there are two forms of ACCase, one is plastidial form which is a multi-enzyme complex comprising several subunits, and the other one is cytosolic ACCase, a single multifunctional protein [28]. It has been widely reported that four subunits of heteromeric ACCase have a positive correlation with SOC [249–252]. Moreover, seed-specific overexpressed homomeric ACCase in *B. napus* could result in a 5 % increase in SOC [253],though the data presented resulted in a computed flux control coefficient of the order of 0.03 according to Fell et al. [108] (Fig. 4). The enzyme has also been used to improve oil accumulation in vegetative tissues such as potato tubers [254]. In fact, in leaf tissue ACCase has been shown to be very important [255]. The lengthening of the acyl chain catalysed by the FAS complex is due to several different condensing enzymes. Among them, KAS III (sometimes called the short-chain condensing enzyme) is the initial one and converts acetyl-CoA to

Table 4
Improving seed oil content (SOC) by regulating important enzymes in *Brassica* oilseed species.

Target gene(s)	Source species	Target species	Increased SOC	FCC	Comment	Ref
BnACC1	*B. napus*	*B. napus*	5 %	»0.03	• Seed-specific over-expression of Homomeric ACCase	[253]
SsG3PDH	*S.cerevisiae*	*B. napus*	up to 40 %	0.16–0.27	• Seed-specific over-expression of cytosolic *G3P DEHYDROGENASE*	[398]
BnLACS2	*B. napus*	*B. napus*	6 % to 8 %		• No effect seed protein content • Constitutive over-expression of *LACS2* • SOC decreased by 3 % to 6 % in BnaLACS2-RNAi lines	[36]
BnLACS9	*B. napus*	*B. napus*	up to 15 %		• Constitutive over-expression of *LASC9* • Increased in chlorophyll content, the number of chloroplast grana lamellae and galactolipids content	[265]
TmLPAAT2	*T. majus*	*B. napus*	25 % to 29 %	0.14–0.17	• Constitutive over-expression of *LPAAT2*	[234]
BnaLPAAT2 and BnaLPAAT5	*B. napus*	*B. napus*	38.9 % to 49.4 %		• Constitutive over-expression of *LPAAT2* and *LPAAT5* • Increased accumulation of oil bodies in the seeds • Increased content of sugar and protein in *lpaat2* and *lpaat5* mutant seeds	[277]
BnaDGAT1	*B. napus*	*B. napus*	up to 14 %	0.076	• Seed-species over-expression of *DGAT1*	[43]
BnaDGAT1	*B. napus*	*B. napus*	2.5 % to 7 %	0.036	• Seed-species over-expression of *DGAT1* in both greenhouse and field conditions	[271]
SsDGAT1	*S. sebiferum*	*B. napus*	12.3 % to 14.7 %		• Constitutive over-expression of *DGAT1* • Decreased in oleic acid levels and increased in α-18:3 levels.	[322]
TmDGAT1	*T. majus*	*B. napus*	3.5 % to 8.0 %		• Seed-specific over-expression of *DGAT1* in HEAR canola cultivar	[319]
AtDGAT1	*A. thaliana*	*B. juncea*	4 % to 14 %		• Seed-specific over-expression of *DGAT1* • Decreased in oleic and linoleic acid content	[324]
BnaSDP1-KD	*N/A*	*B. napus*	up to 8 %		• Seed-specific down-regulation of *SDP1* by RNAi	[64]
AtWRI1 and *BjAGPase-KD*	*A. thaliana*	*B. juncea*	7.5 % to 16.9 %		• Constitutive down-regulation of BjAGPase and the seed-specific expression of *WRI1* • The starch content exhibited a reduction by about 45 % to 53 %	[399]
BnaGPDH, BnaDGAT, BnaGPAT and *ScLPAAT*	*B. napus* and *S. cerevisiae*	*B. napus*	13.6 % to 15.3 %		• Seed-specific over-expression of multiple genes • No effect normal plant growth	[325]
ScGPDH, BnaDGAT, BnaGPAT and *ScLPAAT*	*B. napus* and *S. cerevisiae*	*B. napus*	11.3 % to 13.8 %			

Abbreviations: ACC1, acetyl-CoA carboxylase (ACCase); BjAGPase, endogenous ADP-glucose pyrophosphorylase; DGAT1, diacylglycerol acyltransferase 1; GPAT, *sn*-glycerol-3-phosphate acyltransferase; GPDH, glycerol-3 phosphate dehydrogenases; G3PDH, *sn-3* glycerol-3-phosphate dehydrogenase; HEAR, high erucic acid rapeseed; LACS, long chain acyl-CoA synthetase; LPAAT, lysophosphatidic acid acyltransferase; SDP1, sugar-dependent 1 lipase; WRI1, wrinkled 1. Flux control coefficients are given for those papers that provided the necessary data in [108].

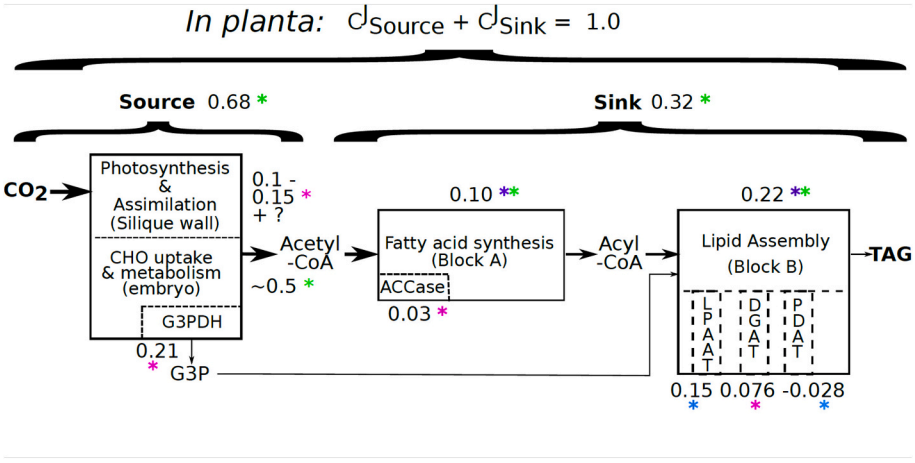

Fig. 4. Indicative distribution of *in planta* flux control coefficients in silique and embryo metabolism from CO_2 to seed triacylglycerol (TAG).
The figure is based on Fig. 5 of [108] where the data sources and methods of derivation are given. Colored asterisks indicate the methodologies behind the estimates: blue, experimental bottom-up Metabolic Control Analysis (MCA) *in planta*; purple, experimental top-down MCA on Blocks A and B of TAG synthesis in isolated embryos; lilac, retrospective MCA calculations on published data; lime green, inferred values to ensure consistency of measurements and MCA principles. Control coefficients assigned to specific enzyme activities are subsumed in the overall values for their containing blocks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mainly a 4-carbon product [256]. However, over-expression of KAS III led to a decreased rate of FA synthesis which caused lower level SOC in *B. napus* [257,258]. KASI carries out the bulk of the condensation reactions, with palmitoyl-ACP as its final product. KASII is used for the final condensation [31]. After generation of 16 or 18C acyl-ACP, the last step is the fatty-acyl acyl carrier protein thioesterase (FAT) which catalyses acyl-ACP hydrolysis to release a non-esterified fatty acid and ACP. Although most studies of FATs focused on the alteration of FA composition [259,260], a few results showed that heterologous over-expression of *fat* can increase the FA content in the seeds or leaves [261–263] and a *fat* double-mutant had reduced TAG content without affecting other non-polar lipids [264]. Besides, 16C- or 18C-FA, long-chain FAs also exist in plants. Elongases, such as long chain acyl-CoA synthases (LACSs), extend acyl chains of 12–18 carbon into longer

products. There are nine LACSs in plants which showed overlapping functions for FA synthesis. Some of these LACSs have been shown to increase SOC in over-expression transgenic lines [36,265]. In addition, other enzymes in fatty acid modification and transportation, such as fatty acid desaturases, can also contribute to SOC (see Section 2 and Fig. 1).

