

Development of *Lepidium campestre* into a new oil and catch crop

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Cover: Seed pods of *Lepidium campestre*
(Photo: Anna Lehrman)

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Abstract

A growing world population and dwindling fossil oil reserves demand an increase in the world plant oil production. The possibility to increase the area of cultivated land is limited due to the shortage of arable land. Also, the possibility for increasing the oil content in the limited number of cultivated oil crops is restricted. New, high-yielding oil crops, which can be grown in areas where no other oil crops can grow and have less environmental impact, need to be developed. It is preferable that such new oil crops would allow us to tailor-make the oil composition *in planta* for food, fuel or industrial applications. In Sweden, the main oil crop cultivated is winter rapeseed, which, due to weak winter hardiness, only can be grown in southern Sweden. Developing a new winter-hardy oilseed crop would extend the plant oil production in Sweden and other cold climate regions. *Lepidium campestre* is a wild *Brassica* species. It is very winter hardy, high-yielding, has an upright stature and synchronous flowering. Moreover, it is biennial, and thus being suitable as a catch crop. However, it needs to be domesticated first so that it possesses all important agronomic traits necessary for being a successful agricultural crop.

The aim of this thesis was to improve some properties of *L. campestre* by genetic engineering with focus on: the seed oil content, pod shatter, seed oil composition and wax ester production in the seed oil. In order to enable genetic engineering of this wild species, a well-functioning regeneration and transformation protocol was first developed, which has greatly facilitated the subsequent genetic improvements of the target traits of the species. Through RNAi-down-regulation of the *FAD2* and *FAEI* genes, transgenic lines with oxidative stable oil high in oleic acid were generated, indicating the potential of the species for being used for food oil purposes. Moreover, transgenic lines with increased seed oil content were developed by expressing either the *AtWR11* or *AtHb2* or *BvHb2* gene. Transgenic lines with pod shatter resistance were produced by RNAi down-regulation of the *IND* gene. Wax esters were produced in this species by expression of the jojoba wax synthesis genes, showing the potential of the species as a new platform for industrial oil production. These transgenic lines are valuable materials for further breeding of this species.

Keywords: *Lepidium campestre*, oleic acid, oil content, pod shatter, wax ester, genetic engineering, RNAi

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Dedication

To my family and friends

Det handlar om att få leka, drömma sig bort, absorberas av en värld man tycker om.

Håkan Hellström

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Ivarson, E., Ahlman A., Li X-Y. and Zhu L-H. (2013). Development of an efficient regeneration and transformation method for the new potential oilseed crop *Lepidium campestre*. *BMC Plant Biology* 13(115).
- II Ivarson, E., Ahlman A., Lager I. and Zhu L-H. (2016). Significant increase of oleic acid level in the wild species *Lepidium campestre* through direct gene silencing. *Plant Cell Reports* 35(10), 2055-63.
- III Ivarson, E., Eriksson N. L., Ahlman A., Kanagarajan S., Bülow L. and Zhu L-H. Effects of overexpressing of *WRI1* and hemoglobin genes on the seed oil content of *Lepidium campestre*.(under revision)
- IV Ivarson, E., Iven T., Sturtevant D., Ahlman A., Cai Y., Chapman K., Feussner I. and Zhu L-H. Production of wax esters in the wild oil species *Lepidium campestre*. (submitted)
- V Ivarson E., Ahlman A., Lager I. and Zhu L-H. Silencing the *INDEHISCENT* gene significantly reduced the pod shatter in *Lepidium campestre* (manuscript)

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The contribution of Emelie Ivarson to the papers included in this thesis was as follows:

- I Performed some of the experimental work, the molecular analyses and participated in writing of the manuscript
- II Participated in the design of the experiment, performed the majority of the experimental work and wrote the manuscript together with the co-authors
- III Participated in the design of the experiment, performed the majority of the experimental work and wrote the manuscript together with the co-authors
- IV Performed some of the experimental work and wrote the manuscript together with the co-authors
- V Participated in the design of the experiment, performed the majority of the experimental work and wrote the manuscript together with the co-authors

Abbreviations

ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
Acyl-CoA	acetyl-Coenzyme A
ALC	ALCATRAZ
CPT	choline phosphotransferase
CRISPR	clustered regularly interspaced short palindromic repeat
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DZ	dehiscence zone
ER	endoplasmic reticulum
FAE	fatty acid elongase
FAD2	fatty acid desaturase 2
FAR	fatty acyl-CoA reductase
FAS	fatty acid synthase
FUL	FRUITFULL
GM	genetically modified
G3P	glycerol-3-phosphate
GPAT	glycerol-3-phosphate acyltransferase
IND	INDEHISCENT
LACS	long-chain acyl-CoA synthases
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
MCMT	malonyl-CoA:acyl carrier protein malonyltransferase
MUFA	monounsaturated fatty acid
nsHb	non-symbiotic hemoglobins
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PDAT	phospholipid:diacylglycerol acyltransferase
PGR	plant growth regulator
PUFA	polyunsaturated fatty acid
RPL	REPLUMLESS
SHF	SHATTERPROOF

TAG	triacylglycerol
WRI1	WRINKLED 1
WS	wax synthase
WT	wildtype
12:0	lauric acid
16:0	palmitic acid
18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid
22:1	erucic acid

1 Introduction

Our society relies highly on the fossil oils. As the world population increases and the fossil oil reserves decline, replacing fossil oils has become more urgent now than ever before. As a renewable source, plant oil is believed to be able to partially replace the fossil oils. However, the plant oil production is currently limited and needs to be greatly increased to meet the future demand for food, fuel and industrial feedstocks. Starting 1960, the green revolution brought about a trebling of the cereal production during the following 50 years. This was mainly due to genetic improvements and an increase in fertilization, water and pesticide input (Pingali, 2012; Tilman *et al.*, 2002). The world population is expected to reach 9.1 billion people by 2050 (FAO, 2009), demanding an increase in the world food production similar to the one generated by the green revolution. The possibility to increase the area of cultivated land is limited. Also, such an increase would infer negative environmental consequences, and would be at the expense of natural lands such as forests (Fita *et al.*, 2015; Zhang *et al.*, 2006). Thus, to increase the world plant oil production, both the yield per hectare and the oil content of the oil crops need to be increased (Carlsson *et al.*, 2011). However, the rates of yield growth in agricultural production have slowed down. Alston *et al.* (2009) have reported that the productivity growth rate was slower between 1990 and 2007 than during 1961 and 1990. One of the reasons behind this could be that the investments in the research and development (R&D) have mainly been utilized in preventing yield losses instead of increasing the yields (Alston *et al.*, 2009). Thus, more focus needs to be put on how to increase the production, and new methods to do so need to be introduced. Apart from continuing the improvement of the existing oil crops, new and high-yielding crops with less environmental impact need to be developed to ensure more production of plant oils.

Plant oil is an important human dietary source of energy but has also been utilized as biofuel and a feedstock for replacing fossil oils in the chemical industry. Plants can function as natural factories for producing different oil

qualities for various applications. Plants produce fatty acids, oils and waxes, which are well-suited in replacing petrochemicals in the chemical industry, since the linear carbon chains exhibit molecular structures similar to the industrial chemical feedstocks used today (Vanhercke *et al.*, 2013b). Plant-derived oil can be utilized in products such as soaps, detergents, lubricants, paints, surfactants and solvents (Carlsson, 2009). Today, around 20 % of the plant oil produced is used as industrial feedstocks, a share that has the possibility to be increased. By optimizing the oil composition *in planta* and making the oil as close to ready-to-use as possible, the downstream processing costs are minimized and thus making plant oil a desirable alternative in the chemical industry.

