

Optimisation of Trierucin Content in Oilseed Rape

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1 Introduction

1.1 High Erucic Acid Rapeseed

Rapeseed (*Brassica napus*) is one of the most important oil seed crop in Europe. Rapeseed oil ranks at third position on the world's oil market after Soyabean and Palm oil. The seed oil from traditional rapeseed cultivars is rich in erucic acid (*cis*-13-docosenoic acid, 22:1), which comprises 45 – 60 % in the total fatty acid mixture. Genetic studies show that in rapeseed, which arose from a spontaneous cross between *B. rapa* (A genome) and *B. oleracea* (C genome), the 22:1 content is controlled by the additive effects of two gene loci *E1* and *E2*, which encodes E2 subunit of the plastid pyruvate dehydrogenase complex (Harvey and Downey 1963, Stefansson 1983, Lühs *et al.* 1999, Hill 2004). Due to its detrimental nutritional effects, 22:1 is undesirable in edible oils. This led to the breeding of low erucic acid rapeseed (LEAR, Canola) cultivars which are almost devoid of 22:1 in their seed oils by introducing recessive alleles at the two loci controlling 22:1 synthesis (Loof 1972, Stefansson *et al.* 1961).

More recently, high erucic acid rapeseed (HEAR) cultivars have regained interest for industrial purposes because 22:1 is a valuable industrial feedstock with a wide range of applications (Leonard 1994; Sonntag 1995). Currently, 22:1 derivatives are used as slip agents in the manufacture of polyethylene sheets but 22:1 itself and its cleavage products have potential in other commercial products such as plasticizers, surfactants and nylon 1313 (Kleiman 1990). Moreover, HEAR oil is directly used in lubricants, especially where high heat stability is required because the long carbon chain affects a greater affinity to metal surfaces and better lubricates than mineral oils. In addition, it is easily biodegradable which makes it especially appealing for its use in environmentally sensitive areas (Sovero 1993). Thus, 22:1 and its derivatives are important renewable raw materials, especially if 22:1 is available in sufficient quantities and in sufficiently pure form.

The present level of 45 - 60 % 22:1 in the seed oils of the HEAR cultivars is, however, not sufficient to compete well with the alternative source for petrochemicals because of the high cost required for the purification of 22:1. An increase in the 22:1 content to more than 80 mol% would make it an attractive alternative source and would appreciably improve its industrial applicability (Ohlrogge 1994; Sonntag 1991, 1995; Murphy 1996). Hence, combined efforts in plant breeding and genetic engineering are in progress to

maximize the 22:1 content in the seed oil of HEAR cultivars. As outlined below, efforts especially focus on the identification and transgenic expression of genes that encode enzymes involved in the biosynthesis of 22:1 and the channeling of this acyl group into the seed oil (Lassner *et al.* 1996, Frentzen 1998, Han *et al.* 2001, Mietkiewska *et al.* 2004; Wilmer *et al.* 2003)

1.2 Triacylglycerol

Lipids represent the major form of carbon storage in the seeds of many plant species constituting up to 60 % of the dry weight of such seeds. They usually consist of triacylglycerols (TAGs), which accumulate during the midstage of seed development due to temporal specificity of expression of the genes involved (Fehling *et al.* 1990). Within the cells of developing embryos or endosperm, TAGs are synthesized at the endoplasmic reticulum (ER) and deposited in discrete spherical organelles, the oil bodies that bud off from the ER. Their core is made up of TAG surrounded and stabilized by a half-unit membrane.

Unlike membrane lipids, the structures of fatty acids esterified in TAGs have been found to be remarkably diverse throughout the plant kingdom (Van de Loo *et al.* 1993). Many plant species synthesize unusual fatty acids in their developing seeds that are specifically channeled into TAG but largely excluded from the membrane lipids. Seed oils from many species of Araceae, Lauraceae, Lythraceae and Ulmaceae, for example, contain saturated medium chain acyl groups ranging from C₈ to C₁₄. Some plants produce seed oils with acyl chains containing functional groups other than methylene-interrupted double bonds, such as conjugated double bonds, triple bonds, hydroxy, epoxy, keto or cyclopentenyl groups. The seed oils of the Brassicaceae, such as *Brassica napus*, *Crambe abyssinica*, *Sinapis alba* and *Lunaria annua* usually contain 40-60 % 22:1, while the seed oil of nasturtium (*Tropaeolum majus*) contains as much as 80 % 22:1 (Mattson and Volpenhein 1961). Moreover, the seed oils of *Limnanthes* species or *Simmondsia chinensis* (jojoba) contain more than 90 % very long chain fatty acids (Miller *et al.* 1964). The current list of fatty acids found in seed oils contains well over 300 entries indicating a broad biosynthetic flexibility with regard to both fatty acid and TAG biosynthesis (Voelker and Kinney 2001).

1.3 Overview of Fatty Acid Biosynthesis

In plants, the reactions of *de novo* fatty acid synthesis are located in plastids (Fig. 1, Ohlrogge *et al.* 1979). Priming and elongation of nascent acyl chains requires acetyl-CoA and malonyl-CoA respectively, as precursors. The enzyme acetyl-CoA carboxylase (ACCase) catalyzes the first reaction of the fatty acid biosynthetic pathway, namely the formation of malonyl-CoA from acetyl-CoA and CO₂.

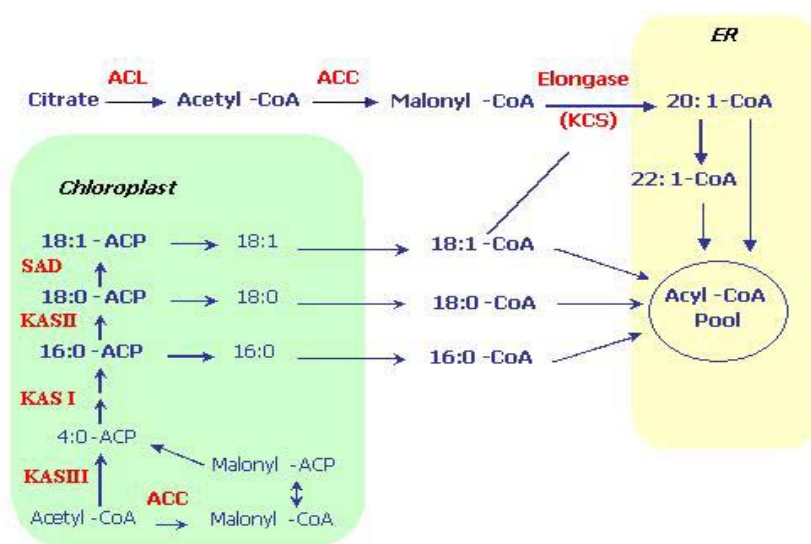


Fig. 1 Scheme presenting overview of fatty acid biosynthesis in plants (ACL, ATP:citrate lyase; ACC, Acetyl-CoA Carboxylase; KCS, β -Ketoacyl-CoA Synthase; SAD, stearoyl-ACP desaturase ; KAS, 3-ketoacyl-ACP synthases).

Two different types of ACCase are observed in plants. Type I referred to as prokaryotic type present in plastids consists of four separate subunits assembled into a 700 kD complex while type II or eukaryotic ACCase is a homodimer of a single large multifunctional polypeptide that is located in the cytosol but in plastids of certain plant species as well (Sasaki *et al.* 1993; Alban *et al.* 1994, Ohlrogge and Jaworski 1997, Thelen and Ohlrogge 2002). The eukaryotic ACCase is needed in the cytosol to supply malonyl-CoA for a variety of

pathways, including fatty acid elongation, mevalonate pathway and flavonoid biosynthesis. Within the cytosol an ATP:citrate lyase (ACL) has been shown to provide the ACCase with acetyl-CoA (Fig. 1). To this end, citrate generated in the mitochondria is exported into the cytosol via a tricarboxylate transporter and subsequently converted into acetyl-CoA by ACL activity (Evans *et al.* 1983). Unlike ACL of vertebrates which is a homotetramer of 110 kDa subunits, ACL of plants consists of two subunits of 45 and 65 kDa, probably in a A₄B₄ stoichiometry indicating that a evolutionary fusion of the *ACLA* and *ACLB* genes probably occurred early in the evolutionary history of vertebrates (Fatland *et al.* 2002). In higher plants, ACL occurs both in the cytosol and plastids, but the distribution of activity changes according to the species (Rangasamy and Ratledge 2000a). In rapeseed and spinach, for instance, ACL activity is predominantly located in plastids whereas in pea and tobacco about 80 % of the total ACL activity is located in the cytosol (Ratledge *et al.* 1997, Rangasamy and Ratledge 2000a). Hence, in addition to acetyl-CoA synthetase and pyruvate dehydrogenase, ACL can significantly contribute to the biosynthesis of acetyl-CoA within plastids required for *de novo* fatty acid synthesis (Ohlrogge and Jaworski 1997, Rangasamy and Ratledge 2000a). On the other hand, the cytosolic ACL supply the acetyl-CoA substrate for the cytosolic ACCase, which generates malonyl-CoA for the synthesis of various compounds (Rangasamy and Ratledge 2000b).

In mammals, ACCase is regulated by several biochemical mechanisms, including phosphorylation, activation by citrate, and feedback inhibition by acyl-CoA. None of these mechanisms has yet been shown to occur in plants, but complex biochemical regulation occurs in plants as well (Hunter and Ohlrogge 1998, Ohlrogge *et al.* 1993, Ohlrogge and Browse 1995, Sasaki *et al.* 1997, Savage and Ohlrogge 1999, Shintani and Ohlrogge 1995). ACCase rapidly extracted from light-incubated chloroplasts, for instance, is two-four fold more active than that from dark-incubated chloroplasts, even when *in vitro* conditions and cofactors are identical (Ohlrogge *et al.* 1993, Ohlrogge and Browse 1995). In addition, fatty acid synthesis in tobacco suspension cells is subject to feedback inhibition by exogenously provided lipids and this feedback appears to act at the level of ACCase activity (Shintani and Ohlrogge, 1995).

The plastidial ACCase from plant species apart from grasses is a complex comprising of four different subunits (Ohlrogge and Jaworski 1997), namely biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and α - and β -carboxyltransferase (α -CT and β -CT).

The BC subunit catalyzes the ATP-dependent carboxylation of the biotinyl moiety on BCCP and α -CT and β -CT subunits catalyze the transfer of activated carboxyl groups from BCCP to acetyl-CoA forming malonyl-CoA. Recently, Thelen and Ohlrogge (2001) provided evidence that the ACCase complex is associated with envelope membranes through non-ionic interactions to the CT subunits. In that way ACCase might perceive cytosolic demand for fatty acids and coordinate *de novo* fatty acid synthesis through a feedback control mechanism (Shintani and Ohlrogge, 1995). Assembly of an ACCase complex requires coordination of cytosolic and plastid production of the subunits because the β -CT subunit is plastome encoded whereas the other three subunits are nuclear encoded (Ohlrogge and Jaworski 1997). Little is known about this coordination and assembly. One early effort to increase ACCase activity was to over-express the BC subunit using a CaMV 35S promoter in tobacco. Although BC protein increased threefold in leaves, there was no accompanying increase in the amount of other ACCase subunits and ACCase activity (Shintani *et al.* 1997). Thus for ACCase, unlike some other multienzyme complexes, the over-expression of just one subunit does not increase the amount of the remaining subunits (Shintani *et al.* 1997). Evidence that increased malonyl-CoA pools can increase fatty acid production was obtained by targeting a homodimeric ACCase to rapeseed plastids (Roesler *et al.* 1997). Under the control of a seed-specific promoter, this chimeric protein resulted in higher ACCase activities and increased oil yield by 3–5 % on a seed dry-weight basis. More recently it was confirmed that increasing the pool size of malonyl-CoA precursor could quantitatively enhance seed oil. The small increase in oil yield pointed to additional control points for fatty acid and triacylglycerol synthesis (Hill, 2004).

In the course of *de novo* fatty acid synthesis at least three separate condensing enzymes, also known as 3-ketoacyl-ACP synthases (KAS), are required to produce an 18-carbon fatty acid (Fig. 1). KAS III catalyzes the first condensation reaction namely the condensation of acetyl-CoA with malonyl-ACP (Jaworski *et al.* 1989). A second condensing enzyme, KAS I, is responsible for producing chain lengths from six to sixteen carbons. Finally, elongation of the 16-carbon palmitoyl-ACP to stearyl-ACP requires KAS II. The initial product of each condensation reaction is a 3-ketoacyl-ACP. Three additional reactions occur after each condensation to form a saturated fatty acid (Fig. 1). The 3-ketoacyl-ACP is reduced at the carbonyl group by the 3-ketoacyl-ACP reductase, which uses NADPH as the electron donor. Next, a hydroxyacyl-ACP dehydratase catalyzes the dehydration of the

hydroxyacyl group. Finally, each round of fatty acid synthesis is completed by an enoyl-ACP reductase, which uses NADH or NADPH to reduce the *trans*-2 double bond of enoyl-ACP. The combined action of these four reactions leads to the lengthening of the precursor fatty acid by two carbons while it is still attached to ACP.

The first *cis* double bond is introduced by the soluble stearoyl-ACP desaturase present in plastids. This fatty acid desaturase is unique to the plant kingdom in that all other known desaturases are integral membrane proteins (Shanklin and Cahoon, 1998). In addition, unlike the stearoyl-ACP desaturase, all other fatty acid desaturases of plants utilize acyl groups esterified to membrane lipids as substrates. A certain part of the long chain acyl groups formed in the plastids is used as acyl-ACP thioesters for the plastidial glycerolipid biosynthesis but the major part is exported usually into the cytosol. To this end, an acyl-ACP thioesterase located in the stroma hydrolyzes the acyl-ACP and releases free fatty acids. The free fatty acids are then reactivated by an acyl-CoA synthetase, an integral membrane protein of the outer envelope membranes so that acyl-CoA thioesters become available for glycerolipid biosynthesis and fatty acid modifications in the endoplasmic reticulum. Very long chain fatty acids such as 22:1 are synthesized by membrane-bound, multienzyme fatty acid elongation complexes (elongase) located at the ER. They catalyze the addition of two carbon units from malonyl-CoA to an acyl-CoA similar to the reaction sequence of the plastidial fatty acid synthetase system (Fehling and Mukherjee 1991). By two elongation cycles 18:1-CoA is converted via eicosenoyl, 20:1-CoA to 22:1-CoA (Fig. 1).

1.4 Overview of Triacylglycerol Biosynthesis

TAG biosynthesis, at least in some species, has been proposed to take place by the comparatively straightforward glycerol-3-phosphate pathway (Fig. 2). As in the pathway for the biosynthesis of the major membrane glycerolipids, the acyl groups are sequentially transferred from acyl-CoA to the *sn*-1 and *sn*-2 position of glycerol-3-phosphate by acyltransferase activities located in the endomembrane system, especially the ER.

The first step of triacylglyceride biosynthesis is the acylation of glycerol-3-phosphate at the *sn*-1 position by glycerol 3-phosphate acyltransferase (GPAT) forming 1-acylglycerol-3-phosphate (lysophosphatidic acid). Lysophosphatidic acid acyltransferase (LPAAT), also known as 1-acylglycerol-3-phosphate acyltransferase, catalyzes the acylation of lysophosphatidic acid at the *sn*-2 position resulting in the formation of the central metabolite,

phosphatidic acid (PA). This product is then dephosphorylated to form diacylglycerol, which is acylated at the *sn*-3 position by diacylglycerol acyltransferase (DAGAT) to form triacylglyceride, the only enzymatic reaction unique to TAG biosynthesis (Fig. 2)

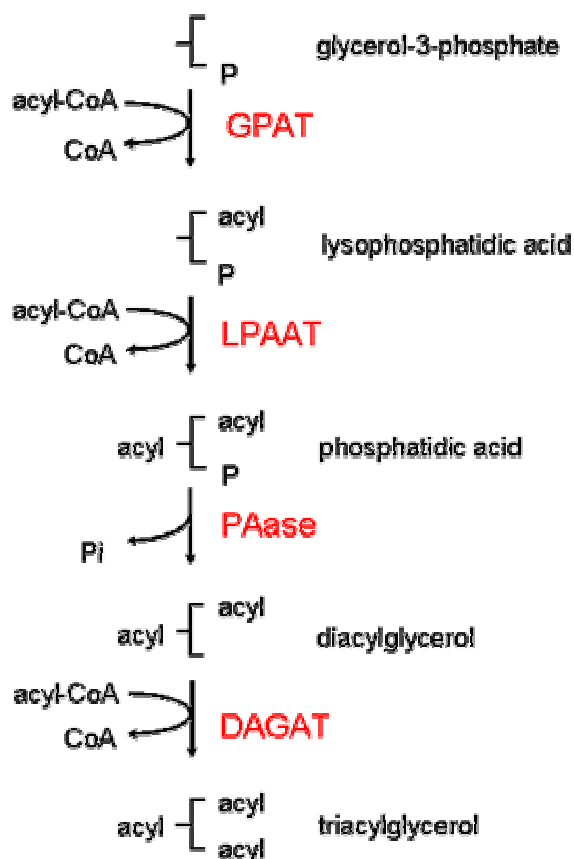


Fig. 2 Scheme for triacylglycerol bioassembly via the glycerol-3-phosphate pathway in developing oilseeds (GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PAase, phosphatidic acid phosphatase; DAGAT, diacylglycerol acyltransferase; Pi, inorganic phosphate).

The assembly of three fatty acids onto a glycerol backbone is, however, not always as straightforward as outlined for the glycerol-3-phosphate pathway. In many oilseeds, most fatty acids produced in the plastid are not immediately available for TAG biosynthesis. Instead, the acyl chains enter into phosphodiacylglycerols, especially phosphatidylcholine (PC), where they become desaturated or otherwise modified. Fatty acids from PC may then become available for TAG synthesis by different mechanisms (Fig. 3).

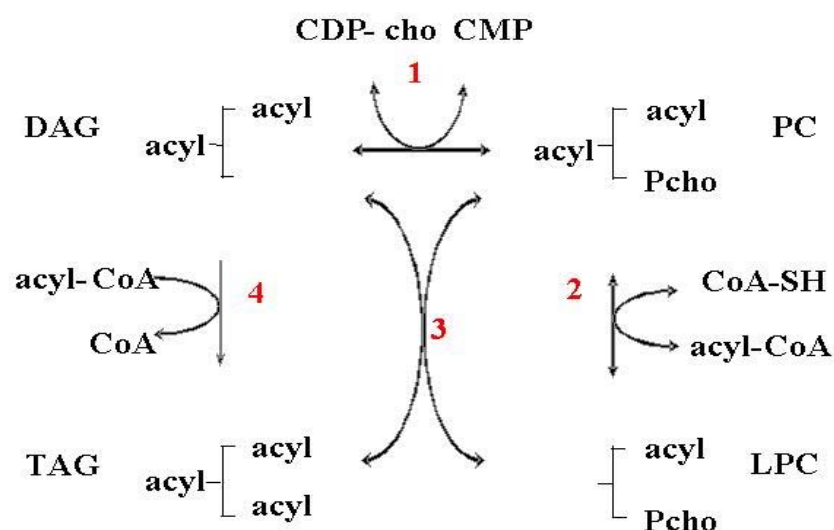


Fig. 3 Scheme showing channeling of acyl groups from PC into TAG (CDP-cho, cytidinediphosphocholine; CMP, cytidinemonophosphate, PC, phosphatidylcholine; PC synthase, PCS (1); LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase (2); PDAT, phospholipid:diacylglycerol acyltransferase (3); DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase (4); TAG, triacylglycerol).

In one mechanism, a fatty acid attached to CoA and a fatty acid on PC may essentially change places. Such an acyl exchange occurs by the combined reverse and forward lysophosphatidylcholine acyltransferase (LPCAT) reaction (Fig. 3, Stymne and Stobart 1987). The resulting acyl-CoA can then be used as an acyl donor in TAG synthesis. The exchange reaction allows 18:1 newly produced and exported from the plastid to enter PC, while desaturated or otherwise modified fatty acids depart for TAG or other lipids. A further mechanism by which PC can participate in TAG biosynthesis is by donation of its entire DAG portion (Stymne and Stobart 1987). In many plants, the synthesis of PC from DAG and CDP-choline catalyzed by a CDP-choline phosphotransferase (PC synthase, PCS) appears to be rapidly reversible (Slack *et al.* 1983). The reversibility of this reaction allows the DAG moiety of PC, including any modified fatty acids, to become available for TAG synthesis. Recently, a novel enzyme reaction for TAG biosynthesis involving the transacylation of acyl groups from phospholipids to DAGs was described in yeast and plants (Fig. 3, Dahlqvist *et al.* 2000, Oelkers *et al.* 2000, Sandager *et al.* 2002, Ståhl *et al.* 2004). The enzyme was thus

termed as phospholipid:diacylglycerol acyltransferase (PDAT). In developing seeds PDAT has been shown to play an important role in the channeling of unusual acyl groups from PC to TAG (Dahlqvist *et al.* 2000). The enzyme can utilize both *sn*- positions of PC but has a pronounced preference for the *sn*-2 position. It shows a significant sequence similarity to LCAT (Dahlqvist *et al.* 2000, Ståhl *et al.* 2004).

1.5 Biosynthesis and Channeling of 22:1 into TAG

In most members of the Brassicaceae and specifically in natural *B. napus*, 22:1 and other very long chain fatty acids are present in the *sn*-1 and *sn*-3 position of TAG but excluded from the *sn*-2 position due to the properties of the acyltransferases involved in seed oil synthesis (Frentzen 1993). This obstacle prevents the biosynthesis of trierucoylglycerol (trierucin) and restricts the 22:1 content in rapeseed oil to a theoretical maximum of 66.7 %. To overcome this biochemical limitation, genes encoding a 22:1-CoA specific LPAAT of *Limnanthes* species were cloned (Hanke *et al.* 1995, Lassner *et al.* 1995) and over-expressed in HEAR cultivars (Lassner *et al.* 1995, Brough *et al.* 1996) or resynthesized rapeseed (RS) lines (Weier *et al.* 1997). Among the various developed transformants those derived from RS lines showed the highest trierucin content of up to 13% and the highest 22:1 level of about 40 % at the *sn*-2 position of the seed oil (Weier *et al.* 1997), which can be attributed to the higher 22:1 synthesizing capacity of these lines as compared to conventional HEAR cultivars used in similar experiments (Lassner *et al.* 1995, Brough *et al.* 1996). Seed specific antisense inhibition of the endogenous rapeseed LPAAT in transgenic RS lines which over-expressed a 22:1-CoA specific LPAAT gene lead to about 50 % 22:1 in the *sn*-2 fatty acid composition accompanied by a slight increase of trierucin content (Münster *et al.* 1998). In spite of the high levels of 22:1 esterified at the *sn*-2 position, none of the transformants contained total 22:1 proportions higher than the range found in untransformed *B. napus* material (Lassner *et al.* 1995, Brough *et al.* 1996, Weier *et al.* 1997, Münster *et al.* 1998). These data strongly suggest that in HEAR cultivars as well as in the RS lines the 22:1 content is not limited by the properties of the LPAAT involved in seed oil synthesis, but by the elongase activities producing 22:1-CoA. Hence, the development of HEAR lines that can accumulate trierucin is a necessary step towards increasing the 22:1 level of rapeseed oil also in addition the biosynthesis capacity for 22:1-CoA has to be improved.