In addition to the main pathway of FA synthesis and editing, enzymes involved in other pathways (such as starch synthesis) have also been recognized as good candidates for increasing FA content [108]. These two pathways share common carbon backbones generated via photosynthesis. Blocking starch synthesis means enhanced carbon flux into FA, which will increase the lipid content [266]. ADP-glucose pyrophosphorylase (AGPase) catalyses an important step in starch biosynthesis [267]. In plants, down-regulation of *ADP-glucose pyrophosphorylase* has been shown to result in a significant decrease in starch content and increase in SOC [268].

Although FAs are the main precursors for TAG biosynthesis, they are also important for the formation of membrane lipids. Therefore, suppressing this competing pathway for TAG assembly could be a promising approach to partitioning FAs into TAG synthesis. One noticeable target enzyme is monogalactosyldiacylglycerol synthase 1 (MGD1), which transfers a galactosyl residue from UDP-galactose to DAG yielding monogalactosyl diacylglycerol (MGDG), which is the major component of chloroplast thylakoids [269]. Knockdown of *MGD1* in tobacco significantly reduced the MGDG content and increased oil content in the leaves [270]. However, regulation of MGDG content mainly focused on vegetative tissues which do not usually contain much oil.

Compared to FA biosynthesis, TAG assembly has a substantial control over SOC in *B. napus* [58]. DGAT catalyses the acyl-CoA-dependent acylation of DAG to produce TAG and represents an important step in the Kennedy pathway. Therefore, manipulating DGAT has been used as a common method to increase the flow of acyl chains into TAG biosynthesis. Many groups have demonstrated that over-expression of *DGAT* in oleaginous crops can stimulate oil accumulation in seeds and leaves [271–273]; this aspect is extensively reviewed in the next section. In addition to DGAT, other acyltransferases, like GPAT [39,274,275,278] and LPAAT [234,276,277,282] have also been manipulated to enhance SOC. There are ten GPATs in plants, of which one is a soluble GPAT localised in the plastid, whereas the others are membrane-bound enzymes detected in the ER or mitochondria (for a review, see [263]). GPAT9 is considered to be important for TAG biosynthesis [38,39]. Jain et al. [274] expressed cDNAs encoding *Escherichia coli* or safflower GPATs in Arabidopsis. They removed the plastidial targeting sequence of the safflower GPAT to allow cytosolic production in Arabidopsis and reported increases in oil production with either cDNA. On the other hand, when Liao et al. [60] studied over-expression of *GPAT9* in *B. napus* they found that overexpressing lines had similar lipid biosynthesis rates and MALDI-MS distribution characteristics to WT plants. They concluded that GPAT does not seem to have a major role in regulating SOC in canola varieties of *B. napus* (see [108]). Recently, Liu et al. [279] have reported characteristics of four homologous genes of GPAT9 in *B. napus* seeds. In erucate-containing cultivars, functional divergence of the four genes contributes to the erucic acid content.

Compared with GPAT and DGAT, LPAAT has the highest activity in developing seeds of *B. napus* [280]. Over-expression of *LPAAT2* and *LPAAT5* in *B. napus* increased the SOC of transgenic seeds by 39 % to 49 % [277]. Interestingly, the results indicated that BnaLPAAT2 and BnaLPAAT5 promote SOC accumulation by different mechanisms. BnaLPAAT2 increased DAG synthesis to increase total lipid content, whereas BnaLPAAT5 promoted PA synthesis more to generate membrane lipids. Previously, Zou et al. [281] reported an increase in the SOC of a high-erucate *B. napus* (cv. Hero) when they expressed a mutated gene encoding a yeast LPAAT (sphingolipid compensation mutant; *SLC1–1*). When T4 or T5 generations of cv. Hero were grown in field studies, relative increases in SOC of up to 14 % were found [267].

When a cDNA encoding *Tropaeolum majus* LPAAT was over-expressed in *B. napus* it resulted in a 25–29 % relative increase in TAG accumulation, despite its low flux control coefficient. This was explained by the exponential nature of lipid accumulation which increased the effect of a small flux control coefficient [234]. It is also important to note that increased levels of PA may have led to stimulation of DGAT1 [40]. Further coverage of this aspect is presented in Section 6. There were also changes in the distribution of FAs within lipid classes as revealed by MALDI-MS and the authors also reported a new, novel application of flux control analysis.

In addition to the enzymes in the Kennedy pathway, other acyltransferases have been used to increase SOC. Monoacylglycerol acyltransferases (MGATs) catalyse the conversion of monoacylglycerol (MAG) to DAG in the animal intestine, but this path does not exist in higher plants [283]. The first reported study of using MAG to generate DAG in plants is over-expression of mammalian *MGAT1* and *MGAT2* in tobacco. The over-expression lines resulted in an higher leaf TAG content compared to expressing *AtDGAT1* in the same system [284].

Although DGATs are the major contributors for TAG assembly in oilseeds, the flux through PC also contributes to oil accumulation. In the acyl-CoA-independent reaction of TAG biosynthesis, the acyl chain used in the conversion of DAG to TAG, via the catalytic action of PDAT, is derived from PC. Because of the difference in substrates between DGAT and PDAT, over-expression of these two enzymes leads to a differential FA composition in the oil [285]. In addition to this, DGAT1 and PDAT seem to have different efficiencies in utilising PC-derived DAG pools [286]. Although PDAT has been demonstrated to have a complementary function to DGAT in *Arabidopsis* [56], simple over-expression of *PDAT* did not result in an increase in SOC and may even have caused a slight, but not significant, lower oil level [55,59]. Nevertheless, PDAT is still a target enzyme to increase SOC in a multiple gene strategy. Introducing *PDAT1* into a mutant of *ADP-glucose pyrophosphorylase* has been shown to enhance TAG biosynthesis by consuming sugars [287]. As mentioned above, PCs can also act as acyl donors, so PC turnover is important for TAG synthesis as well. In plants, a mechanism named acyl-editing refers to de-acylation and re-acylation of PC. In this cycle, acyl chains in the acyl-CoA pool are exchanged with those on PCs, and can contribute to the routing of PUFA into TAG [288]. The two major enzymes responsible for the hydrolysis and regeneration of PC are phospholipase A (PLA) and LPCAT, respectively [28]. Knockout of *LPCAT* and *PLA* has been shown to decrease TAG content, while over-expression promoted lipid accumulation [289–291].

TAG hydrolysis has been shown to cause a decrease in SOC (see Section 2). Protecting TAG by disrupting TAG lipases involved in TAG breakdown, such as SDP1, is another approach to increasing SOC accumulation. SDP1 can directly catalyse the release of FAs from TAG; silencing the gene encoding this enzyme has been shown to significantly increase SOC in oilseed crops [64,292,293]. Nevertheless, a recent study revealed that rapeseed *sdp1* lines had a 2.2–2.4 % absolute increase of SOC in field trials, but this slight oil increase was accompanied with negative agronomic traits such as reduction in seedling emergence [294], indicating that further studies are required for using this gene in BOS breeding.

6. Diacylglycerol acyltransferase as a target for increasing seed oil content

Numerous metabolic engineering interventions to boost SOC in developing seeds of oleaginous plants have focused on the over-expression of various *DGAT* genes [25,40]. Therefore, it seems appropriate to devote a major section of this review to this enzyme category. Thus, this section deals with the detailed properties of DGATs from BOS, examples of *DGAT* over-expression in *B. napus* and the use of directed evolution to produce high performance variants of *B. napus* DGAT1. Both the knowledge of the detailed properties of DGAT1 and DGAT1 variants showing increased performance could lead to new strategies for increasing SOC in BOS.