The global plant oil production is estimated to reach 187 million metric tons during 2016/2017 (Figure 1) (USDA, 2016). The major sources are oil palm (33 %), soybean (29 %), rapeseed (16 %), sunflower seed (9 %), and palm kernel (4 %). The remaining 9 % are constituted by coconut, cottonseed, olive and peanut (Figure 1). Oil from these plants are mainly constituted by linear chain fatty acids of 16 and 18 carbons, such as palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids esterified to glycerol molecules. The shorter-chain fatty acid lauric acid, C12, is found in the seed kernels of coconut and oil palm (Gunstone & Harwood, 2007). Wild plant species, on the other hand, produce various types of unusual fatty acids such as fatty acids with double or triple bonds, epoxy-, and hydroxyl groups (Gunstone & Harwood, 2007; Metzger & Bornscheuer, 2006).

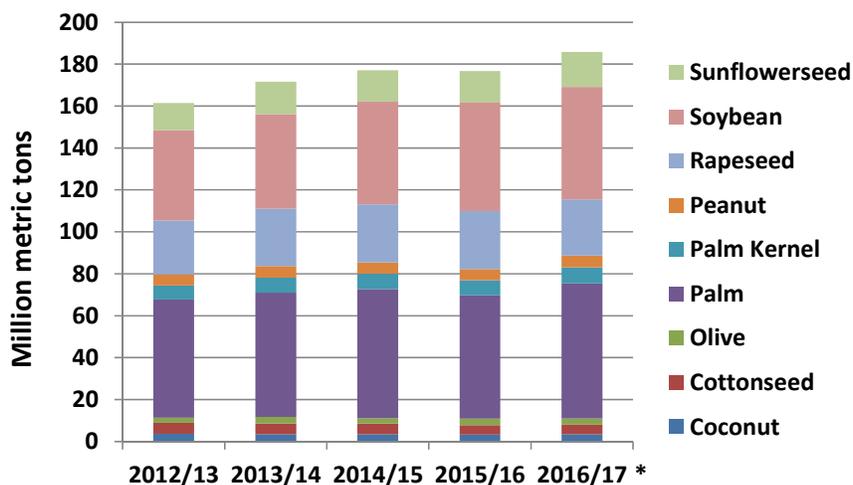


Figure 1. Major vegetable oil supply and distribution, 2012/13 – 2016/17. *Forecast. (Data from USDA, 2016)

The possibility to increase the oil content in the existing cultivated oil crops is limited for various reasons. The oil contents of rapeseed and oil palm are already high, which makes a further increase in the oil content more difficult. Soybean has a relatively low oil content, ca. 20 %, thus a substantial increase in oil content would be theoretically possible. However, in soybean, the protein is the component of greatest economical value. A significant increase in the oil component would likely be on the expense of the protein content, which is probably not a feasible alternative (Carlsson *et al.*, 2011). Development of new, high-yielding oilseed species, preferably with a combination of tailored oil composition and favorable agronomic characteristics, which can be grown with less negative environmental impact, can be a part of the solution.

A new winter-hardy oilseed crop with the possibility of being cultivated as a catch crop, for instance, undersown in a spring cereal, would enable more efficient use of cultivated land and would be environmentally favorable.

Catch- or cover crops provide several potential ecosystem benefits; such as increasing the soil organic C concentration, by additional biomass C inputs both above and below ground (Blanco-Canqui, 2013). Moreover, soil erosion and NO₃ leaching can be reduced, and hence reduction in losses of nutrients and subsequently reduction in pollution of lakes, rivers and other water sources, which have caused serious problems to aquatic organisms (Galloway, 2001). Also, cover crops can suppress weeds, both by direct competition for resources and by physical and chemical suppression (Blanco-Canqui *et al.*, 2015), which will reduce the use of chemicals such as herbicides, and thus contributing positively to the environment.

Rapeseed, the main *Brassica* oil crop cultivated in Sweden has weak winter hardiness, thus the plant oil production is limited to the southern parts of Sweden (Nilsson *et al.*, 1998). Developing a new, winter hardy oilseed- and catch crop that could survive the winter in colder climate regions would thus provide a better possibility for further increasing the plant oil production in Sweden and other cold climate areas in the world.

2 Background

2.1 *Lepidium campestre* – a promising new oil crop for the future

Lepidium campestre (shown in Figure 2) is a wild species belonging to the Brassicaceae family. Comprising approximately 230 species, *L. campestre* is one of the largest genera within the Brassicaceae family (Al-Shehbaz *et al.*, 2006; Mummenhoff *et al.*, 2001). *Lepidium* is distributed all over the world, but is most commonly found in temperate and sub-tropical regions. It can be found in the mountains in tropical areas, but only scarcely in Arctic climates (Al-Shehbaz, 1986). It is a self-fertilizing and diploid species ($2n = 16$) with some superior agronomical characteristics, making it a good candidate for being domesticated into a future new oil crop. The plant has an upright stature, relatively synchronous flowering, branches only in the upper part of the stem and is resistant to the pollen beetle. It is very cold hardy, as recently shown in the field trial performed within the Mistra Biotech program at <https://www.slu.se/en/Collaborative-Centres-and-Projects/mistra-biotech/> and would thus enable oil production in regions with colder climates than what winter rapeseed can tolerate (Merker & Nilsson, 1995). *L. campestre* has shown a high yield potential with over 4.8 tons/ha (Merker *et al.*, 2010), which is approximately 30 % higher compared to the yield of the winter oilseed rape. Moreover, it is biennial, and thus being suitable as a catch crop. Sown together with a spring cereal it has the potential of increasing the yield of the spring cereal, which has been shown in the trial where *L. campestre* was sown together with barley (Merker *et al.*, 2010). Since *Lepidium* over-winters, no tilling is needed after harvesting the spring cereal, which reduces the production costs. Also, it covers the ground during winter, and thus preventing nutrients from leaching. However, *Lepidium* has a relatively low seed oil content of around 20 %, but it has a protein content of about 20 % and a dietary

fiber content of 40 %, which will make the seed cake a valuable source as animal feed (Andersson *et al.*, 1999; Nilsson *et al.*, 1998). The seed oil is not suitable for food consumption as it is composed of a high level of the unhealthy fatty acid erucic acid (20 %). It also contains a high level of the heat unstable fatty acid linolenic acid (ca. 40 %), making it unsuitable for food processing, although it has ca. 11 % of the heat stable fatty acid oleic acid (Ivarson *et al.*, 2016). Moreover, *Lepidium* is prone to pod shattering, which seriously affects yields.

In order to develop *Lepidium* into an economically viable oil crop, several important agronomic traits, as mentioned above, need to be improved. Besides, other important traits like resistance to biotic/abiotic stress need to be carefully studied and evaluated.



Figure 2. Flowers (left), plant (centre) and seed pods (right) of *Lepidium campestre* (Photos by Anna Lehrman)

2.2 Genetic engineering – a powerful tool for genetic improvements

Plant genetic improvements can be achieved by conventional and genetic engineering or biotechnological methods. Both biotech crops and traditionally bred crops are the results of alterations in the genetic constitution of plant genomes of the target genotypes. Broadly speaking, in biotech crops, the genetic change is generally small and more defined as the target gene/s is/are more specific, compared to traditionally bred crops. Biotechnology enables targeted and precise genome or gene modifications, while a hybrid plant is the result of a mixture and reorganization of two parental genomes. Thus, both desired and undesired genes are transmitted into the offspring, and sometimes desirable traits may be lost, which require extensive back crossing to get them

back. Genetic engineering offers possibility to introduce genes from non-crossable species into a target plant genome, to overcome the species barrier and broaden the genetic diversity (Ronald, 2011). Through the use of inducible promoters, genetic engineering enables more precise control of the temporal and spatial expression of the target genes, and thus enabling tissue-, development-, stress- and organ-specific gene expression for achieving the desirable traits (Datta, 2013).

