Towards the Domestication of *Lepidium campestre* as an Undersown Oilseed Crop

Dennis Eriksson
Faculty of Landscape Planning, Horticulture and Agricultural Science
Department of Plant Breeding and Biotechnology
Alnarp

Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp 2009

Acta Universitatis agriculturae Sueciae
2009:65

Cover: *Lepidium campestre* field trial in Lönnstorp.
(photo: D. Eriksson)

ISSN 1652-6880
ISBN 978-91-576-7412-8

Print: SLU Service/Repro, Alnarp 2009

Towards the Domestication of *Lepidium campestre* as an Undersown Oilseed Crop

Abstract

Lepidium campestre (field cress) has been selected for domestication. It is proposed to be cultivated as a catch crop in a spring cereal and produce a seed oil of industrial quality the following year(s). This crop system will help to alleviate the problem of nutrient leaching from agricultural soils. The oil produce will provide an alternative to fossil oil in certain industrial applications. This thesis addresses the issues of seed dispersal, seed oil quality and also includes a field trial evaluation of *L. campestre*.

A shoot regeneration protocol has been developed for *L. campestre* in order to enable a transformation procedure. The most important factors include a pretreatment on 2,4-D and a temporary starvation treatment. The combination of plant growth hormones in the regeneration medium was of little importance. For transformation, a number of factors for co-cultivation with *Agrobacterium tumefaciens* have been evaluated. So far, however, there has been little success.

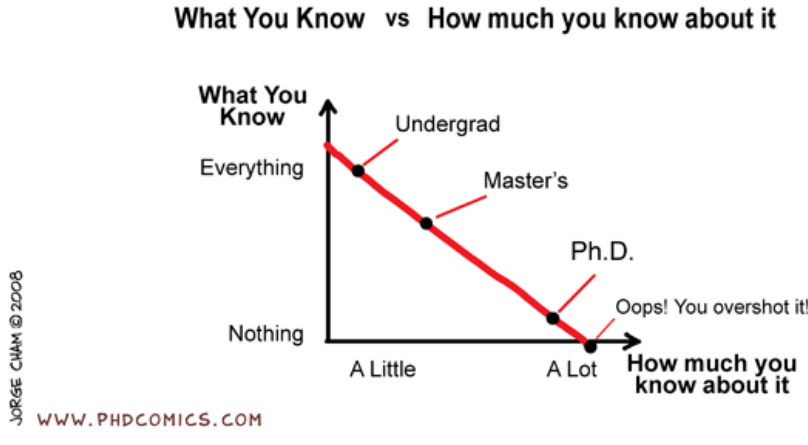
Microscopical studies on *L. campestre* fruits have identified a basic architecture as well as a dehiscence zone (DZ) that are important for seed dispersal. A gene controlling the development of the DZ, the *INDEHISCENT*, has been cloned from *L. campestre*. The function of the gene has been confirmed through functional complementation of the corresponding mutant, as well as RNAi down regulation, in *A. thaliana*.

To modify the seed oil quality, the genes encoding two important enzymes, the FATTY ACID DESATURASE 2 and the FATTY ACID ELONGASE 1, have been cloned from *L. campestre*. The functions of these genes have been confirmed through functional complementation of the corresponding *A. thaliana* mutants.

A three-year field trial with *L. campestre* undersown in spring barley demonstrated the high seed yield potential of this species. A positive effect on the barley yield was recorded when *L. campestre* was undersown with short row distance.

Keywords: Lepidium campestre, *Arabidopsis thaliana*, domestication, oil crop, perennial, pod shatter, fatty acid, shoot regeneration, transformation

Author's address: Dennis Eriksson, , Department of Plant Breeding and Biotechnology, P.O. Box 101, 230 53 Alnarp, Sweden
E-mail: Dennis.Eriksson@ltj.slu.se


What You Know vs How much you know about it
What You Know
Everything
Nothing
Undergrad
Master's
Ph.D.
Oops! You overshot it!
A Little
A Lot
How much you know about it
JORGE CHAM © 2008
WWW.PHDCOMICS.COM

Contents

List of Publications

This doctoral thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Eriksson D, Merker A. 2009. An efficient adventitious shoot regeneration protocol for *Lepidium campestre* (L.) R. BR.. Propagation of Ornamental Plants (9:2), 78-83.

II. Eriksson D, Merker A. 2009. Identification of a gene involved in pod shatter in the novel oil crop *Lepidium campestre*. (Manuscript).

III. Eriksson D, Merker A. 2009. *Lepidium campestre FAD*2 and *FAE*1 cDNAs complement the corresponding *fad*2 and *fae*1 mutations in transgenic *Arabidopsis* plants. (Manuscript).

IV. Merker A, Eriksson D, Bertholdsson N-O. 2009. Barley yield increases with undersown *Lepidium campestre*. Acta Agriculturae Scandinavica, Section B – Soil and Plant Science, published online 13 July.

Papers I and IV are reproduced with permission from the publishers.

The contribution of Dennis Eriksson to the papers included in this thesis was as follows:

Paper I Planned and performed all the experiments, evaluated the data and wrote the paper.

Paper II Planned and performed all the experiments (except for performing the transformations of *A. thaliana* and the SEM procedure), evaluated the data and wrote the paper.

Paper III Planned and performed all the experiments (except for performing the transformations of *A. thaliana*), evaluated the data and wrote the paper.

Paper IV Assisted with the field trials.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
bHLH	basic helix-loop-helix
cDNA	complementary DNA
CDS	coding sequence
CIM	callus induction medium
DNA	deoxyribonucleic acid
DZ	dehiscence zone
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
FAD	fatty acid desaturase
FAE	fatty acid elongase
GC	gas chromatography
GUS	beta-glucuronidase
IAA	indole-3-acetic acid
KCS	3-ketoacyl-CoA synthase
LaNe RAGE	lariat-dependent nested PCR for rapid amplification of genomic DNA ends
LM	light microscopy
mRNA	messenger RNA
NAA	napthaleneacetic acid
NCBI	national center for biotechnology information
ORF	open reading frame
PCR	polymerase chain reaction
PGR	plant growth regulator
PUFA	polyunsaturated fatty acid
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR
SEM	scanning electron microscopy
SIM	shoot induction medium
SR	shoot regeneration
UTR	untranslated region
VLCFA	very long chain fatty acid
WT	wildtype

Background

Introduction

The potential for domesticating new plant species is great. Of the more than two million known plant species, an estimated 300.000 are edible (Simpson and Ogorzaly, 2001) and many more could have some kind of technical application. However, only about 150 species have entered into world commerce (Simpson and Ogorzaly, 2001). Hence there is a vast number of plant species that have the potential to provide us with useful commercial products, such as fruits, seeds, leaves or roots for direct human or animal consumption, oils or fibres for technical applications or carbohydrates for fuel.

There are several benefits from domesticating new plant species. One is the prospect of providing environmentally friendly alternatives to certain industrial commodities, such as e.g. plastics. Another benefit is the potential to develop more environmentally sustainable and high yielding cultivation systems. Increasing the agrobiodiversity has the potential to increase the resistance and/or resilience of the agricultural system to pests, diseases and adverse weather conditions, with a resulting decrease in pesticide use as well as more stable harvests (Thrupp, 2002).

A domestication program has been initiated to develop the field cress, *Lepidium campestre*, as a new, perennial and undersown oil crop. This plant species belongs to the diverse Brassicaceae family, to which numerous other familiar crop plants belong, such as rapeseed, swede, cabbage, broccoli and cauliflower, mustard and radish. The primary objectives of this domestication program are to develop a perennial cropping system that will reduce soil nutrient leaching, and also to provide a vegetable oil that can replace fossil oil in certain industrial applications.

First issue: preventing nutrient leaching

An environmental problem of modern agriculture in the temperate regions is the leaching of nutrients into ground and surface water. One of the main causes of this problem is the predominant growing of annual crops that leaves the ground uncovered in the winter season (Jensen, 1991). It was estimated that an average of 18.1 kg/ha nitrogen (N) and 0.52 kg/ha phosphorus (P) leached from Swedish agricultural soils in the year 2005. The leaching of N and P has nevertheless been reduced with 12% and 4.4%, respectively, in Sweden over a ten year-period from 1995 to 2005. A large part (21%) of the reduction in N leaching can be attributed to the growing of catch crops (Johnsson *et al.*, 2008). Catch crops are established by undersowing in a spring cereal to reduce N leaching and to increase available N for the successive crop (Känkänen and Eriksson, 2007). Two important characteristics of a suitable catch crop are the ability to efficiently take up nitrogen during the autumn and winter periods and to not significantly influence the yield of the main crop (Jensen, 1991). An additional advantage of catch crops is that they allow for no-tilling practices without the corresponding increase in the use of herbicides (Cox *et al.*, 2002). Tilling is one of the contributing causes of nutrient leaching from soils with no plant cover, especially in the autumn.

Perennial crops would have the same benefits as traditional catch crops regarding nutrient conservation and no-till farming. However, all of our major grain crops are annuals, and no current perennial species produce sufficiently high yields. Despite considerable breeding efforts for perenniality in several of the major grain crops, highly productive perennial grains will surely not be developed in the near future (Cox *et al.*. 2002). Domesticating suitable wild perennial plant species may be a more feasible approach than recovering the perennial growth habit in our already heavily domesticated annual crops.

Second issue: replacing fossil oil

Our society today is completely dependent on fossil oil. This dependency, however, must come to an end. Not only a limited and non-renewable resource, fossil oil is also a major source of atmospheric pollution. Environmentally friendly and renewable oil produced by plants has the potential to replace fossil oil in many applications. Already, 10-15% of all vegetable oil produced in the world is consumed as fuel or in the oleochemical industry (Stymne, 2005). A few examples include the use of oil from rapeseed, soybean and oil palm as an additive to diesel (Durrett *et al.*, 2008), linseed oil in paint (Somerville and Bonetta, 2001) and epoxidised soybean oil as plasticiser (Pyper, 2003). Other applications, of many, where vegetable oils may replace fossil oil include also lubricants and inks (Dyer *et al.*. 2008). Vegetable oils are mainly obtained from seeds, where the oil serves as storage energy. The oil is composed of a mix of several different fatty acids, and it is the specific composition of fatty acids that determines the inherent industrial and/or nutritional value of the oil (Thelen and Ohlrogge, 2002). Vegetable oils have for many years been too expensive compared to fossil oil in most technical applications. However, the price gap has been rapidly closing the last years. Furthermore, plant biotechnology is steadily advancing, and genetically engineered optimized plant-derived oleochemicals will for sure be economically competitive in a not too distant future (Stymne, 2005).

Why *Lepidium campestre*?

Late 1980s and early 1990s

Late in the 1980s, a large number of accessions of mainly cruciferous wild species were screened and evaluated for their properties as potential perennial oilseed crops. From this screen, biennial and perennial species from the genera *Lepidium* and *Barbarea* were chosen because of their good plant type, useful oil qualities and excellent winter hardiness. Two consecutive field trials were conducted in 1992-1994 at the Swedish University of Agricultural Sciences (SLU) in Uppsala with the selected species *L. campestre*, *B. stricta*, *B. vulgaris* and *B. verna*, and seed yield, seed size, germination, winter hardiness, plant height, flowering and self-fertility were scored (Merker and Nilsson, 1995).