As discussed earlier in this review, MCA analysis of storage lipid biosynthesis in rapeseed has validated DGAT as a useful target for manipulation of SOC. Even before MCA was used in probing lipid biosynthesis, there were already hints in the literature that DGAT may be a suitable target. Indeed, in the 1990s, research demonstrated that the DGAT-catalysed reaction exhibited a substantial effect on flow of carbon into TAG [94,95,280]. Next to TAG, DAG was shown to be the next prevalent metabolite in the Kennedy pathway. In addition, DGAT activity was the lowest of the four enzymes of the Kennedy pathway; these observations implied that an increase in DGAT activity could potentially lead to increased SOC. Also in the 1990s, it was demonstrated that DGAT activity reached a maximum in developing seeds of *B. napus* during the active phase of oil formation implying the important contribution of this enzyme activity in the oil formation process [70,295]. Moreover, over-expression of *DGAT1* in *B. napus* increased oil accumulation [43,271]. More recently, Chen et al. [296] have reported that among six lines of canola-type *B. napus*, middle to high SOC lines exhibited increased PAP and DGAT activities. Thus, there was a positive association between increased SOC and DGAT activity. In addition, in a study involving 34 *B. napus* inbred lines, a higher expression of *BnaDGAT1* genes was associated with higher SOC [297].

In plants, various gene families encoding proteins with DGAT activity have been identified [40]. DGAT1 and DGAT2, which share no homology and are ER-bound enzymes, have been the most extensively studied. *B. napus* has been shown to have four highly homologous gene forms (homeologous genes) of *DGAT1* and *DGAT2* [297–302]. Partial length and full-length cDNAs encoding one of the *DGAT1* gene forms were first cloned from microspore-derived cell suspension cultures of *B. napus* L. cv Jet Neuf [303–305]. This gene form was later categorized as *BnaA.DGAT1.b* [301]. The truncated version of *BnaA.DGAT1.b* originally reported by [304] may represent a mutation generated via a duplication event which occurred over numerous years of sub-culturing [303]. Transcriptome analysis during seed development in *B. napus* has shown that expressed sequence tags representing *DGAT1* are more abundant than those corresponding to *DGAT2* [80].

An intricate network of TFs is operative during seed development in oleaginous plants [86]. In a comparative study of gene expression in two NILs of *B. napus* differing in SOC, a number of *TF*-genes were up-regulated in the high SOC line, including *ABSCISIC ACID INSENSITIVE (ABI) 4*, *ABI5* and *FUSCA3* [179]. FUSCA3 is a master regulator of seed development [306]. *DGAT1* and *DGAT2* were also up-regulated. As mentioned previously, a TF (BnaZFP1) has been shown to interact with the promoter region of BnaDGAT1 [88]. In addition, the TF WAX INDUCER1/SHINE1 has been shown to interact with the promoter region of a BnaDGAT2 [307]. Modulating TF action may represent a way of increasing *DGAT* expression thus providing another avenue to increase SOC.

Unlike DGAT1 and DGAT2, DGAT3 is a soluble enzyme [40,308–310]. During Arabidopsis seed development, *DGAT3* has been shown to exhibit a similar expression pattern to *DGAT1* but with higher expression at later stages of seed maturation [84]. Recently, two *DGAT3* genes were identified and cloned from *B. napus* ZS11 [302]. Rani et al. [311] have also reported on another soluble protein with DGAT activity in Arabidopsis encoded by the gene known as *DEFECTIVE CUTICULAR RIDGES (DCR)*. The investigators hypothesized that DCR was involved in the transient formation of a hydroxy-TAG precursor in support of cutin biosynthesis.

The bifunctional wax synthase-diacylglycerol acyltransferase (WSD) is yet another protein category exhibiting DGAT activity. WSD-related proteins were first identified in bacteria and Arabidopsis [312,313] with further identification in numerous other plant species [310,314]. Recombinant Arabidopsis WSD1 was shown to exhibit a 10-fold lower level of DGAT activity than wax synthase activity [313]. In the same study, sub-cellular localisation experiments indicated that WSD1 was associated with the ER in leaf epidermal cells. Despite having some DGAT activity, the physiological role of WSD1 appears to be in catalysing wax ester synthesis. Recently, Yang et al. [315] identified two splice variants of *WSD1* from turnip (*B. rapa* L. var. *rapa*). The recombinantly-produced variant WSD1-X2 was more effective in synthesising wax esters than WSD-X1 and may be important in stem wax synthesis during drought stress.

Over-expression of selected *DGAT* genes has been shown to increase SOC in numerous oil-producing crops [40]. As discussed earlier, seed-specific over-expression of *DGAT1* during seed development in *B. napus* was shown to reduce the control exerted by the TAG assembly block of storage lipid biosynthesis. Seed-specific over-expression of *BnaA.DGAT1.b* in *B. napus* L. cv Westar resulted in a relative increase in SOC of about 12 % when plants were grown under greenhouse conditions [43,316]. Analysis of developing seeds obtained four weeks after flowering revealed a four-fold increase in DGAT specific activity and a substantial decrease in the DAG:TAG ratio when seed from the transgenic line was compared to the WT. In a field study, seed-specific over-expression of Arabidopsis *DGAT1* in *B. napus* L. cv Quantum was also shown to reduce the penalty on SOC caused by drought [43]. Further investigations with over-expression of Arabidopsis *DGAT1* in cv Quantum and *BnaA.DGAT1.b* in *B. napus* double haploid line DH12075, under both greenhouse and field conditions, resulted in SOC increases ranging from 2.5 % to 7 % on an absolute basis [271]. Analysis of the transcriptome of developing seeds of transgenic Quantum over-expressing Arabidopsis *DGAT1* revealed transcriptional changes that were not limited to storage lipid biosynthesis, thus indicating unintended side effects [317]. In addition, antisense suppression of *DGAT1* in line DH12075 resulted in a decrease in SOC along with severe development abnormalities and reduced seed yield [318]. Therefore, DGAT1 also appears to be important in normal seed development in *B. napus*. The seed oil from the antisense line also exhibited a significant decrease in α-18:3 content and significant increase in 16:0 content. An increase in 18:1 content and decrease in 18:2 content was also observed in the antisense line, but the changes were not significant.

Seed-specific over-expression of *DGAT1* from garden nasturtium (*Tropaeolum majus*) in a HEAR line has also been shown to increase SOC [319]. In the same study, the performance of TmDGAT1 was enhanced through site-directed mutagenesis to convert serine-197 to an alanine residue. Serine-197 represented a putative phosphorylation target of sucrose non-fermenting-1-related kinase (SnRK1). It was hypothesized that phosphorylation of serine-197 kept the enzyme in a down-regulated state. SnRK1 kinases are thought to play a role in the global regulation of carbon metabolism in plants [320,321]. Over-expression of the mutated gene encoding the altered TmDGAT1 (S197A) in Arabidopsis resulted in relative increases in SOC ranging from 20 % to 50 % on a per seed basis [319]. In another study, *DGAT1* from the woody oil tree *Sapium sebiferum* was over-expressed in *B. napus* L. *W10* under the control of the constitutive CaMV 35S promoter resulting in relative SOC increases ranging from 12.3 % to 14.7 % when transgenic lines were compared to the WT [322]. Heterologous expression of *SsDGAT1* also resulted in decreased 18:1 content and increased α-18:3 content. In addition, *DGAT1* from the microalga *Chlorella ellipsoidea* was expressed in Arabidopsis and *B. napus* L. cv Westar under the control of a *NOPALINE SYNTHASE* promoter [323]. Net relative increases in 1000-seed total lipid content ranged from 25 % to 50 % in both transgenic Arabidopsis and *B. napus* when compared to the WT. The FA composition of the seed oil, however, was not affected. Seed-specific over-expression of Arabidopsis *DGAT1* in Indian mustard (*B. juncea*) under the control of an Arabidopsis *OLEOSIN* promoter has also been shown to increase both SOC and seed weight, but the increase in SOC was accompanied by a large decrease in 18:1 content [324]. The changes in FA composition associated with heterologous over-expression of *DGAT1* may be related to the substrate selectivity properties of the newly introduced DGAT. *BnaDGAT1* has also been over-expressed in *B. napus* in combination with three other genes encoding enzymes involved in TAG assembly resulting in a synergistic effect in increasing SOC to levels beyond what could be achieved with individual over-expressors [325].