For the last 30 years, genetic engineering has been used for improving plant properties in many species and a large number of improved plant lines or genotypes have been generated (Moeller & Wang, 2008). Since the first publications about genetic engineering in plants in 1983 (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983), a number of biotechnological methods have been developed for improving plant properties, of which transgenesis is the method that has been most utilized (Baltes & Voytas, 2015). There are different methods developed for producing transgenic plants, of which *Agrobacterium*-mediated transformation is the most commonly used method. This method utilizes the tumour inducing (Ti) soil bacterium *Agrobacterium tumefaciens* as a vector for transferring a DNA segment into a plant genome. The bacterium hosts a Ti-plasmid that, through infection, transfers a DNA segment, called transfer DNA (T-DNA), into the plant nuclear genome, where it is integrated and transcribed (Binns & Thomashow, 1988; Bevan *et al.*, 1983).

It is worthwhile to mention that in recent years, the development of site-directed mutagenesis techniques, particularly CRISPR/Cas9, enables more precise mutation of DNA sequences than genetic engineering, namely it allows specific modification of a target gene and insertion of transgenes into defined spots in the genome (Podevin *et al.*, 2013; Mahfouz *et al.*, 2011). These new technologies will bring more possibilities for genetic modification of plant properties in the future.

2.3 Plant oil biosynthesis

2.3.1 *De novo* fatty acid biosynthesis in seeds

De novo fatty acid (FA) synthesis takes place in the plastids, where acetyl-CoA is converted to malonyl-CoA catalyzed by the enzyme acetyl-CoA carboxylase (ACCase). Catalyzed by malonyl-CoA:acyl carrier protein malonyltransferase (MCMT), the malonyl group is transferred from malonyl-CoA to a protein cofactor, acyl carrier protein (ACP) before being utilized by the multi subunit complex, fatty acid synthase (FAS) (Brown *et al.*, 2006; Ohlrogge & Browse, 1995). Malonyl-ACP functions as a carbon donor in the subsequent elongation

reactions, where it adds two carbon units at each elongation cycle. Each cycle consists of four separate reactions, where the first reaction is the condensation of acetyl-CoA with malonyl-ACP to form 3-ketobutyl-ACP and CO₂, catalyzed by the β -ketoacyl synthase III (KAS III). The condensation is followed by a reduction to 3-hydroxyl-ACP, dehydration to an enoyl-ACP and another reduction where the elongated 4:0-ACP is formed (Voelker & Kinney, 2001). The enzyme KAS I catalyzes the formation of carbon chain lengths between 6-16 carbons. The saturated 16:0 acyl-ACP can either be hydrolyzed by the FATB acyl-ACP thioesterase, or elongated to 18:0-ACP by the KAS II enzyme before it is saturated by a stromal Δ^9 stearoyl-ACP desaturase (SAD) to 18:1 ^{Δ^9} -ACP and finally hydrolyzed by FATA acyl-ACP thioesterase. The resulting free fatty acids are activated to CoA esters on the outer envelope membrane of the chloroplast by long-chain acyl-CoA synthetases (LACS) before they are exported to the endoplasmic reticulum (ER) (Baud & Lepiniec, 2010). The acyl-CoAs can be further elongated on the ER by a fatty acyl-CoA elongase (FAE). Elongated acyl-CoAs together with plastidially exported acyl-CoAs constitute the acyl-CoA pool (Weselake *et al.*, 2009).

In most plants and tissues, the dominating free fatty acids exported from the plastid are 16:0 and 18:1, with their relative proportions decided by the activities of FATA, FATB, SAD and KASII enzymes (Bates *et al.*, 2013). By manipulating the expression of any of these four enzymes, the chain length and saturation of the fatty acids can be modified (Cahoon *et al.*, 2010).

2.3.2 Molecular basis of oil biosynthesis

The seed oil biosynthesis, mainly in the form of triacylglycerol (TAG), occurs in the (ER). Acyl-CoA esters together with glycerol-3-phosphate (G3P) serve as primary substrates in the TAG-yielding Kennedy pathway. Glycerol-3-phosphate acyltransferase (GPAT) transfers the acyl chain from CoA to the *sn*-1 position of G3P to form lysophosphatidic acid (LPA). The enzyme lysophosphatidic acid acyltransferase (LPAAT) is catalyzing the transfer of the acyl chain from CoA to the *sn*-2 position, forming phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) removes the phosphate, generating the formation of diacylglycerol (DAG). Diacylglycerol acyltransferase (DGAT) finally catalyzes the transfer of an acyl group from acyl-CoA to the *sn*-3 position of DAG to form TAG (Voelker & Kinney, 2001).

Another more complex pathway of TAG assembly involves the membrane lipid phosphatidylcholine (PC). Polyunsaturated fatty acids (PUFAs) are formed by ER-localized desaturases that use phosphatidylcholine (PC) as a substrate. The delta-12 FA desaturase (FAD2) enzyme desaturates oleoyl-PC (18:1) to linoleate (18:2), and the 18:2-PC is further desaturated to linolenoyl

(18:3)-PC by the delta-15 desaturase (FAD3) enzyme (Okuley *et al.*, 1994; Arondel *et al.*, 1992; Stymne & Appelqvist, 1978). The PUFAs formed on PC are incorporated into TAG by **three possible routes**. In the acyl editing route, FAs synthesized in the plastid are incorporated into PC, and FAs that have been modified on PC can enter the acyl-CoA pool by the reverse reaction of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) (Lager *et al.*, 2013; Stymne & Stobart, 1984) from which they can be incorporated into TAG via the Kennedy pathway (Bates *et al.*, 2009; Bates *et al.*, 2007). A second pathway involves the activity of phospholipid:diacylglycerol acyltransferase (PDAT), where PDAT catalyzes the transfer of the acyl chain from the *sn*-2 position of PC to DAG, generating TAG and lyso-PC (Dahlqvist *et al.*, 2000). In the third route, PC-derived DAG is utilized as a substrate for TAG synthesis. PC-derived DAG is generated by the action of *sn*-1,2-diacylglycerol:cholinephosphotransferase (CPT) (Slack *et al.*, 1985; Slack *et al.*, 1983) and/or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu *et al.*, 2009).

2.3.3 Wax ester biosynthesis

Seed oils normally exist in the form of TAG, however, the desert plant jojoba (*Simmondsia chinensis*) is unique in that it accumulates mainly wax esters as an energy reserve in the seed oil (Miwa, 1971). Wax esters consist of one fatty acid molecule esterified to one fatty alcohol molecule. The wax ester synthesis consists of two steps, namely, conversion of fatty acids to fatty alcohols by a fatty acyl-coenzyme A (CoA) reductase (FAR) and the esterification of the fatty alcohols to fatty acids by a wax synthase (WS) to form wax esters (Vanhercke *et al.*, 2013b). The wax ester biosynthesis starts by the synthesis of oleic acid (C18:1) in the plastid. The oleic acid is then exported from the plastid, converted to a fatty acyl-CoA ester before the carbon chain is elongated to C20:1, C22:1 and C24:1 acyl-CoAs by FAE. The generated very long chain (more than 18 carbons) monounsaturated acyl-CoAs are precursors in wax ester synthesis (Metz *et al.*, 2000).

2.4 Alterations of plant oil quality and quantity by genetic engineering

2.4.1 High oleic acid

Vegetable oils having a high portion of oleic acid (18:1) are desirable both in the food and chemical industry. Oleic acid is a monounsaturated fatty acid (MUFA) with long shelf-life and high oxidative and temperature stability, which enables direct use of high oleic acid oils as deep-frying oils in the food

industry, without any need for hydrogenation (Li *et al.*, 2015). In the chemical industry, oleic acid is a desirable component of cosmetics, biodiesel blends (Durrett *et al.*, 2008), bio-based lubricants (Wagner *et al.*, 2001) and as a starting material for a great number of oleochemicals (Mol & Buffon, 1998).