Among the constituent four enzymes of an elongase complex, the condensing enzyme, the β -ketoacyl-CoA synthase (KCS), is regarded to catalyze the first and rate-limiting step determining the acyl chain length of the reaction products (Cassagne *et al.* 1994, Lassner *et al.* 1996, Millar and Kunst 1997, Millar *et al.* 1998, Cahoon *et al.* 2000, Moon *et al.* 2001, Katavic *et al.* 2001, Mietkiewska *et al.* 2004). The genes encoding seed-specific KCS were first characterized in *Arabidopsis thaliana* (James *et al.* 1995) and jojoba (Lassner *et al.* 1996). Subsequently a couple of homologous sequences were isolated from *B. napus* (Clemens and Kunst 1997, Barret *et al.* 1998, Fourmann *et al.* 1998, Han *et al.* 1998) and other plant species (Venkateswari *et al.* 1999, Cahoon *et al.* 2000, Moon *et al.* 2001, Mietkiewska *et al.* 2004). With regard to the improvement of 22:1 content in *B. napus*, the role of KCS has been investigated by identifying and characterizing molecularly more effective 22:1 alleles (Lühs *et al.* 1999) and introducing additional KCS genes (Han *et al.* 1998, Mietkiewska *et al.* 2004, Lassner *et al.* 1996).

Following a genetic engineering approach described by Lassner *et al.* (1996), the effect of specific KCS genes or alleles can be studied in LEAR cultivars that show an almost complete abolishment of 22:1 synthesis. Seed specific over-expression of KCS genes in LEAR cultivars was found to restore elongase activity and thus, to convert LEAR into HEAR lines (Lassner *et al.* 1996, Han *et al.* 1998, Wilmer *et al.* 2003, Mietkiewska *et al.* 2004, Katavic *et al.* 2001). These data clearly demonstrate the essential role of KCS within the microsomal fatty acid elongation process. In addition, KCS co-expression with a 22:1-CoA specific LPAAT in HEAR lines resulted in the accumulation of trierucin in rapeseed oil, but gave total 22:1 levels of 60 to 70 % at most (Han *et al.* 1998, Wilmer *et al.* 2003). Consequently, these data provide strong evidence that high KCS activities in developing seeds and the ability to produce trierucin are necessary for optimising the 22:1 content in rapeseed but still not sufficient. The 22:1 level is further limited by other factors such as the substrate pools available to the elongase or the strength of lipid sinks (Bao *et al.* 1998, Domergue *et al.* 1999, Zou *et al.* 1997, Lardizabal *et al.* 2000).

1.6 Diacylglycerol acyltransferase (DAGAT)

Within the oil-storing cells TAG is synthesized at the endoplasmic reticulum via the glycerol-3-phosphate pathway conserved in nature. The acyl-CoA dependent acylation of

DAG catalyzed by DAGAT, the only enzyme in the glycerol-3-phosphate pathway that is exclusively committed to TAG biosynthesis. In developing and germinating seeds of oilseed plants, TAG accumulation and DAGAT activity have been shown to be associated and are also present with the endoplasmic reticulum (high-speed microsomal fraction) (Cao and Huang 1986; Stymne and Stobart 1987; Frentzen 1993; Settlage *et al.* 1995; Lacey and Hills 1996) and is also present in oil bodies. The biochemical properties of microsomal DAGAT have been examined in a number of plant systems (Frentzen 1993), including developing seeds (Cao and Huang 1987; Bernerth and Frentzen 1990; Vogel and Browse 1996) and embryo cultures (Taylor *et al.* 1991, 1992; Weselake *et al.* 1991; Little *et al.* 1994) of *Brassica napus*. In general, studies with developing seeds indicate that DAGAT activity increases rapidly during the active phase of oil accumulation and then decreases markedly as seed lipid content reaches a plateau (Tzen *et al.* 1993; Weselake *et al.* 1993). A number of studies suggest that DAGAT may catalyze a rate-limiting reaction in TAG bioassembly in developing seeds (Ichihara *et al.* 1988; Perry and Harwood 1993a, 1993b; Settlage *et al.* 1995; Perry *et al.* 1999). For example, developing seeds of *B. napus* have been shown to produce significant levels of DAG during the active phase of oil accumulation (Perry and Harwood, 1993a, 1993b, 1999). The DAGAT activity is the lowest of the four Kennedy pathway enzymes. In addition the alteration in carbon flux resulted in changes of the acyl quantity of the DAG pool but of no other intermediates (Perry *et al.* 1999). The data collectively suggest that the DAGAT reaction may regulate the flow of carbon into TAG at times of high lipid accumulation.

Two sequence-unrelated genes, *DGAT1* and *DGAT2*, have been shown to encode DAGATs (Bouvier-Nave *et al.* 2000, He *et al.* 2004, Hobbs *et al.* 1999, Lardizabal *et al.* 2001, Nykiforuk *et al.* 2002, Routaboul *et al.* 1999, Zou *et al.* 1999). Various studies provided evidence that DAGAT1 that show sequence similarity to ACAT plays an important role in TAG biosynthesis in developing seeds. *Arabidopsis thaliana* mutants deficient in DAGAT1 activity can only accumulate 55 - 75% of seed TAG of the wild type (Katavic *et al.* 1995, Routaboul *et al.* 1999, Zou *et al.* 1999). DAGAT1 protein was found to be most abundant in developing seeds and its amount correlated with TAG content (Lu *et al.* 2003, Nykiforuk *et al.* 2002). Furthermore, over-expression of *DGAT1* genes in plants resulted in a marked increase in TAG levels (Bouvier-Navé *et al.* 2000, Jako *et al.* 2001). The analysis of

Arabidopsis thaliana mutants deficient in DAGAT1 activity also showed that not only the level of TAG but also the level of very long chain fatty acids was significantly reduced (Katavic *et al.* 1995, Routaboul *et al.* 1999, Zou *et al.* 1999) although the acyl-CoA specificity of DAGAT1 was not affected by the mutation (Jako *et al.* 2001). These data suggest that DAGAT1 can be a critical determinant not only of oil yield but also of the fatty acid composition of TAG and hence, oil quality. The latter is in line with the properties of DAGATs determined in microsomal fractions of developing seeds. DAGATs from plant species accumulating high levels of unusual fatty acids in their seed oils possess pronounced preferences for the respective acyl groups so that the unusual acyl groups are effectively channeling into TAG but excluded from the membrane lipids (Frentzen 1993). Thus, DAGAT1 genes might be useful for manipulating oil yield and quality of oilseed crops by genetic engineering.

1.7 Scope of the Thesis

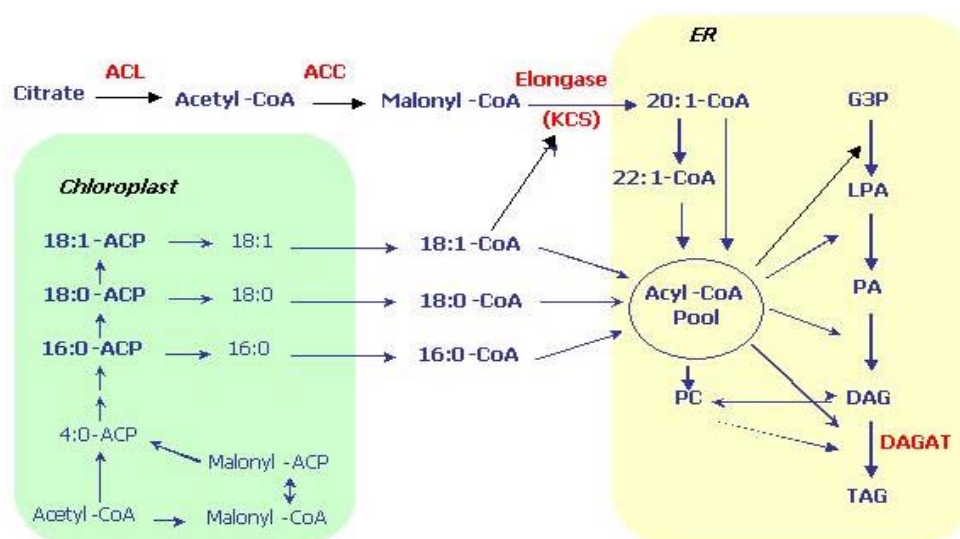


Fig. 4 Scheme for triacylglycerol biosynthesis showing the approach taken for scope of the thesis (Enzymes in Red). ACL, ATP-Citrate Lyase; ACC, Acetyl-CoA Carboxylase; KCS, β -Ketoacyl-CoA synthase; DAGAT, 1,2-Diacylglycerol acyltransferase.

In recent years, combined efforts in plant breeding and genetic engineering have been undertaken to increase the 22:1 content of rapeseed oil to more than 80 % as desired for industrial uses. Various transgenic HEAR lines expressing LPAAT and KCS genes have

been developed which gave promising results, but the analysis of these lines also revealed that additional genes have to be inserted to obtain HEAR cultivars accumulating trierucin as major TAG species. As outlined above, the data so far available suggest that the substrate pools available to the elongase or the strength of lipid sinks can limit the 22:1 content. The data of Domergue *et al.* (1999) suggest that the cytosolic pool of malonyl-CoA can limit 22:1-CoA synthesis while Rangasamy and Ratledge (2000b) show that over-expression of an ACL gene can stimulate fatty acid biosynthesis in plants. Therefore, increasing the availability of acetyl-CoA as substrate for ACCase and stimulating ACCase activity should be beneficial for the biosynthesis rate of 22:1-CoA. In addition, strengthening of the neutral lipid sinks appears to be a promising strategy for optimizing 22:1 levels (Zou *et al.* 1997, Lardizabal *et al.* 2000).

This was the starting point of the thesis. In order to optimize the trierucin levels in transgenic rapeseed plants, the first approach was to increase the cytosolic malonyl-CoA pool available to the elongase systems (Fig. 4). In this regard two genes, namely ACL and ACCase genes (Fig. 4) should be cloned and over-expressed in RS lines. In a second approach the neutral lipid sink should be strengthened by over-expressing a gene encoding a DAGAT with properties optimally suited for the channeling of 22:1 into TAG. In that way, the analysis of the various transgenic lines should contribute to our understanding of the biosynthetic pathway of 22:1 in *B. napus* and the regulatory mechanisms involved.

2 Materials and Methods

2.1 Instruments, Disposable Materials, Chemicals and Enzymes

2.1.1 Instruments

Instruments	Model	Company
Centrifuges:		
Centrifuge	5810 R	Eppendorf
Centrifuge	5417 R	Eppendorf
Centrifuge	RC-5B	Sorvall
Centrifuge	5417 C	Eppendorf
Ultra Centrifuge	L-50	Beckmann
Rotors	SS34, HB6, GS3, SLA3000	Sorvall
Gaschromatograph:		
	GC 6890	Hewlett Packard
	GC Injector 6890	Hewlett Packard
	J & W DB23, Capillar column (30m x 0,530 mm x 0,5 µm)	Agilent
Evaluation Software	GC Chemstation A06.03	Hewlett Packard
Other Devices:		
Electrophoresis chamber for horizontal agarose gel-electrophoresis	19 cm x 13 cm	
Liquid scintillation counter	LS 5000 TD	Beckmann
Power supply unit	300	BioRad
Waring Blendor		Waring
pH-Meter	HI 9321	Hanna Instruments
Photometer Smart Spec	3000	BioRad
Thermocycler	Primus 96	MWG Biotech
Western Blotting Apparatus "semi dry"	V20 SDB	Carl Roth
Thermal-Shaker	Thermomixer compact	Eppendorf
Sonicator	Sonoplus GM70	Bandelin
Gel-documentation-System	UVT-28M	Herolab
SDS-Gel-electrophoresis apparatus	Mini-Protean®3 Cell	BioRad
Rotary Evaporator	Rotavapor R-200	Büchi
Imaging system	Fla2000	Raytest
	Las1000	

2.1.2 Disposable Materials

Disposables		
Silica Gel Plates	Kieselgel 60, 0.25 mm	Merck
Nitrocellulose membrane	Protran BA 85, 0.45 µm	Schleicher & Schüll
Sterile filter	0.2 µm	Sartorius

2.1.3 Chemicals, Enzymes and Kits

If otherwise not mentioned, the chemicals used for this work were purchased from the following companies:

AppliChem (Darmstadt), BD Biosciences (Heidelberg), Biomol (Darmstadt), Duchefa (Haarlem, The Netherlands), Fluka (Neu-Ulm), Invitrogen (Karlsruhe), VWR (Darmstadt), Roche Diagnostics (Mannheim), Carl Roth (Karlsruhe), Serva (Heidelberg), Sigma-Aldrich (Deisenhofen), Vivascience (Hannover), WAK Chemie (Bad Soden), Gibco BRL (Neu Isenburg), MBI Fermentas (Vilnius), New England Biolabs (Schwalbach/Taunus), Roche Diagnostics (Mannheim), Machery and Nagel (Düren), Promega (Mannheim), Perkin Elmer (USA), Stratagene (Amsterdam Zuidoost, The Netherlands) and Qiagen (Hilden).

2.1.4 Labeled-substrates

[1-¹⁴C] labeled compounds (Oleoyl-CoA, Erucic Acid, Eicosenoic Acid and Malonyl-CoA) were bought from Amersham Pharmacia Biotech (Freiburg), or Perkin Elmer (USA).

2.2 Biological materials

2.2.1 *Escherichia coli* (*E. coli*)-strains

XL1-Blue MRF' (Invitrogen, Karlsruhe, Germany) (Bullock *et al.* 1987)

$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 } supE44 \text{ thi-1 } recA1 \text{ gyrA96 } relA1$
 $lac[F'proAB, lacI^qZ\Delta M15, Tn10(tet^r)]$

SCS110 (Stratagene, Heidelberg, Germany)

$rpsL(Str^r), thr, leu, endA \text{ thi-1 } lacY \text{ galK } galT \text{ ara } tonA \text{ tsx } dam \text{ dcm } supE44 \Delta(lac-proAB \text{ l}) [F' traD36 proAB lacI^qZ\Delta M15]$

DH5 alpha (Invitrogen, Karlsruhe, Germany) (Hanahan 1983)

$F-, deoR \text{ recA1 } endA1 \text{ hsdR17 } (rk- \text{ mk}+) \text{ supE44 } lamda\text{-thi-1 } gyrA96 \text{ relA1}$

BL21 DE3 (Stratagene)

$B, F-, dcm \text{ ompT } hsdS(rb- \text{ mb-}) \text{ gallammda(DE3)}$

Sure cells (Stratagene)

el4- (Mcr Aö) delta (mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 (Kan r) uvrC [F'proab lac qZ deltaM15 Tn10 (Tet r)]

2.2.2 *Agrobacterium tumefaciens* (*A. tumefaciens*)-strains

ATHV C58C1, Rif^r (Hood *et al.* 1986)

The strain contains the helper plasmid pEHA101 and has a chromosomal rifampicin resistance.

2.2.3 *Saccharomyces cerevisiae* (*S. cerevisiae*)-strain

BY4742 (Euroscarf, France)

MAT α ; *his3* Δ 1; *leu2* Δ 0:*lys2* Δ 0:*ura3* Δ 0:*YOR245c::kanMX4*

2.2.4 Plant material

2.2.4.1 *Brassica napus* cultivars/line

RS306: resynthesized rapeseed line, 60% erucic acid. (Lühs and Friedt, 1994)

Maruca: Rapeseed line, 45% erucic acid

Rapeseed plants were cultivated in a greenhouse (25 °C, 16 h light). Harvested plant material was immediately frozen in liquid nitrogen and stored at –80 °C or used directly.

2.2.4.2 *Tropaeolum majus*

Tropaeolum plants were cultivated in the botanical garden during the summer season. Harvested plant material was immediately frozen in liquid nitrogen and stored at –80 °C or used directly.

2.3 Vectors

2.3.1 Bacterial transformation vectors

<u>Plasmid</u>	<u>Selection marker</u>	<u>Reference</u>
pBluescript SK [–]	<i>amp</i> ^R	Stratagene
pBluescript KS [–]	<i>amp</i> ^R	Stratagene

pGEM-T	<i>amp^R</i>	Promega
pGEM-Nap, Nos	<i>amp^R</i>	Weier <i>et al</i> (1997)
pTE200	<i>amp^R</i>	Hemmann & Martini (1995)
pUC18	<i>amp^R</i>	Yanish-Perron <i>et al.</i> (1985)

2.3.2 Yeast Transformation Vectors

<u>Plasmid</u>	<u>Selection marker</u>	<u>Reference</u>
pYES2	<i>amp^R/URA3</i>	Invitrogen
pESC	<i>amp^R/TRP</i>	Invitrogen

2.3.3 Plant Transformation Vectors

pRE1	<i>str^R/spe^R/npt II</i>	Weier <i>et al.</i> (1997)
pNKAT	<i>str^R/spe^R/npt II</i>	Han <i>et al.</i> (1998)
pPZP111	<i>Cm^R/nptII</i>	Hajdukiewicz <i>et al.</i> (1994)

2.4 Cultivation of microorganisms

2.4.1 *E. coli* cultivation

The cultivation of the *E. coli* strains was carried out according to Sambrook *et al.* (1989). Wild type and transformed *E. coli* strains were grown at 37 °C in LB medium or on LB-agar plates supplemented for selection with the appropriate antibiotics.

LB medium (Luria & Bertani, see Sambrook *et al.* 1989)

10 g/l Trypton
 5 g/l Yeast extract
 5 g/l NaCl
 pH 7.5, adjust with NaOH
 for LB plates : +15 g/l agar
 Sterilisation: autoclave at 120 bar for 20 min

LB-Selection media: prepare sterile LB-medium, after cooling to 50 °C,
 add appropriate antibiotics from sterile stock solutions (filter sterile)

Antibiotic	stock solution	final concentration
Carbenicillin (Carb)	50 mg/ml in H ₂ O dest.	50 mg/l
Tetracyclin (Tet)	25 mg/ml in H ₂ O dest.	25 mg/l

2.4.1.1 Preparation of glycerol stocks:

Glycerol stocks were prepared by mixing 500 µl fresh cultures in 500 µl of sterile glycerol solution [25mM Tris-HCl, pH 7.5; Glycerol 70% (w/v)]. Glycerol stocks were stored at –80 °C.

2.4.1.2 Optical density of bacterial cultures

The amount of absorbance can be determined by using a spectrophotometer, which measures what fraction of the light passes through a given solution and indicates on the absorbance display the amount of light absorbed compared to that absorbed by a clear solution. To determine the absorbance of bacterial cultures, the wavelength is preset to 600 nm. The blank sample used to standardize the measurement is sterile growth medium, therefore 1 ml of the sterile medium was pipetted into a micro-cuvette. After the absorbance for this blank sample was set, 1ml of the bacterial culture was pipetted into a second micro-cuvette, and the absorbance OD_{600nm} was read and recorded.

For *E. coli* cultures 1 OD_{600nm} approximates 1×10^9 cells (Sambrook *et al.* 1989)

2.4.2 *A. tumefaciens* cultivation

Wild type and transformed *A. tumefaciens* C58C1 ATHV cells were cultured at 28 °C in YEP medium supplemented with the appropriate antibiotics (Walkerpeach & Velten 1994).

YEP medium (An *et al.* 1988)

- 10 g/l Pepton
- 10 g/l Yeast extract
- 5 g/l NaCl
- pH 7.2

For YEP plates: +15 g/l agar

YEP-Selection media: prepare sterile YEP-medium, after cooling to 50 °C add appropriate antibiotics from sterile stock solutions (filter sterile)

Antibiotic	Stock solution	Final concentration
Rifampicin (Rif)	50 mg/ml in methanol	100 mg/l
Streptomycin (Str)	100 mg/ml in H ₂ O dest.	100 mg /l

Spectinomycin (Spe)	150 mg/ml in H ₂ O dest	300 mg /l
Chloramphenicol (Cm)	100 mg/ml in H ₂ O dest	100mg/l

2.4.3 *S. cerevisiae* cultivation

YPD medium 10 g/l Bacto-yeast extract (Gibco)
 20 g/l Bacto-peptone (Gibco)
 20 g/l Bacto-agar and a piece of solid NaOH (for solid medium)
 autoclave, then add:
 50 g/l Glucose (filter sterile)

Dropout powder	Adenine (Hemisulfate)	2.5 g
	L-Arginine (HCl)	1.2 g
	L-Aspartate	6.0 g
	L-Glutamate (Na-salt)	6.0 g
	L-Lysine (HCl)	1.8 g
	L-Methionine	1.2 g
	L-Phenylalanine	3.0 g
	L-Serine	22.5 g
	L-Threonine	12.0 g
	L-Tyrosine	1.8 g
	L-Valine	9.0 g
mix well		

Selection media:

Complete minimal dropout medium (CMdum, Ausubel *et al.* 1995).

Complete drop out medium

1.155 g Dropout powder
 765.1 ml H₂O dest
 20 g/l Bacto-agar and a piece of solid NaOH (for solid medium)
 Autoclave, and add:

for CMdum uracil: 8.3 ml Histidine (240 mg/100 ml, filter sterile, storage at 4°C)
 8.3 ml Leucine (720 mg/100 ml, filter sterile, storage at RT)
 8.3 ml Tryptophan (480 mg/100 ml, filter sterile, storage at 4°C)

for CMdum trp	8.3 ml Histidine (240 mg/100 ml, filter sterile, storage at 4°C)
	8.3 ml Leucine (720 mg/100 ml, filter sterile, storage at RT)
	8.3 ml Uracil (480 mg/100 ml, filter sterile, storage at 4°C)
for CDdum trp,uracil	8.3 ml Histidine (240 mg/100 ml, filter sterile, storage at 4°C)
	8.3 ml Leucine (720 mg/100 ml, filter sterile, storage at RT)
	then add to all
	100 ml Raffinose or Glucose (20 g/100 ml, filter sterile)
	100 ml YNB- (6.7 g/100 ml, filter sterile, Difco)

Complete minimal dropout uracil medium was used for the growth of yeast cells transformed with pYES vector constructs, which have uracil selection. Complete minimal dropout tryptophan medium was used for the growth of yeast cells transformed with pESC vector constructs, which have tryptophan selection. Complete minimal dropout tryptophan, uracil medium was used for the growth of yeast cells co-transformed with pYES and pESC vector constructs which have uracil and tryptophan selection, respectively.