1994-1996

L. campestre, *B. vulgaris* and *B. verna* were then selected as the most promising candidates for domestication and a thorough analysis of seed oil content and fatty acid composition in these three species was carried out. The seed oil content of *L. campestre* was found to be 20%, with little variation. The seed oil is dominated by linolenic acid (C18:3, 34-39%) and erucic acid (C22:1, 22-25%), with significant levels also of oleic acid (C18:1, 12-16%) and linoleic acid (C18:2, 8-11%) (table 1). The seed oil content of *B. vulgaris* and *B. verna* was found to be around 30%, and the dominating fatty acid is erucic acid in both species (25-32% and >50%, respectively) with considerable levels also of oleic acid (>20% and 13%, respectively) and linoleic acid (>20% and 14%, respectively). This is a fatty acid composition that resembles that of old rapeseed varieties (Nilsson *et al.*, 1998).

1996-1997

Next, the broad chemical compositions of the three selected candidates were analysed to evaluate the technical and nutritional qualities of the seeds. All three were found to contain a lower level of oil and protein and a higher level of dietary fibre in the seeds, compared to rapeseed. Oil and protein are the desirable products of an oil crop and thus, the levels of them have to be increased in order to obtain more satisfactory cultivars. The three species have an amino acid composition suitable for human consumption. However, the high level of erucic acid in all three species, and especially in *B. verna* (50%), makes them unsuitable for food or feed. The heat stability of the long-chain erucic acid makes it suitable rather as a technical oil, e.g. in production of erucamide, a slipping agent, or as a component of lubricants. The antinutritional and/or toxic glucosinolates found in the three species are, for our aims, not of major concern since the primary goal is to develop an oil crop for industrial applications rather than direct consumption or feed (Andersson *et al.*, 1999).

1996-1999

To evaluate the potential of the three species as catch crops that could yield a commercial product the consecutive year(s), and to investigate on the technical aspects of cultivation, a series of field trials were conducted at three different locations in Sweden (Uppsala, 59°N, Kristianstad, 56°N and Svalöv, 55°N). All the three species were successfully established as catch crops sown in the spring together with barley (fig 1a). *L. campestre* and *B. verna* were harvested only the following year, whereas *B. vulgaris* produced a reasonable harvest up to three consecutive years without resowing. They were all found to be adapted to the climate of central Sweden, with very little reduction in the stands after overwintering. The effect of plant density and nitrogen supply on seed yield and seed quality parameters was investigated. At harvesting with a combine-harvester, a strong tendency to seed dispersal was observed in all three species. None of the *Barbarea* species were found to be susceptible to the pollen beetle, a severe pest in *Brassica* crops. *L. campestre* was excluded from the experiments with pollen beetle exposure since the small buds (<2 mm) makes it an inappropriate host plant for this insect (Börjesdotter, 1999).

2004-present

Of the three evaluated candidate species, *L. campestre* was eventually chosen for the proposed domestication attempt that is being advanced in this thesis. This species has a very good agronomic plant type with branching only in the upper part of the upright stems (fig 1b,c). It is a spring germinating biennial with closely related perennials. It has a winter hardiness far better than the *Brassica* oil crops and it is resistant to the pollen beetle. It also has a high seed yield and a suitable seed size (fig 1d), with seeds being about half the size of rape seeds but much bigger than the *Barbarea* seeds (Börjesdotter, 2000). The seed oil has a high level of linolenic acid (table 1), which suggests a technical quality similar to linseed oil. Self-fertilisation and diploidy are other important advantages enabling simple pure-breeding of characters. The cultivation of *L. campestre* as a biennial/perennial oil-producing catch crop undersown in a spring cereal would combine the advantages pictured in the two previous sections on environmental issues.

Fatty acid	Carbon chain length	Double bonds	Level in *L. campestre* (%)[1]	Commercial use
Oleic acid	18	1	12-16	Biofuel[2], margarine and frying oil[3]
Linoleic acid	18	2	8-11	
Linolenic acid	18	3	34-39	Paints, drying oils[4]
Eicosenoic acid	20	1	5-6	
Erucic acid	22	1	22-25	Slipping agent[5], lubricants and nylon[6]

Table 1. *Properties of five common plant fatty acids, their respective levels in L. campestre seed oil and commercial uses. [1]Nilsson et al., 1998; [2]Durrett et al., 2008; [3]Liu et al., 2002; [4]Jaworski and Cahoon, 2003; [5]Dyer et al., 2008; [6]Millar and Kunst, 1997.*

However, there are mainly four problems that have to be overcome in *L. campestre* in order to make it a suitable cultivar for the proposed cropping system:
1. The tendency to seed dispersal.
2. Modification of the fatty acid profile of the seed oil, to obtain an optimal technical quality.
3. Increasing the low oil content (20%) of the seeds. The large variation in the genus can possibly be utilised to improve this trait.
4. Extension of the biennial to a perennial state through hybridisations with perennial relatives. This is not an absolut requirement for the cropping system but would extend the environmental benefits.

Figure 1a-d. Field trials with *L. campestre* at the Lönnstorp experimental station. a) First season; emerging rosette leaves from *L. campestre* undersown in barley (4 July 2006), b) Second season; maturing *L. campestre* flowers/fruits (29 May 2007), c) Second season; stands of *L. campestre* (29 May 2007), d) Second season; harvest of *L. campestre* seeds.

Seed dispersal

The sessile nature of plants has caused them to evolve several different ways to spread their seeds. Many species in the Brassicaceae family manage this through a kind of springload mechanism, where tension in the valves causes the pods to burst and the seeds to fall out (Mummenhoff *et al.*, 2009). An advantage to wild plants, seed dispersal is nevertheless a nuisance in some domesticated plants. Resistance to seed dispersal occurred early in the Neolithic revolution for many of the common crops (Li et al., 2006). However, seed dispersal often causes severe problems for farmers cultivating some of the more recently domesticated Brassicaceae oilseed crops, e.g. with losses ranging between 10% and 50% in oilseed rape (*Brassica napus*) depending on the weather conditions (Summers et al., 2003).

Several species in the Brassicaceae family develop fruits (pods) that are composed of two fused carpels. The carpel valves (pod walls) are separated by a central lamella called septum. The replum, to

which the valves attach, is the outer margin encircling the septum. The dehiscence zone (DZ) develops along this valve-replum margin and runs along the entire length of the silique/silicle (fig 2a-c) (Spence *et al.*, 1996). There are mainly three cell layers involved in the opening of the pod: a valve layer (endocarp *b*, en*b*) and a valve margin layer that both become lignified, and a separation layer that disintegrates due to the activity of hydrolytic enzymes (fig 2c) (Ferrándiz, 2002). It is believed that the lignification indirectly causes the tension responsible for the springload mechanism in these plants and that the disintegration of the separation layer facilitates the detachment of the valves (Liljegren *et al.*, 2004).

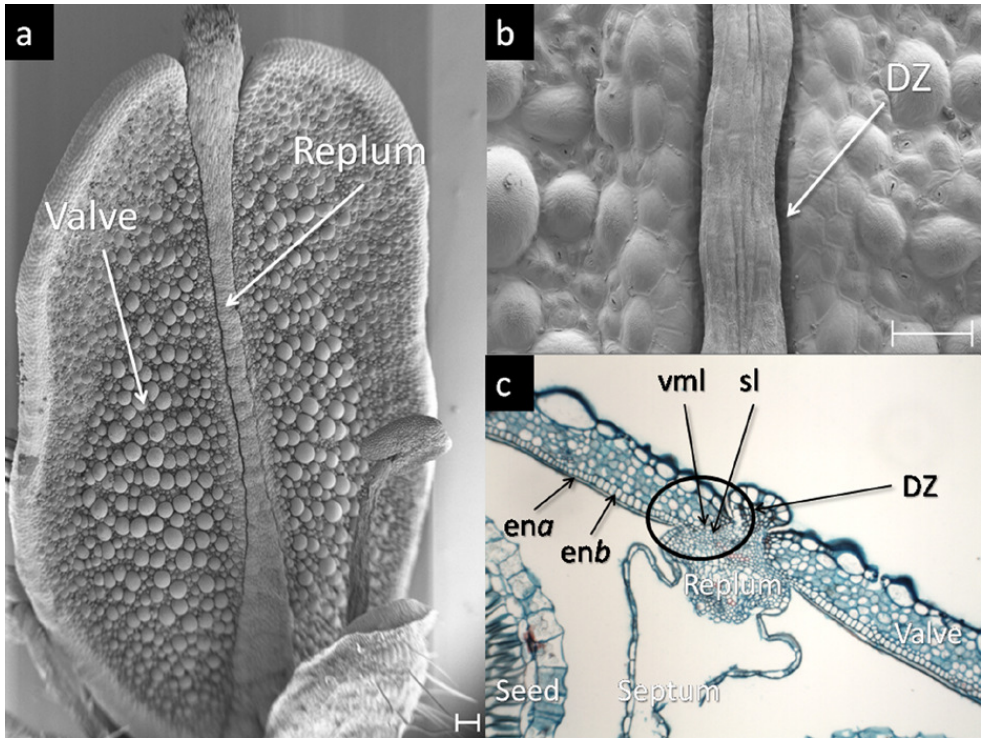


Figure 2a-c. Anatomical and histological study of the *L. campestre* fruit. a) SEM picture of the *L. campestre* fruit at post fertilisation stage 16 (Ferrándiz *et al.* 1999), b) SEM picture of the *L. campestre* fruit at post fertilisation stage 17, indicating the DZ at the valve-replum margin, c) Cross section of the *L. campestre* fruit at post fertilisation stage 16-17A, showing the distinct endocarp *a* (en*a*) and endocarp *b* (en*b*) layers in the valves as well as the valve margin layer (vml) and the separation layer (sl) in the dehiscence zone (DZ). The cross section was stained with the general stain alcian-blue and the lignin-specific red stain safranin-o. There is a tendency to lignin formation in the vascular bundle of the replum, however, the en*b* and the vml are not yet lignified. Scale bar = 100 μm.

The genetic regulation of pod shatter has been studied thoroughly in *Arabidopsis thaliana* over the last ten years. Liljegren *et al.* (2000) showed through double mutant analysis that the closely related SHATTERPROOF (SHP1) and SHATTERPROOF2 (SHP2) MADS-box transcription factors redundantly control valve margin development. The same research group also showed that the FRUITFULL (FUL) MADS-box transcription factor, necessary for valve differentiation, is a negative regulator of *SHP* expression (Ferrándiz *et al.*, 2000). Similarly, the REPLUMLESS (RPL) homeodomain protein was shown to specify replum development by negative regulation of *SHP* (Roeder *et al.*, 2003). Rajani and Sundaresan (2001) then described the myc/bHLH transcription factor ALCATRAZ (ALC) and its function to specify the separation layer. Finally, Liljegren *et al.* (2004)

identified the INDEHISCENT (IND) bHLH protein involved in specification of both cell layers in the valve margin required for pod shatter.