Learning about structure/function relationships in *B. napus* DGATs could lead to the development of additional strategies for increasing SOC. Among the four isoforms of Bna.DGAT1 identified in *B. napus* line DH12075 (BnaA.DGAT1.a, BnaC.DGAT1.a, BnaA.DGAT1.b, BnaC.DGAT1.b), BnaC.DGAT1.a was the most highly produced in a yeast line (H1246) devoid of TAG synthesis [301]. The *Saccharomyces cerevisiae* mutant strain H1246 has four genes encoding enzymes with DGAT activity knocked out and thus represents an excellent host system for introducing various *DGAT* genes for functional characterisation experiments [326].

Recombinant BnaC.DGAT1.a has been solubilized from yeast microsomes and highly purified [327]. This was the first report on the purification of a DGAT1 in active form to apparent homogeneity. A number of recent studies involving purified recombinant BnaC.DGAT1.a, yeast microsomes containing recombinant BnaC.DGAT1.a and various recombinant truncations of the enzyme have vastly contributed to our understanding of structure/function in this enzyme [40,301,327–333]. Earlier insight into the biochemical properties of the BnaDGAT1 family were based on a study of the purified cytosolic hydrophilic N-terminal domain (NTD) of BnaA.DGAT1.b with C-terminal poly-histidine tail attached (BnaA.DGAT1.$_{b(1-113)}$His$_6$) [334,335]. Unlike members of the DGAT2 family, members of the DGAT1 family feature a polypeptide with a hydrophilic NTD followed by several transmembrane domains (TMDs) [40]. The poly-histidine-tagged BnaA.DGAT1.b hydrophilic NTD was recombinantly produced using a bacterial expression system, purified and shown to interact with acyl-CoA in a positive cooperative fashion and to self-associate into dimers and tetramers [334]. Both cross-linking experiments and size-exclusion chromatography provided evidence to support self-association of the hydrophilic NTD. These observations suggested that BnaA.DGAT1.b was an allosteric enzyme. Later, dimeric and tetrameric forms of solubilized full-length isoform BnaC.DGAT1.a were also demonstrated using size-exclusion chromatography [327] thereby corroborating the earlier observations on self-association of the NTD of BnaA.DGAT1.b. Further investigation, using recombinant BnaC.DGAT1.a and its truncations, revealed that the hydrophilic NTD (common to all isoforms of BnaDGAT1) contained both an intrinsically disordered region and a folded segment that could interact with acyl-CoA and CoA [329]. The intrinsically disordered region had an autoinhibitory function and served as a dimerization interface to mediate cooperativity. As cellular acyl-CoA concentration increases, the non-catalytic site in the NTD facilitates homotropic allosteric activation. Under limiting cellular acyl-CoA concentration, however, CoA acts as a non-competitive feedback inhibitor which contributes to promoting a less active form of the enzyme. In addition, at higher concentrations of acyl-CoA, beyond 5 μM, the enzyme became less active suggesting yet another mechanism for down-regulating the enzyme [329,333]. In essence, the NTD of the BnaDGAT1 family members acts as a sensor for cellular acyl-CoA/CoA status to regulate enzyme activity accordingly.

Further investigation of the biochemical properties of BnaC.DGAT1.a has shown that the enzyme is also feed-forward activated by PA [328]. PA action was associated with a direct interaction with the hydrophilic NTD which resulted in decrease in the autoinhibition of the enzyme by the NTD. PA action shifted the response to increasing acyl-CoA concentration from a sigmoidal response to a response that was more hyperbolic. In addition, the apparent maximum velocity of the enzyme increased about 7-fold in the presence of PA and the enzyme was desensitised to inhibition at higher concentrations of acyl-CoA beyond 5 μM. A yeast two-hybrid assay was used to demonstrate a direct interaction between BnaLPAAT2 and BnaC.DGAT1.a suggesting that co-localisation of the two Kennedy pathway enzymes facilitated channelling of the PA product of the LPAAT-catalysed reaction to the activation site on BnaC.DGAT1.a.

In addition, hydrolysis of PA by PAP generates the other substrate of DGAT1, DAG, whose increase will also stimulate its activity [328]. Thus, LPAAT has two feed-forward activation routes that ensure BnaDGAT1 adjusts its activity to the incoming flow of carbon within the Kennedy pathway whilst reducing the individual changes in DAG and acyl-CoA required to achieve this. Just as MCA has shown that feed-back inhibition lowers an enzyme's potential flux control coefficient, so feed-forward activation should act to increase the flux control coefficient of LPAAT at the expense of that of DGAT1. Indeed, measurement of LPAAT's flux control coefficient via the increase in *B. napus* SOC on raising its activity through over-expression of *T. majus LPAAT* [234] yielded an *in planta* value of 0.15 compared with an estimated value [108] of 0.076 for DGAT1 (Fig. 4) derived from measurements by Weselake et al. [43,336] and Taylor et al. [271]. Fell et al. [108] also calculated that parallel increases in the activity of LPAAT and DGAT1 would increase SOC by more than either separately. As mentioned previously in Section 4, joint increases of the enzyme activities should minimise changes in the concentrations of the intermediates between them, PA and DAG, which would thereby limit the disturbance to other parts of lipid metabolism, in relation to OB, with which they are involved.

Interestingly, Arabidopsis CTP:phosphocholine cytidylyltransferase (CCT) 1 has also been shown to have an autoinhibitory region that interacts with PA resulting in enzyme activation [337]. CCT is a key regulatory enzyme in PC biosynthesis which catalyses the formation of CDP-choline for use by CPT in the conversion of DAG to PC [338]. Therefore, co-activation of DGAT1 and CCT1 may ensure that synthesis of nitrogenous phospholipids keeps pace with TAG and OB formation in the developing zygotic embryo. Therefore, it would be of interest to over-express both *DGAT1* and *CCT1* as a possible means of further increasing SOC.

As discussed previously, *T. majus* DGAT1 was shown to have a putative site for SnRK1-catalysed phosphorylation [319]. Caldo et al. [328] directly demonstrated that BnaC.DGAT1.a was a substrate of SnRKI, which catalyses the phosphorylation of the enzyme, resulting in a less active form. Presumably, an unknown phosphatase is responsible for catalysing the removal of phosphate from phosphorylated DGAT1 to up-regulate the enzyme under appropriate cellular conditions. Therefore, along with its role as an energy sensor of the cell, SnRKI also appears to regulate the production of TAG. The observations on the effects of PA and SnRK1 action on BnaC.DGAT1.a were used to further refine the model proposed for the allosteric regulation of BnaC.DGAT1.a. Interestingly, Caldo et al. [339] have also reported that Arabidopsis CCT1 is down-regulated by SnRK1-mediated phosphorylation. Thus, co-phosphorylation of *B. napus* DGAT1 and CCT1, catalysed by SnRK1 might represent a post-translational-based mechanism for modulating TAG biosynthesis in relation of OB formation.