2.4.3.1 *S. cerevisiae* cultivation and induction

For lipid analysis of transformed *S. cerevisiae* cells expressing DAGAT sequences from different organisms alone or together with the *Brassica napus* KCS-sequence, the strain BY4742 Δ YOR245c lacking most of the internal DAGAT activity (Brachmann *et al.* 1998; Sorger and Daum 2002) and harbouring the empty expression vector pYES2 or one of the following constructs pBndg1, pBndg2, pBndg3 and pTmdg, pLldg1, pLldg2 alone or in combination with pBnKCS was used.

These transformed cells were cultured in the appropriate selection media containing glucose as a carbon source. Adding galactose to the medium induced the transcription of the introduced sequence from the GAL1 promoter. Briefly, 50 ml selective medium was inoculated with a single colony and incubated at 30 °C for 2d. The culture was pelleted, resuspended and induced in 200 ml selective medium and incubated at 30 °C with shaking (250 rpm) for 24/48 hrs. The cells were harvested by centrifugation (4000 x g, 10 min, 4 °C), washed with H₂O dest and the resulting pellet was stored for further use at -20 °C.

2.5 Transformation of microorganisms

2.5.1 Transformation of *E. coli*

2.5.1.1 Preparation of competent *E. coli* cells (Inoue *et al.* 1990)

5 ml LB-medium were inoculated with a single bacterial colony and cultured overnight at 37 °C under continuous shaking (200 rpm). 1 ml of this pre-culture was inoculated into 100 ml SOB medium and the cells were grown at 37 °C with vigorous shaking (225-250 rpm) to an OD₆₀₀ of ~ 0.5. After 10 min incubation on ice, the cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C in a sterile tube. The cells were resuspended in 40 ml ice-cold TB buffer, incubated on ice for 10 min and centrifuged again as described above. The supernatant was discarded and the cell pellet was gently resuspended in 8 ml ice-cold TB buffer supplemented with DMSO to a final concentration of 7 %. After 10 min incubation on ice, microfuge tubes with 200 µl aliquots of the competent cells were frozen in liquid nitrogen and stored at -70 °C.

TB buffer	10 mM Piperazine-N, N'-bis [2-ethanesulfonic acid] (PIPES), pH 6.7 adjust with KOH autoclave after cooling add: 55 mM MnCl ₂ , filter sterile 15 mM CaCl ₂ , filter sterile 250 mM KCl, filter sterile
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2.5.1.2 Transformation of competent *E. coli* cells

Competent cells were thawed on ice and, after adding 10-50 ng plasmid DNA or 10 µl of a ligation reaction; the cells were incubated on ice for 20 min. After a 90 sec heat shock in a water bath at 42 °C the cells were quickly transferred to an ice bath for 2 min. 0.8ml SOB medium was added and cells were incubated at 37 °C with shaking for 45 min. 50-200 µl of

the cells were spreaded on LB selection plates supplemented with the appropriate antibiotics, and incubated overnight (~16 h) at 37 °C for colony formation.

SOB medium	20 g/l Peptone
	5 g/l Yeast extract
	10 mM NaCl
	2.5 mM KCl
	autoclave, then add:
	10 mM MgCl ₂ , filter sterile
	10 mM MgSO ₄ , filter sterile

2.5.1.3 Selection of transformed *E. coli* cells by α -complementation (blue-white screening) (Ullmann *et al.* 1967)

Many vectors carry coding information for the first 146 amino acids of the β -galactosidase gene. Without disrupting the β -galactosidase ORF a polycloning site is embedded in the coding region, into which insert DNA is cloned. When expressed, this 146 amino acid fragment of the β -galactosidase protein is incapable of using the chromogenic substrate (X-gal). But when expressed in appropriate host cells (e.g. XL1Blue, DH5 α) which express the carboxyl terminal fragment of the β -galactosidase protein, these two protein fragments can associate to form an enzymatically active protein. This is called α -complementation and such cells turn blue when plated on plates containing X-gal. If insert DNA is ligated into the polycloning site, it almost invariably results in the production of an amino-terminal fragment that is not capable of α -complementation and hence, those colonies remain white.

IPTG stock solution:	100 mM Isopropyl-1-thio- β -D-galactoside (IPTG) in H ₂ O dest.
	filter sterile
	final concentration: 200 μ M

X-Gal stock solution: 2% (w/v) 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal)
in Dimethylformamide
final concentration: 80 μ g/ml

2.5.2 Transformation of *A. tumefaciens*

2.5.2.1 Preparation of competent *A. tumefaciens* cells (An *et al.* 1988)

5 ml YEP-medium were inoculated with a single bacterial colony and cultured for 2 d at 28 °C under continuous shaking (250 rpm). 1-2 ml of this pre-culture were inoculated into 50 ml YEP medium and cells were grown at 28 °C under constant shaking to an OD₆₀₀ of ~0.5. The cells were harvested by centrifugation at 3000 $\times$ g for 5 min. The cell pellet was resuspended in 1 ml ice-cold 20 mM CaCl₂ and 100 μ l aliquots in microfuge tubes were quickly frozen in liquid N₂ and stored at -80 °C.

2.5.2.2 Transformation of competent *A. tumefaciens* cells

1 μ g plasmid DNA (e.g., pRE1, pPZP111) were added to the competent cells and incubated in a 37 °C water bath for 5 min. 0.5 ml YEP-medium were added and for regeneration the cells were incubated at 28 °C for 2-4 h with gentle rotating. The cells were plated on YEP selection plates containing the appropriate antibiotics and incubated at 28 °C for 2 d.

2.5.3 Transformation of *S. cerevisiae*

2.5.3.1 Preparation of competent *S. cerevisiae* cells by a modified lithium acetate (LiAc) method (Ausubel *et al.* 1995).

A single colony of the yeast strains YOR245c was inoculated in 10 ml YPD-medium and incubated at 30 °C for 1 d. 2 ml of the pre-culture was added to 200 ml YPD medium and the cells were grown at 30 °C to an OD₆₀₀ of ~0.5 (6-8 h). The cells were harvested by centrifugation (4000 $\times$ g, 5 min, 20 °C) and washed with 10 ml sterile H₂O dest., followed by centrifugation, as described above. The cells were washed with a LiAc solution and after centrifugation the cell pellet was resuspended in 1 ml LiAc solution. The competent cells were directly used for transformation or could be stored at 4 °C for 1-2 weeks.

LiAc-solution: 1 ml 10 x TE (0.1 M Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0)

1 ml 1 M LiAc, pH 7.5, adjust with acetic acid

8 ml H₂O dest

filter sterilisation

make fresh before using

2.5.3.2 Transformation of competent *S. cerevisiae* cells

For the transformation 1-2 µg plasmid (for co-transformation add 1 µg of each plasmid) and 100 µg sheared salmon sperm carrier-DNA were added to the competent yeast cells and incubated for 30 min at room temperature with shaking. After adding 450 µl PEG solution, the cells were incubated at room temperature with shaking for 30 min. A heat shock was given at 42 °C for 15 min. The cells were sedimented by centrifugation at 6000 rpm for 2 min, resuspended in 200 µl H₂O dest., spread on plates with the appropriate selection medium and incubated at 30 °C for 2-3 d.

Yeast strain BY4742-YOR245c was transformed with yeast vector constructs (refer to 2.4.3.1)

Salmon Sperm

DNA preparation: Prepare immediately before use.

Denature by boiling 10 min

10 mg/ml Salmon Sperm DNA in TE Buffer

(0.1 M Tris-HCl, pH 7.5; 10mM EDTA, pH 8.0)

PEG solution: 8 ml 50 % (w/v) PEG 4000 (Merck), filter sterile

1 ml 10 x TE (0.1 M Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0)

1 ml 1 M LiAc, pH 7.5, adjust with acetic acid, filter sterile

2.6 Plasmid isolation

2.6.1 Isolation of plasmid DNA from *E. coli*

2.6.1.1 Analytical isolation of plasmid DNA (Riggs & McLachlan, 1986)

1.5 ml LB selection medium was inoculated with a single *E. coli* colony and incubated at 37 °C overnight under constant shaking. This overnight culture was transferred into a microfuge tube and centrifuged at $14000 \times g$ for 20 sec. The sedimented cells were resuspended in 0.2 ml BF buffer with lysozyme (final concentration 1 mg ml^{-1}), boiled in a water bath for 1 min and shortly cooled on ice. Cell debris, denaturated proteins and chromosomal DNA were sedimented by centrifugation at $14000 \times g$ for 30 min at 20 °C. Plasmid DNA was precipitated from the supernatant by adding 480 μl Isopropanol-Mix and sedimented after 15-30 min incubation at room temperature by centrifugation at $14000 \times g$ for 20 min at 20 °C. The DNA pellet was washed with 0.5 ml 70 % (v/v) ethanol and was again centrifuged for 5 min at $14000 \times g$. The plasmid DNA was air-dried, dissolved in 100 μl TE buffer containing RNaseA (final concentration 10 mg/ml) and incubated for 45 min at 37 °C.

For subsequent restriction and electrophoretic analysis 2 μl of the plasmid DNA solution were used. For sequence analysis the isolated plasmid DNA was further purified. The plasmid DNA solution was supplemented with 200 μl TE buffer, and extracted twice with 200 μl phenol/chloroform (1:1) and once with an equal volume of chloroform. The purified plasmid DNA was precipitated with 0.1 V 5 M ammonium acetate and 3 V ethanol and sedimented after 20 min incubation at 20 °C by centrifugation at $14000 \times g$ for 20 min. The plasmid DNA pellet was washed with 70 % (v/v) ethanol; air-dried and redissolved in 50 μl H₂O dest.

BF buffer:	8 % (w/v) Sucrose
	0.5 % (w/v) Triton X-100
	50 mM EDTA, pH 8.0
	10 mM Tris-HCl, pH 8.0

Lysozyme solution:	20 mg/ml in H ₂ O dest.
	Storage at -20 °C

Isopropanol-Mix:	400 μl Isopropanol
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80 µl Ammonium acetate (5 M)

TE buffer: 10 mM Tris-HCl, pH 7.6
 1 mM EDTA, pH 8.0

2.6.1.2 Preparative isolation of plasmid DNA (Qiagen)

Depending on the required amount of plasmid DNA the preparative isolation of plasmid DNA was carried out with the Qiagen Plasmid Midi Kit (for 100 µg plasmid DNA) or a Qiagen Plasmid Maxi Kit (for 500 µg plasmid DNA). The plasmid purification with the kit is based on the interaction between the negatively charged phosphate residues of the DNA and the positively charged groups of diethylaminoethanol on the resin surface. The preparations were performed according to the manufacturer's protocol.

2.6.1.3 Quantitation of nucleic acids

The concentration of nucleic acids was determined using a spectrophotometer (BioRad) at 260 nm (Sambrook *et al.* 1989).

DNA :	1 OD _{260 nm} = 50 µg/ml
RNA :	1 OD _{260nm} = 40 µg/ml
Oligonucleotide :	1 OD _{260 nm} = 33 µg/ml

The ratio of OD_{260 nm}/OD_{280 nm} was used for estimating the purity of the nucleic acid preparation. A pure DNA preparation should give a ratio of about 1.8 and a pure RNA preparation should have a ratio of about 2.0.

2.6.2 Isolation of plasmid DNA from *A. tumefaciens*

Plasmid DNA was isolated from *A. tumefaciens* using an alkali lysis method.

3 ml YEP selection medium was inoculated with a single *A. tumefaciens* colony and incubated at 28 °C overnight. 1-2 ml of this pre-culture were added to 5-10 ml YEP selection medium. After reaching the stationary growth phase, the cells were sedimented by centrifugation (3000 x g, 15 min) and resuspended in 200 µl TE/sarcosyl buffer. The suspension was diluted with 1.3 ml TE/sarcosyl buffer, transferred to a fresh 2 ml microfuge tube and centrifuged for 10 min at 20 °C. The sedimented cells were resuspended in 100 µl

GTE buffer with lysozyme (final concentration 5 mg ml⁻¹) and incubated for 5 min at 20 °C. 200 µl NaOH/SDS solution was added and the suspension was mixed gently by inverting. After 10 min at 20 °C 150 µl prechilled KAc solution (4°C) was added to the cell lysate and thoroughly mixed by inverting. After centrifugation at 14000 × g for 10 min at 20 °C, the supernatant was transferred into a new 1.5 ml microfuge tube and extracted twice with 1 V phenol/chloroform and once with 1 V chloroform. The plasmid DNA was precipitated from the supernatant by adding 1 ml ethanol. After incubation at -70 °C for 10 min the plasmid DNA was sedimented by centrifugation at 14000 × g for 10 min at 20 °C. The plasmid DNA pellet was washed with 500 µl 70 % ethanol, air dried, resuspended in 40-50 µl H₂O dest. and stored at -20 °C.

TE/sarcosyl buffer	50 mM Tris-HCl, pH 8.0 20 mM EDTA, pH 8.0 0.05 % (v/v) Na-sarcosyl
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GTE buffer	50 mM Glucose (filter sterile) 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0 make fresh
------------	---

KAc solution	294 g l ⁻¹ Potassium Acetate 115 ml l ⁻¹ acetic acid add H ₂ O dest. to final volume pH 4.8
--------------	---

NaOH/SDS solution:	0.2 N NaOH 1 % SDS use fresh
--------------------	------------------------------------

2.6.3 Isolation of plasmid DNA from *S. cerevisiae*

Plasmid DNA was isolated from yeast cells using a glass bead method described by Ausubel *et al.* (1995)

2 ml appropriate selection medium was inoculated with a single *S. cerevisiae* colony and the cells were grown at 30 °C with shaking until the culture reached the stationary growth phase. The cells were harvested by centrifugation (14000 x g, 5 sec) in a 1.5 ml microfuge tube and resuspended in 200 µl phenol/chloroform/isoamylalcohol (25:24:1). The cell suspension was vortexed, with glass beads added to it in equal amounts as the weight of the pellet, at high speed for 2 min and centrifuged at 14000 x g for 5 min. The supernatant was transferred to a fresh microfuge tube and 2 µl of the supernatant were used to transform competent *E. coli* XL1-Blue cells. Plasmid DNA was isolated from transformed *E. coli* cells grown on LB selection medium as described in chapter 2.4.1 and used for restriction and sequence analysis.

2.7 Plasmid DNA analysis with restriction endonucleases

Approximately 200 ng plasmid DNA and 1U restriction endonuclease were used for restriction analysis according to the conditions described by the manufacturer. The molecular weight of the restricted DNA fragments was determined by horizontal agarose gel electrophoresis.

2.7.1 Agarose gel electrophoresis

Separation of isolated DNA and RNA fragments was carried out using horizontal agarose gel electrophoresis. Depending on the size of the expected nucleic acid fragments, 0.7 – 1.6 % agarose gels in 1 x TBE buffer containing 30 ng/ml ethidium bromide were used. The DNA samples were spiked with 0.1 V 10 x loading buffer, applied to the sample wells and separated in 1 x TBE buffer at 50 – 60 V (4 V/cm). The molecular weight of the DNA fragments was calculated in comparison to appropriate molecular weight standards.

The DNA was visualized by excitation of intercalated ethidium bromide by UV light and documented with a videocamera system (Herolab) and a Video-Printer (Herolab).

10 x TBE buffer	890 mM Tris
	890 mM Boric acid
	20 mM EDTA, pH 8.3
10 x Loading buffer	0.25 M EDTA, pH 8.0

10% (w/v) Ficoll
0.25% (w/v) Bromophenol blue
0.25% (w/v) Xylene cyanol

Molecular weight standards:

GeneRuler™ 1kb DNA Ladder, 14 fragments (in bp): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.

GeneRuler™ 100bp DNA Ladder Plus, 14 fragments (in bp): 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.

2.8 Isolation of DNA fragments from agarose gels

DNA fragments for subcloning were purified from agarose gels using the QiaexII kit (Qiagen) according to the manufacturer's instructions.

2.8.1 Modification of DNA fragments

2.8.1.1 Creation of blunt end DNA fragments

Fill-in of 5'-overhangs and removal of 3'-overhangs of DNA fragments to form blunt ends was carried out using the polymerase and 3'- 5' exonuclease activity of the Large DNA Polymerase I (Klenow) fragment according to the manufacturers protocol.

The enzyme was removed from the sample by phenol/chloroform extraction (chapter 2.12.1).

2.8.1.2 Dephosphorylation of DNA fragments

Alkaline phosphatase was used for the catalytic removal of 5' phosphate groups from DNA ends according to the protocol supplied by the manufacturer. Since CIP-treated fragments lack the 5' phosphate termini required by ligases, they cannot self-ligate and therefore the vector background in ligation and transformation experiments can be decreased.

The enzyme was removed from the sample by phenol/chloroform extraction and precipitated with ethanol (Chapter 2.12.1).

2.9 Ligation of sticky and blunt end DNA fragments

The formation of phosphodiester bonds between 5' phosphate and 3' hydroxyl groups of DNA fragments was catalysed by T4 DNA ligase according to the instructions of the manufacturer.

Sticky end ligation: vector (approximately 50 ng) and insert fragment in a molar ratio of 1:3, were ligated with 1 Weiss unit T4 DNA ligase at 16 °C for 4 h

Blunt end ligation: vector (approximately 50 ng) and insert fragment in a molar ratio of 1:3, were ligated with 1 Weiss unit T4 DNA ligase at 16 °C overnight.

2.10 DNA Sequence analysis

Sequence analysis of DNA was carried out by the Sequencing service unit of the RWTH Institute for Biology VII using the ABI PRISM® 3700 Genetic Analyzer (Dr. Jost Muth).

The sequence data were analysed using the following programmes:

BLAST (Altschul *et al.* 1990)

CLUSTAL X (<http://alfredo.wustl.edu/msa/clustalold.cgi>)

TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>)

GeneDoc (Nicholas & Nicholas 1997; www.cris/~ketchup/genedoc.shtml).

2.11 PCR

2.11.1 Primer

Oligonucleotides (synthesized by MWG Biotech (München)) were used as primers for the amplification of DNA by the polymerase chain reaction (PCR) method.

The melting temperature (T_m) of oligonucleotides was calculated according to Suggs *et al.* (1981): $T_m = 2^{\circ}\text{C} \sum (\text{dA} + \text{dT}) + 4^{\circ}\text{C} \sum (\text{dG} + \text{dC})$

Tab. 2.9.1 Sequences of the oligonucleotide primers used.

Primer	Sequence	$T_m(^{\circ}\text{C})$
A1	5'tagaattcccatgtcggccaagg	57.1 °C
A2	5'ataagctttgttacatgctcatgtg	52.8 °C
A3	5'catccggagcaatgatatggattt	54 °C
A4	5'taccatggcaggatccattaatgggaatgatcata	62.1 °C
A5	5'gcctgcaggatcgatcagtaaacat	58 °C
A6	5'taccatggcaggatccattaatgggaatgatcata	62.1 °C
K1	5'taggatccatgacgtccgtaacgtaaag	60.1 °C
K2	5'tactcgagtaggaccgacgttttgac	62.9 °C
T1	5'tactcgagtcacttttctttagatttatcaggtea	67.1 °C
T2	5'taggatccatggcgggtggcagagtcgtcac	67.1 °C

B1	5'taatggcgattttggattctggaggcgtcg	63 °C
B2	5'tatcaggacatggatcctttgcggttcatc	61.6 °C
S1	5'ttgcaaaactaatcatattcactgg	51.1 °C
S2	5'ccagtgaatatgattagtttgcaa	51.1 °C
S3	5'tggattcatgggatttataatag	48.1 °C
S4	5'ctattataaatcccatgaatcca	48.1 °C
S5	5'atagaacaatatataaacccaattg	47.1 °C
S6	5'caatagggtttatataattttctat	47.1 °C
S7	5'agcatccttgaaaggggatcttc	55.3 °C
S8	5'gaagatccccgtttcaaaggatgct	55.3 °C
S9	5'gaggtttggttcaacggtgggc	58.6 °C
S10	5'gccacccgttgaaaccaaaccctc	58.6 °C

2.11.2 Standard PCR reactions

Either *Taq* DNA polymerase or *Pfu* DNA polymerase was used for the standard PCR reactions depending on the application. *Taq* DNA Polymerase can produce highly accurate amplicons with careful control of reaction parameters, as *Taq* DNA Polymerase does not have a proofreading function. *Pfu* DNA Polymerase is possibly the best known of all the proofreading polymerases and has fidelity approximately almost 8-fold higher than *Taq* DNA polymerase.

Standard PCR reaction using *Taq*-Polymerase

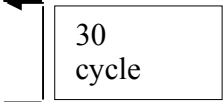
5 µl	10 x PCR buffer
1.5 µl	50 mM MgCl ₂
1 µl	10 mM dNTP
1 µl	20 µM Primer 1
1 µl	20 µM Primer 2
1 µl	2 units/µl <i>Taq</i> DNA polymerase
X µl	10-50 ng DNA template
<u>X µl</u>	H ₂ O dest.
50 µl	total reaction volume

Standard PCR reaction using *Pfu*-polymerase

5 µl	10 x PCR buffer
1 µl	10 mM dNTP
1 µl	20 µM Primer 1

1 μ l	20 μ M Primer 2
1 μ l	2 units/ μ l Pfu DNA polymerase
X μ l	10-50 ng DNA template
<u>X</u> μ l	H ₂ O dest.
50 μ l	total reaction volume

Temperature profile

1. Denaturation	94°C	3 min	
2. Denaturation	94°C	30 sec	
3. Annealing	T _a °C	30 sec (T _a = T _m – 5°C)	
4. Polymerization	72°C	(1 min for ~1 kb)	
5. Elongation	72°C	5 min	
6. Termination	4°C	∞	

For the amplification of the DAGAT sequence from *Tropaeolum majus* the annealing temperature used was equal to the calculated T_m. All other PCR reactions were carried out as described above. PCR amplification was analysed by agarose gel electrophoresis (Chapter 2.7.1)

2.11.3 Cloning of PCR fragments

Amplified PCR products were purified from agarose gels using QiaexII DNA Isolation Kit (Qiagen, Hilden) and cloned into either the pGEM-T vector (Promega, Madison, USA) or the pUC18-vector of the Sureclone[®] Ligation Kit (Pharmacia Biotech, Freiburg) according to the manufacturers instructions.

The pGEM-T vector is prepared by cutting pGEM[®]-5Zf (t) with *EcoRV* and adding a 3' terminal thymidine to both ends. Thus the PCR fragment amplified by Taq polymerase can be directly ligated to pGEM-T, since Taq polymerase has a terminal transferase activity and adds dATP to the terminal end.

The pUC18 vector of the Sureclone[®] Ligation Kit is digested with *SmaI* and dephosphorylated, so that it is suitable for cloning blunt PCR fragments amplified by Pfu polymerase.

2.12 Isolation of nucleic acids from plants

2.12.1 Isolation of genomic DNA from *B. napus*

Genomic DNA was isolated from leaf tissue with some modifications according to Dellaporta *et al.* (1983).