These discoveries have led to the establishment of a model for genetic regulation of pod shatter (fig 3), where SHP1/2 promote the expression of *ALC* in the separation layer and of *IND* in the entire valve margin. FUL and RPL negatively regulates the *SHP/IND/ALC* cluster to prevent their expression in the valves and replum, respectively, and thereby limiting their expression to the valve margin (Dinneny and Yanofsky, 2004; Østergaard, 2009). The usefulness of this model for applied crop research has been demonstrated. With the assumption that the genetic regulatory network controlling pod shatter is conserved between *Arabidopsis* and *Brassica*, Østergaard *et al.* (2006) expressed the *A. thaliana FUL* gene constitutively from a 35S promoter in *B. juncea* to produce pod shatter resistant plants. In *B. napus*, down regulation of each of the two *IND* orthologues (*Bn*1*IND* and *Bn*2*IND*) found in this species caused a complete prevention of pod shatter (Vancanneyt *et al.*, 2002).

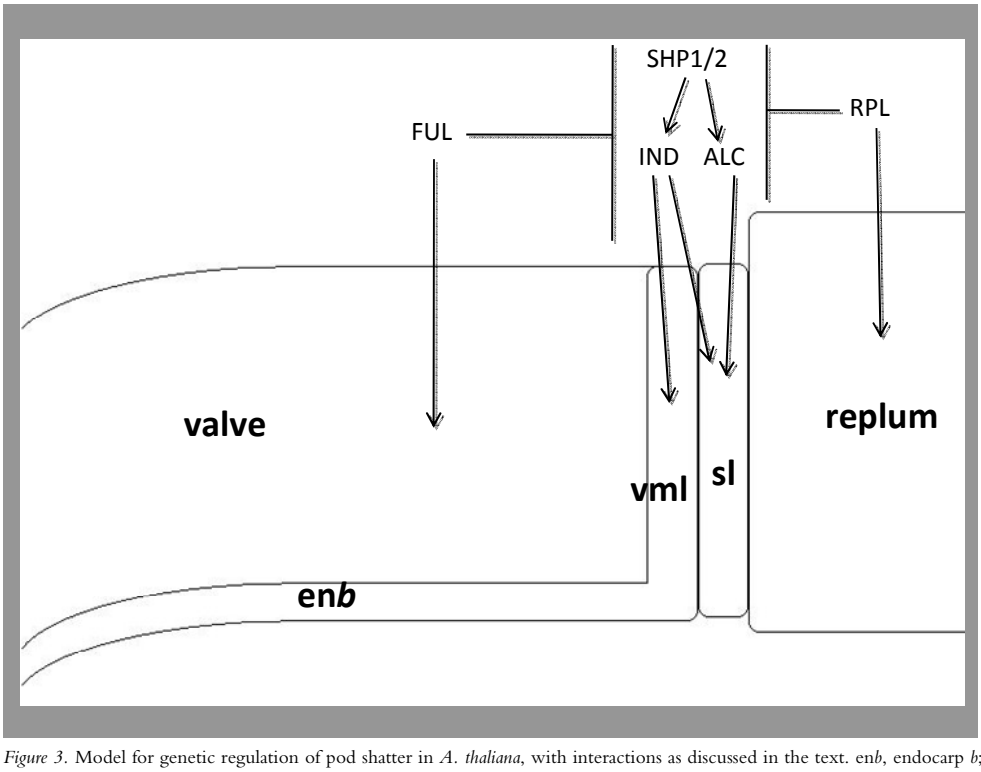


Figure 3. Model for genetic regulation of pod shatter in *A. thaliana*, with interactions as discussed in the text. en*b*, endocarp *b*; vml, valve margin layer; sl, separation layer.

Seed oil properties

More than 200 naturally occurring fatty acids have been identified in the seed oil from different plant species. These fatty acids are classified mainly according to the carbon chain length, the number and position(s) of double or triple bonds and of various functional groups, such a hydroxy or epoxy groups (Buchanan *et al.*, 2006). The five most abundant fatty acids that occur in the common oilseed crops, such as soybean, oil palm, rapeseed and sunflower, are the saturated palmitic (C16:0) and stearic acids (C18:0), the monounsaturated oleic acid (C18:1) and the polyunsaturated linoleic (C18:2) and linolenic acids (C18:3) (Singh *et al.*, 2005). Species of the Brassicaceae family, such as rapeseed and mustard, also

commonly contain high levels of the monounsaturated, very long chain erucic acid (C22:1) (Mikolajczak *et al.*, 1961).

For many industrial purposes, it is desirable to have a purified fraction of a specific fatty acid. However, to separate the different fatty acids after seed harvest and oil extraction is very expensive. It is therefore advantageous to have the plant itself producing as high level as possible of one single, industrially useful fatty acid (Murphy, 1996). The synthesis of fatty acids in the plastid, and further modifications in the cytosol, is controlled by several different enzymes (fig 4). Since the early 1990s, many of these enzymes have been cloned and characterised, and it is now possible to modify the fatty acid composition of several plant seed oils for a specific end use (Singh *et al.*, 2005). Pioneer Hi-Bred has developed a soybean cultivar, introduced to the market in 2009, with a very high level of oleic acid (>75%) that circumvents the need for hydrogenation and yields nutritionally as well as industrially superior oil (Pioneer 2009). Already in 1995, Calgene launched a rapeseed variety with a high level of lauric acid (40%) that can be used for soaps and detergents (Murphy, 1996).

Desaturation

A desaturated fatty acid has one or more double bonds in the carbon chain. The introduction of these double bonds is controlled by different fatty acid desaturases (FAD). The soluble stearoyl-ACP 9-desaturase (SAD) uses stearic acid as substrate in the plastid to introduce a double bond and produce oleic acid (Los and Murata, 1998). Much of the oleic acid is exported to the cytosol, where the endoplasmic reticulum (ER)-localised enzyme oleoyl-phosphatidylcholine(PC) Δ12-desaturase (FAD2) introduces a second double bond to form linoleic acid (Stymne and Appelqvist, 1978; Okuley *et al.*, 1994). Further desaturation is controlled by the ER-localised enzyme linoleoyl-PC Δ15-desaturase (FAD3) and produces linolenic acid (Yadav *et al.*, 1993).

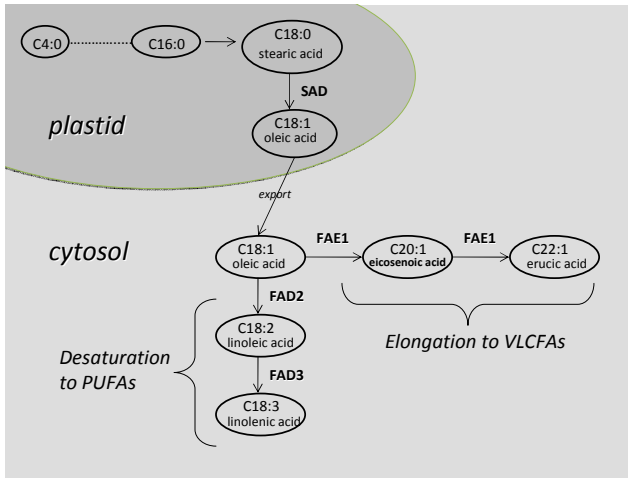


Figure 4. Simplified overview of fatty acid biosynthesis in the plastid and PUFA/VLCFA biosynthesis in the cytosol. In the plastid, SAD catalyses the introduction of a double bond in the carbon chain when stearic acid is converted to oleic acid. In the cytosol (on the endoplasmic reticulum), FAD2 catalyses the introduction of a second double bond when oleic acid is converted to linoleic acid. FAD3 catalyses the introduction of a third double bond when linoleic acid is converted to linolenic acid. FAE1 catalyses the elongation of oleic acid to eicosenoic acid and erucic acid. SAD, stearoyl-ACP desaturase; FAD2, fatty acid desaturase 2;, FAD3, fatty acid desaturase 3; FAE1, fatty acid elongase 1.

Elongation

The synthesis of very long chain fatty acids (VLCFAs) with 20 (eicosenoic acid) or 22 (erucic acid) carbon atoms in the acyl chain is catalysed by an acyl-coenzyme A (acyl-CoA) elongase complex. This is an ER-localised enzymatic complex composed of four distinct enzymes: a condensing enzyme, two reductases and one dehydrase. The initial step in the elongation reaction is the condensation of an oleoyl-CoA with a malonyl-CoA by the 3-ketoacyl-CoA synthase (KCS) (Bao *et al.*, 1998). The gene encoding the main KCS responsible for VLCFA synthesis in the storage oil of Brassicaceae seeds (Rossak *et al.*, 1997) was cloned by transposon tagging in 1995 by James *et al.* and named *FATTY ACID ELONGATION* 1 (*FAE*1).

Aims and Design of this Study

The goal of this thesis is to establish a platform for future progress in the domestication attempt of *L. campestre*. As a domestication attempt of any plant species is a huge endeavour, it was clearly necessary for me to define the limits of my work at a very early stage. I decided to focus on the two problems of seed dispersal and of modifying the fatty acid profile. The problems of perenniality and of increasing the seed oil content are outside the scope of this thesis. Also included, however, is a three-year field trial to evaluate the yield potential of *L. campestre* and the influence of the undersown *L. campestre* on the main crop.

The problems of seed dispersal and of modifying the fatty acid profile can be dealt with in at least two different ways. One way is to use transgenic technology to target specific genes that are involved in pod development and in fatty acid biosynthesis. Another approach is mutation breeding, where a very large number of plants would be mutagenised and subsequently screened for suitable traits. The relatively new method TILLING would very much facilitate the mutation approach (Colbert *et al.*, 2001). However, I decided to use the transgenic approach for a number of reasons. One reason is the ease of handling a much smaller number of plants. *L. campestre* is a relatively big plant and the mutation approach would require a very large greenhouse and/or field area. Another is that I reckoned the chance of success was higher, since the mutation approach does not guarantee that the desired gene(s) will be targeted nor manifested in a phenotype (unless an extremely high number of plants are treated). However, a drawback of the transgenic approach is that the current regulations of genetically modified organisms (GMO) in the European Union (EU) would require a long and expensive process to be granted market approval for a transgenic *L. campestre* (Jaffe, 2004). However, considering the worldwide development and adoption of GM crops, it is likely that the EU regulations will be revised and the restriction be eased well in time before *L. campestre* reaches the market.

Regeneration (Paper I) and transformation

A prerequisite for the transgenic approach is to have an established protocol for transformation and for *in vitro* tissue culture. This was therefore the very first thing I started to work on. A number of experiments were initially set up to identify the factors most important for successful shoot regeneration in *L. campestre*. After a number of factors had been identified, they were further analysed in carefully designed experiments to work out the most efficient shoot regeneration protocol for this species. For transformation of plants, a number of methods have been developed, including *Agrobacterium tumefaciens*-mediated gene transfer, microprojectile bombardment with DNA and direct DNA transfer into cells (Sharma *et al.*, 2005). I decided to focus my efforts on using *A. tumefaciens*, which is by far the most commonly used method (de la Riva *et al.*, 1998). The conditions of *A. tumefaciens* treatment would subsequently be integrated with the regeneration protocol. An alternative transformation method, called floral dipping, that avoids *in vitro* tissue culture has been developed for a number of plant species, including *A. thaliana* and *B. rapa* (Grabowska and Filipecki, 2004). I also decided to evaluate if this method would be possible to apply on *L. campestre*.