As stated earlier, the key genes controlling the seed oil composition of most oil crops is *FAD2* and, in Brassicaceae, also *FAEI*. By regulating the expression levels of *FAD2* and *FAEI*, the level of oleic acid can be effectively altered.

The oleic acid content in the seed oil has been modified in several species. By silencing *FAD2*, the oleic acid level was increased in flax (Chen *et al.*, 2015), cotton (Liu *et al.*, 2002) and soybean (Zhang *et al.*, 2014). Simultaneous silencing of both *FAD2* and *FAEI* genes led to increased levels of oleic acids in *Crambe abyssinica* (Li *et al.*, 2015), *Brassica napus* (Peng *et al.*, 2010) and *Camelina sativa* (Nguyen *et al.*, 2013).

2.4.2 Seed oil content

Oil content is a quantitative trait, and is controlled by a number of genes. Among the enzymes analyzed for their capacity to affect seed oil content, DGAT, the enzyme which catalyzes the acylation of *sn*-1,2-DAG to form TAG, is the most extensively studied one (Weselake *et al.*, 2009).

DGAT has been suggested to be a rate-limiting enzyme in the TAG synthesis and the accumulation of storage lipids (Perry *et al.*, 1999; Perry & Harwood, 1993; Ichihara *et al.*, 1988). Most plants have two types of DGAT: DGAT1 and DGAT2, which belong to two non-closely related gene families (Lardizabal *et al.*, 2001). Their respective roles are determined by their temporal expression and localization within the organism (Shockey *et al.*, 2006). In an Arabidopsis mutant (AS11) with reduced DGAT activity, the phenotype showed reduced oil content and an alteration in the seed oil composition (Katavic *et al.*, 1995). The AS11 mutant was attributed to a mutation in the *DGAT1* gene (Routaboul *et al.*, 1999; Zou *et al.*, 1999). The possibility to enhance TAG accumulation in plants by overexpression of DGAT has been investigated in a number of studies. By constitutively expressing the Arabidopsis *DGAT1* (*AtDGAT1*) in tobacco, Bouvier-Navé *et al.* (2000) got transformants with oil contents increased by up to 7 times in the leaves in comparison to the wildtype. When *AtDGAT1* was seed-specifically overexpressed in Arabidopsis, the seed oil content of the homozygous lines was increased by up to 28 % (Jako *et al.*, 2001). By expressing a codon-optimized form of the soil fungus *Umbelopsis romanniana* *DGAT2A* in soybean, the absolute seed oil content of mature seeds was increased by 1.5 % (Lardizabal *et al.*, 2008). A 3 % increase in seed oil content of the seed weight

in soybean was achieved by overexpression of an improved variety of soybean *DGAT1* (Roesler et al., 2016). Hatanaka *et al.* (2016) achieved an even higher increase in soybean oil content of 4 % by expressing a *DGAT* from *Vernonia galamensis*, without reducing the amount of seed protein.

Regulating transcription factors (TFs) involved in the oil biosynthesis is another possible way of altering the oil content. Transcription factors function as master regulators of multiple processes simultaneously. By themselves, or as part of a protein complex, they bind to DNA and decide whether target gene expression is activated or repressed (Century *et al.*, 2008).

Several important TFs have been shown to play an important role in plant seed oil biosynthesis. For instance, *LEAFY COTYLEDON 1 (LEC1)* and *LEAFY COTYLEDON 2 (LEC2)* are two important TFs, which have been shown to be involved in regulation of fatty acid biosynthesis, apart from their key roles in other developmental processes (Stone *et al.*, 2001; Lotan *et al.*, 1998; West *et al.*, 1994). Mu *et al.* (2008), showed that overexpression of *LEC1* in Arabidopsis resulted in upregulation of fatty acid biosynthetic genes and an increase in the accumulation of major fatty acid species. By expressing *LEC2* in leaves of Arabidopsis, accumulation of seed-quality like TAGs in leaves was achieved (Santos Mendoza *et al.*, 2005).

The transcription factor *WRINKLED1 (WRI1)* belongs to the AP2/EREBP domain family, and has been shown to be involved in regulation of genes associated with glycolysis and fatty acid synthesis (Cernac & Benning, 2004; Focks & Benning, 1998). Focks and Benning (1998) found that a mutation in *WRI1* resulted in incompletely filled seeds with a wrinkled seed coat appearance and a seed oil content reduction of 80 %. *WRI1* has successfully been utilized to alter the seed oil content of Arabidopsis and maize. Expression of a *WRI1*-like gene from *B. napus* in Arabidopsis resulted in an increase in seed oil content of between 10-40 % in the transgenic lines (Liu *et al.*, 2010). Overexpression of maize *WRI1* in maize embryo and scutellum resulted in transgenic lines with up to 46 % increase in oil content (Shen *et al.*, 2010).

Overexpression of plant hemoglobin genes have been reported to increase the seed oil content (Vigeolas *et al.*, 2011). The plant hemoglobins, now called phytohemoglobins, are divided into two types: the symbiotic and the non-symbiotic hemoglobins. The symbiotic hemoglobins are primarily found in the legume root nodules that are infected by a nitrogen-fixing bacterium (Appleby *et al.*, 1988).

The non-symbiotic hemoglobins (nsHbs) can be found in different plant species and tissues (Anderson *et al.*, 1996). They are further divided into two classes, namely class 1 (nsHb1) and class 2 (nsHb2), based on their phylogenetic characteristics, gene expression patterns and capacity to bind

oxygen (Vigeolas *et al.*, 2011). Class 1-nsHbs have an extremely high affinity for oxygen, while class2-nsHbs have a much lower affinity. Vigeolas *et al.* (2011) overexpressed endogenous class2-nsHb *AtHb2* in Arabidopsis, which resulted in a seed oil content increased by 40 %. The increase was explained to be a consequence of an elevated energy state and sucrose content in the seeds.

2.4.3 Wax esters

Wax esters are important industrial feedstocks due to their outstanding lubricating properties and high resistance to hydrolysis. Properties such as melting temperature, oxidation stability and pressure stability are dependent on the chain length and the degree of unsaturation of the fatty acids and fatty alcohols, which in turn determines the utilization of the wax esters (Aslan *et al.*, 2014). Lubricants should preferably have low melting points and be oxidative stable, thus species of medium or long chain lengths with maximum one double bond in each part of the molecule is desirable (Heilmann *et al.*, 2012).

Before the ban, spermaceti oil from the spermaceti whale was the main resource of wax esters, which had extensive use in lubrication and transmission fluids in the past. Today, wax ester sources are very limited. As stated above, plants do not normally produce wax esters in their seed oils, and jojoba is thus unique in this sense. However, since jojoba is a low yielding desert shrub not suitable for agricultural production, the wax esters from jojoba are often highly priced and used in exclusive products such as cosmetics. Synthetic wax esters can be obtained through refining petrochemicals or by chemical conversion of fatty acids, but these approaches are not optimal since the chemical procedures are costly and the wax esters obtained might be mixtures not suitable as some technical lubricants (Heilmann *et al.*, 2012). Efficient production of wax esters in plants that are suitable for extensive cultivation is thus desirable.

Biotechnology enables introduction of the jojoba *FAR* (*ScFAR*) and the jojoba *WS* (*ScWS*) genes into the genome of a plant suitable for cultivation, and thus providing the possibility of generating a crop with high levels of wax esters in the seed oil. By introducing the *ScFAR* and *ScWS* genes together with *Lunnaria annua* *FAE1* (*LaFAE1*) into Arabidopsis, Lardizabal *et al.* (2000) obtained transgenic lines with 49 % wax esters in the seed oil. In a study by Iven *et al.* (2015) transgenic lines of Arabidopsis and *C. sativa* expressing *ScWS* and *Marinbacter aquaeolei* *FAR* (*MaFAR*) genes produced 59 % and 21 % wax esters in the seed oil, respectively. By seed specifically overexpressing *ScWS* and *ScFAR* with or without expression of *ScFAE1* in *C. abyssinica*, *C. sativa* and *B. carinata* Zhu *et al.* (2016) have produced transgenic lines with

high amounts of wax esters in these species, showing the possibility of using industrial oilseed species as biotechnological platforms for wax ester synthesis.