B. napus leaves (500–600 mg fresh weight) were ground in liquid nitrogen to a fine powder and transferred to a 2 ml microfuge tube. 660 µl extraction buffer and 72 µl 10 % (w/v) SDS were added and the solution was mixed by vortexing. The samples were incubated in a water bath at 65 °C for 20 min. 360 µl ice-cold 5 M KAc was added and the samples were cooled on ice for 15 min. After centrifugation at 14000 for 10 min, the supernatant was transferred into a fresh 2 ml tube, supplemented with RNase (final concentration 40 µg/ml) and incubated at 37 °C for 1 h. The samples were extracted twice with phenol/chloroform (1:1) and once with an equal volume of chloroform. The aqueous and organic phase were separated by centrifugation at 14000 × g for 5 min. The supernatant was transferred to a new 1.5 ml tube and adding 0.1 volume 3 M NaAc and 1 volume isopropanol precipitated genomic DNA. After incubation at –20 °C for 2 h the DNA was sedimented by centrifugation at 4000 × g for 10 min. The DNA pellet was washed with 500 µl 70 % ethanol followed by centrifugation (14000 × g, 10 min), air-dried and resuspended in 50 µl TE buffer. The quality of the DNA was analysed by separation of 1-2 µl of the genomic DNA solution on a 0.7 % agarose gel by electrophoresis. The quantity of the analysed DNA was calculated in comparison to a λ-DNA marker (MBI Fermentas, Vilnius)

DNA extraction buffer	100 mM Tris-HCl, pH 8.0
	50 mM EDTA, pH 8.0
	500 mM NaCl
	H ₂ O dest.
	Make fresh
10 % (w/v) SDS	
TE buffer:	10 mM Tris
	1 mM EDTA

pH 7.5

DNA marker: Lambda DNA/EcoRI+HindIII Marker, 13 fragments (in bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125

2.12.2 Isolation of total RNA from plant tissues

Total RNA was isolated from plant tissues using LiCl method. Plant seed tissues were ground in liquid nitrogen to a fine powder and transferred to a 50 ml Falcon tube. Extraction buffer (10 ml/g FW) and 10 % SDS (2 ml/g FW) was added and the suspension was mixed by vortexing for 20 min. The mixture was extracted with an equal volume of phenol, phenol/chloroform and chloroform for 15 min each, and the phases were separated by centrifugation at $3000 \times g$ for 15 min, respectively. RNA was precipitated from the supernatant by adding 1 volume 5 M LiCl and incubation at 4 °C overnight in a corex tube. The RNA was sedimented by centrifugation at $6000 \times g$ for 10 min at 4 °C. The RNA pellet was washed twice with 0.5 ml 70 % ethanol, air dried and resuspended in 100 µl DEPC-H₂O. The RNA was analysed by agarose gel electrophoresis and the concentration was determined photometrically (chapter 2.6.1.3). The RNA samples were stored at -70 °C.

RNA extraction buffer	50 mM Tris-HCl, pH 9.0
	150 mM NaCl
	5 mM EDTA, pH 8.0
	DEPC-H ₂ O
	Make fresh

5 M LiCl	21.2 g LiCl
	5 ml 1M Tris-HCl, pH 8.0
	DEPC-H ₂ O to 100 ml

DEPC-H₂O:

Add 100 µl diethyl pyrocarbonate (DEPC) to a bottle containing 100 ml H₂O. Cap, shake well, and incubate 2 hr at 37 °C. Autoclave 15 min on liquid cycle. Store at room temp.

DEPC is a suspected carcinogen. Handle with care.

2.12.3 mRNA isolation

mRNA isolation from 100 µg total RNA was carried out using the Chemagen mRNA purification kit (Chemagen, Baesweiler, Germany) following the manufacturers instructions. The mRNA concentration was measured photometrically (1 OD_{260nm} (1 cm path length) $\approx$ 40 µg RNA/ ml).

2.12.4 First strand cDNA synthesis

For the synthesis of first strand cDNA, approximately 1 µg of mRNA in a sterile RNase free microcentrifuge tube with 2 pmol each of gene specific primers in a total volume of 14 µl was incubated at 70 °C for 5 min, then cooled quickly on ice for 5 min. MMLV reverse transcriptase (Promega, Mannheim) was used for first strand cDNA. The following components were added to the annealed primer/template:

dH ₂ O (to 25 µl final volume)	6.75 µl
M-MLV RT Reaction buffer	5.00 µl
10 mM dNTP mix	1.25 µl
M-MLV-RT (H-) (100 units)	1.00 µl

The reaction was incubated at 50 - 55 °C for 1 hour followed by inactivation at 70 °C for 15 min. The cDNA was further used as a template for amplification by PCR. For a negative control reaction the enzyme reverse transcriptase was not added, but the conditions were otherwise identical.

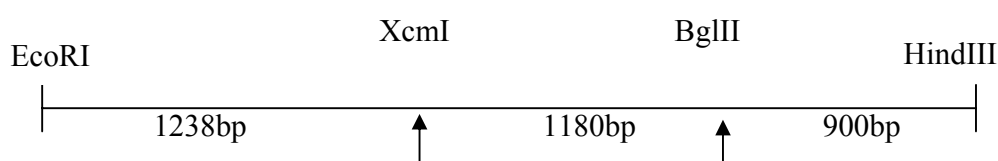
2.12.5 Second strand cDNA synthesis and PCR amplification

5 to 10 µl of the first-strand cDNA reaction were used in subsequent PCR amplification using gene specific primers (50 pmol), 1x reaction buffer, 0.2 mM dNTP, 3 U *Pfu* polymerase (Promega, Mannheim) and H₂O dest. to a final volume of 50 µl.

2.13 Preparation of chimeric gene constructs for functional expression studies

2.13.1 Preparation of chimeric ATP: citrate lyase (ACL) gene constructs

The published mammalian ACL sequence was 3.3 kb in length (NCBI accession number XM 012684). mRNA isolated from human liver was kindly provided by Dr. J. Gruber. Three primer sets were designed and RT-PCR was conducted to obtain three subclones of the ACL sequence having respective restriction sites, so that these could be joined to get a full-length cDNA in pGEM-T. The identity of the sequence was verified by sequencing.



The diagram above shows ACL gene was divided into three fragments. The first, second, third subclones started at the EcoRI, *XcmI* site, *XcmI* site ended at the *XcmI* site, *BglII* and *HindIII* respectively.

2.13.2 Preparation of chimeric ACL constructs for plant transformation

To overexpress the ACL in *B. napus*, three plant constructs were prepared. Two were single constructs containing the ACL sequence under the control of the napin promotor and the nos terminator with or without selection. The triple construct contained the ACL-, the BnKCS- and the LdLPAAT-sequences, all three under the control of the napin promotor. The ACL sequence, cloned in pGEM-T (refer to 2.13.1), was excised using *EcoRI* and *HindIII* and was made blunt by a klenow fill in reaction. Simultaneously, the pUC18 vector containing the Napin promotor and the Nos terminator (kindly provided by Dr. J. Gruber) was cleaved with *SalI* and *XhoI*, blunted and ligated with the blunted ACL insert giving the vector pACL2. pACL2 was cut with *MssI*, this enzyme cuts out the whole insert again, simultaneously, pPZP111 (plant vector) was cut with *SmaI* and ligated with the excised ACL2 cassette resulting in the single construct pACL.

A double construct, pNKAT, containing the BnKCS and the LdLPAAT sequences was provided by Dr. J. Han and the *MssI* excised ACL2 cassette was ligated with the *SmaI* cut pNKAT vector resulting in the triple construct named pTriACL. The vector maps of constructs pACL and pTriACL are shown in the appendix.

2.13.3 Chimeric constructs for functional expression studies of DAGAT and KCS sequences in *S. cerevisiae*

To identify erucoyl-CoA specific DAGAT sequences, cDNAs encoding DAGATs were isolated from the resynthesized *B. napus* line RS306 (Lühs and Friedt, 1994), *Lesquerella lindheimeri* and *Tropaeolum majus*.

Gene specific primers B1 and B2, having the respective restriction sites (*PaeI* and *KpnI*) to allow subsequent cloning steps, were designed from known sequence of DAGAT, AF164434 in the NCBI database (Nykiforuk *et al.* 1999). Two ~ 1.5 kb putative DAGAT sequences namely DAGAT1 and DAGAT2 were obtained from seeds of *B. napus* line RS306 by performing RT-PCR using gene specific primers, that is B1 and B2. The two DAGAT cDNAs thus amplified were restricted with *KpnI/PaeI* and ligated into pYES2. This resulted in pBndg1 and pBndg2. The respective vector maps are shown in the appendix (5.2).

Similarly, gene specific primers were designed from known sequences of *T. majus* DAGATs in the databases (Mietkiewska *et al.* 2002). RT-PCR was performed and a putative DAGAT sequence was obtained from *Tropaeolum majus* seeds using the primers T1 and T2 having *BamHI* and *XhoI* restriction sites. It was cloned into pYES giving pTmdg. The vector map is shown in the appendix. Dr. T. Roscoe provided the DAGAT gene of *B. napus*, isolated from B002, a LEAR variety (Barret *et al.* 1998). This was also cloned into pYES2 having *BamHI* and *XhoI* restriction sites giving pBndg3. pLldg1 and pLldg2, cloned by Dr. Müller (unpublished data) are two respective DAGAT constructs with the DAGAT sequences obtained from *Lesquerella lindheimeri*, cloned in pYES2. BnKCSa was provided by Dr. J. Han (Han *et al.* 1998). The restriction sites *BamHI* and *XhoI* were introduced and the BnKCS was ligated behind the GAL1 promoter in the yeast expression vectors pYES2 and pESC (Invitrogen, CA), respectively.

2.13.4 Preparation of chimeric constructs for plant transformation

2.13.4.1 Chimeric DAGAT2 of *L. lindheimeri* construct for plant transformation

To overexpress the *L. lindheimeri* DAGAT2 in transgenic *B. napus* plants, the DAGAT2 sequence was introduced into the polycloning site of the pNKAT vector to get a triple construct, containing BnKCS, LdLPAAT and DAGAT2, first two under the control of the

strong seed specific napin promotor and DAGAT2 under USP promotor. For this purpose a pBS-DAGAT2 construct, pBSdg2 was provided by Dr. Müller. The pBSdg2 construct was cut with *Sma*I and the nos terminator sequence was ligated behind the DAGAT sequence giving the intermediate pdg2nos construct. Next, a pGEM-T construct with the USP promotor was restricted with *Nhe*I and blunted by a fill-in reaction with the Klenow enzyme. The DAGAT and nos terminator sequence was excised from pdg2nos by *Sma*I restriction and ligated into the *blunted* *Nhe*I site of the pGEM-T-USP1 vector giving pUSPdg2nos. This construct was cut with *Not*I to excise the DAGAT sequence along with the promoter and terminator sequence, the resulting fragment was blunted and ligated into the *Sma*I cut pNKAT vector, yielding the triple construct, pDAGAT3. The vector construct map is shown in the appendix.

2.13.4.2 Chimeric DC3 Ω –BnACC construct for plant transformation ACCase

The genomic ACCase sequence coding for a plastidial ACCase was kindly provided by Prof. R.Töpfer (BAZ Siebeldingen).

In order to develop a transformation construct for the cytosolic expression of the ACCase in transgenic *Brassica napus* seeds, the N-terminal plastidial transit peptide sequence comprising 100 amino acids had to be removed. Therefore, the 5' portion of the genomic ACC sequence was amplified using the specific primers A3 and A4 (having *Nco*I and *Msc*I sites respectively). This fragment was cloned into the *Hinc*II site of pBS and the sequence was verified by sequencing. The DC3 Ω promoter was amplified with additional *Sbf*I and *Nco*I restriction sites included in the primers A5 and A6 (refer to 2.9.1) and the integrity were also verified by sequencing. The amplified 5'-ACC fragment was ligated to the DC3 Ω promoter using *Nco*I and *Xho*I resulting in p5'M-DC3 Ω . Next, the ACC was excised from bnacc10 with *Not*I and p5'M-DC3 Ω was cut with *Xho*I, both DNA fragments were blunted and ligated to each other giving rise to pBS-ACC1. This vector was cut with *Nco*I and religated giving rise to pBS-ACC2. The expression cassette was then excised using *Not*I and *Sbf*I, blunted and cloned into the *Sma*I site of pPZP111 giving rise to pPZP-ACC1. The respective vector construct map is shown in the appendix.

2.13.4.3 Chimeric USP–BnACC construct for plant transformation ACC

The 5'ACC fragment was excised from the pBS-5'ACC construct (refer to 2.12.4.2) with *NcoI* and *MscI* and blunted. This fragment was ligated into the blunted *NheI* site of pGEM-T+USP1 (provided by Dr. J. Gruber). Next, the promotor USP1 - 5'ACC fragment was excised with *SbfI* and *MscI* to replace the DC3 Ω promoter and the respective 5'ACC region present in pBSACC1. This construct, pBSACC2 was cut with *NcoI* resulting in pBSACC3. pBSACC3 was cut with *BssHII*, blunted and ligated into the *SmaI* cut vector pPZP111 to give rise to pPZP-ACC2. The respective vector construct map is shown in the appendix.

2.14 Lipid analysis

2.14.1 Preparation and GC analysis of Fatty acid methyl esters (FAME)

2.14.1.1 Fatty acid methyl esters (FAME) preparation of total fatty acids from *S. cerevisiae*.

The cultivation of *S. cerevisiae* was done as explained in chapter 2.4.3.1. The cell pellets of the respective yeast strains harbouring the respective constructs were harvested after induction and were resuspended in 5 ml MeOH/H₂SO₄ solution. This was transferred to a 12 ml Sovirel glass tube and incubated at 80 °C for 1 h for fatty acid methyl ester (FAMES) formation. Twice adding 2.5 ml petrolether and 2.5 ml H₂O dest. and vortexing extracted FAMES and the phases were separated by centrifugation (1200 x g, 3 min). The supernatant (~5 ml) from two extractions was collected in a fresh glass tube and washed with 2.5 ml H₂O dest. by vortexing and centrifuging (1200 x g, 3 min). FAMES were concentrated by under nitrogen, redissolved in 100 μ l hexane and stored at –20 °C.

2.14.1.2 Fatty acid methyl ester (FAME) preparation of lipids from *S. cerevisiae*

For triacylglycerol analysis 0.5 g cells were washed with water and boiled for 20 mins at 80 °C. Afterwards an equal amount of glass beads (0.5 g) was added and the cells were vortexed for 5 min. 5 ml methanol/chloroform (1:1) were added and this suspension was shaken for 2 hr at room temperature. The organic phase containing the extracted lipids was separated from the aqueous phase by adding salt (1 ml 0.9 % NaCl), so that the lower organic phase could be easily extracted. The organic phase was concentrated under nitrogen and redissolved in

100 µl hexane which was loaded onto Silica gel plate (Merck). The plate was developed in diethylether/acetic acid (40:1) for 50 min and the separated lipids were visualized under UV light by spraying the plate with dichlorofluorescein. The bands corresponding to TAG and DAG standards (Sigma-Aldrich, Deisenhofen) were scraped off the plate, and the acyl groups were transesterified into FAMES as described in 2.14.1.1.

Methanolic Sulphuric acid

MeOH/H ₂ SO ₄	100 ml methanol (water-free)
	2 ml Dimethoxypropane
	0.5 M H ₂ SO ₄

FAMES from total fatty acids and triacylglycerol samples were analysed by gas chromatography. 2 µl of the FAME solution were injected into a gas chromatograph. Signals were identified by comparing with the corresponding FAME standards (Sigma-Aldrich (Deisenhofen)). The FAME standards were derived from the following fatty acids: Palmitic acid (C16:0), Palmitoleic acid (C16:1n9c), Heptadecanoic acid (C17:0), Stearic acid (C18:0), Oleic acid (C18:1n9c), Linoleic acid (C18:2n6c), Arachidic acid (C20:0), cis11Eicosenoic acid (C20:1), Linolenic acid (C18:3n3), Erucic acid (C22;1n9)

2.14.2 Fatty acid methyl esters (FAME) preparation of total fatty acids from plants

Fatty acid methyl esters (FAME) analysis of total lipids was also done with developing seeds of *B. napus*. 10 developing seeds (*B.napus* RS 306) were homogenised in 2 ml MeOH/H₂SO₄ solution and heated at 80 °C for 1 h for fatty acid methyl esters (FAMES) formation. Methyl esters were extracted as described before and used for GC analysis.

2.14.3 GC analysis of FAMES

GC-conditions

Gas chromatograph:	HP 6890 GC
Carrier gas:	Nitrogen
Column:	J&W DW23 50 % Cyanopropyl/methylsiloxane, 30m, 0,5mm Diameter
Injector:	HP GC injector

Detector:	Flame ionisation detector, 250 °C
Oven temperature:	170-220 °C
Autosampler	HP 7673, Injection amount: 2µl
Temperature gradient	2.5 °C/min

2.15 Acyl-CoA isolation from developing seeds of *B. napus*.

About 10 developing seeds from *Brassica napus* (RS 306) were homogenised in 3 ml chloroform/methanol (2:1). 1 ml chloroform and 1 ml water were added and the suspension was vortexed for 30 sec. After centrifugation for 10 min at 5000 rpm at 4 °C the sample was separated into three phases, the upper and lower phases were discarded and the interphase was dried under nitrogen. Then 200 µl extraction buffer, 5 µl saturated ammonium sulphate and 600 µl methanol/chloroform (2:1) were added to the dry interphase and the suspension was vortexed for 3 min and incubated for 20 min at room temperature. After centrifugation at 5000 rpm for 5 min the supernatant was transferred to a 1ml eppendorf vial with a pasteur pipette. The supernatant was centrifuged again at 14000 rpm for 2 min and the now obtained supernatant was used for methyl ester formation and GC analysis, as mentioned in 2.14.

Extraction buffer:	2 ml Isopropanol
	50 µl Glacial acetic acid
	80 µl BSA (50 mg/ml)
	2 ml 50 mM KH ₂ PO ₄

2.16 Membrane isolation

2.16.1 Membrane preparation from *S. cerevisiae*

Membranes were isolated from the transformed *S. cerevisiae* strain BY4742 harbouring the respective DAGAT constructs (refer to 2.12.3) or the empty vector pYES2, as a control. A single colony was inoculated in 10 ml selection medium (CMdum) containing glucose as a carbon source and cultured overnight at 30 °C. The preculture was used to inoculate a 200 ml culture, that was grown for 36 h with shaking at 30 °C. The culture was pelleted and suspended in 400 ml induction medium with galactose as a carbon source and cultured for 24 or 48 h with shaking at 30 °C. The cells were sedimented and washed with water and then

with buffer A. The pellet was suspended in buffer A (5 ml/g wet weight cells) containing 10 mM DTT and was incubated at 30 °C with shaking for 20 min. After centrifugation, the cells were washed with buffer B. Next, suspended in 5.7 ml buffer B and 5 mg Zymolase/g cells and incubated at 30 °C for 1h. After this step, the cells were twice washed with cold buffer B and suspended in 1 ml buffer A/g cells containing 1mM PMSF and an equal amount of glass beads as the weight of cells. The suspension was vortexed for 5 min and incubated on ice for 5 min, and centrifuged at 4000 rpm for 10 min at 4 °C. The resulting supernatant was centrifuged at 9500 rpm for 10 min, at 4 °C and the supernatant thus obtained was ultracentrifuged at 40,000 rpm for 1h at 4 °C. The membrane pellet was suspended in 500 µl buffer A with 20 % glycerol and frozen at –20 °C until further use. The protein concentration of the membrane fraction was estimated with the Bradford method (2.17.1).

Buffer A : 1 M Tris H₂SO₄ (pH 7.4)

Buffer B : 1.2 M sorbitol, 20 mM KH₂PO₄, pH 7.4

1 M PMSF (Phenylmethylsulfonylfluorid, Boehringer Mannheim) was dissolved in DMSO

2.16.2 Preparation of membrane fractions from developing plant seeds

Developing seeds from *B. napus* and *T. majus*, stored at –70 °C, were grounded with a pestle and mortar using liquid nitrogen. Five times the volume of freshly prepared homogenisation buffer was added and the homogenate was passed through double layered mira cloth. The filtrate was centrifuged at low speed (2500 rpm, 4 °C, 5 min). The supernatant was centrifuged at 14000 rpm at 4 °C for 20 min and the pellet was suspended in 200 µl suspension medium and stored at –20 °C until further use. The protein concentration of the membrane fraction was estimated with the Bradford method (2.17.1).

Homogenisation Buffer: 100 mM Hepes-KOH, pH 7.5
0.5 M Sucrose
2 mM DTT

Suspension Buffer : 20 mM Hepes-KOH, pH 7.5
20 % Glycerol
2 mM DTT

2.17 Acyl-CoA Synthesis

The Acyl-CoA substrates for DAGAT assays were synthesised using a protocol described by Taylor *et al.* (1990). 1 μ mole of the respective fatty acid was pipetted into a glass-vial. The organic solvent was evaporated under nitrogen and the fatty acids were dissolved in 450 μ l H₂O dest. 10 μ l 10 % Triton X-100, 100 μ l 100 mM MgCl₂ and 100 μ l 1 M MOPS buffer pH 7.5 were added and the suspension was sonicated for 5 min. 70 μ l 70 mM CoA (Sigma-Aldrich, Deisenhofen), 100 μ l 100 mM ATP (Sigma-Aldrich, Deisenhofen), 100 μ l 100mM DTT (Sigma-Aldrich, Deisenhofen) and 0.25 units Acyl-CoA synthetase (Sigma-Aldrich, Deisenhofen) in a final volume of 1 ml were added. The glass-vial was sealed after addition of a micro-stir bar and flushing with nitrogen. The suspension was stirred at 35 °C for 2 h. In parallel a RP varian sep C18 column was washed with 3.6 ml methanol with a flow rate of 1 drop per second. The column was equilibrated with 3.6 ml 100 mM MOPS pH 7.5 before the sample was applied to the column. The column was washed with 1ml 100 mM MOPS pH 7.5 and then with 2 ml Methanol/H₂O dest (1:1). The collecting tube was changed before elution of the respective acyl-CoA with 20 ml methanol. The methanol was evaporated under nitrogen and the synthesized acyl-CoAs were dissolved in 500 μ l 10mM MES, pH 6.0. 70 mM CoA was prepared in 10 mM MES pH 6.0. 100 mM ATP and 100 mM DTT were prepared in milliQ water.