Pod shatter-resistance (Paper II)

The mechanism and genetic control of pod shatter has been studied thoroughly in the Brassicaceae model species *A. thaliana* as well as in the common crop plant *B. napus* (rapeseed). As the fruit of *L. campestre* is similar in architecture to *A. thaliana* and *B. napus*, my hypothesis was that the genetic control of pod shatter would also be similar. Taking advantage of the sequence information from *A. thaliana* and *B. napus*, I then decided to intent to clone from *L. campestre* the ortholog of *INDEHISCENT* (*IND*), which is one of the central genetic factors controlling pod shatter. This clone would subsequently be used to make an RNAi (RNA interference) construct that, after transformation into *L. campestre*, would down regulate the function of *IND*, producing pod shatter-resistant plants.

Modification of the seed oil quality (Paper III)

For modification of the seed oil quality, I followed the same approach as for pod shatter-resistance. Taking advantage of the vast information accumulated on fatty acid biosynthesis in oilseed plants, I decided to clone *L. campestre* genes coding for two main factors in the production of polyunsaturated fatty acids (PUFAs) and very long chain fatty acids (VLCFAs). PUFAs and VLCFAs are very important quality traits in commercial plant oil products and I hope that my approach will enable the future breeding of a commercially interesting *L. campestre* cultivar.

Field trials (Paper IV)

The efforts of Börjesdotter and her collegues (Börjesdotter, 1999) were followed up with a three-year field trial of *L. campestre* to evaluate the potential seed yield and to study the effect of an undersown *L. campestre* on the yield of barley as the main crop. The field trials were carried out during the years 2004-2007 at the Lönnstorp experimental station located at Global Positioning System (GPS) coordinates N55°40´9´´ E13°6´10´´. *L. campestre* and barley seeds were sown at the same time in a randomized block design with three treatments: no undersowing, long *L. campestre* row distance (25 cm) and short *L. campestre* row distance (12.5 cm). The barley was harvested in August the same year and *L. campestre* was harvested in July or August the following year. The harvested plot size was 2.75 m^2 for *L. campestre* and 4.8 m^2 for the barley. Besides yield, a number of other factors were measured, such as thousand-grain/thousand-seed weight, straw/stalk stiffness and stand of both species as well as flowering period and plant height of *L. campestre*. These field trials give valuable information on the performance of *L. campestre* as a catch crop undersown in a spring cereal.

Results and Discussion

Micropropagation (Paper I)

In vitro regeneration protocols as well as transformation protocols have been established for several species in the Brassicaceae family. However, the variation in response to various treatments is very big between different species (Poulsen, 1996), and it is therefore difficult to draw any general conclusions. In order to identify the factors most important for shoot regeneration (SR) of *L. campestre*, a series of initial experiments were therefore performed. One of these experiments indicated that cotyledons are the most regenerative explant type in this plant species. Hypocotyls completely failed to regenerate any shoots whereas true leaves had a considerably lower regeneration frequency. It was also determined that younger explants tended to have a higher SR frequency, and thus the cotyledons were excised at the earliest practical stage, which was after five days of germination. One thing which was concluded early on was that induction of cell division and callus formation on the strong auxin 2,4-D was an absolute requirement to achieve adventitious SR. Another observation was that wounding of the tissue before the subculture on 2,4-D seemed to further enhance the SR response, probably because of the increase in callus formation on the cut surfaces. After the initial experiments I decided to more carefully evaluate the following factors: callus induction with 2,4-D, plant growth regulators (PGR), and a temporary desiccation treatment. Finally, the five available accessions were compared using the optimized adventitious SR protocol.

Callus induction with 2,4-D

Cotyledons were subject to a preculture on callus induction medium (CIM) containing 2,4-D for three, six or nine days, or no preculture, before transfer to shoot induction medium (SIM). The treatment without preculture on CIM completely failed to regenerate any shoots. There was a small but statistically significant tendency of increased SR when the length of CIM culture was extended from 6 days to 9 days, with an SR average of 48.8% and 60.0%, respectively (fig 5a). However, the regeneration after 9 days of subculture on 2,4-D was not significantly better than after 3 days. There were no statistically significant differences between the subculture regarding the average number of shoots per regenerating explant. The nearly equal SR response already after 3 days motivated an attempt to minimize the 2,4-D subculture since this strong auxin is also known to cause mutations at high exposure (Mullison, 1982). Another reason to minimize the exposure to 2,4-D is the relatively long half-life of this auxin.

Defining shoot induction medium

In the first experiment, designed to simultaneously evaluate the 2,4-D subculture and various PGRs, I saw no statistically significant difference between any of the SIM, neither for SR frequency nor number of shoots per regenerating explant. A wide range of zeatin from 0 to 3 mg l-1 was tried in the following experiment. No statistically significant difference in SR frequency was found between any of

the zeatin concentrations in the SIM, with frequencies ranging from 40.0% on 2 mg l-1 zeatin to 55.8% on 0.5 mg l-1 zeatin. Even a medium which contained no PGR at all showed a SR frequency of 51.7% (fig 5b). The lack of a clear response in my experiments with various combinations of PGRs in the SIM is intriguing. Regeneration protocols for species within the Brassicaceae family most often use a combination of cytokinins (especially BAP, zeatin or kinetin) and auxins (especially NAA, IAA or 2,4-D) to induce shoot regeneration (Ullah *et al.*, 2004; Christey and Earle, 1991; Feldman and Marks, 1986; Hadfi and Batschauer, 1994; Pande *et al.*, 2002). Normally the cytokinins that stimulate cell division are particularly important in this respect. An explanation to my result might be that there is residual 2,4-D in the calluses from the subculture and this overshadows the effect of the cytokinins and auxins in the SIM.

Desiccation treatment

A desiccation treatment, where the explants after two weeks on SIM simply were put on empty Petri dishes, had a very clear and statistically significant effect on the adventitious shoot regeneration frequency. The average frequency had its maximum of 68.3% after 2 days of desiccation, but after 3 days the average frequency decreased again to 52.5% (fig 5c). Apparently, the tissues were damaged severely by a prolonged period of desiccation. This stress-induced response has previously been shown in carrot (*Daucus carota*) where production of somatic embryos from callus cultures was increased up to 20-fold after a similar desiccation treatment. Further experiments with carrot indicated that this stress-induced increase in somatic embryogenesis was due both to the withdrawal of nutrients, and especially sucrose, as well as to the decrease in relative humidity (Lee *et al.*, 2001). However, increased somatic embryogenesis in carrot has been shown also on high levels of sucrose (Kamada *et al.*, 1989). What is not clear in my study is if the stress response is caused mainly by the loss of water or by the withdrawal of nutrients, or a combination of both. An alternative explanation, not discussed in the study of Lee *et al.* (2001), for the adventitious shoot regeneration response might also be that the endogenous concentration of sugar in the cells increases as the tissue loses water when removed from the medium. However, this remains to be tested.

Accession

Finally, I evaluated the adventitious SR response in the five available accessions of *L. campestre*, using the optimized adventitious SR protocol. There was a statistically significant difference between some of the accessions, with Öland and Budapest having the highest SR frequency of 60.8% and 64.2%, respectively, and Arild and Bergianska having the lowest of 41.7% and 46.7%, respectively (fig 5d). There was no statistically significant difference between any of the accessions for the number of shoots per regenerating explant, with values ranging from 1.8 for Bergianska to 2.3 for Budapest.

Transformation attempts

Transient transformation

After having developed an efficient shoot regeneration protocol, the next step in my attempts to develop a transformation protocol was to evaluate certain factors in the co-cultivation procedure of *L. campestre* tissue with an *A. tumefaciens* culture. Initially I did this by a transient transformation procedure, where I co-cultivated *L. campestre* cotyledons with an *A. tumefaciens* strain carrying a GUS construct with an intron for prevention of bacterial expression. The cotyledons were screened for GUS expression six days later. The factors I evaluated were the following: 1) concentration of the bacterial inoculum, 2) duration of co-cultivation, 3) co-cultivation in temporal relation to the 2,4-D pretreatment and 4) addition to the co-cultivation

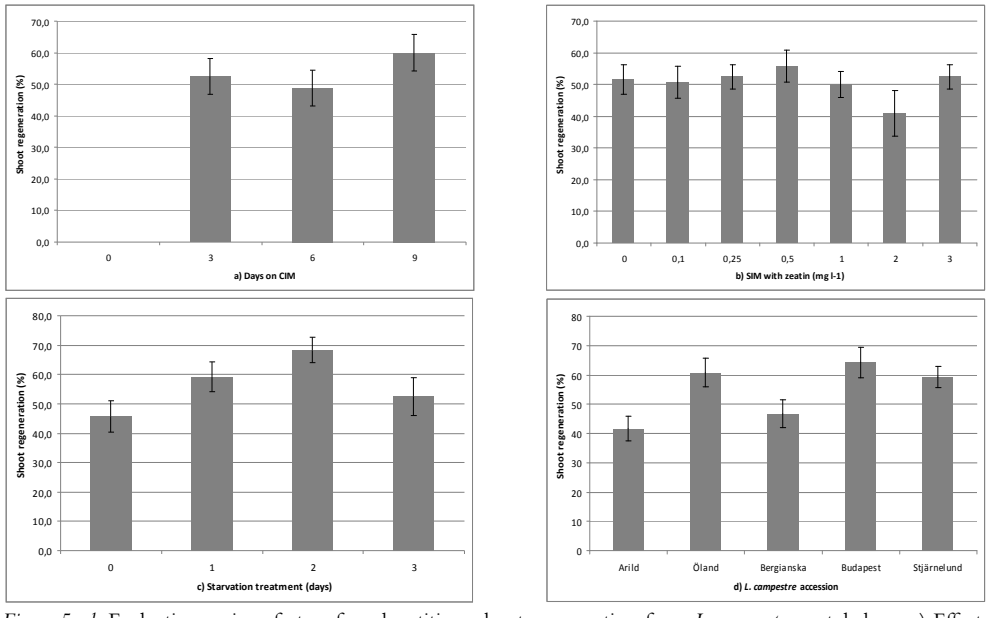


Figure 5a-d. Evaluating various factors for adventitious shoot regeneration from *L. campestre* cotyledons. a) Effect on shoot regeneration of a pretreatment on callus-inducing medium (CIM) containing 1.0 mg l-1 2,4-D. b) Effect on shoot regeneration of zeatin in the shoot regeneration medium. c) Effect of a temporary starvation treatment on shoot regeneration. d) Comparing the shoot regeneration response of five different accessions of L. campestre. Vertical bars show standard error.