2.5 Molecular mechanisms of pod shatter control and alteration

Fruit-bearing plants have developed mechanisms that ensure efficient dispersal of their seeds, and hence optimized the chances for survival of the coming generation. For *Brassica* species, such as *Arabidopsis* and other important oil crops, the seed dispersal is accomplished through a process called fruit dehiscence or pod shatter (Spence *et al.*, 1996). In wild, the mechanism is an advantage, but in cultivation pod shatter can result in yield losses of up to 50 % (Summers *et al.*, 2003). Apart from lost harvests, the shattered seeds that fall to the ground might germinate and hence contaminate future crops (Ostergaard *et al.*, 2006). Pod shatter resistance is thus an important target trait in oilseed breeding.

The *Arabidopsis* fruit is called silique, and is composed by four major distinct tissues: the valves, the septum, the replum and the valve margins. The two fruit valves enclose the developing seeds (Figure 3). On the interior of the valves, two layers of endocarp cells form *the inner epidermis*, called endocarp a (*ena*) and a second endocarp layer called endocarp b (*enb*) or the lignified valve layer. The *ena* layer breaks down when the fruit matures, but the *enb* layer becomes lignified late in the fruit development (Roeder & Yanofsky, 2006). The septum and the replum divide the fruit into two halves, where the septum stretches from one replum to the other (Roeder & Yanofsky, 2006) and the valve margins (or dehiscence zones) connect the replum to the valves. The valve margins consist of two layers: the lignified layer, consisting of lignified cells, and the non-lignified separation layer, which is made up by small, spherical shaped parenchyma cells (Ostergaard *et al.*, 2006). At fruit maturity, the parenchyma cells secrete cell-wall degrading enzymes which degrade the middle lamella between dehiscence zone cells generating a breaking zone between the valve and the replum (Meakin & Roberts, 1990). The ripening fruit dries and shrinks, and since the lignified structures in the lignified layer and the *enb* layer within the pod stay rigid, a tension is built within the pod. The pressure forces the valves to separate from the replum at its weakest point; the breaking zone (or separation layer), thus generating shattering of the seeds (Spence *et al.*, 1996).

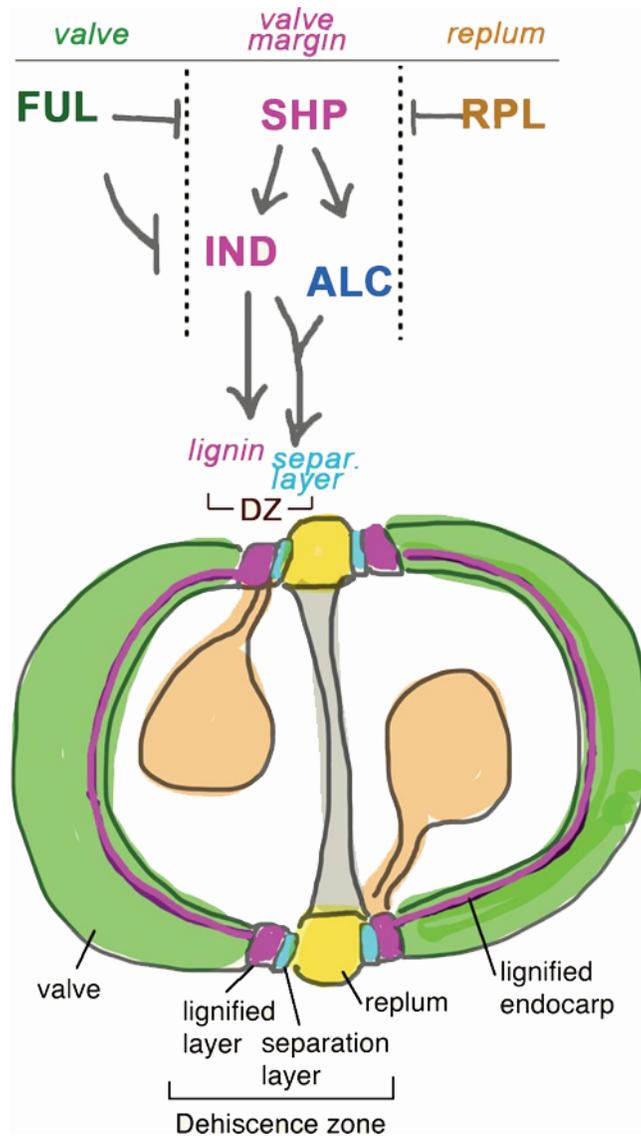


Figure 3. Simplified genetic model for the development of the DZ in *Arabidopsis*. The cartoon represents a transversal section through the mature ovary, where valves are coloured in green, the lignified layer and the lignified endocarp in pink, the separation layer in blue, and the replum in yellow. Figure reproduced from Ferrándiz and Fourquin (2014) with kind permission from Oxford University Press.

The development of a functional dehiscence zone (DZ) is dependent on the expression of several TF genes (Liljegren *et al.*, 2004; Rajani & Sundaresan, 2001; Ferrándiz *et al.*, 2000). The MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) are required for differentiation of the

lignified layer and the separation layer of the valve margin. The two genes share 87 % identity at the amino acid level, and act redundantly, since *shp1* or *shp2* single mutants exhibit no abnormal phenotypes. However, mutations in both *SHP* genes results in indehiscent fruits (Liljegren *et al.*, 2000). The *SHP* genes activate the expression of the basic helix-loop-helix (BHLH) genes *INDEHISCENT (IND)* and *ALCATRAZ (ALC)* (Roeder & Yanofsky, 2006; Liljegren *et al.*, 2004; Rajani & Sundaresan, 2001). The *ALC* gene is involved in differentiation of the separation layer. In *alc* mutants, the seeds are imprisoned due to an incorrect formation of the separation layer (Rajani & Sundaresan, 2001). The *IND* gene is involved in valve margin development, and *ind* fruits are severely affected due to absence of both lignified layer and separation layer throughout the fruit (Liljegren *et al.*, 2004). *FRUITFULL (FUL)* is a MADS-box gene required for normal growth and differentiation of valve cells (Ferrándiz *et al.*, 2000; Gu *et al.*, 1998). Mutation in *FUL* results in short fruits with ectopic lignification of the internal mesocarp cells in the valves (Ferrándiz *et al.*, 2000). The *FUL* gene restricts the expression of the valve margin identity genes *SHP*, *IND* and *ALC* to the valve margin (Figure 3). In a *ful* mutant, the valve margin identity genes are ectopically expressed in the valves, thus, the valves partially adopt a valve margin identity, resulting in valve cell lignification (Liljegren *et al.*, 2004; Ferrándiz *et al.*, 2000). The *REPLUMLESS (RPL)* gene negatively regulates the valve margin identity genes from the replum side (Figure 3) (Roeder *et al.*, 2003). In *rpl* mutants, the outer cell layers of the replum adopt the characteristics of valve margin cells instead of replum cells. This is due to ectopic expression of the *SHP*, *IND* and *ALC* genes in the replum (Roeder & Yanofsky, 2006).

3 Aims and objectives of the thesis

The goal of this work was to make significant progress towards the ultimate goal of developing *L. campestre*, into a new oil and catch crop that can be grown in cold regions in the world.