2.18 DAGAT-Assay

The DAGAT assay was performed in a final volume of 50 μ l containing 100 mM MOPS, pH 7.2, 3 mM DAG, 15 μ M [1-¹⁴C]oleoyl-CoA (dissolved in 10 mM MES, pH 6.0), and membrane enzyme fraction (from 5 μ g - 40 μ g). The reaction was incubated for 5 min at 25 °C and stopped by adding 240 μ l Chloroform:Methanol (1:1) containing 4 μ l of 25mg/ml TAG and 100 μ l salt solution (1 M KCl, 0.2 M H₃PO₄). The lipophilic assay components were extracted into the organic phase by vortexing, and the phases were separated by centrifugation. 80 μ l of the organic phase were loaded onto a TLC plate, and the chromatograms were developed in an ascending manner with the solvent system diethyl ether-acetic acid (40:1). The separated lipids were visualized on the TLC plates under UV light by staining with DCF-reagent and the bands were scraped off the plates into

scintillation vials containing 5 ml scintillation liquid (OptiPhase II 'Hisafe', Perkin Elmer). Radioactivity was measured with a liquid scintillation counter (Beckmann). 3mM DAG was sonicated for 30 seconds in buffer solution.

DCF reagent: 0.1% 2,7-dichlorofluorescein (DCF) in 97% methanol

2.18.1 Bradford protein assay

Protein concentrations were determined as described by Bradford (1976). In the typical assays for estimation of protein concentration, 2 μ l to 10 μ l protein solution were gently mixed with 1.0 ml Bradford reagent and incubated for 5 min at RT. The absorbance at 595 nm wavelength was measured against a blank containing 2 – 10 μ l of the corresponding storage buffer in 1 ml Bradford reagent. Bovine serum albumin (BSA, Sigma-Aldrich, Deisenhofen) was used as standard for calibration assays in the range of 5 μ g to 20 μ g BSA protein.

2.19 Site Directed Mutagenesis

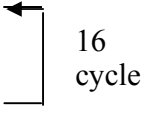
Site directed mutagenesis was carried out according to the QuickChange® Site-Directed Mutagenesis Instruction manual (Stratagene). Five sets of two complimentary oligonucleotides were synthesized, S1-S10, containing the desired mutation in the middle, flanked by unmodified nucleotide sequence.

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The control reaction was prepared as indicated below:

- 5 μ l of 10 $\times$ reaction buffer
- X μ l (5–50 ng) dsDNA template
- X μ l (125 ng) oligonucleotide primer #1
- X μ l (125 ng) oligonucleotide primer #2
- 1 μ l dNTP mix
- ddH₂O to a final volume of 50 μ l
- then added
- 1 μ l Pfu DNA polymerase (2.5 U/ μ l)
- 50 μ l total reaction volume

Temperature profile

1. Denaturation	94 °C	30 sec	
2. Denaturation	94 °C	30 sec	
3. Annealing	55 °C	30 sec	
4. Polymerization	68 °C	(1 min for ~1 kb)	
5. Elongation	68 °C	5 min	
6. Termination	4 °C	∞	

After amplification, 10 µl of the PCR reaction was restricted with *DpnI* for 3 h at 37 °C and an aliquot of 10 µl was used for bacterial transformation (refer to 2.4.1).

2.20 Elongase and β -ketoacyl-CoA synthase (KCS) assays with membrane fractions from developing seeds of resynthesized *B. napus* line RS306.

2.20.1 Elongase and KCS enzyme preparation (Wilmer *et al.* 1998)

Developing seeds from the resynthesized *B. napus* line RS306 were frozen in liquid nitrogen and grounded in a mortar. 5 ml/g tissue homogenisation buffer was added. The homogenate was passed through a double layer of mira cloth and centrifuged at 2000 rpm for 5 min. The supernatant was recentrifuged at 14000 rpm for 25 min to obtain the membrane pellet. The pellet was resuspended in a buffer containing 80 mM HEPES, pH 6.8 and 10 mM β -mercaptoethanol. Protein was quantified with the Bradford assay with BSA as calibration standard (2.17.1).

Homogenisation Buffer: 80 mM Hepes-KOH, pH 6.8
 0.32 M Sucrose
 10 mM β -Mercaptoethanol
 50 mg/ml PVPP (Polyvinylpyrrolidon)

2.20.2 Elongase and KCS enzyme assay

Elongase activity in membrane fractions of developing *B. napus* seeds was assayed using a modified protocol described in Wilmer *et al.* 1998

The assay was performed in a final volume of 100 µl containing 80 mM HEPES, pH 7.2, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM MgCl₂, 150 µM Triton X-100, 8 µM [2-¹⁴C] Malonyl-CoA (60 mCi/mmol, Perkin Elmer) and 20 µM oleoyl-CoA. The reaction was incubated at 30 °C for 30 min and stopped by the addition of 100 µl 5 M KOH, 10 % methanol. The reaction products were saponified for 1h at 70 °C. Next, 100 µl 5 M H₂SO₄ with 10 % malonic acid were added and the reaction mixture was extracted with 2 ml chloroform. The organic phase was washed twice with 2 ml H₂O dest., evaporated under nitrogen and the radioactivity was measured with a liquid scintillation counter.

KCS activity was assayed under the same assay conditions with the only exception, that NADH and NADPH were not added.

Malonyl-CoA and oleoyl-CoA were diluted in 10 mM MES pH 6.0. DTT, MgCl₂, Triton X-100, NADH, NADPH were prepared in milli Q water and stored at -20 °C.

Results and Discussion

3.1 Analysis of developing seeds of transgenic RS306 plants expressing *BnKCS* and *LdLPAAT*

Seed oil analysis of transgenic HEAR plants expressing a *B. napus* KCS and a *L. douglasii* LPAAT showed that the 22:1 content at the *sn*-2 position of the triacylglycerols increased up to 31.7 – 37.5 % compared to less than 1 % in wild-type plants, even though the overall 22:1 content did not exceed 60 % (Han *et al.* 2001). The analysis reflected the effective channeling of 22:1 into the *sn*-2 position of TAGs by the *L. douglasii* LPAAT, but gave no indication of a higher activity of the elongase complex in these transgenic HEAR lines. To investigate whether KCS over-expression resulted in a more active elongase complex in transgenic HEAR plants, KCS and elongase activities were analyzed in developing seeds of wild-type plants and a selected transgenic RS306 line (T306-5), containing one copy each of the introduced KCS and LPAAT sequences.

Therefore, microsomal fractions from developing seeds (about 40 days after flowering) of transgenic and wild-type plants were prepared to assay KCS and elongase activities. As depicted in Fig. 5, microsomal fractions from developing seeds of T306-5 plants showed not only three-fold higher KCS activities but also three-fold higher elongase activities as compared to wild-type.

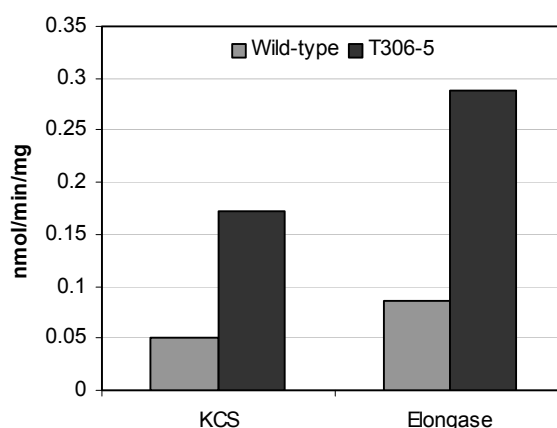


Fig. 5 Specific activities of microsomal β -ketoacyl-CoA synthase (KCS) and elongase from developing seeds of the rapeseed line T306-5 and wild-type plants. Enzyme assays were carried out as described in Material and Methods using 5 to 30 μ g protein of the microsomal fractions from developing seeds.

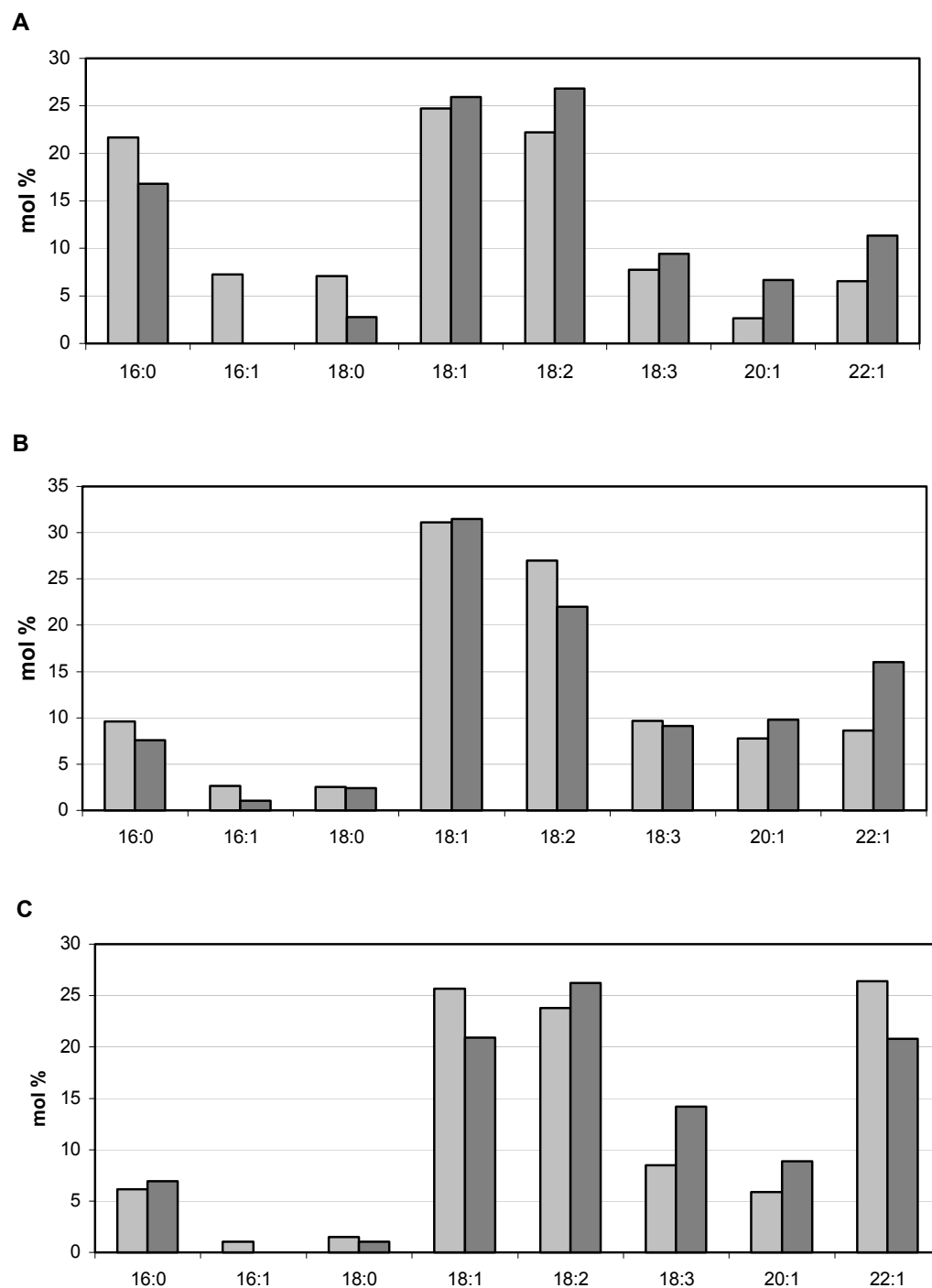


Fig. 6 Fatty acid composition of the acyl-CoA pools (A) and total fatty acid composition (B and C) of developing seeds from T306-5 (dark grey bars) and wild-type (light grey bars) plants [stage of seed development: about 35 days after flowering (A and B) and about 45 days after flowering (C)] (16:0, palmitic acid; 16:1, palmitoleic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:1, eicosenoic acid; 22:1, erucic acid).

According to this, over-expression of the KCS in transgenic HEAR plants was sufficient to overcome the first rate-limiting elongation step and to yield an altogether significantly higher elongase activity. The higher elongase activity in developing seeds from the transgenic T306-5 plants was also reflected in the total fatty acid composition of the developing seeds as well as in the composition of their acyl-CoA pools (Fig. 6A and B).

In comparison to developing seeds of wild-type plants harvested 35 days after flowering (DAF), those of the respective transgenic plants contained higher levels of very long chain fatty acids such as 20:1 and 22:1 in their acyl-CoA pool as well as in their total fatty acid composition (Fig. 6A and B). The differences in these compositions between T306-5 and wild-type seeds, however, were less distinct than those in KCS and elongase activities (Fig. 5). Moreover, during the stage of maximal TAG accumulation (45 DAF) this difference in the total fatty acid composition between transgenic and wild-type plants was no longer observed. In contrast the 20:1 and 22:1 contents were even lower in the seeds of transgenic than in those of wild-type plants (Fig. 6C).

In summary, the presented data clearly showed that the introduced KCS gene was functionally expressed in the developing seeds of T306-5 plants and affected an increase in elongase activity and higher levels of 20:1 and 22:1 in the acyl-CoA pool. Hence, these data are in line with those showing that the KCS and not the further enzymes catalyzes the rate limiting step of the elongase (Cassagne *et al.* 1994, Lassner *et al.* 1996, Millar and Kunst 1997, Millar *et al.* 1998, Cahoon *et al.* 2000, Moon *et al.* 2001, Katavic *et al.* 2001, Mietkiewska *et al.* 2004). During seed maturation, however, the improved elongase activity was no longer reflected in the total fatty acid composition of the seeds. These data suggest that during seed maturation the elongase activity is limited by other factors such as the substrate pools of acetyl-CoA and malonyl-CoA available to the enzyme complex and necessary for elongation.

3.2 Over-expression of ATP:citrate lyase, acetyl-CoA carboxylase in *B. napus* line RS306

In order to improve the substrate pool available to the elongase, chimeric gene constructs of ATP:citrate lyase (ACL) and acetyl-CoA carboxylase (ACCase) in combination with the pHAN2 expression cassettes having a *B. napus* KCS and *L. douglasii* LPAAT gene

were developed. The over-expression of these constructs was supposed to increase the supply of acetyl-CoA and eventually malonyl-CoA for the elongation of 18:1-CoA by the elongase complex at the ER. Together with an increased KCS and LPAAT activity this should result in an elevated 22:1-CoA pool and finally in a higher trierucin content in the seed oil.

To this end, an ATP:citrate lyase sequence was cloned from mammalian tissue and three chimeric gene constructs were prepared using the cloned ACL sequence under the control of the napin promoter from *B. napus* and the nopaline synthase terminator sequence from *A. tumefaciens* (Fig. 7). The pACL and pACL-nptII constructs, with or without the nptII selection marker gene, were developed for co-transformation experiments with pHAN2, whereas the chimeric triple gene construct pTriACL directly contained all three sequences for transformation on one T-DNA. The transformation and co-transformation experiments of HEAR RS306 plants and the selection of nptII ELISA positive transgenic plants were carried out in collaboration with Dr. K. Sonntag (BAZ Groß Lüsewitz) while PCR and seed oil analysis were subsequently done by Dr. M. K. Zarhloul (University Giessen).

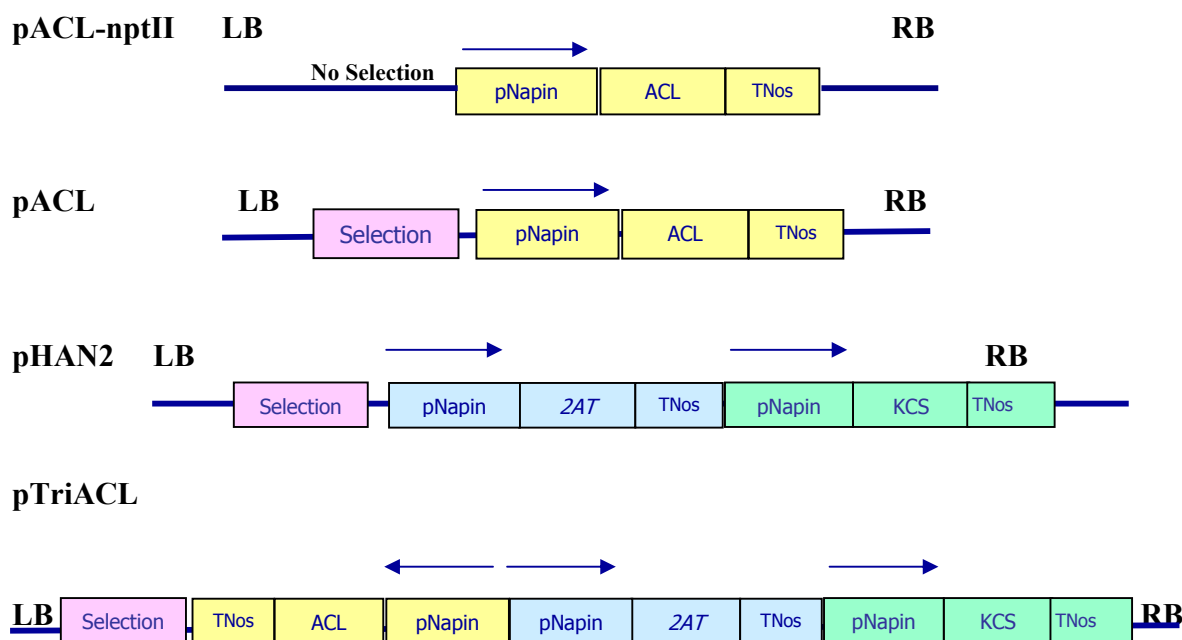


Fig. 7 Schematic representation of the T-DNA region of the different chimeric plant transformation constructs with ACL-, KCS- and LPAAT-sequences. The chimeric single gene constructs pACL-nptII and pACL were used together with the chimeric double gene construct pHAN2 containing a *B. napus* KCS and a *L. douglasii*

LPAAT sequence under the control of the strong seed specific napin promoter for co-transformation experiments. LB, left T-DNA border; Selection, neomycin phosphotransferase sequence under the control of a cauliflower mosaic virus 35S promoter and termination sequence; pNapin, seed specific napin promoter from *B. napus*; ACL, *H. sapiens* ATP:citrate lyase; 2AT, *L. douglasii* lysophosphatidic acid acyltransferase; KCS, β -ketoacyl-CoA synthase from *B. napus*; TNos, transcription termination sequence of the nopaline synthase gene from *A. tumefaciens*; RB, right T-DNA border.

As shown in Table 1, none of the transformants containing the chimeric pTriACL construct had 22:1 levels higher than the wild-type. ACL expression and thereby an increased supply of acetyl-CoA, together with high KCS and LPAAT activity, is not sufficient to increase the 22:1 content in the seed oil significantly. These data suggest that the cytosolic ACCase activity limits the adequate conversion of the surplus of acetyl-CoA to malonyl-CoA, the substrate for the first intermediate step in the elongation of 18:1-CoA catalyzed by the microsomal KCS.

In the seed oil of some of the transgenic plants we observed a significant decrease in the total 22:1 content. This was quite contrary to our expectations, but had already been seen in transgenic HEAR plants harboring the pHAN2 construct (Han *et al.* 2001) and might be explainable by silencing effects evoked by the additional *B. napus* KCS or napin promoter sequences in the transgenic plants. A probable explanation for the strong silencing effect could also be due to co-suppression of the endogenous KCS expression by the additionally introduced *B. napus* KCS gene. It is known that silencing could be triggered by the threshold concentration of transgenic transcripts (Que *et al.* 1997); therefore, the transcript level of the introduced genes should be determined by real-time PCR. Previous reports showed, that the frequency of silencing is positively correlated with the promoter strength of the introduced gene (Que *et al.* 1997). Therefore, by using a weak promoter for the expression of the *B. napus* KCS sequence, we could possibly avoid silencing.

Table 1 Variation range of the erucic acid content (22:1) in T2 seeds of control and transgenic RS306 rapeseed plants transformed with pTriACL and co-transformed with pACL or pACL-nptII and pNKAT55 expressing the introduced selection marker. %22:1, 22:1 content in % of total fatty acids analyzed with pooled T2 seed material; N, number of analyzed T1 plants.

Transformants	% 22:1			
	N	Minimum	Mean	Maximum
pTriACL	33	17.8	46.7*	55.7
pACL/pHAN2	25	48.5	53.1	58.8
pACL-nptII/pHAN2	43	7.2	38.7**	57.4
Control	10	42.5	53.7	59.0
t-test * p<0.05; ** p<0.001				

The co-transformation experiments with pACL or pACL-nptII and pHAN2 also did not result in an increased 22:1 content in the seed oil of transgenic plants (Table 1). But in that case, the chimeric ACL gene could not be detected in the transgenic plants by PCR analysis. Hence, the transgenic plants harbored only the pHAN2 T-DNA. According to this, the results of the seed oil analysis corresponded to the data obtained by Han *et al.* (2001). In comparison to the wild-type, we detected a significant decrease in the 22:1 content in the seed oil of some transgenic plants, most probably due to the already discussed silencing effects caused by the KCS or napin sequences.

To elucidate the impact of the cytosolic ACCase activity by provision of the KCS substrate malonyl-CoA, transgenic rapeseed plants over-expressing an ACCase in the cytosol were developed. The ORF of the multifunctional *Bn*ACCase comprises about 7 kb which is difficult to amplify errorless by RT-PCR. Therefore, we used a genomic ACCase clone kindly provided by our project partner (Prof. R. Töpfer, BAZ Siebeldingen). Given that no full-length genomic ACCase sequence coding for a cytosolic multifunctional ACCase form was available, we used a genomic *B. napus* ACCase sequence coding for a multifunctional plastidial class I ACCase (Schulte *et al.* 1997) and deleted the 5' sequence encoding the transit peptide. Due to the fact that the genomic sequence was around 12 kb long, the cloning

procedure was difficult and required several intermediate steps (see Material and Methods 2.13.4 for a detailed description).

Within the course of cloning we learned, that the short DC3 Ω promoter, our chosen promoter for the ACCase expression studies, confers only very low seed specific expression levels of the controlled gene. Therefore, we additionally utilized the presumably stronger seed-specific USP promoter sequence. The resulting chimeric gene constructs for rapeseed transformation, pDC3 Ω -ACCase and pUSP-ACCase containing the truncated genomic ACCase sequence under the control of the seed specific DC3 Ω - and USP-promoter, respectively, are depicted in Fig. 8.

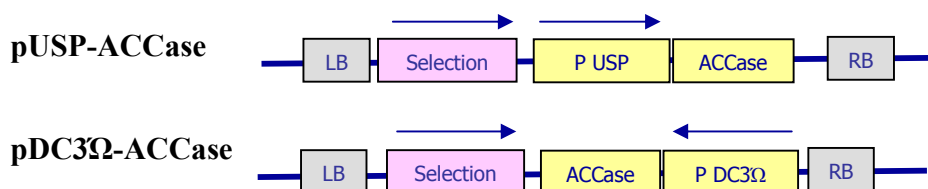


Fig. 8 Schematic representation of the T-DNA region of the different chimeric plant transformation constructs with an ACCase sequence, pDC3 Ω -ACCase and pUSP-ACCase, containing the truncated genomic ACCase sequence under the control of the weak and strong seed specific DC3 Ω - and USP-promoter respectively. LB, left T-DNA border; Selection, Neomycin phosphotransferase sequence under the control of a cauliflower mosaic virus 35S promoter; ACCase, acetyl-CoA carboxylase under the control of the USP or pDC3 Ω promoter; RB, right T-DNA border. The vector maps of the final constructs are given in appendix (5.3).