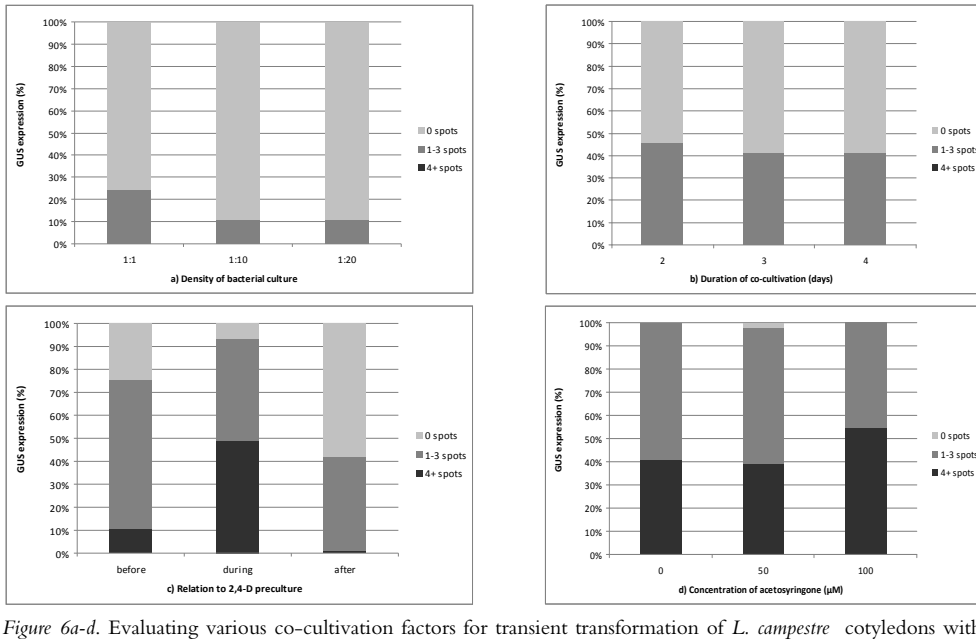


Figure 6a-d. Evaluating various co-cultivation factors for transient transformation of *L. campestre* cotyledons with *A. tumefaciens*. a) Density of bacterial culture. Undiluted at OD_{600} = 0,5 and its 10x and 20x dilutions. b) Duration of co-cultivation (2, 3 or 4 days). c) Co-cultivation before, during or after the 2,4-D preculture. d) Addition of acetosyringone at 0, 50 or 100 µM. Results are given in proportion of the 90 explants in each category of GUS expression. Light grey bar = no blue spots, dark grey bar = 1-3 blue spots, black bar = 4+ blue spots.

medium of acetosyringone, a phenolic compound known to stimulate *A. tumefaciens* virulence (Dion *et al.* 1995). The results are shown in fig 6a-d. The first experiment indicated that a higher concentration of bacterial inoculum produces a higher number of transiently transformed cells. However, it has been reported that a high concentration of *A. tumefaciens* for co-cultivation may result in necrosis of the plant tissue (Chakrabarty *et al.*, 2002), and thus caution must be taken. The second experiment showed that the duration of co-cultivation does not matter, at least not in the range of two to four days. The third experiment demonstrated that the transformation efficiency is by far the highest when co-cultivation is simultaneous with the 2,4-D pretreatment. This makes sense since the plant tissue is considered to be in a very active stage of cell division and callus growth during the treatment with 2,4-D, and thus more prone to take up foreign DNA from *A. tumefaciens*. The fourth experiment showed that addition of acetosyringone has little effect on the transient transformation efficiency.

Combining the regeneration protocol with co-cultivation treatments

The next step was the use the information obtained in the transient transformation experiments to integrate the co-cultivation procedure with the shoot regeneration protocol to produce stably transformed *L. campestre* shoots, using the same GUS construct. A number of factors were evaluated in a series of eight experiments, including the following: 1) *A. tumefaciens* strain, 2) concentration of the bacterial inoculum, 3) length of submersion of plant tissue in the bacterial inoculum, 4) length of co-cultivation and 5) *L. campestre* accession, with combinations as seen in table 2. However, all of these attempts failed to regenerate a single shoot from the treated cotyledon batches. Control batches without submersion, or submersion in water only, always regenerated shoots in a regular fashion. This indicates that the *L. campestre* cotyledon cells are very sensitive to infection by *A. tumefaciens*. Further work is of outmost importance to investigate ways to overcome this obstacle.

Exp #	Agro strain	Agro conc (OD^{600})	Agro subm	Dry on filter	Co-cultivation	Lep acc
1	EHA101	0,70	30 min	no	3 days	Öland
2	EHA101 + GV3850	0,70	30 min	no	2 days	Öland
3	EHA105 + GV3850	0,59-0,67	20 min	no	3 days	Öland
4	EHA105	0,50	20 min	yes	3 days	Öland
5	EHA105	0,75	1 sec-1 min-5 min	yes	6 days	Öland
6	EHA101	0,05-0,10-0,20-0,30	5 min	yes	3 days	Öland
7	EHA105	0,20	3-5 sec	yes	2-3-6 days	all five
8	EHA105	0,20	3-5 sec	yes	1-2-3-4 days	Öland

Table 2. *Evaluating various factors for transformation of L. campestre cotyledons with A. tumefaciens.*

Floral dipping

Floral dipping is a very elegant transformation method that was first demonstrated in *A. thaliana* (Clough and Bent, 1998) and has since been applied to a number of other plant species, such as radish (Curtis and Nam, 2001), pakchoi (Qing *et al.*, 2000), peanut (Rohini and Rao, 2000) and cotton (Li *et al.*, 2004). This method avoids tissue culture by simply dipping the inflorescences of a plant into an *A. tumefaciens* culture. Any bacterial infection occurring in the germline cells, or more specifically the ovules, will produce a stably transformed plant in the next generation (Desfeux *et al.*, 2000). The factors that are the most important for successful transformation via floral dipping is the timing of infection, the addition of a surfactant to facilitate the penetration of the bacterial solution and the application of vacuum at the time of infection (Grabowska and Filipecki, 2004).

I decided to do a preliminary attempt at floral dipping of *L. campestre*. Using a construct with a kanamycin resistance gene in *A. tumefaciens* strain EHA101, I first evaluated the timing of infection and the addition of a surfactant. Plants of three different developmental stages were dipped twice, with seven days interval. Silwet-77 was added to the bacterial culture at a concentration of 0.02% or 0.05%. Six plants were dipped for each treatment. No indication of Silwet sensitivity was observed in any of

the plants. 100 seeds from each plant (in total 3600 seeds) were later screened on selective medium, however, not a single putative transformant was identified. In the second experiment, I used two different strains of *A. tumefaciens* (EHA105 and GV3820) and the Silwet-77 concentration was 0.05%. Four plants for each bacterial strain were dipped a total number of six times, with seven days interval, in order to cover the entire floral development period and to increase the chance of targeting a potential "window of opportunity" for infection of the ovules. After screening of the T1 seeds, 14 putative transformants were identified. However, none of these were confirmed by PCR to be transformed, and it is likely that they were escapes on the selection medium. It would certainly be very informative to do a careful study of gynoecium development in *L. campestre*. It might be that the nature of the gynoecium structure in *L. campestre* prevents any bacterial infection in the ovule, and thereby making transformation by floral dipping a great deal more difficult.

Though somewhat disappointing, it is nevertheless not surprising that I did not manage to develop a complete transformation protocol for *L. campestre* within my PhD project. Even if some plant species, like e.g. *A. thaliana*, are extremely easy to transform (Akama *et al.*, 1992), most other plant species are painstakingly difficult to transform and it may often take several years of laborious research to develop an efficient protocol for a new species (Sharma *et al.*, 2005).

Anatomy of the *Lepidium campestre* fruit (Paper II)

The *L. campestre* fruit (fig 2a) is a silicle with two fused, round carpel valves enclosing the two seeds that are attached to the septum. Microscopy analysis of the valve margin revealed a cellular structure similar to that of *A. thaliana* and *B. napus* siliques described by Spence *et al.* (1996). The carpel valves consist of seven cell layers: the outer epidermis, four intermediate layers of mesophyll tissue and two endoderm layers (en*a* and en*b*) (fig 2c). Staining with safranin-o of *L. campestre* fruits at post fertilisation stage 18, according to the division of fruit developmental stages proposed by Ferrándiz *et al.* (1999), demonstrated the lignification of the en*b* layer as well as the valve margin layer in the DZ (fig 7). The differentiation of the en*a* layer resembles that of *B. napus* fruits. The en*a* cell layer in *A. thaliana* disintegrates completely as the fruit matures, whereas a thickened primary wall lining the fruit locule remains in *B. napus* as the en*a* cells collapse (Spence *et al.*, 1996). These remaining cell walls of en*a* are seen also in the maturing fruits of *L. campestre* (fig 2c, 7). Interestingly, it appears as if also the en*a* of *L. campestre* becomes lignified (fig 7). This is something not observed in neither *B. napus* nor *B. juncea* (Spence *et al.*, 1996).

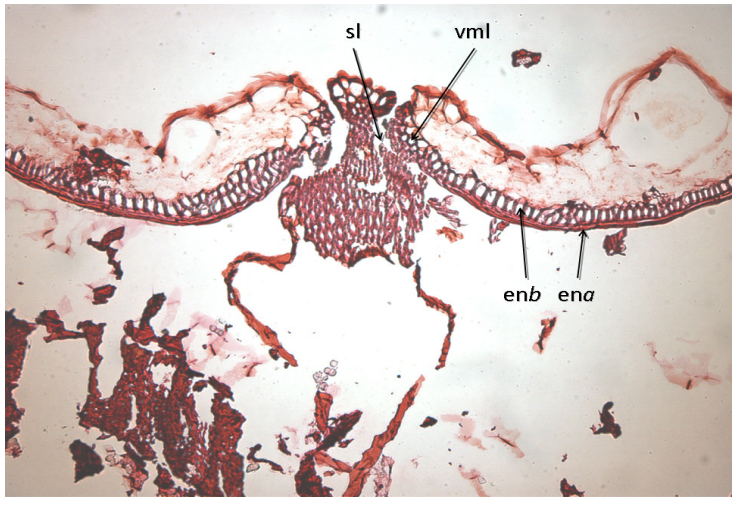


Figure 7. Cross section of *L. campestre* fruit at post fertilization stage 18-19. The cross section was stained with the lignin-specific red stain safranin-o. There is lignification of the cell walls of the slightly elongated en*b* cells and of the valve margin layer (vlm) all the way up to where it meets the exocarp, as well as lignification of the remaining en*a* layer. The separation layer (sl) has already disintegrated to some extent.

Towards pod shatter-resistance (Paper II)

Considering the similarity of *L. campestre* to *Arabidopsis* and *Brassica* in valve margin structure and the presence of a DZ in *L. campestre*, it is likely that the genetic regulation of pod shatter is conserved also in this species. With the aim of generating pod shatter-resistant *L. campestre*, I therefore attempted to clone the orthologous *LcIND* gene in order to down regulate its function through RNA interference (RNAi), a technique commonly used for various applications in basic research and crop improvement (Galun, 2005).

Cloning of LcIND

From the sequences of *A. thaliana IND* (*AtIND*, NCBI accession number NM_116229) and *B.napus IND* genes, I designed degenerate primers with which I amplified a 290 bp fragment from *L. campestre* genomic DNA. From this fragment I used the genome walking method LaNe RAGE (Park, 2005) to amplify fragments covering the entire putative *LcIND* transcriptional region. The *LcIND* ORF is 531 nt, contains no introns and shows 79% homology in the translated amino acid sequence to the AtIND. RACE analysis later confirmed a 43 nt 5´-UTR and a 324 nt 3´-UTR. The *LcIND* sequence has been submitted to the NCBI genbank with accession number FJ907544. RT-PCR analysis of *L. campestre* wildtype (WT) fruits at the approximate developmental stages 13 (at anthesis), 16 (small, green fruits with sepals and petals starting to fall) and 17 (mature, full-size but still green fruits) as well as of tissue from leaves and roots confirmed that *LcIND* is expressed mainly in all the examined stages of developing fruits and not in the other examined parts of the plant.