The more specific objectives were to:

- Develop a functional regeneration and transformation protocol for *L. campestre*
- Alter the oil composition through downregulating two target genes in the seed oil biosynthesis
- Increase the seed oil content through overexpression of three target genes
- Produce wax esters in the seed oil through introducing the jojoba wax synthesis genes into the species
- Reduce the pod shattering by downregulating one target gene involved in pod shatter control

4 Methodology

4.1 Plant transformation

Seeds originating from accession number NO94-7, collected by late professor Arnulf Merker were used in all transformations. Hypocotyl explants excised from 5 days old seedlings were pre-cultured on the MS medium (Murashige & Skoog, 1962) with 30 g L⁻¹ sucrose, 2.5 g L⁻¹ agar and 0.5 g L⁻¹ 2,4-D at pH 5.7 for 2 days before they were immersed in O/N cultured bacterial suspension for 5 min. The explants were then co-cultured on the co-cultivation medium containing MS with 30 g L⁻¹ sucrose, 2.5 g L⁻¹ agar and 1.1 mg L⁻¹ TDZ at pH 5.7 for 4 days. After co-cultivation, the explants were rinsed from excess *Agrobacterium* using MS20 (MS with 20 g L⁻¹ sucrose) and then transferred to the selection medium, which was the same as for co-cultivation, but supplemented with 150 mg L⁻¹ ticarcillin and 15 mg L⁻¹ kanamycin. The selection pressure was increased to 25 mg L⁻¹ kanamycin and later to 30 mg L⁻¹ kanamycin in the subsequent subcultures. Regenerated shoots of about 1 cm were transferred to the shoot proliferation medium, according to Li *et al.* (2010).

4.2 Molecular analysis

4.2.1 PCR and Southern blot analysis

Regenerated shoots growing well on the selection medium for at least 1 month were analyzed through PCR analysis. Total genomic DNA was extracted from the *in vitro* grown shoots by the CTAB method (Aldrich & Cullis, 1993).

Stable integration and the number of transgene copies integrated into the transgenic lines were analyzed by Southern blot analysis. Approximately 20 µg of genomic DNA, extracted from *in vitro* grown shoots using the CTAB method, were digested with the appropriate restriction enzymes. The probes were synthesized using the same primers as for the PCR analysis, according to

Zhu *et al.* (2008). The non-radioactive DIG system was used in the Southern blot hybridization (Zhu *et al.*, 2008).

4.2.2 qRT-PCR analysis

Total RNA was extracted from immature seed pods using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Apart from addition of 4 % polyvinylpyrrolidone (PVP) to the extraction buffer, the protocol provided by the manufacturer was followed. To remove potential traces of genomic DNA, the extracted RNA was DNase treated, using TURBO DNA-free (Ambion, Austin, TX). Using Superscript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, Life Technologies, Carlsbad, CA, USA), cDNA was synthesized from 1000 ng of RNA in a 20 µl reaction, which was used for qRT-PCR analysis using BIO-RAD C1000 Thermal Cycler, CFX 96 Real-Time System (California, USA) with BIO-RAD iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

4.3 Seed oil analysis

4.3.1 Oil content

Seed oil (TAG) was extracted from triplicates of pooled samples. Using an IKA[®] T18 basic (ULTRA TURRAX[®]), the seeds were homogenized in 1 ml 0.15 M acetic acid and 3.75 ml MeOH:CHCl₃ (2:1,v/v) prior to addition of 1.25 ml CHCl₃ and 0.9 ml H₂O. The samples were vortexed and centrifuged before 200 µl of the chloroform phase was transferred to a new glass tube. The samples were dried completely under nitrogen and dissolved in 100 µl hexane. 2 ml methylation solution (2 % H₂SO₄ in methanol) and 100 nmols of internal standard 17:0 (in methanol) were added to the samples. The samples were methylated at 90°C for 1 hour, after which 1 ml H₂O and 600 µl hexane were added. After vortexing and centrifugation, 200 µl of the hexane phase was transferred to a GC vial with an insert. Samples were finally analyzed on an Agilent (model 7890A) gas chromatograph with a WCOT Fused Silica CP-Wax 58 column and a FID detector (Shimadzu Corporation, Kyoto, Japan).

4.3.2 Oil composition

The seed oil composition was analyzed using the half-seed technique as described by Li *et al.* (2012). Sterilized seeds were germinated in light for around 18 h, then the seed coat was removed and the upper parts of the cotyledons were excised for oil analysis. The remaining parts of the seeds were kept on the germination medium until the result from the analysis was known.

The seedlings belonging to the samples with desirable oil composition were kept for seed production of the next generation.

The excised cotyledons were homogenized in 1200 μl hexane using a mortar. The samples were moved to screw cap tubes and dried under nitrogen. The lipids were dissolved in 2 ml methylation solution (2 % H_2SO_4 in methanol) before methylation at 90°C for 45 min. Thereafter, 500 μl hexane and 2 ml H_2O were added. After vortexing and centrifugation, the hexane phase was transferred to GC vials. The sample volumes were reduced under a beam of nitrogen before being analyzed on an Agilent (model 7890A) gas chromatograph with a WCOT Fused Silica CP-Wax 58 column and a FID detector (Agilent Technologies, Santa Clara, California).

4.3.3 Wax ester

Seeds were homogenized in 3.75 ml $\text{MeOH}:\text{CHCl}_3$ (2:1,v/v) and 1 ml 0.15 M HAc using an IKA[®] T18 basic (ULTRA TURRAX[®]). Thereafter, 1.25 ml CHCl_3 and 1.25 ml H_2O were added to the samples, which were then vortexed and centrifuged. After centrifugation, the lower phase was transferred to screw-cap tubes, and allowed to dry completely under a beam of nitrogen. The lipids were dissolved in 200 μl CHCl_3 , and 30 μl of each sample was loaded on TLC plates and developed in hexane:DEE:HAc (90:10:1, v/v/v). The TAG and wax ester areas were scraped off and 200 μl MeOH was added to the samples before they were dried under nitrogen. Methylation solution (2 % H_2SO_4 in methanol) and internal standard 17:0-Me were added before methylation at 90°C for 50 min. 600 μl hexane and 2 ml H_2O were added to each tube, the samples were vortexed and centrifuged, and finally, the upper layer was transferred to a GC vial and analyzed on an Agilent (model 7890A) gas chromatograph as stated above.

4.4 Pod shattering analysis

The pod shatter resistance was analysed using the random impact test according to Bruce *et al.* (2002) and Lenser and Theissen (2013) with minor adjustments. Mature pods were collected from the transgenic and wildtype plants. From each plant, 3 replicates with 20 seed pods per replicate were analysed. The pods were placed in a 50 ml grinding jar together with six 4 mm metal balls, and shaken using a MM 400 mixer mill (Retsch GmbH, Haan, Germany). The pods were shaken in intervals of 5, 10, 20, 40, 80, 160 and 320 sec or until all pods were opened. The number of intact seed pods was calculated after each interval. The agitation force was set to 9 Hertz in T_1

generation, but increased to 15 Hertz in T₂-T₄ generations in order to reduce the time of analysis.

5 Results and discussion

In **paper I**, an efficient regeneration and transformation protocol for genetic modification of *L. campestre* was established. The effects of different types, combinations, concentrations and exposure times of plant growth regulators (PGR), light conditions and types of explants were investigated. The regeneration from hypocotyl explants was superior to that of the cotyledon explants, and so was germination in light compared to that in dark. Most combinations of auxins and cytokinins did not result in regeneration, however, when combining 2.4-D and TDZ, 76.5 % of the hypocotyl explants regenerated shoots (Figure 4). When testing different concentrations and exposure times of the PGRs, preculture on 0.5 mg L⁻¹ 2.4-D for 2 days followed by 1.1 mg L⁻¹ TDZ was the best combination, which was then chosen for the *Lepidium* transformations. An average transformation efficiency of 6 % was achieved using the optimized protocol.