Mature seeds were collected from transgenic self-pollinated nptII ELISA positive RS306 plants containing the pDC3 Ω -ACCase and pUSP-ACCase T-DNA (Dr. K. Sonntag, BAZ Groß Lüsewitz). The pooled T₂ seeds of these transgenic and wild-type plants were used to determine the fatty acid composition of the seed oils (Dr. K. M. Zarhloul, Giessen). As shown in Table 2, a minor, but nevertheless significant increase in the 22:1-content was observed comparing mean values as well as maximum values of seed oils from transgenic and wild-type plants.

Table 2. Levels of erucic acid in the total fatty acid mixtures of seed oil from untransformed rapeseed plants (control) and transgenic ones harbouring the chimeric ACCase gene. % 22:1, 22:1 content in % of total fatty acids analyzed with pooled T2 seed material, N refers to the number of transformants analyzed.

% 22:1				
Transformants	N	Minimum	Mean	Maximum
RS 306				
pDC3ΩACCase	87	39.8	55.6*	60.7
Control	11	41.0	51.0	59.0
RS 306				
pUSP-ACCase	39	43.8	51.1	57.4
Control	2	45.9	(48.8)	51.6
Maruca				
pUSP-ACCase	3	41.1	46.0	49.3
Control	4	35.5	39.3	45.7

t-test: * $p < 0.05$

According to this, an increase in the cytosolic malonyl-CoA pool, due to the cytosolic expression of an additional recombinant ACCase, partly overcomes the limitations of microsomal 18:1-CoA elongation during seed maturation and leads to an overall increase of 22:1 in the seed oil of transgenic plants. This just slight increase is probably caused by a possibly low turnover rate of the recombinant class I ACCase, since the activity of this isoform in developing embryos is less than 10 % of the cellular total ACCase type I activity (Sellwood *et al.* 2000).

Nevertheless, it should be possible to get even higher increases of the 22:1 content in the seed oil of transgenic plants, if a high malonyl-CoA pool would be combined with an adequate supply of acetyl-CoA, a high elongase activity and an effective channeling of 22:1 into the *sn*-2 position of TAGs. Therefore, it would be necessary to cross the best transgenic ACCase over-expressing plants with the best transformants harboring the pTriACL construct or to use them for supertransformation experiments with pTriACL.

3.3 Isolation and sequence analysis of DAGAT clones

In order to increase the levels of 22:1 in TAG on the basis of HEAR phenotypes, it is necessary to increase not only the levels of 22:1 in the seed acyl-CoA pool but also the channeling of 22:1 into the oil and to increase the sink strength for lipid deposition. For this purpose, the idea was to strengthen the neutral lipid sink by over-expressing a chimeric gene encoding a diacylglycerol acyltransferase that effectively channels 22:1 into TAG.

To this end, DAGAT cDNAs were cloned and functionally characterized from plant species almost exclusively carrying very long chain acyl groups at the *sn*-3 position of their seed oils, namely *T. majus*, *B. napus* and *L. lindheimeri*. DAGAT cDNAs from *T. majus* and the resynthesized HEAR line RS306 were amplified by RT-PCR with gene specific primers deduced from the DAGAT sequences available in the data-bases.

Table 3. Survey of the cloned cDNAs from the given plant species encoding a DAGAT

Plant species	Constructs	cDNA	Encoded polypeptide		
	(in pYES)	(bp)	length	mass (kDa)	pI
<i>T. majus</i>	pTmdg	1554	518	58.81	8.88
<i>B. napus</i>	pBndg1	1506	502	57.47	8.75
	pBndg2	1527	509	57.95	8.53
	pBndg3	1512	504	57.24	8.05
<i>L. lindheimeri</i>	pLldg1	1569	523	59.56	8.28
	pLldg2	1509	503	57.64	8.95

Analysis of the sequences revealed that the *T. majus* cDNA has a length of 1521 bp and encodes a polypeptide of 518 amino acids (Table 3). The deduced amino acid sequence of *T. majus* DAGAT cDNA differs by three amino acid groups from the one in the data-base (AY084052), namely Val66Glu, Ser161Asn, and Ala517Glu. These differences are perhaps not merely due to different *T. majus* cultivars used for cDNA cloning because the serine

residue at position 161 that lies in the predicted phosphopantetheine binding domain is conserved in all plant DAGAT sequences suggesting that this residue is important (Fig. 9). The two DAGAT cDNAs of *B. napus* encode polypeptides of 502 and 509 amino acids, respectively, which were termed *BnDAGAT1* and *BnDAGAT2* (Table 3).

These two polypeptides are 89.4 % identical. Their central and C-terminal regions (400 amino acids) possess even higher sequence identity of 94.1 %. A further *B. napus* DAGAT cDNA from the LEAR variety B002 encoding *BnDAGAT3* (Table 3) was kindly provided by Dr. T. Roscoe. *BnDAGAT3* shares a higher sequence identity with *BnDAGAT2* (97.6 %) than with *BnDAGAT1* (88.2 %). The *BnDAGAT1* has 95 % identity with the *BnDAGAT* from jet neuf cultivar in the database while it has 83 % identity with the DAGAT of *Arabidopsis thaliana* in the database. There is a sequence DVR (position 83 of *BnDAGAT1*) present in all the *BnDAGAT* sequences but missing in *A. thaliana* and *L. lindheimeri* DAGATs. In addition, two DAGAT cDNAs from *L. lindheimeri* were cloned by Dr. F. Müller in our laboratory and used in the present study (Table 3). The deduced amino acid sequence of DAGAT1 shares 83% identity with DAGAT2 of *L. lindheimeri*. As given in Table 3, the DAGATs from the different plant species possess very similar molecular masses and isoelectric points. Moreover, the DAGAT sequences are very similar to each other apart from the N-terminal, hydrophilic region that shows a comparatively high degree of variation (Fig. 9). The hydrophobicity profile is also very similar in all the DAGATs which possess 10 conserved hydrophobic domains (Fig. 9)

Within the central and C-terminal regions several amino acid stretches are highly conserved across all DAGATs (Fig. 9) suggesting that they play an important role with regard to structure and function of the DAGATs. As shown by Nykiforuk *et al.* 2002, neither the putative acyl-CoA binding site nor the putative phosphopantethein attachment site are essential for DAGAT activity but may influence the enzymic properties such as acyl-CoA specificity. A conserved domain marked in rose in Fig. 9 is present in the hydrophilic domains situated between two supposed transmembrane helices that are common to both DAGATs and ACATs.

In addition, the invariant serine residue, highlighted in Fig. 9, is conserved in all DAGAT and ACAT sequences. This residue has been shown to be essential for ACAT activity by stabilizing the native conformation of the enzyme (Cao *et al.* 1996, Guo *et al.* 2001, Lu *et al.* 2002).

Fig. 9 Alignment of the amino acid sequences deduced from the plant DAGAT cDNAs using the ClustalX programme (Thompson et al. 1999). Putative functional motifs are highlighted as follows: brown, acyl-CoA binding protein signature; orange, phosphopantetheine-attachment site; turquoise, thiolases acyl-enzyme intermediate signature; pink, tyrosine kinase phosphorylation site; green, fatty acid binding protein signature; rose, conserved domain; blue, diacylglycerol/phorbol ester binding signature. Conserved residues are marked as follows: asterisk, 100% conserved; single dot, at least 75% conserved;

colon, 50–74% conserved. The invariant serine (S) residue is highlighted in red. The amino acid sequence between arrows represents leucine zipper pattern. The grey box represent transmembrane domains

This particular serine residue lies adjacent to a putative tyrosine kinase phosphorylation site that is found in the DAGAT sequences from plants except that from *T. majus* which, however, possesses such a site at position 385 to 392 unlike the other plant DAGATs (Fig. 9). Based on the sequence analysis it is likely that DAGAT activities of plants are regulated by phosphorylation and dephosphorylation in a similar way as the respective enzymes from mammals, but this remains to be determined experimentally.

3.4 Functional expression of plant DAGAT sequences in *Saccharomyces cerevisiae*

For heterologous expression of DAGAT sequences, the respective cDNAs were cloned into the yeast expression vector pYES and transformed into the *S. cerevisiae* strain BY4742 Δ YOR245c lacking most of the internal DAGAT activity (Brachmann *et al.* 1998; Sorger and Daum, 2002). Microsomal fractions were prepared from the yeast cells harboring one of the six different constructs given in Table 3 or the empty vector as a control and used as enzyme source in DAGAT assays with [$1\text{-}^{14}\text{C}$] oleoyl-CoA and dioleoylglycerol as substrates.

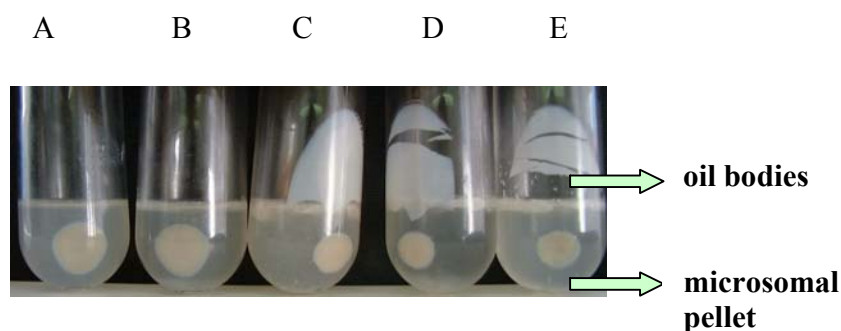


Fig. 10 Oil body formation in the yeast cells expressing a plant DAGAT construct. Tubes obtained after high speed centrifugation of extracts from yeast cells harboring the empty vector (A), pLldg1 (B), pLldg2 (C), pTmdg (D) or pBndg1 (E) are shown. Microsomal membranes are sedimented while the oil bodies float on the supernatants and stick to the sidewalls of the tubes.

During the isolation of the microsomal membranes, it became obvious that yeast cells expressing either pLldg2 or pTmdG or pBndg1 accumulated appreciable amounts of oil bodies (Fig. 10). Yeast cells expressing pBndg2 or pBndg3 gave results very similar to those of pBndg1-cells depicted in Fig. 10. On the other hand, yeast cells expressing pLldg1 unlike those expressing pLldg2, but similar to control cells, contained very low levels of oil bodies (Fig. 10). These observations were supported by the TAG contents determined in the transgenic yeast cells. As depicted in Fig. 11, yeast cells expressing a plant construct contained distinctly higher TAG levels as the control cells. The highest TAG contents were determined in pLldg2-cells, lower ones in pTmdg- and pBndg-cells and the lowest ones in pLldg1- and control cells. These data provide strong evidence that all plant constructs apart from that encoding *L. lindheimeri* DAGAT1 were functionally expressed in yeast cells and caused the biosynthesis of enough TAG to induce oil body formation under *in vivo* conditions.

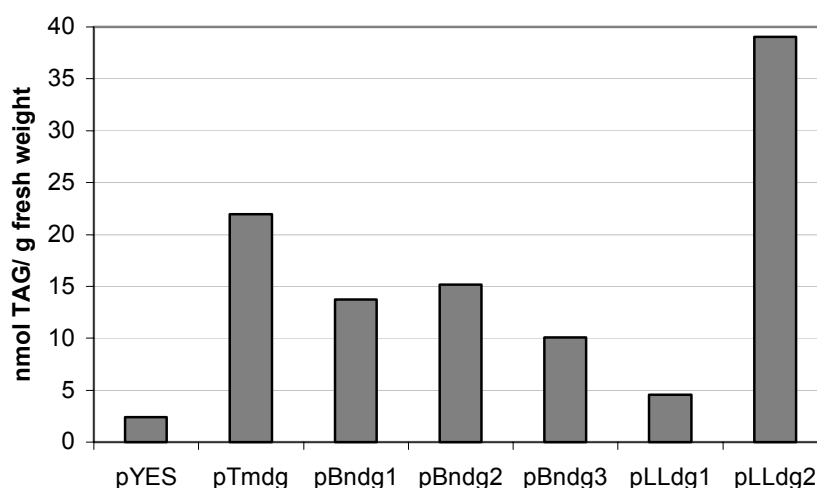


Fig. 11 TAG content of yeast cells expressing a plant DAGAT construct or harboring the empty vector pYES as a control. Yeast cells were harvested 48 h after gene expression was induced by galactose, lipids were extracted from the yeast cells (0.5 g fresh weight), separated by TLC and TAG levels were quantified after transmethylation by GC analysis.

3.5 Effects of various parameters on *T. majus* DAGAT activity

In order to quantify DAGAT activities in the subcellular fractions of transgenic yeast cells, at first the *in vitro* assay conditions were optimized. To this end, microsomal fractions of yeast cells expressing the *T. majus* DAGAT were selected as the enzyme source and [^{14}C] oleoyl-CoA and dioleoylglycerol as substrates. As shown in Fig. 12, hardly any DAGAT activity was detectable in control fractions while the membrane fractions carrying the *T. majus* DAGAT clearly displayed DAGAT activity that increased with increasing amounts of microsomal fractions. These data are in line with the TAG levels observed in the yeast cells expressing pTmdg (Fig. 10 and 11).

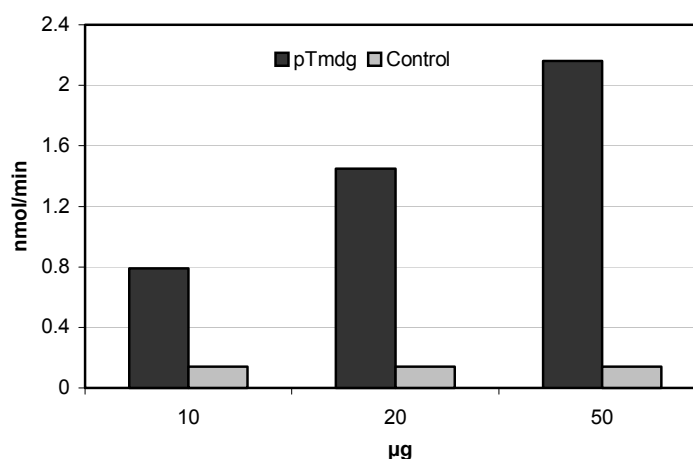


Fig. 12 Functional expression of the *T. majus* DAGAT in yeast cells. Formation rates of TAG from 18:1-CoA and dioleoylglycerol by microsomal fractions of yeast cells harboring either pTmdg or the empty vector as a control are given as a function of the protein concentration.

The dependency of DAGAT activity on pH values is shown in Fig. 13A. Enzyme activity was found to be maximal at pH 7.2. The data are consistent with previous ones obtained with microsomal fractions from developing seeds (Weselake *et al.* 1991; Martin and Wilson, 1983, Bernerth and Frentzen, 1990). Different buffers were tested to select the buffer best suited for DAGAT activity (Fig. 13B). Although a variety of buffers can be used for the DAGAT activity, the buffer, which gave maximum activity, was MOPS.

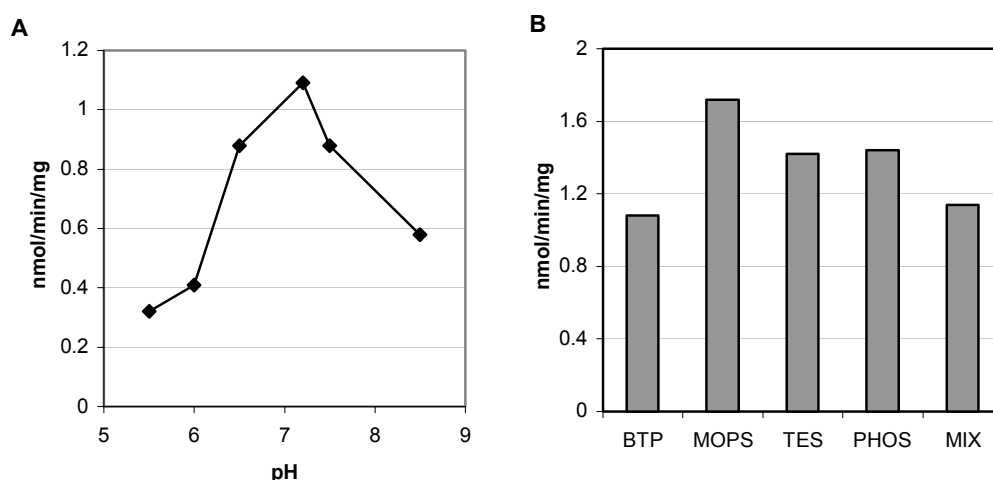


Fig. 13 Activity of *T. majus* DAGAT expressed in yeast as a function of the pH value (A) and the buffer substance (B). A buffer mixture of MES (2-morpholinoethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid) and Tricine (N-[tris(hydroxymethyl)methyl]glycine, N-(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)glycine) was used in A (BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; MOPS, 3-(N-morpholino)propanesulfonic acid; TES, (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), PHOS, phosphate; MIX, buffer mixture used in A).

The analysis of DAGAT activity as a function of the buffer concentration revealed that 100 mM MOPS buffer gave highest DAGAT activity (data not shown). The effect of reaction temperature on the DAGAT activity is shown in Fig. 14. Under standard assay conditions, DAGAT exhibited highest activity at 25 °C. A temperature optimum of 25 °C is consistent with the result of Martin and Wilson (1983) in spinach but differs from the results of Weselake *et al.* (1991) in microspore derived *B. napus* embryos.

Different concentrations of MgCl_2 (1-50 mM) were determined for their effect on the enzymic activity. The results showed that 8 mM were optimal for the DAGAT activity. The activity increased from 1.5 nmol/min/mg without MgCl_2 to 2.3 nmol/min/mg in the presence of 8 mM MgCl_2 . A stimulation of DAGAT activity by Mg^{2+} ions was also observed with microsomal fractions from developing oil palm seeds (Oo and Chew, 1992) as well as membrane fractions from insect cells expressing the DAGAT from *A. thaliana* (Hobbs and Hills, 2000).

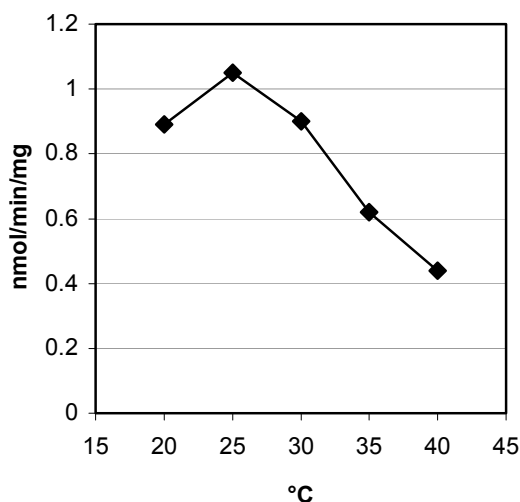


Fig. 14 Effect of temperature on DAGAT activity in microsomes of yeast expressing *pTmdg*.

Detergents are often used to disperse the substrate dioleoylglycerol. Three different detergents were tested namely Tween 20, CHAPS (3-[(3-cholamido-propyl)dimethyl-ammonio]-1-propanesulphonate) and dodecyl maltoside at a final concentration of 0.1% (w/v). The addition of any detergent mentioned above resulted in appreciable decrease of the enzyme activity. The inhibition of DAGAT activity with the addition of detergents was also reported by Weselake *et al.* (1991) and Kwanyeon and Wilson (1986). Therefore detergents were not used in the DAGAT assay.

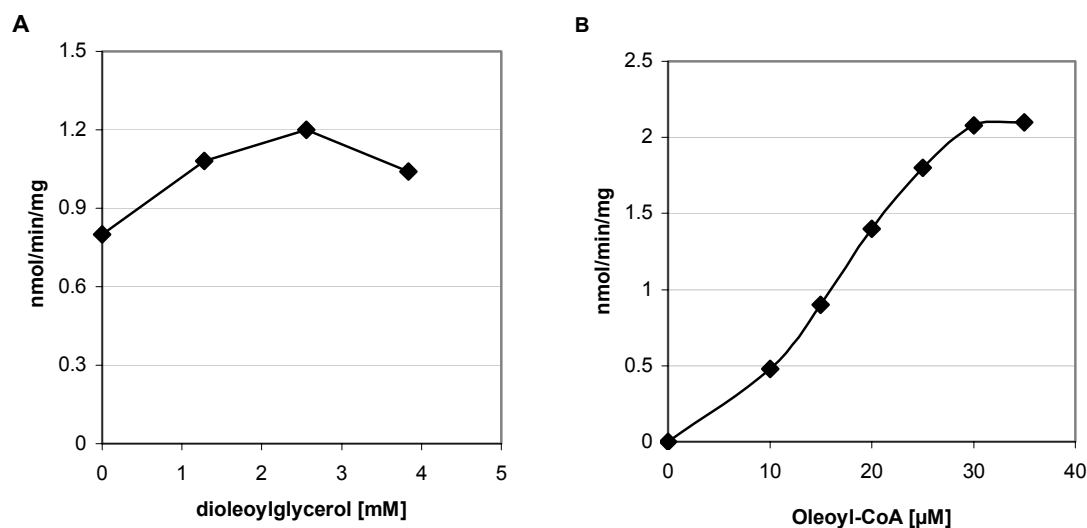


Fig. 15 Activity of the *T. majus* DAGAT over-expressed in yeast as a function of the dioleoylglycerol (A) and oleoyl-CoA (B) concentrations.

Fig. 15 shows the formation rates of TAG as a function of the substrate concentrations. DAGAT activity was clearly detectable without exogenously added dioleolglycerol (Fig. 15A) because DAG is present in ample amounts in the microsomal fractions of yeast cells.

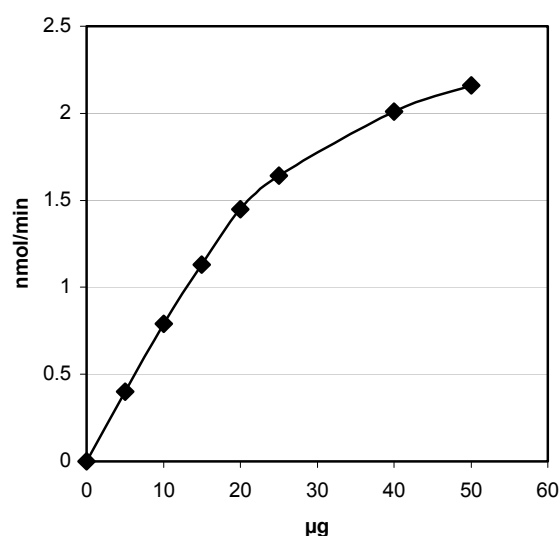


Fig. 16 Activity of *T. majus* DAGAT over-expressed in yeast as a function of the protein concentration.