Functional complementation of Arabidopsis ind mutant

The high sequence homology between the putative *LcIND* and the *AtIND* indicates that *LcIND* may be able to perform its function in an *A. thaliana* genetic background. This is a reasonable assumption, since the *Bn1IND* and *Bn2IND* from *B. napus* have both been used to down regulate the IND function and produce pod shatter-resistant *A. thaliana* (Vancanneyt *et al.*, 2002) and the *A. thaliana FUL* has been shown to work in a *B. juncea* background (Østergaard *et al.*, 2006). To confirm the function of the putative *LcIND*, I therefore designed a construct, named indr, to complement the IND function in an *A. thaliana* loss-of-funtion *ind* mutant (*Atind*) exhibiting a complete loss of pod shatter. The full-length *LcIND* ORF was cloned into the Gateway overexpression vector pK2GW7 and used to transform *Atind*. 15 independent transformed indr T2 lines were generated. Phenotypic observations confirmed that six of them (40%) had completely restored the pod shatter-sensitive phenotype. The other nine lines still retained the pod shatter-resistant phenotype to varying degrees. Three of the pod shatter-sensitive lines (3-1, 18-1 and 30-8) were subject to microscopy analysis. Whereas the *Atind* lacks a clearly defined separation layer in the DZ, staining and light microscopy (LM) of the indr lines showed the recovery of such a separation layer (fig 8a,b). The indr lines more resembled the WT in this respect (fig 8b,c). Scanning electron microscopy (SEM) also showed the lack of a distinct DZ in the *Atind* but the complete restoration of such a DZ in the examined indr lines (fig 9a,b).

Down regulation of Arabidopsis IND

To examine if the putative *LcIND* also can be used to down regulate the IND function, I designed an RNAi construct, named indk, for transformation of WT *A. thaliana*. I targeted a 242 nt region with relatively high homology between the *LcIND* and the *AtIND*, avoiding the conserved bHLH region since this may lead to unspecific targets in the bHLH family of transcription factors being down regulated. 19 independent transformed indk T2 lines were generated. Phenotypic observations showed, however, that only two lines (2-6 and 15-3, 10.5%) displayed a strong pod shatter-resistant phenotype. The other transformed lines still retained a pod shatter-sensitive (WT) phenotype to some extent. Microscopy analysis of fruit samples from these two lines showed that they to some extent resemble the *Atind* fruit, regarding the failure to disintegrate the separation layer. However, there is still the

tendency to lignification of the valve margin layer (fig 8a,d). SEM showed the lack of a distinct DZ in the two indk lines even though there is still a tendency to a valve-replum boundary, which is almost completely absent from the *ind* mutant (fig 9a,d). In any case, the valves in the two indk lines completely failed to detach from the replum, something they do in WT *A. thaliana* (fig 9c,d). My results on down regulation of *IND* is in line with previous reports. Liljegren *et al.* (2004) reported the characterisation of various *A. thaliana ind* mutants. One of the stronger mutants (which is the same mutant that I have used in the functional complementation experiment presented here) showed no lignification in the valve margin, whereas one of the weaker, but nevertheless pod shatter-resistant,

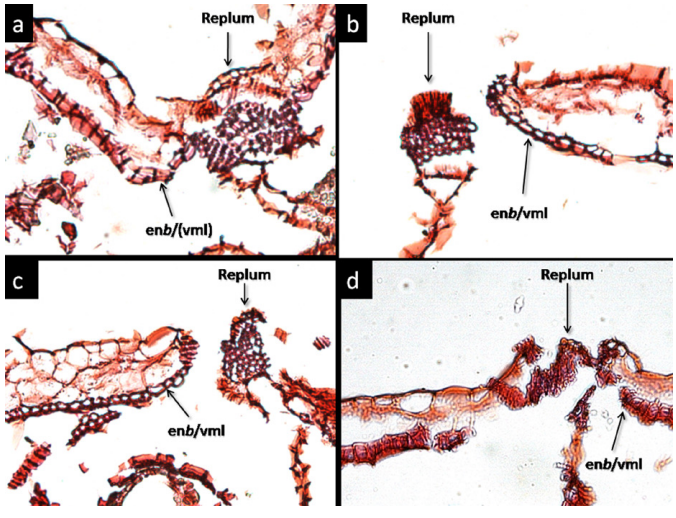


Figure 8a-d. Cross sections of *A. thaliana* fruits stained with the lignin-specific red stain safranin-o. a) *ind* mutant lacking a separation layer (sl) as well as a lignified valve margin layer (vml). The valve remains attached to the replum, b) indr (line 3-1) showing a complete recovery of the sl and the vml, allowing the valves to separate from the replum, c) wildtype showing the lignification of the vml and separation of the valve from the replum, d) indk (line 2-6) lacking a defined sl, however, there is the tendency to lignification of the vml and the valves are not as firmly attached to the replum as in the *ind* mutant.

mutants still had a margin lignification similar to WT. The RNAi method for down regulation of gene expression commonly fails to yield a complete knock-out of gene function (Stoutjesdijk *et al.*, 2002). Kerschen *et al.* (2004) suggested that each target sequence may possess an inherent degree of susceptibility to RNAi, due to factors such as expression level, sequence composition and normal RNA turnover rate. Because of this, it is not surprising that the pod shatter-resistant indk lines generated in these experiments quite resemble a weak *ind* mutant.

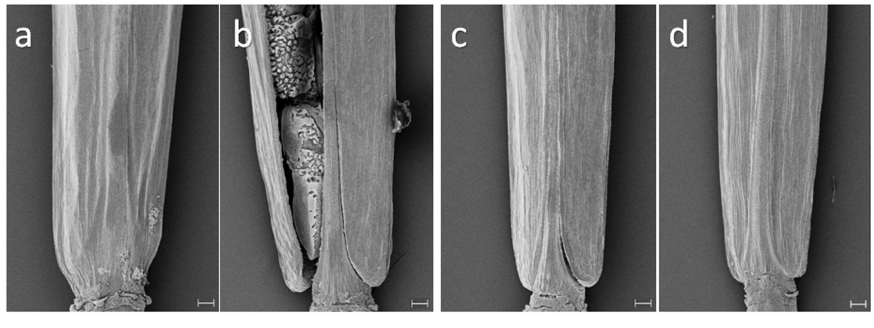


Figure 9a-d. A. thaliana fruits at post fertilisation stage 18-19. a) *ind* mutant showing a lack of a distinct dehiscence zone (DZ) and a strongly pod shatter-resistant phenotype, b) indr line 3-1 (*ind* mutant transformed with the indr construct) showing a recovery of the pod shatter-sensitive phenotype, c) wildtype (WT) with one valve starting to detach from the replum, d) indk line 15-3 (WT transformed with the indk construct) showing only a tendency to develop a DZ and displaying a pod shatter-resistant phenotype. Scale bar = 100 µm.

These results nevertheless show that the putative *LcIND* is able to restore the pod shatter-sensitive phenotype in the pod shatter-resistant *Atind* mutant, and that is an indication that LcIND performs the same function to regulate pod shatter in *L. campestre* as the AtIND in *A. thaliana*. I have also showed that the *LcIND* can be used to down regulate the IND function in an *A. thaliana* background. It is therefore very likely that the targeting of *LcIND* in *L. campestre* will generate pod shatter-resistant plants.

Towards modification of the seed oil quality (Paper III)

Cloning of L. campestre FAE1 and FAD2 cDNAs

From the *A. thaliana*, *B. napus* and *B. juncea* sequences for *FAE1* and *FAD2*, I designed degenerate primers which I used to amplify a 1324 bp putative *LcFAE1* cDNA fragment and a 1080 bp putative *LcFAD2* cDNA fragment from a *L. campestre* developing fruit RACE-ready cDNA library. A RACE-ready cDNA library (a one-tube collection of cDNAs) is a very quick and cheap method to screen for orthologs of known genes in other species. Results are obtained in only a few days, including the sequencing of candidates. The advantage compared to screening directly on genomic DNA is the possibility to narrow down on the target through careful selection of tissue for RNA extraction. The disadvantages may be difficulties in the case of low expression level of the target gene and/or to maintain the integrity of the RNA sample to produce full-length cDNAs from each transcript. Special care has to be taken also in the selection of tissue for RNA extraction and in the design of the primers, to avoid, as much as possible, amplification of closely related genes/cDNAs. Our degenerate primers always amplified multiple (2-5) candidate cDNA fragments, which then had to be cloned and sequence analysed to compare with the template orthologs. From these obtained fragments I then continued with upstream and downstream RACE analysis to obtain the entire coding sequence (CDS) for both genes. I managed to obtain fragments covering the putative start and stop codons of both genes, however only the *LcFAE1* 3´-RACE appeared to yield a complete UTR. One reason for the failure to obtain full-length 5´-UTR and 3´-UTR for *LcFAD2* and 5´-UTR for *LcFAE1* could be minor disintegration of the RNA sample used for cDNA synthesis. Another reason could be incomplete extension by the reverse transcriptase during cDNA synthesis. Subsequent sequence analysis nevertheless identified a 1521 nt putative *LcFAE1* CDS, showing 88%, 84% and 84% homology to the *AtFAE1, BnFAE1 and BjFAE1*, respectively, and a 1152 nt putative *LcFAD2* CDS, showing 91%, 85% and 84% homology to the *AtFAD2, BnFAD2 and BjFAD2*, respectively. Further sequence analysis revealed important active-site residues in *LcFAE1* and *LcFAD2* and PCR analysis showed that both genes lack introns. The *LcFAE1* and *LcFAD2* cDNA sequences have been submitted to the NCBI genbank with accession numbers FJ907545 (*LcFAE1*) and FJ907546 (*LcFAD2*). RT-PCR analysis of *L. campestre* wildtype (WT) fruits at the approximate developmental stages 13 (at anthesis), 16 (small, green fruits with sepals and petals starting to fall) and 17 (mature, full-size but still green fruits) as well as of tissue from leaves and roots confirmed that *LcFAD2* is expressed in each of the examined parts of the plant. For *LcFAE1*, I did not manage to detect any expression in any of the plant tissues examined. The reason for this peculiar failure is not known. The integrity of the RNA samples was maintained at a level sufficient to detect the *LcFAD2* transcript by RT-PCR. However, this may not be a guarantee that the transcript of *LcFAE1* has not been degraded to such an extent that the primers I used failed to amplify a fragment. The RT-PCR reaction itself did not fail, as confirmed by the positive control, and the cloning of the putative *LcFAE1* from the RACE-ready cDNA library showed that there is expression at least in developing fruits/seeds. The experiment must be repeated to clarify this matter.