When designing metabolic engineering experiments involving the introduction of a selected DGAT, it is also important to take into consideration the substrate preferences of the newly introduced enzyme. For example, it would be inappropriate to introduce a DGAT1 isoform with a very low preference for 18:1-CoA into a LEAR line given that 18:1 is the dominant FA in LEAR seed oil. The term acyl-CoA specificity is often used when assessing a range of different molecular species of acyl-CoA in separate reactions and then ranking the enzyme's preferences for the different molecular species of acyl-CoA based on increasing enzyme activity. Only one molecular species of acceptor substrate (DAG) is used. Conversely, DAG specificity can be examined using different molecular species of DAG in different reactions with one molecular species of acyl-CoA. In the developing zygotic embryo of *B. napus,* however, DGAT is presented with a mixture of different molecular species of acyl donors and acceptors present in different molar ratios (see [298]). Under these conditions, the substrate selectivity properties of the enzyme come into play; i.e., what is the order of molecular species of substrate preference in a situation where a choice of molecular species of substrate is presented to the enzyme? Under these conditions, one can envision possible scenarios involving competition of different molecular species of acyl-CoA for the active site and/or possible allosteric effects of certain molecular species of acyl-CoA which alter the activity of the enzyme thus

affecting its operation with other molecular species of acyl-CoA. Earlier studies of TAG biosynthetic enzymes, however, used microsomes prepared from developing seeds or embryos as a source of enzymes [42,336] and, subsequently, insights into substrate molecular species preferences were likely the result of the action of different isoenzyme families and even different isoforms within these families. In the metabolic engineering of *B. napus*, choosing a DGAT with inappropriate substrate selectivity properties may even further reduce the flux control coefficient of the TAG assembly block.

Fortunately, the four microsomal recombinant BnaDGAT1 isoforms (produced in H1246 yeast) from canola-type LEAR effectively utilised a wide range of molecular species of acyl-CoA representing FAs typically found in seed TAG (i.e., 16:0, 18:0, 18:1, 18:2 and α-18:3) [340]. A similar acyl-CoA specificity profile was previously reported in assays with solubilized and highly purified BnaC.DGAT1.a [327]. Despite being able to use a range of molecular species of acyl-CoA, the most effective acyl donor was α-18:3-CoA. In this case, dioleoyl-*sn*-1,2-glycerol served as the acyl acceptor. Greer et al. [301] have categorized the four recombinant BnaDGAT1 isoforms, produced in H1246 yeast, into two clades, with BnaA.DGAT1.a and BnaC.DGAT1.a representing clade I and BnaA.DGAT1.b and BnaC.DGAT1.b representing clade II. Therefore, both the A and C genomes of *B. napus* make contributions to TAG formation. The clade I enzymes, however, exhibited considerably higher enzyme activity than clade II enzymes [340]. Interestingly, Aznar-Moreno et al. [298] reported that clade I *BnaDGAT1* transcripts were also produced at a higher level than clade II transcripts during seed development. All four BnaDGAT1 isoforms have also been shown to exhibit enhanced specificity for 16:0-CoA over 18:1-CoA despite 18:1 being the major FA in seed TAG of LEAR [298,340]. The main difference between clade I and clade II BnaDGAT1s, however, was that clade II enzymes displayed a significantly enhanced preference for 18:2-CoA [340]. Clade I BnaC.DGAT1.a versus clade II BnaA.DGAT1.b were also assessed for acyl-CoA selectivity using an equimolar mixture of 18:2-CoA and α-18:3-CoA [340]. Use of clade II enzyme in this competitive scenario resulted in a significantly higher ratio of 18:2 to α-18:3 in TAG than for the clade I enzyme alone. Since the amino acid residue sequences among the four BnaDGAT1 isoforms were outside of the motifs suggested to be involved in catalysis, it was hypothesized that other regions are involved in governing substrate preferences [340].

Aznar-Moreno et al. [298] further examined the selectivity properties of the four forms of BnaDGAT1 using concentrations of 18:1-CoA and 16:0-CoA that reflected the in vivo ratio of 18:1-CoA to 16:0-CoA during seed development, which was 3:1. Under these in vitro conditions, despite the higher specificities of the BnaDGAT1s for 16:0-CoA, the four isoforms incorporated 18:1 in amounts two- to five-fold higher into TAG than 16:0, thus contributing to the production of seed oil with enhanced 18:1 content. Therefore, the nature of both the in vivo acyl-CoA and DAG pools are also important factors in determining the outcomes of DGAT action.

The situation is even more complicated for HEAR lines. Demski et al. [300] have studied BnaDGATs from a LEAR line (MONOLIT) and a HEAR line (MAPLUS). Microsomes from developing seeds of MAPLUS exhibited 6- to 14-fold higher DGAT activity with 22:1-CoA than microsomes from MONOLIT suggesting that DGAT isoforms with different acyl-CoA selectivities are differentially active in the two cultivars. Results of *BnaDGAT* transcript analysis suggested that expression patterns for *BnaDGAT1* and *BnaDGAT2* isoforms may differ among *B. napus* cultivars. Further experiments were conducted with recombinant forms of BnaDGAT1 and BnaDGAT2 isoforms produced in H1246 yeast. It was, however, necessary to codon optimise *BnaDGAT2* cDNAs in order to achieve effective enzyme production in yeast. This aspect was previously demonstrated for Arabidopsis *DGAT2* cDNA by Aymé et al. [341]. In agreement with previous results [340], the BnaDGAT1 isoforms displayed a broad acyl-CoA specificity. Two of the BnaDGAT2 isoforms, operative in MAPLUS, however, exhibited enhanced acyl donor specificity for 22:1-CoA while all four isoforms could still effectively utilise α-18:3-CoA. Although the low 22:1 content of LEAR lines is generally linked to a defect in the elongation of 18:1-CoA to 22:1-CoA [7], the study of Demski et al. [300] suggested that during the breeding process HEAR lines may have also acquired mutated *BnaDGAT2* genes encoding DGAT2 forms with enhanced 22:1-CoA selectivity. In a follow-up study, involving a range of chimeric enzymes produced from parts of a 22:1-CoA-specific BnaDGAT2 isoform, a region containing two predicted TMDs was revealed which affected 22:1-CoA specificity [342]. Subsequently, the section of a peptide associated with enhanced 22:1-CoA specificity was installed into Arabidopsis DGAT2, thus converting the enzyme into a DGAT with enhanced specificity for 22:1-CoA.

It is interesting to note that the earlier acyl-CoA-binding experiments with BnaA.DGAT1.$_{b(1–113)}$His$_6$ indicated a dissociation constants of about 17 μM and 2 μM, respectively, for interactions of the hydrophilic NTD fragment with 18:1-CoA and 22:1-CoA [334]. Therefore, acyl-CoA ligand selectivity may also be involved in the allosteric modulation of BnaDGAT1 activity. This aspect would add yet another level of complexity. Given the relatively high 18:1 content of the seed TAG of LEAR lines, it is possible that increasing the affinity of the non-catalytic site for 18:1-CoA may result in a BnaDGAT1 that is more responsive to lower concentrations of 18:1-CoA. Perhaps, this could represent yet another strategy to increase SOC.

There are additional questions regarding the physiological relevance of the different isoforms of BnaDGAT1 and BnaDGAT2. Does having more than one isoform within a family serve as a mechanism to protect against possible future deleterious mutations that may de-activate one of the homeologous genes? In other words, is this a protective mechanism to ensure that TAG biosynthesis and normal seed development continue to occur if one of the homeologous genes is knocked out? Do the various isoforms within each DGAT family self-associate to form hybrid dimers and tetramers?

In addition to communication with LPAAT via PA production [328], plant DGAT1 action also appears to involve other processes in lipid biosynthesis. For example, in a study with recombinant acyltransferases from flax, enhancement of the PUFA content of TAG was shown to be linked to biochemical coupling of the LPCAT1-catalysed reverse reaction to the DGAT1-catalysed forward reaction [343]. Recently, in vivo binding data have revealed that flax DGAT1 and DGAT2 are part of a larger transferase interactome which is operative in channelling PUFA from PC into TAG via various mechanisms [344]. A similar PUFA-enrichment system may also be operative during seed development in *B. napus*.