DAGAT activity, however, increased with increasing DAG concentrations and reached a maximum at about 3 mM exogenously added DAG while higher concentrations were found to inhibit the enzymic activity (Fig. 15A). DAGAT activity as a function of the 18:1-CoA concentration gave saturation kinetic and maximal activities were obtained at 30 µM (Fig. 15B). Under optimal conditions the formation rates of TAG was found to be linear for about 5 min and at least 15 µg of microsomal protein (Fig. 16). Specific DAGAT activities of about 1 nmol/min/mg protein were determined. These activities were in the same range as those reported for the DAGATs from *A. thaliana*, tobacco and castor bean over-expressed in yeast (Bouvier-Navé *et al.* 2000, He *et al.* 2004).

The optimized assay conditions were then used to determine the *T. majus* DAGAT activity in microsomal and oil body fractions of yeast cells harvested one or two days after gene expression was induced.

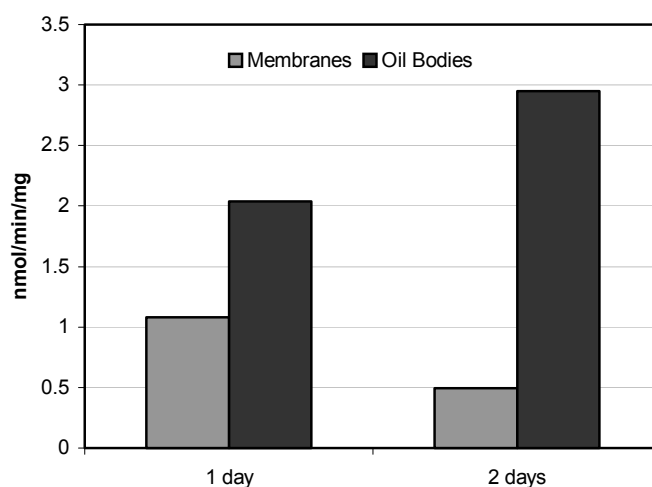


Fig. 17 DAGAT activity in oil body and microsomal fractions of yeast cells harvested after one day and two days, respectively, after pTmdg expression was induced.

The data in Fig. 17 show that *T. majus* DAGAT displayed higher activities in the oil bodies than in the microsomal membranes. This difference became even more distinct in fractions isolated from yeast cells harvested after a prolonged cultivation time after induction (Fig. 17). These data in conjunction with those depicted in Fig. 10 suggest that due to the induced DAGAT activity in the yeast cells more and more oil bodies budded off from the microsomal membranes containing not only TAG but also DAGAT. With regard to the subcellular localization in yeast cells the *T. majus* DAGAT differs from the respective enzymes from *A. thaliana* and castor bean, which showed distinctly higher activities in the microsomal than in the oil body fractions (Bouvier-Nave *et al.* 2000, He *et al.* 2004).

3.6 Properties of the plant DAGAT over-expressed in *Saccharomyces cerevisiae*

After optimizing the assay conditions, the activities, and properties of the DAGATs from *B. napus* and *L. lindheimeri* over-expressed in yeast were analyzed. Membrane fractions of yeast cells expressing one of the constructs given in Table 3, apart from those expressing pLldg1, displayed distinctly higher DAGAT activities with 18:1-CoA as acyl donor than the respective control fractions (Table 4). The highest activities were determined in fractions of yeast cells expressing pLldg2 and lower ones, in those expressing a *B. napus* construct, which were even lower than the activities caused by the expression of pTmdg (Table 4).

Table 4. DAGAT activities in subcellular fractions of yeast cells expressing a plant DAGAT construct or the empty pYES vector as a control as well as the apparent K_m for oleoyl-CoA of the various plant DAGATs.

Constructs	DAGAT activity (nmol/min/mg protein)		$K_m(\mu M)$
	microsomes	oil bodies	
pYES	0.01	0.01	-
pTmdg	0.5	2.95	14
pBndg1	0.78	0.75	41
pBndg2	0.97	0.84	26
pBndg3	0.45	0.25	28
pLldg1	0.22	0.1	-
pLldg2	2.75	3.2	56

Consequently, the subcellular localization in yeast cells not merely correlates with the DAGAT activities but is presumably due to differences in the structure of the DAGAT proteins. Hence, the results of the enzyme assays were in line with the TAG levels determined in the transgenic yeast cells (Fig. 11). Unlike the DAGAT from *T. majus* those from *L. lindheimeri* and *B. napus* showed similar activities in the microsomal and oil body fraction or even higher ones in the microsomal membranes (Table 4).

In view of the fact that DAGAT1 shares a high sequence identity with DAGAT2 from *L. lindheimeri*, the low levels of TAG caused by the expression of pLldg1 in yeast cells (Fig. 11) and the very low DAGAT activity determined in the respective subcellular fractions (Table 4) were surprising. These data suggest that the minor differences between the DAGAT1 and DAGAT2 sequences can cause appreciable alterations in either protein stability or enzymic properties. As outlined below, alterations in the acyl-CoA specificities were found to be the major reason for the observed difference in the DAGAT activities under *in vivo* and *in vitro* conditions.

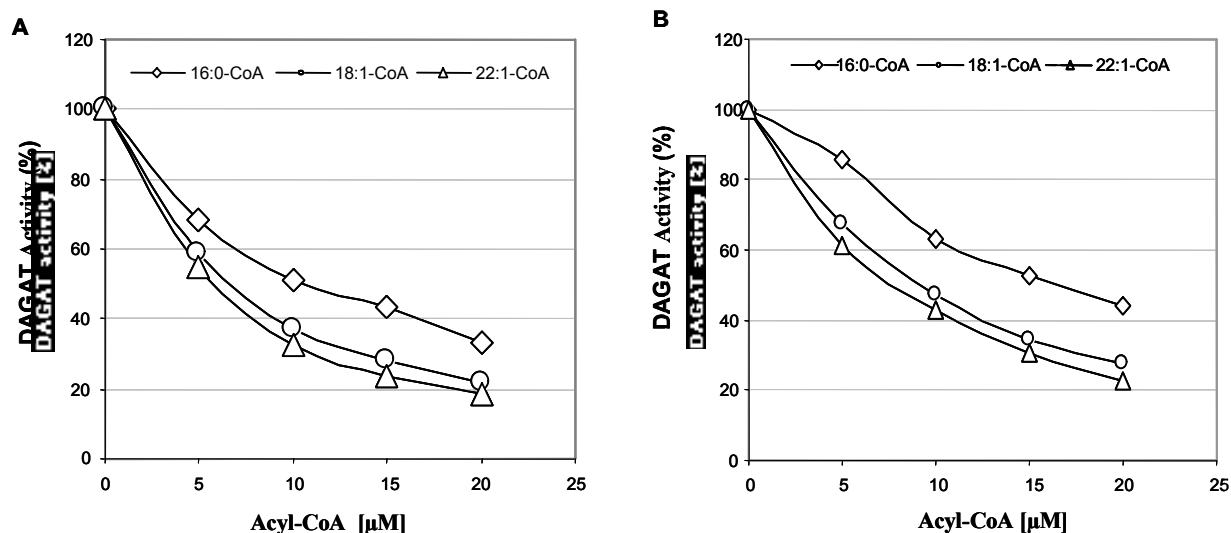


Fig. 18 Fatty acid selectivity of the *T. majus* DAGAT using microsomal fractions of developing *T. majus* seeds (A) or of yeast cells over-expressing pTmdg (B) as enzyme source. The inhibition of the incorporation of labeled 18:1-CoA (15 μM) into TAG by increasing concentrations of the given unlabeled acyl-CoA thioesters is shown.

The results showed that the DAGAT of *T. majus* over-expressed in yeast preferred 18:1-CoA and 22:1-CoA to 16:0-CoA (Fig. 18A). The enzyme, however, displayed a slight selectivity for 22:1-CoA in comparison to 18:1-CoA only. These results were obtained regardless whether the DAGAT over-expressed in yeast or the endogenous DAGAT from developing seeds were used as enzyme source (Fig. 18).

These data show that the enzyme acquires its native conformation in the heterologous environment and that the cloned gene represents the major DAGAT activity in developing seeds. In addition, the data provide strong evidence that DAGAT has to be provided with distinctly higher levels of 22:1-CoA than 18:1-CoA in order to cause the fatty acid composition of TAG in *T. majus* seed oil. Hence, the *sn*-3 fatty acid composition of *T. majus* oil is not primarily determined by properties of the DAGAT but by the composition of the acyl-CoA pool available to the enzyme.

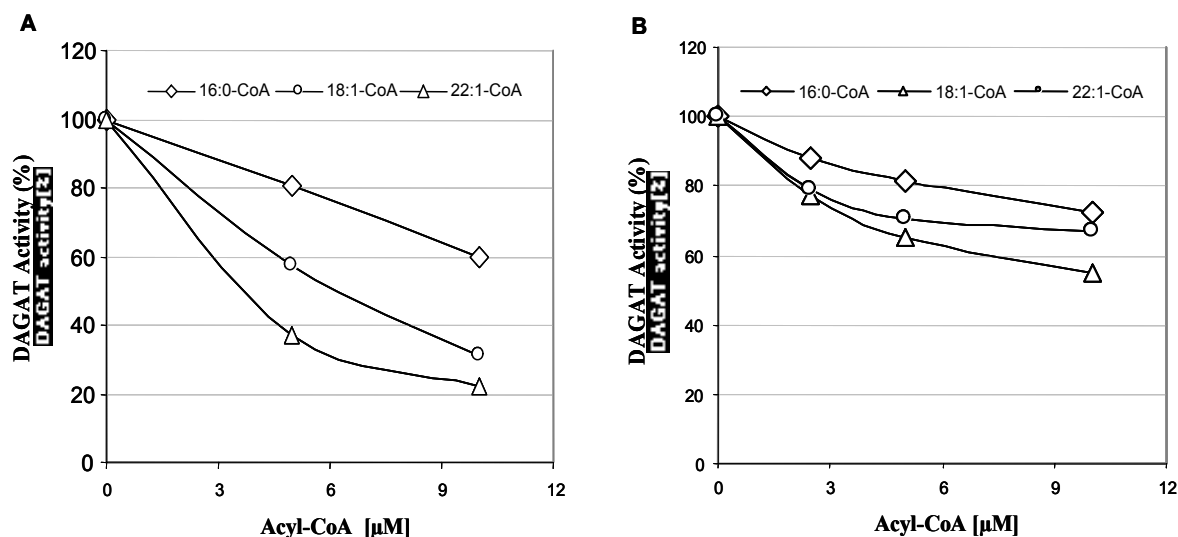


Fig. 19 Fatty acid selectivity of DAGAT1 and DAGAT3 from *B. napus* using microsomal fractions of yeast cells over-expressing pBndg1 (A) or pBndg3 (B) as enzyme source. The inhibition of the incorporation of labeled 18:1-CoA (10 μM) into TAG by increasing concentrations of the given unlabeled acyl-CoA thioesters is shown.

As shown in the Fig. 19B, *B. napus* DAGAT3 isolated from a LEAR line preferred 16:1-CoA to 22:1-CoA or 18:0-CoA. On the other hand, *B. napus* DAGAT1 isolated from a HEAR line took up 22:1-CoA more efficiently than 18:1-CoA and discriminated against 16:0-CoA (Fig. 19A). Results very similar to those depicted in Fig. 19A were obtained with *B. napus* DAGAT2 (data not shown). In addition, the DAGAT activity of microsomal fractions from developing seeds of the HEAR RS306 line, of which the DAGAT1 and DAGAT2 cDNAs were cloned, also showed selectivity for erucoyl-CoA in comparison to 18:1-CoA and 16:0-CoA (Fig. 20). These data indicate that DAGATs from HEAR lines unlike those of LEAR lines preferentially direct 22:1 into the sn-3 position of TAG. This is supported by the data of Bernerth and Frentzen (1990) obtained with DAGAT activities in microsomal fractions from developing seeds from a HEAR and LEAR line different to those used in our experiments.

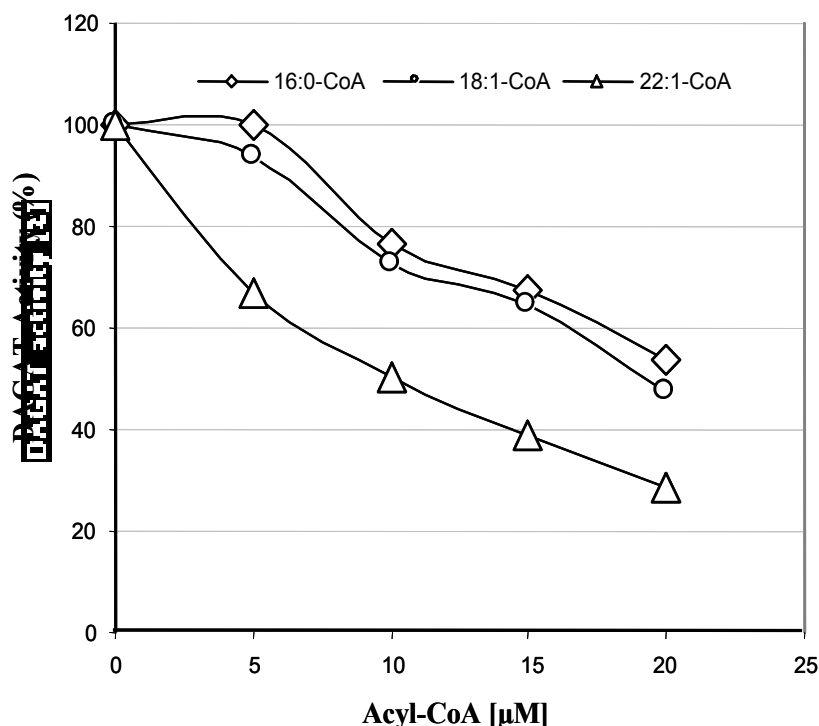


Fig. 20 Fatty acid selectivity of the microsomal DAGAT from developing *B. napus* seeds. The inhibition of the incorporation of labeled 18:1-CoA (15 μ M) into TAG by increasing concentrations of the given unlabeled acyl-CoA thioesters is shown.

On the other hand, Cao and Huang (1987) reported unselective DAGATs from both a HEAR and a LEAR cultivar, whereas Weselake *et al.* 1991 described a 22:1-CoA specific DAGAT from a LEAR cultivar. In summary, these data suggest that the DAGATs from *B. napus*, the seed oil of which is naturally rich in 22:1 at its *sn*-1 and *sn*-3 position, possess selectivity for 22:1-CoA but lost this selectivity by mutations in the DAGAT genes in the course of breeding various LEAR lines and cultivars.

Unlike the DAGATs from *T. majus* and *B. napus*, DAGAT2 from *L. lindheimeri* was found to possess a pronounced selectivity for very long chain monounsaturated acyl groups (Fig. 21). Consistent with the high level of lesquerolic acid (14-hydroxy-11-eicosenic acid, 20:1OH) esterified in the *L. lindheimeri* seed oil, DAGAT2 favored 20:1OH-CoA above 22:1-CoA and 18:1-CoA (Fig. 21) but the enzyme also preferred 22:1-CoA to 18:1-CoA in a similar way as the *B. napus* DAGAT1 (Fig. 19A).

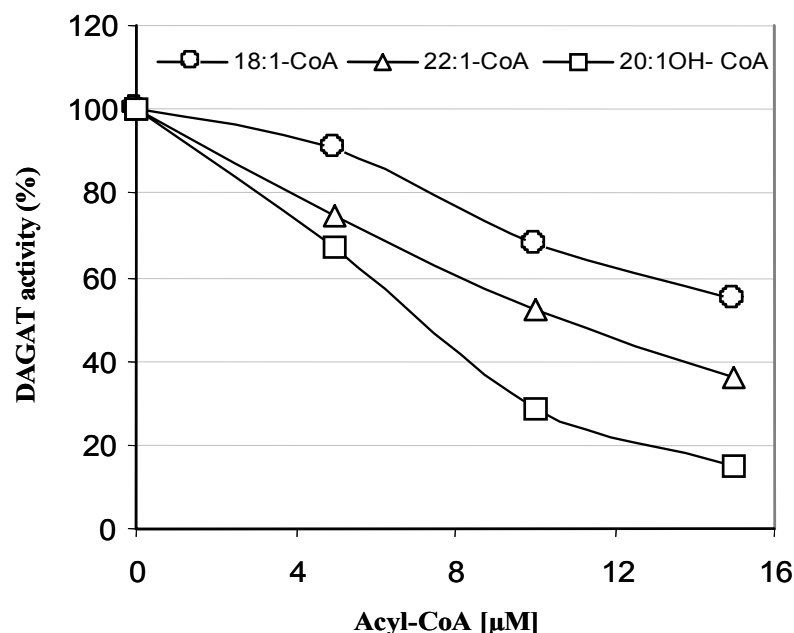


Fig. 21 Fatty acid selectivity of DAGAT2 from *L. lindheimeri* using microsomal fractions of yeast cells over-expressing pLldg2 as enzyme source. The inhibition of the incorporation of labeled 18:1-CoA (15 μ M) into TAG by increasing concentrations of the given unlabeled acyl-CoA thioesters is shown.

These results were obtained regardless whether microsomal or oil body fractions were used as enzyme source showing that the different subcellular localization did not influence the enzymic properties.

Since DAGAT1 of *L. lindheimeri* showed hardly any activity with 18:1-CoA, competition experiments with labeled 18:1-CoA could not be used for determining the fatty acid selectivity of the enzyme. To solve this problem and to elucidate that the low activities of DAGAT2 observed under *in vivo* (Fig. 11) and *in vitro* (Table 4) conditions were caused by its acyl-CoA specificity, labeled 20:1-CoA and 22:1-CoA thioesters were synthesized and used for enzyme assays.

Table 5. Acyl-CoA specificities of DAGAT1 and DAGAT2 from *L. lindheimeri*. DAGAT assays were conducted with microsomal fractions from yeast cells expressing either pLldg1 or pLldg2, unlabeled dioleoglycerol and the given labeled acyl-CoA thioesters at a final concentration of 15 μ M.

Enzyme	Acyl-CoA thioester		
	18:1-CoA	20:1-CoA	22:1-CoA
DAGAT1	0.07	2.60	1.40
DAGAT2	0.45	2.60	0.80

These experiments clearly revealed that DAGAT1 of *L. lindheimeri* was functionally expressed in yeast cells and displayed high DAGAT activity when 20:1-CoA or 22:1-CoA instead of 18:1-CoA were offered as acyl donor (Table 5). With 20:1-CoA DAGAT1 showed the highest activity as DAGAT2 while with 22:1-CoA its activities were even higher than DAGAT2 (Table 5). Hence, DAGAT1 possesses an even more pronounced specificity for very long chain monounsaturated acyl groups than DAGAT2. These data, thus, explain the low levels of TAG caused by the expression of pLldg1 in yeast cells, because these cells produce acyl-CoA thioesters with saturated and monounsaturated C₁₆ and C₁₈ acyl groups, but hardly any 20:1-CoA and 22:1-CoA thioesters. In addition, these data support the results of the competition experiments conducted with DAGAT2 from *L. lindheimeri*.

In summary, the analysis of the properties of the various plant DAGATs over-expressed in yeast provided evidence that the enzymes from *L. lindheimeri* possess the most pronounced specificities and selectivities for very long chain monounsaturated acyl groups. Hence, the respective genes appear to be suited to improve the level of 20:1 and 22:1 in the seed oil of transgenic rapeseed plants.

3.7 Construction and analysis of chimeric and mutated *L. lindheimeri* DAGATs

In spite of the high sequence similarity between DAGAT1 and DAGAT2 of *L. lindheimeri*, the enzymes were found to differ in their acyl-CoA specificity (Table 5). To

elucidate the molecular basis of this difference, initial domain swapping and site directed mutagenesis experiments were conducted.

Table 6. 18:1-CoA dependent enzymic activities of the mutated *L. lindheimeri* DAGAT2 proteins and TAG levels in yeast cells expressing a mutated DAGAT2 construct. The data are given in % of the values determined with the unmutated enzyme.

	Mutations			
	G308R	I315T	L326F	S329D
DAGAT activity [%]	100	25	101	-
TAG level in yeast cells (%)	~100	22	~100	-

The amino acid sequence of the central and C-terminal regions of DAGAT2 differs by a few amino acid groups only from that of DAGAT1 (Fig. 9). Three amino acid groups within and next to the fatty acid binding domain, namely at positions 308, 315 and 326 in the DAGAT1 sequence, were chosen because they are conserved in all plant DAGAT sequences but not in that of DAGAT2 (Fig. 9). In addition, the group at position 329, where DAGAT1 differs from the other sequences, was selected (Fig. 9).

By site directed mutagenesis the DAGAT2 cDNA was altered in such a way that one of the four amino acid groups of the translation product was substituted by the respective group of the DAGAT1 sequence (Table 6). A comparison of mutated DAGAT2 sequences with the unmutated one over-expressed in yeast revealed that the substitutions at position 308 and 326 had no effect on the 18:1-CoA dependent DAGAT activity or the TAG production in the yeast cells (Table 6). On the other hand, the substitution of isoleucine 315 by threonine in the fatty acid binding domain resulted in a fourfold decrease in the 18:1-CoA dependent DAGAT activity (Table 6). The TAG level determined in the transgenic yeast cells supported these data of *in vitro* enzyme assays. This level was distinctly lower in the cells expressing the mutated DAGAT2 sequence than in those expressing the unmutated sequence (Table 6). Hence, isoleucine 315 appears to be of critical importance with regard to protein stability, DAGAT activity or even acyl-CoA specificity.

Surprisingly, the substitution of serine 329 by aspartate resulted in a DAGAT2 protein that was found to be lethal to the yeast cells. The cells harboring the respective construct grew normally in glucose or raffinose containing media, but whenever DAGAT2 gene expression was induced by galactose, they stopped growing and started to die. The reason for the lethality of this mutated DAGAT protein is hard to explain, especially in view of the fact that the aspartate group is conserved in all plant DAGATs apart from DAGAT2 and an expression of none of the other DAGAT sequences affected the growth of the yeast cells.

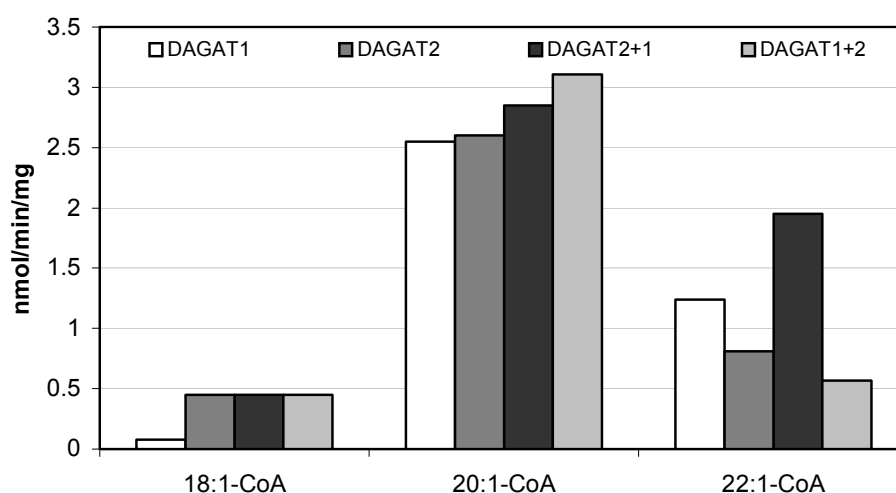


Fig. 22 Acyl-CoA specificities of *L. lindheimeri* DAGAT1, DAGAT2 and two chimerical enzymes DAGAT1+2 and DAGAT2+1 in which the N-terminal half of DAGAT1 and 2 were exchanged. The formation rates of TAG by microsomal membranes of yeast cells (15 μ g protein) expressing the respective *L. lindheimeri* DAGAT construct from 3 mM unlabeled dioleolglycerol and 15 μ M labeled acyl-CoA are given.