The high sequence homology between the putative *LcFAE1* and *LcFAD2* and their orthologs in *A. thaliana* indicates that these *L. campestre* genes may be able to perform their function in an *A. thaliana* genetic background. To confirm the functions of the putative *LcFAE1* and *LcFAD2*, I therefore designed constructs, named fae1r and fad2r, to complement the *FAE1* and *FAD2* functions in *A.*

thaliana loss-of-function *fae1* and *fad2* mutants (*Atfae1* and *Atfad2*) exhibiting a greatly reduced synthesis of eicosenioc acid (20:1) and of linoleic acid (18:2), respectively. The full-length *LcFAE1* CDS and *LcFAD2* CDS were each cloned into the Gateway overexpression vector pK2GW7 and used to transform their respective loss-of-function *A. thaliana* mutant. 20 independent transformed fae1r T2 lines and 23 independent transformed fad2r T2 lines were generated. Seeds from all of these lines were harvested at a mature stage and 100-150 seeds, with three independent plant repetitions, of each line were subject to gas chromatography (GC) analysis to examine the fatty acid profile of the seed oil.

Expression of the LcFAE1 cDNA restores oleate elongase activity in an A. thaliana fae1 mutant line

Of seeds from the 20 fae1r T2 lines analysed by GC, seven exhibited a significant recovery of the elongated eicosenoic acid in the seed oil. However, only one line (6-1) contained wildtype level, at 16.9% of the total fatty acid content compared to the wildtype control with 15.6%. The other six lines contained between 2.9% and 5.6% eicosenoic acid, which was low but still significantly more than the *Atfae1* mutant control with 0.4%. The remaining 13 lines were not significantly different from the *Atfae1* mutant and contained between 0.3% and 2.0% eicosenoic acid (data not shown). However, only fae1r T2 line 6-1 displayed the wildtype lower levels of oleic and linoleic acid, whereas the other six lines with significant, but low, production of eicosenoic acid still showed similar-to-mutant levels of oleic and linoleic acid (fig 10). No phenotypic abnormalities were observed in any of the plants.

Fae1r line 6-1 clearly demonstrates that the *LcFAE1* CDS is able to perform the function of VLCFA production in an *A. thaliana* genetic background. However, the reason why so few (one of 20) of the transformed lines for functional complementation of the loss-of-function mutant managed to recover the wildtype fatty acid profile may be the use of a 35S promoter for expression of the *LcFAE1*. This promoter is considered to be ubiquitously expressed, however an expression study by Sunilkumar *et al.* (2002) suggested that the 35S promoter is not expressed at an early stage of the embryogenesis. For the purpose of strong gene expression in the seeds, it is therefore better to use a seed specific promoter like the commonly used napin storage protein promoter of *B. napus*, which is known to be expressed at a very high level in seeds (Ellerström *et al.*, 1996). Perhaps an even better alternative would be to clone the *LcFAE1* promoter, which is also expressed only in seeds. Rossak *et al.* (2001) argues that the *AtFAE1* promoter is superior to the napin promoter because of its higher *in vivo* activity and better timing for lipid accumulation. However, for my purpose of confirming the function of the putative *LcFAE1* clone, the use of the 35S promoter has been sufficient.

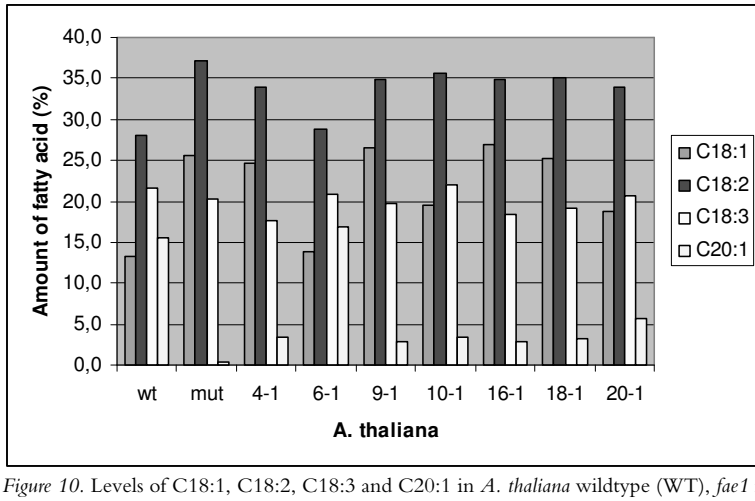


Figure 10. Levels of C18:1, C18:2, C18:3 and C20:1 in *A. thaliana* wildtype (WT), *fae1* mutant and seven fae1r lines. All of the fae1r lines display C20:1 recovery to some extent, compared to the *fae1* mutant. However, only fae1r line 6-1 displays a fatty acid profile similar to WT.

Expression of the LcFAD2 cDNA restores oleate-Δ^{12} desaturase activity in an A. thaliana fad2 mutant line

Of the seeds from 23 fad2r T2 lines analysed by GC, ten exhibited a significant recovery of the polyunsaturated linoleic and linolenic acid in the seed oil, as well as a decrease in the level of monounsaturated oleic acid. However, only two lines (8-1 and 11-1) contained wildtype levels of linoleic acid, at 27.3% and 26.3%, respectively, of the total fatty acid content compared to the wildtype control with 28.1%. The other eight lines contained between 13.9% and 23.2% linoleic acid. This was still considerably more than the *Atfad2* mutant control with 4.1% linoleic acid. The remaining 13 lines that were analysed were not significantly different from the *Atfad2* mutant and contained between 3.2% and 6.0% linoleic acid (data not shown). The lack of Δ^{12}-desaturation in the *fad2* mutant also causes higher levels of oleic and eicosenoic acid as well as a lower level of linolenic acid. In the fad2r 8-1 and 11-1 lines, the levels of oleic acid, linolenic acid and eicosenoic acid were not significantly different from wildtype, with lower levels than the mutant. All of the other eight lines with less-than-wildtype production of linoleic acid nevertheless also contained wildtype levels of linolenic acid and considerably lower levels of oleic acid and eicosenoic acid compared to the *Atfad2* mutant (fig 11). No phenotypic abnormalities were observed in any of the examined plants.

These results clearly demonstrate that the *LcFAD2* CDS is able to perform the function of PUFA production in an *A. thaliana* genetic background. The range of linoleic, oleic and eicosenoic acid levels seen in the ten lines which were significantly different from the *Atfad2* mutant is common and expected in this kind of functional complementation experiment. One of the reasons for obtaining such a range may be differences in expression levels of the introduced gene due to positional effects in the genome (Birch, 1997).

For future production of *L. campestre* transgenic lines with modified fatty acid compositions in the seed oil, it will be necessary to use a seed-specific promoter to avoid adverse effects on the overall plant development. This is especially important for downregulation of the *FAD2*. PUFAs are very important membrane components and play a crucial role to maintain membrane fluidity when plants acclimatise to lower temperatures (Nishida and Murata, 1996). Reducing the level of PUFAs in vegetative parts would make the plants extremely cold sensitive and make them less suitable for cultivation, especially at the more northern latitudes. Modifying the fatty acid content of only the storage oil in the seeds would probably not have any adverse effect on the cold tolerance of the plants.

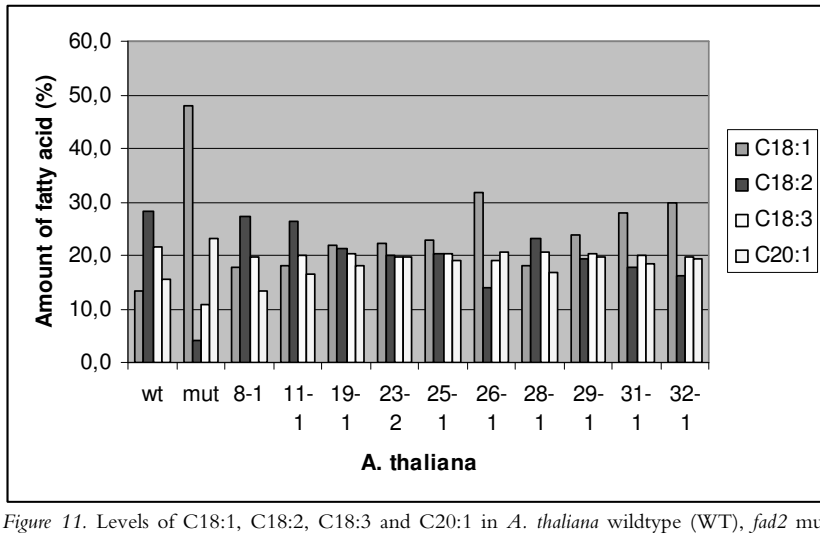


Figure 11. Levels of C18:1, C18:2, C18:3 and C20:1 in *A. thaliana* wildtype (WT), *fad2* mutant and ten fad2r lines. All of the fad2r lines display C18:2 and C18:3 recovery and also a C18:1 reduction, compared to the *fad2* mutant.

Field trials (Paper IV)

The average barley grain yield over the three years was significantly higher when *L. campestre* was undersown at the shorter row distance of 12.5 cm than without undersown *L. campestre*. The longer *L. campestre* row distance of 25 cm had no significant effect on the barley grain yield (Table 3). These results are highly interesting since many undersown legume and grass species have been reported to have a neutral or significantly negative effect on barley grain yield (Känkänen and Eriksson, 2007). To the contrary, our field trials indicated a positive effect of undersown *L. campestre* on the barley grain yield. The data presented in Paper IV do not offer any explanation, but a suggestion for future field trials is e.g. to investigate on the water and nitrogen balance in the soil.

The overall average *L. campestre* seed yield was 4848 kg/ha. However, the variation between the averages each year was very big, from as much as 6295 kg/ha in 2005 to 3614 kg/ha in 2006 (Table 4). The low yield of 2006 was reflected also in a low barley yield the same year (Table 3). It is explained by the severe weather conditions that year, with a very dry summer affecting agricultural yields negatively in the whole region. Hence, the true yield potential of *L. campestre* may be considerably higher than the close-to-five metric tons per hectare presented here as the overall average. However, some caution has to be taken in drawing conclusions from these results, since the relatively small plots may cause border effects. On the other hand, quite some shattering of seeds took place before and in connection with harvest. The measured seed yield is nevertheless extraordinary, considering that it is a wild species that has not been subject to any domestication and breeding.

Over the three years there was no significant difference in *L. campestre* seed yield between the two row distances. However, in 2006, the short row distance out-yielded the long row distance (Table 4). As a consequence of the weather conditions, the stands were not full this year (80% on average) and this probably had a more severe effect on the longer row distance. The short row distance had a better ability to compensate for uneven emergence and stand. Together with the positive yield effect on the barley, this clearly speaks in favor of the shorter row distance for sowing of *L. campestre* in this cropping system.