High performance variants of BnaDGAT1, generated through directed evolution, may also prove useful in the development of *B. napus* lines with increased SOC [40,333,345,346]. In addition, these enzyme variants allow the investigator to link specific amino acid residue substitutions to aspects of function. The high-throughput directed evolution system to produce BnaDGAT1 variants with enhanced performance was based on the use of error-prone polymerase chain reactions to introduce mutations into *BnaDGAT1* cDNA and large-scale screening of yeast transformed with mutated *BnaDGAT1* cDNA for TAG production using Nile red [346–348]. A mutated cDNA encoding an active recombinant DGAT1 variant complemented TAG biosynthesis and restored growth of *S. cerevisiae* H1246 in the presence of oleic acid [348]. In the absence of an active DGAT, the oleic acid was toxic and inhibited growth of the yeast. Characterisation of several BnaC.DGAT1.a variants indicated that TAG production in yeast was affected by different mechanisms, including enhanced enzyme activity, increased DGAT1 variant polypeptide accumulation and possibly reduced substrate inhibition by high cellular concentrations of acyl-CoA [333]. One of the variants (I447F), where I447 represents the native enzyme, was introduced into tobacco (*Nicotiana benthamiana*) leaf tissue using a transient expression system involving co-expression of Arabidopsis *WRI1* [330]. Over-expression of the cDNA encoding variant I447 led to a significant relative increase in leaf TAG content of about 33 %. The FA composition of TAG produced using the I447 variant was similar to the FA composition of TAG from

the control. Site saturation mutagenesis [349] at position I447 resulted in a collection of 19 variants representing the effects of 19 other amino acid residues at this position [330]. In addition to I447F, the variants I447L and I447V resulted in substantially increased TAG accumulation in H1246 yeast in comparison to the native form of the enzyme (I447) indicating that the hydrophobicity of the amino acid side chains was critical in contributing to higher enzyme activity. Analysis of the apparent kinetic parameters of a few of the BnaC.DGAT1.a variants indicated substantial increases in the apparent maximum velocity and a shift from a sigmoidal response to increasing acyl-CoA concentration to a more hyperbolic response [333]. Although variant I447F did not exhibit a marked change in apparent maximum velocity, kinetic analysis revealed a loss of substrate inhibition at higher concentrations of acyl-CoA (beyond 5 μM) when compared to the native enzyme (I447). Thus, variant I447F may prove useful for increasing seed TAG content under physiological conditions where acyl-CoA concentrations are increased. The structure of BnaC.DGAT1.a has been modeled based on the 3D structure of human DGAT1 and the functional relevance of amino acid residue substitutions of several BnaC.DGAT1.a variants were analysed based on the 3D model [40]. Within this context, residue I447F was shown to point directly into a hydrophobic central cavity suggesting that the substitution of an isoleucine residue with a phenylalanine residue increased the affinity of the enzyme for lipid substrates in the reaction center.

7. Other modifications of seed oil production in Brassica oilseed species

7.1. Transcription factors

In the previous section, it was discussed briefly how certain TFs can interact with the promoter regions of *B. napus DGAT* genes. Over-expression of genes encoding TFs represents another way of manipulating SOC and both knockout and over-expression studies have been made to confirm which TFs can be useful for controlling lipid accumulation [350]. Most of these studies have been made using Arabidopsis, which is useful since it is closely related to rapeseed. Nevertheless, it should always be borne in mind that they are not identical and important differences in lipid metabolism between the two species have been noted [177].

Among the TFs, some are known as master regulators because they alter the functions of other TFs. The encoding genes include *ABI3, LEAF COTYLEDON (LEC1, LEC2)* and *FUSCA3*. They control various aspects of seed development. ABI3, LEC2 and FUSCA3 are related proteins containing a common 'B3' DNA-binding domain [351]. Details of the action of these TFs in Arabidopsis are given in [25].

One of the most studied TFs is encoded by the *Arabidopsis WRINKLED 1 (WRI1)* gene. Loss-of-function mutations in *WRI1* caused an 80 % reduction in SOC [352]. Examination of gene expression of WT type versus mutant *wri1* seeds confirmed that the TF was involved in seed oil production [353]. Cernac and Benning [354] gave further details of *WRI1* action which was due to it being a target for LEC2 and, therefore, of the latter's regulation of FA metabolism [355]. In *B. napus*, two *BnaWRI1* genes were identified and sequenced. Over-expression of *BnaWRI1* in Arabidopsis gave up to 40 % relative increase in SOC as well as enlarged seed size and weight [356]. Because of the increase in SOC by WRI1, this TF is often used as part of a multiple gene over-expression technique to boost seed oil accumulation. Thus, for example, van Erp et al. [357] over-expressed *WRI1* and *DGAT1* in Arabidopsis while reducing expression of *SDP1* gene. Plants expressing all three constructs had significantly greater SOC than WT or when each gene was changed on its own. In addition, Vanhercke et al. [246] co-expressed Arabidopsis *WRI1* and *DGAT1* in tobacco where the expression showed a synergistic effect. A more recent paper by the same group discussed step changes for increasing oil production which they labeled 'push-pull-package-protect' [358]. Similar studies have been made by others in Arabidopsis [359]. Such techniques could, obviously, be applied (and have been) to increasing SOC in BOS.

Mechanisms controlling the activity of WRI1 have been studied in some detail (see [360]) and by Shanklin's group. Phosphorylation by KIN 10 (the major sucrose non-fermentation 1-related kinase), acting as a post-translational regulator, causes its proteosomal degradation [361]. Moreover, WRI1 regulates biotin attachment domain-containing (BADC) proteins [362]. BADC proteins were first discovered when Thelen's group [363] identified a novel family of proteins in Arabidopsis following co-precipitation analyses. These proteins were found to interact with the biotin-carboxyl carrier protein (BCCP) component of the heteromeric ACCase. In fact, BADCs resembled BCCP but were not biotinylated and were suggested to be ancestral BCCPs. BADC proteins significantly inhibit ACCase activity [363,364]. Moreover, gene silencing of the BADC isoform 1 in Arabidopsis increased seed oil content by up to 11 % on a dry weight basis or by up to 25 % on a per seed basis [363]. The importance of ACCase for de novo FA biosynthesis and the identification of BADC orthologs in many oil crops stresses the possible use of BADCs in genetic engineering [363].

Another possible way of increasing FA production involves carboxyltransferase interactors (CTIs). These are small plastidial proteins in the envelope membrane that interact with the α-carboxyltransferase unit of heteromeric ACCase [365]. Knockouts of CTI show high rates of FA biosynthesis and a marked increase in leaf TAG (4-fold). Furthermore, WRI1 regulates CTI1 expression by direct binding to its promoter. The 'docking' of ACCase to the envelope membrane is an important regulatory mechanism in leaves [365] and could possibly be used to promote lipid accumulation in BOS.

Other *B. napus* genes shown to be associated with increased SOC are *SHOOTMERISTEMLESS* [366] and *GRF2-LIKE* [178]. Over-expression of *SHOOTMERISTEMLESS* in *B. napus* plants increased SOC without affecting total protein levels. The over-expression was accompanied by induction of genes encoding BnaLEC1, BnaLEC2 and *BnaWRI1* as well as a decrease in seed glucosinolate [366]. The effect on *BnaGRF2a* expression on increasing SOC in *B. napus* was covered previously in Section 3.2. In further regard to *BnaGRF2a* expression, photosynthesis was increased and it was concluded that cell number as well as photosynthesis contributed to increased seed weight and oil content [178].