Figure 4. *In vitro* regenerated shoots of *Lepidium campestre* grown in Petri dishes in the climate chamber (Photo by Anna Lehrman)

In **Paper II**, the *LcFAE1* and *LcFAD2* genes were simultaneously and seed-specifically RNAi down-regulated in *L. campestre*. Stable transgenic lines were generated showing clearly altered seed oil compositions (Table 1). The level of 18:1 was increased from 11 % in the wildtype (WT) to up to over 80 % in the transgenic lines, while the oxidative unstable linolenic acid was decreased from 40 % in WT down to 2.6 % in the transgenic lines, due to the down-regulation of the *FAD2* gene. Moreover, the unhealthy erucic acid was reduced from 20 % down to 0.1 %, due to the down-regulation of the *FAE1* gene. Decreases in the amounts of 18:2, 20:1, 20:2, 22:0 and 24:1 were also observed, and the total amount of PUFAs in the transgenic lines with the highest levels of 18:1 was decreased to less than 4 %. The low PUFA content makes the oil oxidatively stable in high temperatures, and the oil is thus suitable as processing oil in the food industry or as a feedstock for applications in the chemical industry.

The high oleic acid trait has been maintained over several generations, indicating a stable integration and silencing effect of the transgenes (Table 1).

Under biotron conditions, the transgenic lines grew normally with normal flowering in general, however, some transgenic plants did not flower at all, while some did flower, but set fewer seeds. The reason behind the sterility or the poor seed setting is not clear. Since this was found already in the heterozygous T₁ plants, it is unlikely caused by homozygosity of the transgene.

Table 1. Levels (%) of 18:1, 18:3 and 22:1 fatty acids in the seed oil of different generations of selected *LcFAD2-LcFAE1*-RNAi lines of *L. campestris*

Line	No. seeds analyzed	18:1			18:3			22:1		
		Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
WT	10	11.7 ± 0.7 d	12.8	10.8	39.4 ± 2.8 a	42.6	32.4	20.4 ± 0.6 a	21.5	19.7
T ₁ -1	124	71.1 ± 14.3 c	82.3	14.2	4.2 ± 4.9 c	34.9	2.4	6.2 ± 11.3 b	47.2	0.1
T ₂ -1-1	64	72.6 ± 8.3 bc	81.9	47.3	3.6 ± 0.5 c	5.0	2.6	3.4 ± 6.3 c	22.3	0.1
T ₃ -1-1-1	15	76.5 ± 2.5 abc	80.0	70.0	4.0 ± 0.9 c	6.9	3.3	0.1 ± 0.1 c	0.3	0.1
T ₃ -1-1-2	15	71.4 ± 17.2 bc	79.1	9.6	6.8 ± 8.3 b	36.6	3.1	1.5 ± 5.0 c	19.4	0.1
T ₃ -1-1-3	15	77.6 ± 1.5 ab	80.6	75.5	3.4 ± 0.5 c	4.9	2.7	0.1 ± 0.1 c	0.2	0.1
T ₂ -1-2	31	79.4 ± 2.8 a	83.4	71.5	3.6 ± 1.1 c	7.9	2.3	0.3 ± 0.5 c	2.9	0.1
T ₃ -1-2-1	15	74.9 ± 1.6 abc	77.5	72.2	5.0 ± 1.2 bc	7.6	3.4	0.2 ± 0.2 c	1.0	0.1

Lines that do not share the same letter are significantly different at $p = 0.05$. WT wildtype; T₁-1 T₁ generation; T₂-1-1 and T₂-1-2 T₂ generation; T₃-1-1-1, T₃-1-1-2, T₃-1-1-3 and T₃-1-2-1 T₃ generation. Table reproduced from Ivarson *et al.* (2016) with kind permission from Springer.

In **paper III**, the possibility to increase the seed oil content of *L. campestre* by seed specific overexpressing either *AtWR11* or class2-nsHbs *AtHb2* or *BvHb2* was investigated. The analysis of the oil content in WT and transgenic lines revealed that overexpression of the *AtWR11*, *AtHb2* and *BvHb2* resulted in increased seed oil contents by 18.3 %, 20.2 % and 16.7 % respectively, in the T₃ generation lines.

In T₁, the highest increase was found in the lines overexpressing the *BvHb2* gene, with the oil content increased by up to 25.9 %. This increase was not kept in the subsequent generations, indicating that this trait was not maintained.

Seed oil increases caused by overexpression of *WR11* has been accompanied by increases in seed weights in *BnWR11* overexpressing lines of Arabidopsis (Liu et al., 2010) and *AtWR11* overexpressing lines of camelina (An & Suh, 2015). No such correlation could be detected in our study, where the changes in seed weights were inconsistent among the transgenic lines.

AtHb2 expression in Arabidopsis caused an alteration in the seed oil composition (Vigeolas *et al.*, 2011). In our study, neither the expression of the *AtHb2* gene nor the expression of the *BvHb2* gene resulted in any changes in the oil composition of transgenic lines of *L. campestre*.

In order to explore the possibility of producing wax esters in *L. campestre*, 3 jojoba wax ester synthesis genes in two different constructs were introduced into this species (**paper IV**). The first construct harbored the *ScWS* and *ScFAR* genes, while the *ScFAE1* gene was added together with the *ScWS* and *ScFAR* genes in the second construct. Introduction of the wax ester synthesis genes resulted in transgenic lines with varying amounts of wax ester accumulation in the seed oil. The wax ester profile differed between the lines harboring the different constructs. Addition of the *FAE1* gene yielded longer-chained wax ester species compared to the lines lacking the *FAE1* integration. The most abundant wax ester species found in the *ScFAR/ScWS* lines were the C40-C46, accounting for almost 98 % of all wax ester species, while the C42-C48 WEs were the most abundant ones in the lines harboring the *ScFAR/ScWS/ScFAE1* genes, accounting for close to 97 % of all wax ester species (Figure 5).

The seeds accumulating high levels of wax esters had an inhibited seed germination, a tendency also found in the study by Zhu *et al.* (2016), where the wax ester accumulation in the seeds of *C. abyssinica* and *C. sativa* was negatively correlated with the germination capacity. The reason behind this is unclear and needs to be further studied.

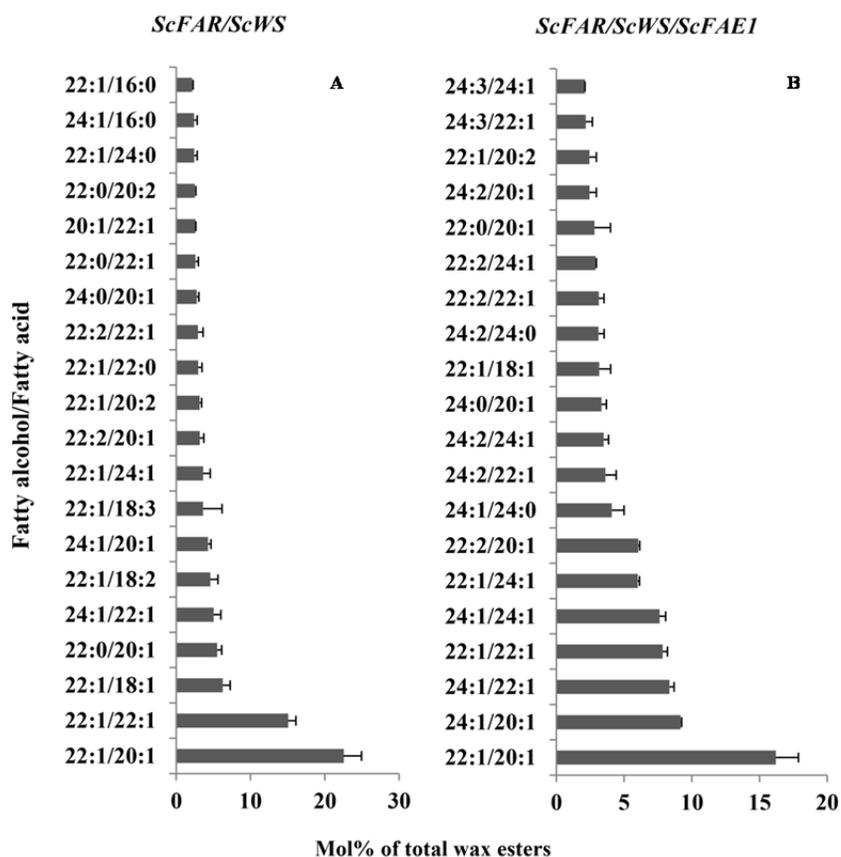


Figure 5. The 20 most abundant wax ester species of the transgenic lines of *L. campestre* analysed by nanoESI-MS/MS. (A = *ScFAR/ScWS*, B= *ScFAR/ScWS/ScFAE1*). The standard errors were calculated from 3 individual seeds (n=3).