As outlined before, the N-terminal regions of the DAGAT sequences from plants display the lowest sequence similarities and this also holds true with regard to the two DAGATs from *L. lindheimeri* (Fig. 9). To investigate whether this region is critical for DAGAT activity and substrate specificity, the 3'-regions of the DAGAT1 cDNA (encoding the residues 1 to 308) and DAGAT2 cDNA (encoding residues 1 to 287) were exchanged giving the constructs pLldg1+2 (5'-region of pLldg1 and 3'-region of pLldg2) and pLldg2+1. The expression of these constructs in yeast cells showed that the chimeric DAGATs caused

the formation of TAG in levels appreciably higher than those affected by pLldg1 expression but similar to those affected by pLldg2 expression (Fig. 10). These data suggest that the exchange of the N-terminal region of DAGAT1 by that of DAGAT2 improves its 18:1-CoA dependent activity while the respective exchange in DAGAT2 hardly affect its activity with 18:1-CoA. This was further supported by enzymic investigations. As shown in Fig. 22, both chimeric DAGATs possessed 18:1-CoA dependent activities very similar to that of DAGAT2 but distinctly higher than that of DAGAT1. In addition, the exchange of the N-terminal region appears to improve the 22:1-CoA dependent activity of DAGAT1 but reduces that of DAGAT2.

In summary, the analysis of chimeric and mutated *L. lindheimeri* DAGATs provided evidence that the N-terminal region containing the acyl-CoA binding domain and perhaps the fatty acid binding domain can essentially determine acyl-CoA specificities.

3.8 Expression of a chimeric *L. lindheimeri* DAGAT gene in transgenic *B. napus* plants

The seed oil analysis of our transgenic plants expressing ACCase or ACL with KCS and LPAAT indicated that it is necessary to increase not only the levels of 22:1-CoA in the seed acyl-CoA pool but also to improve the channeling of 22:1 into the TAGs, in order to achieve a higher 22:1 content in HEAR seed oils and trierucin synthesis. To improve the sink/source relationship, a chimeric gene construct combining the expression cassette of pHAN2 comprising the *B. napus* KCS and the *L. douglasii* LPAAT gene with the DAGAT2 gene of *L. lindheimeri* under the control of the USP promoter was constructed. The resulting triple gene construct, pNKATDG, shown in Fig. 23 was introduced into the *B. napus* cultivars ‘Maruca’ (45 % 22:1 in the seed oil) and the RS306 (about 60 % 22:1 in the seed oil).

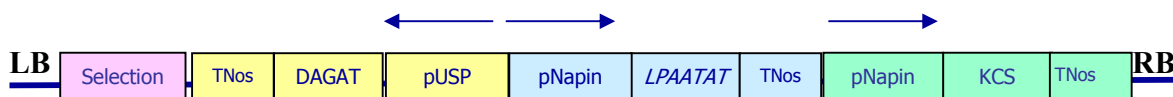


Fig. 23 Schematic representation of the T-DNA region of the plant transformation triple construct pNKATDG containing a *L. lindheimeri* DAGAT2, a *B. napus* KCS and a *L. douglasii* LPAAT sequence all under the control of the strong seed specific promoters (LB, left T-DNA border; Selection, neomycin phosphotransferase sequence fused with a cauliflower mosaic virus 35S promoter and termination sequence; pUSP or pNapin, seed specific USP or napin promoter, respectively; DAGAT, *L. lindheimeri* DAGAT2; LPAAT, *L. douglasii*

LPAAT; KCS, β -ketoacyl-CoA synthase from *B. napus*; TNos, transcription termination sequence of the nopaline synthase gene from *Agrobacterium tumefaciens*; RB, right T-DNA border).

The data from the seed oil analysis of primary transformants are summarized in Table 7. So far, only a few transgenic plants were available for seed oil analysis. According to these data, pNKATDG expression in RS306 and ‘Maruca’ plants did not improve the 22:1 level of the seed oils. These preliminary data suggest that the biosynthesis rate of 22:1-CoA rather than the channeling of 22:1 into TAG primarily controls the 22:1 content of the seed oil from the RS306 line. Hence the data are in line with those of transgenic plants described before (see chapter 3.2).

Table 7. Variation range of the 22:1 content in T2 seeds of control and transgenic RS306 and ‘Maruca’ plants transformed with pNKATDG (% 22:1, 22:1 content in % of total fatty acids of the seed oil extracted from pooled T2 seeds; N, number of analyzed T1 plants; pRNfad2irb, RNAi construct for a $\Delta 12$ -Desaturase [Spiekermann 2005] used for co-transformation with pNKATDG).

Transformant	% 22:1			
	N	Minimum	Mean	Maximum
‘Maruca’ pNKATDG	1	--	--	4.3
‘Maruca’ control	2	42.0	--	45.6
RS pNKATDG	25	9.2	36.0*	55.3
RS control	3	55.1	57.4	60.7
‘RS’pNKATDG, +pRNfad2irb	11	24.1	52.6	59.3
RS control	3	55.1	57.4	60.7
‘Maruca’pNKATDG, +pRNfad2irb	3	12	43.9	57.9
‘Maruca’ control	2	42.0	--	45.6

t-test, * $p < 0.05$

As already observed for pTriACL, certain transgenic plants even contained appreciably lower 22:1 levels in the total fatty acid composition of their seed oils than the controls due to silencing effects as discussed before.

Since 18:1-CoA acts as the substrate for the microsomal elongase complex, the inhibition of the desaturases producing polyunsaturated C₁₈ acyl groups should also help to enlarge the substrate pool for the KCS. Therefore, ‘Maruca’ and RS306 plants were co-transformed with pNKATDG and pRNfad2irb, an RNAi construct with the *B. napus* fad2 desaturase sequence, developed in the group of Prof. Heinz (University Hamburg).

Three nptII-ELISA positive transgenic plants were obtained and used for seed oil analysis and one transgenic ‘Maruca’ plant showed a significant increase in 22:1-content (Table 7). Inhibition of the Δ 12 desaturase gene expression possibly increased the substrate pool for the KCS, respectively the elongase, and together with the expression of the acyltransferase facilitated an effective channeling of 22:1-CoA into TAGs and thus increasing the sink strength for lipid deposition. But analysis of only three transgenic plants allows no statistically relevant interpretation of the results. Therefore, further transgenic plants have to be developed and to be analyzed for erucic acid content.

For the development of a chimeric DAGAT gene construct, we used the DAGAT2 gene from *L. lindheimeri*, showing a high specificity for 22:1-CoA. Since the domain shuffling experiments showed, that the fusion of the N-terminal domain of LIDAGAT2 with the C-terminal part of the LIDAGAT1 gave rise to an DAGAT enzyme with even higher 22:1-CoA specificity, it should be possible to get an even higher increase in the 22:1 content of the seed oil by using the LIDAGAT2+1 fusion for the development of transgenic rapeseed lines. A further obstacle to improve the 22:1 content in TAGs may lie in the seed specific acyl-exchange mechanisms catalyzed by a phospholipid:diacylglycerol acyltransferase (PDAT), utilizing acyl-CoAs from phosphatidylcholine (PC) and DAG to synthesize TAGs.. Therefore, the introduction of suitable PDAT could enhance/optimize these lipid exchange mechanisms and possibly further increase the lipid sink.

Eccleston and Ohlrogge (1998) showed that in transgenic *B. napus* plants producing high levels of 12:0-CoA, β -oxidation was induced during seed maturation, and resulted in

enhanced fatty acid degradation. In seeds, there was a high accumulation of 12:0 in spite of β -oxidation. Therefore, the carbon flux into β -oxidation should also be monitored in our transgenic plants over-expressing different enzymes involved in acyl-CoA elongation or TAG biosynthesis in order to avoid the possibility of futile acyl-CoA cycling.

The results from the seed oil analysis of our different transgenic lines clearly showed, that, although appreciable effects can be achieved by expressing single transgenes, only the concerted action of several key enzymes involved in elongation and TAG biosynthesis can lead to the development of rapeseed oils with very high 22:1 contents (Luhs *et al.* 1999). By combining the effects of ACCase, ACL and KCS over-expression together with fad2 inhibition, it should be possible to increase the 22:1-CoA pool significantly. Subsequently, 22:1-CoA specific LPAAT and DAGAT as well as PDAT can effectuate the efficient channeling of 22:1 into TAGs, thereby storing 22:1 in the neutral oil sink. Concomitant monitoring of the acyl-CoA composition and concentration in the transgenic seeds would be important to achieve a better understanding of the impact of recombinant enzymes on the complex regulation mechanisms of TAG metabolism.

Summary

High erucic acid rapeseed (HEAR) cultivars have regained interest for industrial purposes because erucic acid (22:1) is a valuable feedstock with a wide range of applications especially if it is available in sufficient quantities and in sufficiently pure form. In HEAR oil, however, the 22:1 content is limited to 67 % because 22:1 is excluded from the *sn*-2 position of the glycerol backbone. To overcome this theoretical limit in HEAR oil and, thus, to improve its oleochemical properties combined efforts in plant breeding and genetic engineering have been undertaken. Co-expression of an 22:1-CoA specific lysophosphatidate acyltransferase gene with a β -ketoacyl-CoA synthase (KCS) gene in developing seeds of HEAR plants resulted in the channeling of 22:1 into each position of the glycerol backbone but hardly improved the total 22:1 content.

The enzymic investigations with microsomal fractions of developing seeds conducted in the present study clearly showed that the introduced KCS gene was functionally expressed in the developing seeds of the transgenic HEAR plants and affected a threefold increase in KCS as well as elongase activity catalyzing the malonyl-CoA dependent elongation of oleoyl(18:1)-CoA via eicosenoyl(20:1)-CoA to 22:1-CoA. These data provide further evidence that KCS rather than the further three enzymes of the elongase systems catalyzes the rate limiting step. Analysis of the acyl-CoA and fatty acid composition in the course of seed development, however, revealed that during the phase of maximal oil accumulation the improved elongase activity of the developing seeds from the transgenic HEAR plants was no longer reflected in their 22:1 levels. These data suggest that the elongase activity and, thus, the formation rate of 22:1-CoA is limited by other factors such as the substrate pools available to the elongase complex.

In order to improve the cytosolic acetyl-CoA and malonyl-CoA pool, chimeric gene constructs encoding ATP:citrate lyase and acetyl-CoA carboxylase were developed and introduced into HEAR lines. An overall increase in the 22:1 content of seed oil from transgenic plants was achieved by the over-expression of the chimeric acetyl-CoA carboxylase gene. According to these data it is likely that a further increase in the 22:1 content can be achieved by the co-expression of the acetyl-CoA carboxylase gene with the ATP:citrate lyase, KCS and 22:1-CoA specific lysophosphatidate acyltransferase gene. In addition, the sink-source relationship is important for the increase of unusual fatty acids in

seed oils. Oil yield is decisively determined by the activities of the diacylglycerol acyltransferase (DAGAT) which catalyzes the acylation reaction at the *sn*-3 position of diacylglycerol in the final step of TAG synthesis. Hence, to improve the source strength by over-expressing a DAGAT that effectively channels 22:1 into TAG, DAGAT cDNAs were cloned and functionally characterized from *Brassica napus*, *Tropaeolum majus* and *Lesquerella lindheimeri*, the seed oils of which almost exclusively carry very long chain acyl groups at the *sn*-3 position. Six cDNAs, namely three of *B. napus*, two of *L. lindheimeri* and one of *T. majus*, were analyzed. The DAGATs encoded by these cDNAs possess very similar molecular masses, and apart from their N-terminal hydrophilic regions, their sequences are very similar to each other. The analysis of the properties of the DAGATs over-expressed in yeast revealed differences between the isofunctional enzymes especially with regard to their acyl-CoA specificities and selectivities. The DAGAT from *T. majus* displayed the lowest and those from *L. lindheimeri* the highest preference for very long chain acyl groups. Moreover, in spite of the high sequence identity between the two DAGATs of *L. lindheimeri*, DAGAT1 showed a more pronounced specificity for 20:1-CoA and 22:1-CoA in comparison to 18:1-CoA than DAGAT2.

In order to elucidate the molecular basis of the different acyl-CoA specificities of the two DAGATs from *L. lindheimeri*, initial domain swapping and site directed mutagenesis experiments were conducted. The analysis of the acyl-CoA specificities of the chimeric and mutated DAGATs compared to DAGAT1 and DAGAT2 provided evidence that the acyl-CoA binding domain within the N-terminal region and isoleucine 315 in the fatty acid binding domain, which is substituted by a threonine in DAGAT1, can essentially determine acyl-CoA specificity. Moreover, these experiments resulted in the development of a DAGAT with an improved 22:1-CoA specificity. Consequently, the respective sequence appears to be better suited for improving the 22:1 content of HEAR oil than the DAGAT2 sequence used for rapeseed transformation in the present study in combination with a *B. napus* KCS and a 22:1-CoA specific lysophosphatidate acyltransferase gene.

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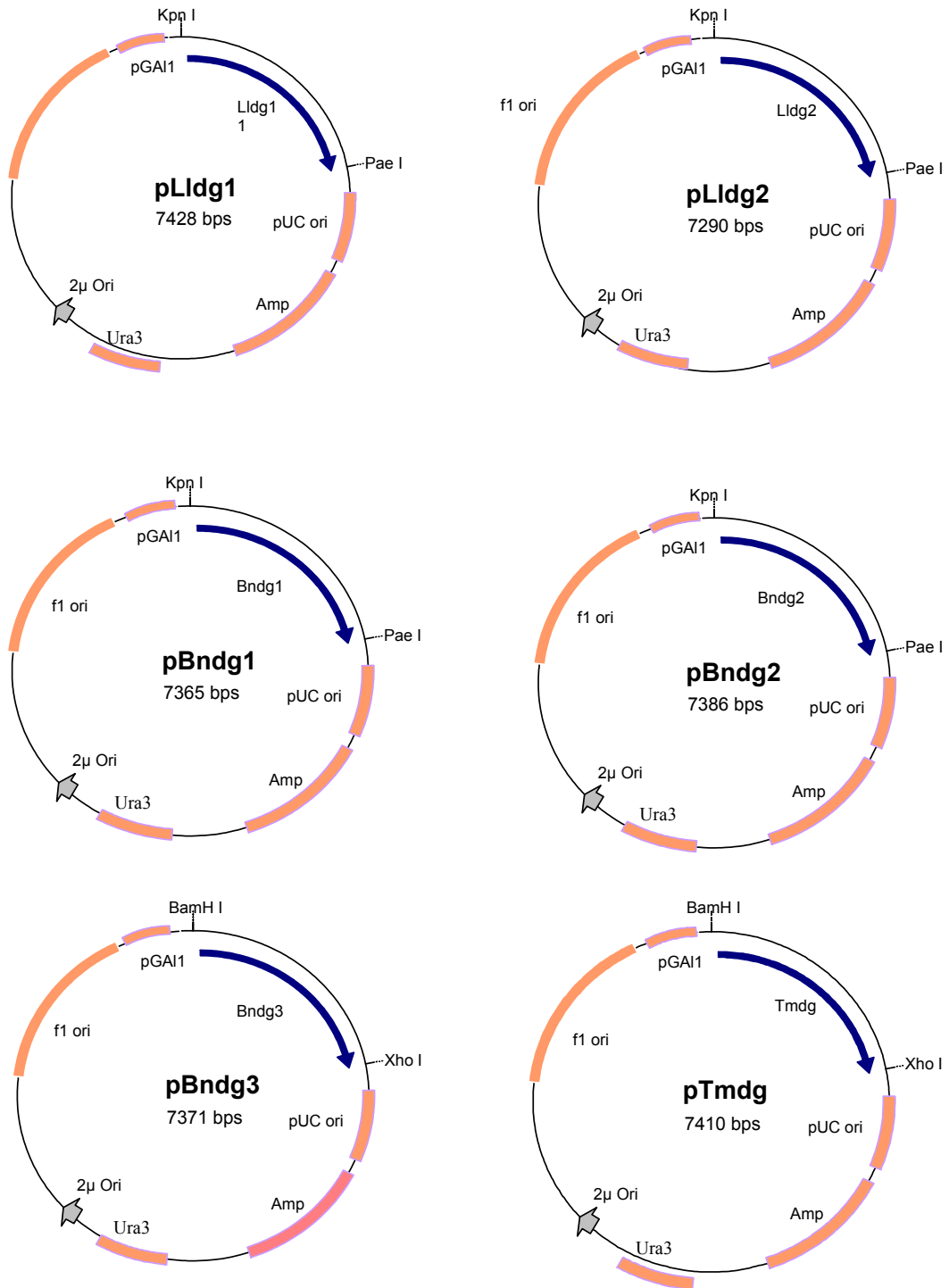
Appendix

6.1 List of abbreviations

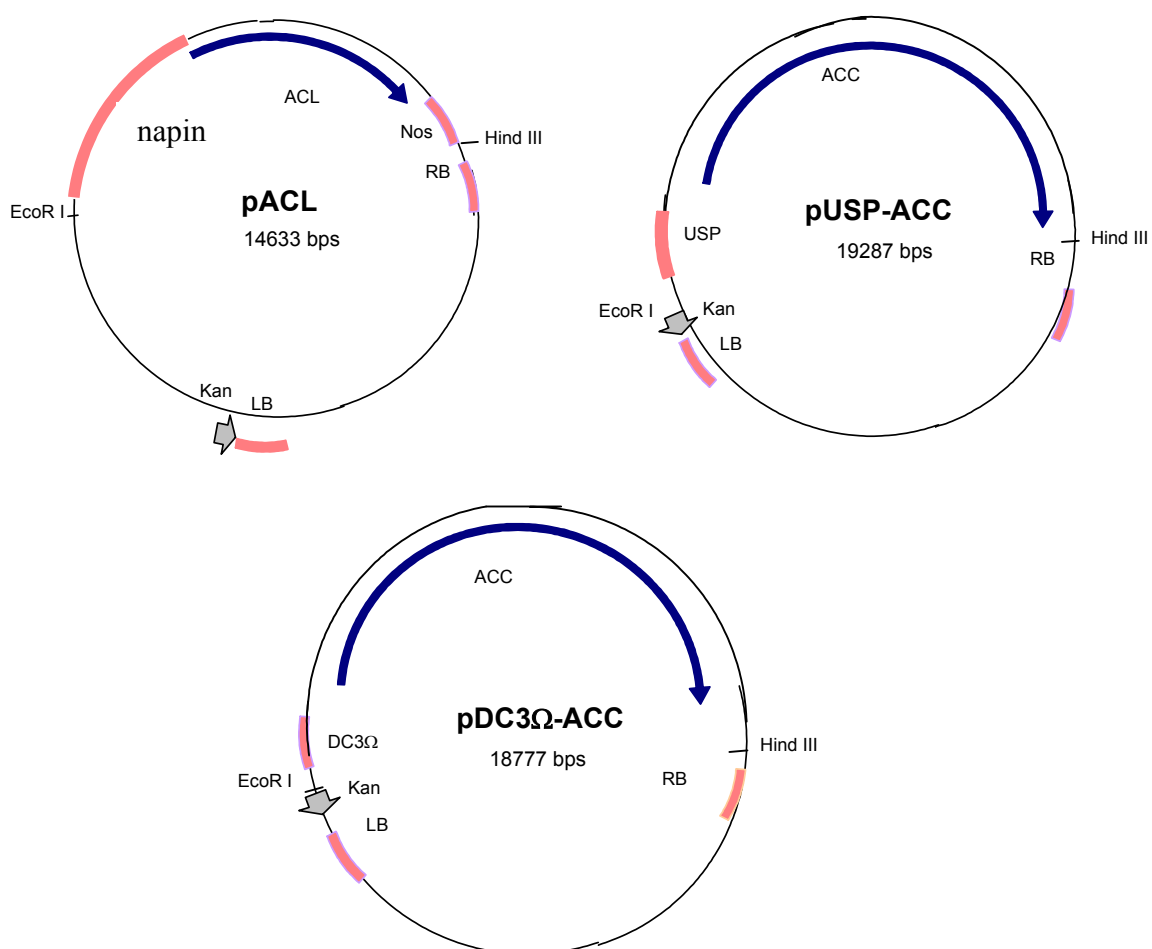
22:1	<i>cis</i> -13-docosenoic acid, erucic acid
LEAR	Low Erucic Acid Rapeseed
HEAR	High Erucic Acid Rapeseed
CoA	CoenzymeA
ACCase	Acetyl-CoA Carboxylase
ACL	ATP:Citrate Lyase
KCS	β -Ketoacyl-CoA Synthase
SAD	Stearoyl-ACP Desaturase
KAS	3-ketoacyl-ACP Synthases
ACP	Acyl Carrier Protein
BCCP	Biotin Carboxyl Carrier Protein
BC	Biotin Carboxylase
CT	Carboxyl-Transferase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NADH	Nicotinamide Adenine Dinucleotide
GPAT	Glycerol 3-phosphate acyltransferase
LPAAT	Lysophosphatidic acid acyltransferase
DAGAT	Diacylglycerol acyltransferase
PC	Phosphatidylcholine
LPCAT	Lysophosphatidylcholine acyltransferase
CDP-cho	Cytidinediphosphocholine
CMP	Cytidinemonophosphate
DAG	Diacylglycerol
LPC	Lysophosphatidylcholine
PDAT	Phospholipid:diacylglycerol acyltransferase
PCS	Phosphatidylcholine synthase
G3P	Glycerol 3 phosphate
IPTG	Isopropyl-1-thio- β -D-galactoside
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

LiAc	Lithium acetate
OD	Optical Density
PEG	Polyethyleneglycol
SDS	Sodium dodecylsulfate
DEPC	Diethyl pyrocarbonate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	N- [2-hydroxyethyl]-piperazine-N0- [2-ethanesulphonic acid]
IPTG	Isopropylthiogalactopyranoside
MES	2-[N-morpholino]-ethanesulphonic acid
PMSF	Phenylmethylsulfonylfluoride

6.2 Chimeric construct maps for *S. cerevisiae* expression



6.3 Chimeric single gene construct maps for *B. napus* transformation



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