7.2. Carbon partitioning into lipid accumulation

Although oil crops, in general, show a preference for oil production rather than starch deposition in their seeds, there have been several studies on carbohydrate metabolism that are relevant to SOC. In fact, our knowledge of the interactions between lipid and carbohydrate metabolism in oil crops is still rather lacking [367].

For *B. napus*, photosynthesis in the silique wall is the main source of carbon during seed development [111,122]. As expected, Rubisco activity is critical [111] and gives a control coefficient of 0.15 for oil accumulation [108]. Moreover, activities of Rubisco in siliques (but not in leaves) showed a positive correlation with SOC in different *B. napus* lines [111]. In addition to a key role for silique photosynthesis in providing carbon for lipid accumulation, other reactions of carbohydrate metabolism have been estimated to provide about half of the control of TAG accumulation in *B. napus* plants [108].

Some specific enzymes have been investigated in more detail. Tomlinson et al. [368] examined sucrose catabolism (involving invertase or hexokinase) in developing tobacco seeds. These two enzymes were over-expressed singly or in combination but neither manipulation had any effect on seed oil levels.

Starch branching enzymes (SBEs) are one of important enzyme groups that are used for starch biosynthesis. SBEs have been studied in some detail in *B. napus* where there are six *BnaSBE* genes [369]. When endogenous gene forms of *SBE* were substituted with endosperm-expressed maize *SBEs*, increased branching was seen which resulted in greater silique numbers. This caused a doubling of TAG production per

plant [370]: the authors are trying to apply this unexpected (and promising) result to canola-type *B. napus*.

The importance of WRI1 for seed oil accumulation was mentioned in Section 7.1. and Zhai et al. [371] noted that trehalose 6-phosphate increased FA synthesis by stabilizing this TF. More recently, this positive effect was confirmed when trehalose 6-phosphate synthase was up-regulated in developing silique walls of *B. napus*. Various genes encoding enzymes involved in oil accumulation were increased in expression and seed oil production was enhanced [372].

Linkage of glycolysis to the TCA cycle is provided by mitochondrial pyruvate dehydrogenase complex. PDCK down-regulates pyruvate dehydrogenase activity in this complex and seed-specific anti-sense repression has been shown to result in increased SOC and seed weight in Arabidopsis [181,373] and *B. napus* [25]. Radiolabeling experiments using [3-^{14}C]pyruvate showed that there was an increased supply of acetyl-CoA from mitochondria [181]. In agreement, Li et al. [177] found that the gene encoding mitochondrial PDCK was one of the genes down-regulated in *B. napus* lines with high SOC. Other papers detailing the effects of repressing expression of the gene encoding mitochondrial PDCK on growth and productivity in Arabidopsis are Leonardos et al. [374] and Weraduwage et al. [375].

Co-expression of *DGAT1* with repression of *PDCK* has been used to provide both 'push' and 'pull' for seed oil synthesis in *B. napus* [231]. The experiments included *B. napus*, Arabidopsis and *T. majus DGATs* and Arabidopsis or *B. napus* mitochondrial *PDCK* as well as a mutated *T. majus DGAT1* encoding a DGAT1 variant with enhanced activity. The napin gene promoter was usually employed to drive seed-specific expression. In general, single expression of *DGAT* gave 6–11 % relative increase in SOC while silencing mitochondrial *PDCK* gave relative increases in the range of 12–16 %. For lines with stacked genes, a relative increase of around 24 % in SOC was found, demonstrating clearly the advantage of this strategy for enhancing seed oil production in *B. napus*.

Glycolysis takes place in both the cytosol and the plastid in plants. Pyruvate kinase is important for pyruvate production and Baud et al. [376] showed that mutations in genes encoding the Arabidopsis plastidial enzyme depleted SOC. In addition, disruption of the gene encoding the beta1 subunit of the enzyme in Arabidopsis caused a 60 % relative reduction in SOC. The oil levels were restored by expression of a cDNA encoding the *beta1* subunit [377]. Moreover, the importance of pyruvate kinase was confirmed when [177] found that *B. napus* lines with high SOC had increased enzyme levels.

Provision of reducing power (NADPH) for FA biosynthesis can come from the catalytic action of glucose 6-phosphate dehydrogenase in the OPPP. Single or double mutants of the two cytosolic forms of this enzyme led to an increase in carbon substrates for oil accumulation and, for the double mutant, higher SOC [378].

Stable isotope studies by Schwender and his coworkers have already been referred to in Section 2 of this review. Papers that describe data into the carbon flux in photosynthetic embryos of *B, napus* are particularly relevant ([96–98,115,379], see also [25], for a summary).

Inactivation of ADP-glucose pyrophosphorylase gives rise to the starchless phenotype in Arabidopsis. *PDAT 1* over-expression in leaves of such mutants enabled an increase in TAG accumulation. It diverts FA flux from membrane lipids to TAG, enabling the latter to act as a source of energy rather than sugars [287]. These and other studies from the same group addressed the general question of diversion of carbon from carbohydrate metabolism to FA and TAG biosynthesis [287,361].

Apart from carbohydrate metabolism, amino acid metabolism and protein synthesis may influence oil production in the Brassicaceae. In an Arabidopsis mutant showing reduced de novo FA synthesis, there was increased storage protein and amino acid synthesis [380]. In addition, when seed oil accumulation is reduced by silencing of *DGAT1* in *B. napus*, there was a reciprocal increase in protein and sugar content [318]. Indeed, in a meta-analysis of the effects of heat and drought on canola-type cultivars of *B. napus*, Secchi et al. [381] highlighted trade-offs between seed oil and protein. Nevertheless, the trade-offs were not always quantitatively reciprocal. This was partly due to the variable effects at different development stages and partly due to oil accumulation in seeds following a sigmoid pattern, whereas protein increased in a substantially linear fashion [69].

7.3. Modification of fatty composition

In the previous sections, this review has concentrated mainly on describing ways in which total SOC can be manipulated in BOS. As discussed in Section 6, the over-expression of specific *DGAT* genes in various BOS can also lead to changes in the FA composition of seed TAG. Given this, there have been numerous studies where the main intent has been to modify FA composition (see [13]). These studies have involved changing the proportions of both saturated and unsaturated FAs, and also generating FAs which are not naturally produced in the target BOS.

Different ways of increasing saturated FAs in *B. napus* have been reviewed by Stoll et al. [15]. This could have applications for both industrial or edible uses [16]. Increasing 18:0 in *B. napus* seed oil by down-regulating expression of the gene encoding stearoyl-ACP desaturase [14] or by the introduction of a modified acyl-ACP thioesterase [382] are two contrasting methods. More recently, Sun et al. [16] used artificial microRNAs to increase saturated FA levels.

B. napus has also been engineered to accumulate medium-chain FAs which are usually sourced from tropical crops such as coconut or oil palm [11]. Thus, seed-specific engineering of LEAR varieties gave production of oils with 11 % caprylic (8:0) and 27 % capric acid (10:0) [17]. By introducing an unusual acyl-ACP (12:0-ACP) thioesterase from California bay laurel, Voelker et al. [18] obtained over 50 % laurate (12:0) in the seed oil. However, further increases were limited by competing beta-oxidation and glyoxylate cycle activity [383]. Nevertheless, introduction of a lauryl-CoA-preferring LPAAT (from coconut) did augment the 12:0 content of *B. napus* seed oil to 60 % [18].

In contrast, ways of lowering saturated FA levels in canola-type *B. napus* have been discussed which include the manipulation of desaturase activity [13,384]. Canola varieties of *B. napus* already contain 60 % oleate in their oil, which is considered a good attribute for an edible oil, particularly when combined with its favorable 18:2/α-18:3 ratio. The oleate content can be increased further by using reduced expression of *FAD2* [21,22]. Other manipulations have included mutated *FAD3* genes in *B. oleracea* [385] or suggestions for manipulating genes encoding other lipid synthetic enzymes [13].