In paper V, the RNAi-*IND* construct was introduced into *L. campestre* to investigate whether the pod shatter could be reduced. Two constructs were used in this study, one with the *Brassica* RNAi-*IND* under the constitutively expressed 35S promoter, and one with RNAi-*LcIND* under the *AtIND* promoter. By analyzing the seed pods from the transgenic lines using the random impact test, the half-life of dehiscence could be calculated, i.e. the time needed for 50 % of the pods to open up. The results showed that the half-life of dehiscence was significantly longer in the *IND* down-regulated transgenic lines, compared to WT (Figure 6). The transgenic lines exhibit different levels of pod shatter resistance, with both highly resistance and intermediate resistance, providing the possibility to choose the lines with optimal pod shatter resistance for avoiding problems when threshing the seeds after harvest.

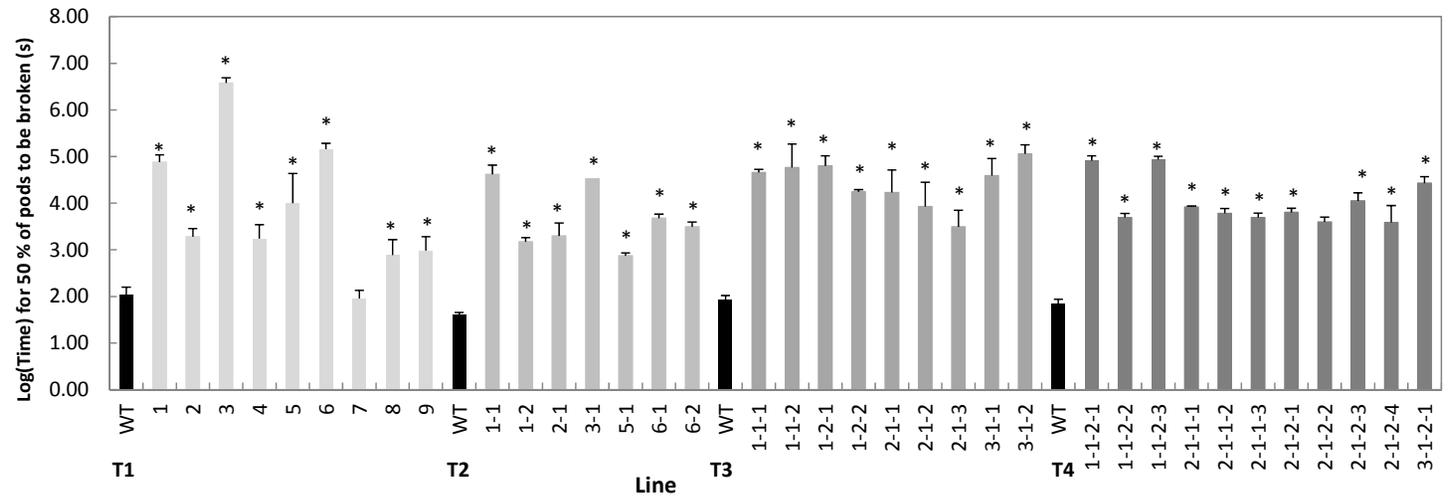


Figure 6. Time for 50 % of pods to be broken for the wildtype plants (WT) and transgenic plants from four generations determined by the random impact test. T1 generation was agitated at 9 Hz, but T2-T4 were agitated at 15 Hz. Results are means of three replicates per plant. Error bars represent standard deviation (SD). Lines marked with * are significantly different from the WT at P = 0.05

6 Conclusions and future perspectives

In this study, we have developed a well-functioning regeneration and transformation protocol for the wild species *Lepidium campestre* through optimizing different factors affecting regeneration, such as explant type, and concentration, combination and exposure times of plant growth regulators. The protocol has provided a sound base for successful performance of the subsequent parts of the project.

By RNAi down-regulating the *FAD2* and *FAE1* genes we obtained transgenic lines with a significantly altered seed oil composition. A high level of the oxidative stable oleic acid together with negligible amounts of the unhealthy erucic acid make the oil highly suitable as processing oil for the food- or chemical industry.

Through expressing either *AtWR11* or *AtHb2* or *BvHb2* gene, we have developed transgenic lines with significantly increased oil contents. Further increase in the oil content of *L. campestre* is still desirable for making the species more viable as the future oil crop. A multigene engineering through utilizing a push-pull-protect-approach (Vanhercke *et al.*, 2014; Vanhercke *et al.*, 2013a) might be a promising approach for increasing the seed oil content in the future.

By down-regulating the *IND* gene we generated transgenic lines with significantly reduced pod shatter. In the future, it would be interesting to evaluate whether the expression levels of other dehiscence zone genes were affected by the *IND* down-regulation. Moreover, it would be interesting to study the *IND* silencing effect on the fruit anatomy by microscopic studies.

The wax ester accumulation achieved in the transgenic lines of *L. campestre* in this study shows the potential for tailor-making the oil composition in this species for various industrial applications. Moreover, the difference in wax ester species generated by the two different gene constructs shows that different wax ester species can be produced for different purposes. One concern is that the transgenic lines with high wax ester contents showed

reduced germination capacity, which will limit its future cultivation. It is thus necessary to study the reasons underlying the hampered germination capacity, as well as the threshold value of wax ester content without negative effect on the germination.

In conclusion, we have shown in this thesis, that genetic engineering can improve target agronomic traits in the wild species *L. campestre* to achieve the desirable results, which would contribute substantially to the domestication of the species. Currently, all improved traits reside in individual lines for confirming the gene functions in this wild species. Next, it would be necessary to combine these improved traits into single breeding lines either by multigene engineering approach, or by cross breeding, or by a combination of both methods. Site-directed mutagenesis techniques, such as CRISPR/Cas9, offer a good alternative to the RNAi technique for gene silencing in *L. campestre*, which provides the possibility to only mutate the target genes without extra DNA insertion in the plant genome.

Apart from the traits studied in this thesis, there are other important agronomic traits of *L. campestre* that need to be improved, such as resistance to biotic and abiotic stresses. Moreover, by reducing the glucosinolate level in the seeds, the seed cake could be utilized directly as animal feed, and thus adding a surplus value for cultivating the species. Moreover, by increasing the seed yield per plant, the seed oil production per hectare could be increased.

It should be mentioned that this thesis work is part of the Mistra Biotech program (<http://www.slu.se/mistrabiotech>). The accession line we used was a wild collection without any property improvement. In parallel to this thesis work, there is also breeding work carried out on this species through non-GM approach by selections and intra- or interspecific hybridizations. Some selected lines or hybrids have been shown to have increased potential in seed yield and better growth as well as improved pod shatter resistance. Such lines could also be used as starting material for further genetic engineering or gene editing in this species in the near future. Through combination of both GM and non-GM approaches, the domestication of this species will be speeded up.

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