The use of gene stacking has already been referred to with regard to the over-expression of other genes in combination with *WRI1*. Indeed, stacked traits are important features of many commercial biotech crops [386,387]. For improvements in oil crops, including rapeseed, there are many examples (see [388]). In canola-type *B. napus*, stacking of *DELTA6* and *DELTA12 DESATURASE* genes from *Mortierella alpina* gave over 40 % γ-linolenic acid [389] while stearidonic acid (18:4$\Delta^{6cis,9cis,12cis,15cis}$) accumulated at up to 23 % when the same *M. alpina* desaturases were stacked with the *B. napus FAD3* gene [390].

Multiple genes have been stacked to allow long chain (20 or 22C) PUFAs to be formed in Brassicaceae. Transgenic *B. carinata* was engineered to allow production of up to 25 % eicosapentaenoic acid (EPA; 20:5$\Delta^{5cis,8cis,11cis,14cis,17cis}$) [391]. More recently, Arabidopsis has been engineered to synthesize up to 12 % DHA in its seed oil [284,392]. Exploitation of this research has resulted in the marketing of commercial *B. napus* oil with useful levels of DHA ('Aquaterra' oil).

8. Closing comments and future perspectives

Conventional plant breeding has resulted in a steady incremental increase in the SOC of LEAR cultivars since their introduction several decades ago [385]. More recently, breeding efforts have been further enhanced through the incorporation of molecular techniques such as marker-assisted selection and identification of QTL associated with

increased SOC. Comparative omics approaches have also been useful in identifying functional proteins associated with high SOC lines when compared to low SOC lines. Comparative omics essentially represents a 'guilt by association' approach. Nonetheless, several functional proteins identified using comparative omics approaches have led to some successful genetic interventions for further enhancing SOC in various BOS. Several biochemical reactions which represent potential targets for increasing SOC in *B. napus* are apparent from MCA of storage lipid biosynthesis and other aspects of metabolism (outlined in Fig. 4). It should be noted, however, that many genetic interventions to increase SOC in BOS have been made in the absence of robust MCA data, using comparative omics data (as previously indicated) and other types of biochemical data. In other words, other lines of evidence have suggested some useful targets.

Going forward, we suggest that investigators who are interested in increasing SOC in BOS (and other oil crops) consider performing MCA prior to selecting the most promising target genes for increasing SOC. Thus, MCA would act as a guide to metabolic engineering. The DGAT1-catalysed reaction already represents a key target which has been validated via TDCA of *B. napus* over-expressing *DGAT1* versus the WT [43,58,316]. Bottom-up MCA has also identified LPAAT as an enzyme with control over SOC [234]. Thus far, it is important to note that MCA has only been directly applied to LEAR cultivars [43,59,234] and not HEAR cultivars which represent the predecessors to LEAR cultivars [7]. Nevertheless, we were recently able to retrospectively analyse some published experimental results on increasing SOC (including some cited earlier in this review) to infer indicative flux control coefficients for G3PDH, ACCase, silique wall photosynthetic rate and silique wall Rubisco [108], and these have been included in Fig.4. These estimates have their limitations, having been culled from experiments in different laboratories and on various cultivars, but they do demonstrate that similar targeted experiments within a cultivar could be performed to refine the estimates.

Further knowledge of the detailed structure/function properties of BnaDGAT1 (including the four isoforms) should in turn result in new strategies involving DGAT1 to further increase SOC. For example, activation of BnaDGAT1 by PA [328] has suggested that over-expression of *T. majus LPAAT* to increase SOC in a LEAR *B. napus* may involve enhanced production of PA for activation of BnaDGAT1 [40]. Directed evolution has been used to generate high performance variants of BnaDGAT1 with many of the variants exhibiting increased apparent maximum velocity [330,333]. Interestingly, one of these variants (L441P) still exhibited high activity at higher concentrations of acyl-CoA (> 5 μM), whereas the WT enzyme was inhibited, suggesting that variant L441P may prove useful in further increasing TAG content under conditions where endogenous acyl-CoA concentrations are relatively high. This strategy may be particularly effective where the developing seed has been engineered to produce higher local concentrations of acyl-CoA [333]; a possible acyl-CoA enrichment strategy has been investigated in Arabidopsis by Yurchenko et al. [393]. Other functional proteins shown to increase SOC in BOS, via metabolic engineering, may also benefit from further improvement by directed evolution. Indeed, improved Arabidopsis LACS9 variants have been generated using a directed evolution approach based on the method for generating high performance variants of BnaDGAT1 [394].

There have also been examples where DGAT manipulation has been combined with another intervention(s). One notable example was the over-expression of *DGAT1* in combination with the partial suppression of mitochondrial *PDCK* which represented a synergistic approach to increasing SOC in *B. napus* [231]. The increased co-expression of *DGAT1* and *CCT1* (encoding CCT1 involved in PC synthesis) has already been proposed by Caldo et al. [328]. Under these conditions, it was envisioned that PA produced in the Kennedy pathway would serve to co-activate both DGAT1 and CCT1. Thereby, OB production could effectively keep pace with TAG accumulation and the availability of a half-unit membrane required for structural integrity of OBs. In turn, if this strategy was combined with over-expression of *LPAAT*, there would be more PA available as a substrate for PAP and as an activator for both DGAT1 and CCT1, possibly resulting even in more OBs. Gene editing technologies would be of further use in the introduction of amino acid residue substitutions in DGAT1 known to produce a more active enzyme variant based on the results of directed evolution. Further studies on the soluble [2Fe—2S] DGAT3, recently investigated in Arabidopsis by Aymé et al. [308], would also be worthwhile pursuing in relation to strategies for increasing SOC in BOS. With respect to the further improvement of HEAR cultivars to enhance SOC containing higher levels of 22:1, it would be important to explore the use of BnaDGAT2, which has been shown to display enhanced specificity for 22:1-CoA [300,342]. Similar strategies would also be applied in the modification of other enzymes in seed oil biosynthesis and accumulation.

Recent reports on a TAG remodeling process in *P. fendleri* [61,62] suggest that it may be worthwhile exploring if anything of this nature is operative in BOS. Certainly, from a metabolic engineering perspective, the introduction of such systems into BOS may represent another way of altering the FA composition of the seed oil.

Other interventions, applied outside of storage lipid biosynthesis, for increasing SOC and overall oil content are also possible. One such possible genetic intervention would be to increase Rubisco activity in the zygotic embryo in the situation where Rubisco operates outside of the Calvin cycle to provide 20 % more acetyl-CoA for FA synthesis combined with a 40 % reduction in carbon loss as carbon dioxide [115]. This review has mainly focused on methods for increasing SOC at an essentially fixed size and amounts of seed per plant. Other approaches to increase overall seed oil yield per plant in BOS could come from studies on increasing seed yield such as size/weight (e.g. [324,395]) and the number of siliques per plant (e.g. [396]). Thus, a myriad of approaches to increase oil accumulation in BOS, such as *B. napus*, are available. Many of these approaches may also be applicable to increasing SOC in other Brassicaceae such as *B. carinata*, camelina (*Camelina sativa*) and pennycress (*Thlaspi arvense*). They promise that future constraints on vegetable oil production due to limited agricultural land, may be alleviated substantially by such methods.

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Randall J. Weselake: Writing – review & editing, Writing – original draft, Conceptualization. **David A. Fell:** Writing – review & editing, Writing – original draft, Conceptualization. **Xiaoyu Wang:** Writing – review & editing, Writing – original draft. **Simon Scofield:** Writing – review & editing, Writing – original draft. **Guanqun Chen:** Writing – review & editing, Writing – original draft. **John L. Harwood:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

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

Data availability

No new data was created during this study.

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