Undersowing	2004	2005	2006	Average
None	5651[a]	5494[a]	4062[a]	5069[a]
25 cm	5625[a]	5703[a]	4063[a]	5130[a]
12.5 cm	5755[a]	5755[a]	4479[a]	5330[b]
Average	5677	5651	4201	5176

Table 3. *Barley grain yield (kg x ha^{-1}) without undersown L. campestre, with 25-cm row distance and with 12.5-cm row distance of undersown L. campestre the years 2004-2006. Different letters in each column denote significant differences according to the Duncan test at 95% significance level.*

Row distance	2005	2006	2007	Average
25 cm	6136[a]	3182[a]	4773[a]	4697[a]
12.5 cm	6454[a]	4045[b]	4500[a]	5000[a]
Average	6295	3614	4636	4848

Table 4. *L. campestre seed yield (kg x ha^{-1}) with 25-cm and 12.5-cm row distance the years 2005-2007. Different letters in each column denote significant differences according to the Duncan test at 95% significance level.*

Conclusions and Future Prospects

The results presented in the three first papers of this thesis provide a platform for developing a pod shatter-resistant *L. campestre* cultivar that will provide a seed oil of technical quality. The fourth paper demonstrates the suitability of *L. campestre* in the proposed cropping system, with a biennial/perennial catch crop undersown in a spring cereal.

The cultivation of perennial catch crops would not only lead to the reduction in nutrient leaching described in the Background. Additional advantages include a reduction in fuel consumption due to the lower required number of sowing, harrowing and tilling, using tractor and machinery. The reduction in the impact from tractor and machinery on the soil would also generally lead to an improvement in the soil fauna (Kladivko, 2001).

The development of a new oilseed crop suited for cultivation at the more northern latitudes is also of great need. The cultivation of rapeseed (*B. napus*) is problematic in Scandinavia due to the weak winter hardiness of this species (Nilsson *et al.*, 1998). Rape turnip (*B. rapa* ssp. *oleifera*) has a better winter hardiness (Persson, 2003), but is a marginal crop due to its low yield (SCB, 2009). These Brassica cultivars have been introduced to Scandinavia from the Mediterranean region and are therefore poorly adapted to the northern climate (Merker and Nilsson, 1995). The advantage of domesticating a native plant species, such as *L. campestre*, is that the cultivar would not suffer the adaptation problems of exotic plant species. In the field trials described by Merker and Nilsson (1995), the *L. campestre* plants survived the winter without any reduction in the stands, while the *B. rapa* control had a reduction of 30%.

Thus, the intriguing question is now *when* we can expect to see the field cress growing on farm land around southern and central Scandinavia? The truth is that is very difficult to predict. In the best of circumstances, that is, if everything goes well with the further experiments and also sufficient funding is provided to take laboratory specimens through a breeding program, we can possibly expect something like 15-20 years from now. There are yet many things that have to be done.

The most urgent matter is to develop a transformation procedure. Once this has been achieved, it will be a simple feat to generate on one hand a pod shatter-resistant line and on the other hand a line with a suitable seed oil quality. These lines can subsequently be crossed to obtain the desired traits in a single line. Care must be taken, however, in the generation of a pod shatter-resistant line. It is possible that the indk construct used in the experiments on *A. thaliana* presented in this thesis will generate a line that will actually be too resistant to pod shatter. If the fruit valves close too tightly around the seeds, it may present difficulties in threshing the fruits to harvest the seeds. Probably several RNAi constructs will have to be designed that will potentially generate a quantitative range in terms of pod shatter-resistance. These can later be screened to find an optimal balance between field resistance to seed dispersal and ease of breaking the pods to harvest the seeds. Once the transgenic *L. campestre* lines, that contain all the desired traits, have been generated it is of course also necessary to run several repeated field trials to check the sustainability of the modified traits.

As described in the Background, it is also necessary to increase the oil content of the seeds to make *L. campestre* a commercially interesting alternative for the farmers. This can possibly be done through a

simple mass selection procedure. Most of the seeds have a thick seed coat with a dark brown colour (fig 1d), indicating a high proportion of dietary fibre (Merker *et al.*, 2009). However, there is also a lot of variation with many seeds displaying a more light brown to reddish colour. The lighter colour indicates a thinner seed coat, which in turn should be an indication of a higher proportion of oil to fiber in the seed (Chen *et al.*, 1988). Selection on these seeds would possibly yield an improved *L. campestre* line with higher seed oil content.

Another thing which is highly desirable is to introduce perenniality in *L. campestre*, as mentioned in the Background. This would extend the advantages of the proposed cropping system. *L. campestre* has several close relatives which are perennial and crossing with these could potentially yield a perennial *L. campestre*. At our department we have already performed a number of interspecific hybridisations between *L. campestre* and its perennial relatives *L. hirtum* and *L. heterophyllum*, and several seeds have been recovered from these hybridisations. Due to the nature of the trait though, it will take at least three seasons to evaluate the material in the field.

Further field trials are also important to give information on effective measures for weed management as well as the susceptibility of *L. campestre* to various pests and diseases. In the field trials conducted by Merker and Nilsson (1995) it was observed that the pollen beetle (*Meligethes aeneus*), which is an important insect pest in Brassica oilseed crops, is attracted to the inflorescences of *L. campestre* but does not cause any damage to the buds. Börjesdotter (1999) speculates that the reason for this is the small buds (<2 mm) making it an inappropriate host plant for this insect. In a field trial in Uppsala, club root disease (*Plasmodiophora brassicae*), vascular wilt (*Verticillium longisporum*), black spot (*Marssonina rosae*), root rot, a species of weevil (*Ceutorhynchus sulcicollis*) and flea beetle were all found on *L. campestre* (Börjesdotter, 2000). Future field trials may also focus on water and nitrogen balance, in order to get some clues to the observed positive effect of undersown *L. campestre* on the main cereal crop.

Provided that sufficient funding can be obtained, it may also be a good idea to follow an induced mutation procedure, in parallel to the transformation procedure, for the improvement of pod shatter, seed oil quality and seed oil content. For perenniality though, this approach is unlikely to be successful, given that this complex trait is often influenced by several genes (Cox *et al.*, 2002; Hu *et al.*, 2003). Mutation breeding has the advantage of being an uncontroversial method for plant breeding that has been used for the production of more than 2700 commercial crop varieties from 170 different plant species (IAEA, 2009). The method of genetic transformation, commonly called genetic modification (GM), has been subject to much debate since the advent of this technique in the 1980s and many environmental organisations have expressed their concerns regarding the use of GM on crop plants (Greenpeace, 2009; Friends of the Earth, 2009). The generation of *L. campestre* lines through mutation breeding might generate less controversy and also facilitate market approval of future *L. campestre* cultivars. Some initial work has already been carried out at our department by a Master student, and an EMS-treated (at LD^{50}) M1 population of 466 plants has been generated (Halder, 2007). Pod shatter-resistance would be an easy trait to screen for in the field. TILLING (Colbert *et al.*, 2001) would also be an alternative screening procedure for both the pod shatter-resistance and for the seed oil quality, now that I have cloned the corresponding genes for these traits.

While improving these traits in the laboratory and in field trials, it is of outmost importance to develop contacts with the plant breeding industry. Marketing of a *L. campestre* cultivar is not the business of an academic department. Even so, there are many obstacles to overcome before a *L. campestre* cultivar can be available on the seed market. First of all, there has to be a steady market for the product, the seed oil. Chemical manufacturers may be reluctant to venture into new raw materials, especially if the supply cannot be guaranteed. Moreover, farmers, who often struggle to make ends meet, tend to be somewhat conservative when it comes to trying new crops (Princen, 1979). It is also difficult to predict e.g. pest and disease resistance in a new crop that have not yet been cultivated on a large scale.

References

Akama K, Shiraishi H, Ohta S, Nakamura K, Okada K, Shimura Y. 1992. Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. *Plant Cell Rep* 12: 7-11.

Andersson AAM, Merker A, Nilsson P, Sorensen H, Åman P. 1999. Chemical composition of the potential new oilseed crops Barbarea vulgaris, Barbarea verna and Lepidium campestre. *J Sci Food Agric* 79: 179-186.

Bao X, Pollard M, Ohlrogge J. 1998. The biosynthesis of erucic acid in developing embryos of *Brassica rapa*. *Plant Physiol* 118: 183-190.

Birch RG. 1997. Plant transformation: Problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol* 48: 297-326.

Buchanan BB, Gruissem W, Jones RL. 2006. *Biochemistry & molecular biology of plants*, sixth impression. American Society of Plant Biologists: Craft Print Int. Ltd., Singapore. ISBN 0-943088-39-9.

Börjesdotter D. 1999. Potential oil crops, cultivation of *Barbarea verna*, *Barbarea vulgaris* and *Lepidium campestre*. *PhD thesis*, SLU.

Börjesdotter D. 2000. Oljehaltiga fånggrödor – vårgyllen, sommargyllen och fältkrassing. *Fakta Jordbruk* 9, SLU Publikationstjänst.

Chakrabarty R, Viswakarma N, Bhat SR, Kirti PB, Singh BD, Chopra VL. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J Biosci* 27(5): 495-502.

Chen BY, Heneen WK, Jönsson R. 1988. Inheritance of seed colour in *Brassica campestris* L. and breeding for yellow-seeded *B. napus* L.. *Euphytica* 59: 157-163.

Christey MC, Earle ED. 1991. Regeneration of *Brassica oleracea* from peduncle explants. *Horticultural Science* 26: 1069-1072.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* 16(6): 735-743.

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S. 2001. High-throughput screening for induced point mutations. *Plant Physiol* 126: 480-484.

Cox TS, Bender M, Picone C, Van Tassel DL, Holland JB, Brummer EC, Zoeller BE, Paterson AH, Jackson W. 2002. Breeding perennial grain crops. *Critical Reviews in Plant Sciences* 21(2): 59-91.

Curtis IS, Nam HG. 2001. Transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey. by floral-dip method – plant development and surfactant are important in optimizing transformation efficiency. *Transgenic Res* 10: 363-371.

de la Riva GA, González-Cabrera J, Vázquez-Padrón R, Ayra-Pardo C. 1998. *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electronic J. of Biotech.* 1(3): 118-133.

Desfeux C, Clough SJ, Bent AF. 2000. Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the Arabidopsis floral-dip method. *Plant Physiol* 123: 895-904.

Dinneny JR and Yanofsky MF. 2004. Drawing lines and borders: how the dehiscent fruit of *Arabidopsis* is patterned. *BioEssays* 27: 42-49.

Dion P, Baclanger C, Xu D and Mohammadi M. 1995. Effect of acetosyringone on growth and oncogenic potential of *Agrobacterium tumefaciens*. *Meth in Mol Biol* 44: 37-45.

Durrett T, Benning C and Ohlrogge JB. 2008. Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J* 54: 593-607.

Dyer JM, Stymne S, Green AG, Carlsson AS. 2008. High-value oils from plants. *Plant J* 54: 640-655.

Ellerström M, Stålberg K, Ezcurra I, Rask L. 1996. Functional dissection of a napin gene promoter: identification of promoter elements required for embryo and endosperm-specific transcription. *Plant Mol Biol* 32(6): 1019-1027.

Eriksson D, Merker A. 2009. An efficient adventitious shoot regeneration protocol for *Lepidium campestre* (L.) R. BR. *Prop Ornam Plants* 9(2): 78-83.

Feldman KA, Marks MD. 1986.. Rapid and efficient regeneration of plants from explants of *Arabidopsis thaliana*. *Plant Science* 47: 63-69.

Ferrándiz C, Pelaz S, Yanofsky MF. 1999. Control of carpel and fruit development in Arabidopsis. *Annu Rev Biochem* 68: 321-354.

Ferrándiz C, Liljegren SJ, Yanofsky MF. 2000. Negative regulation of the *SHATTERPROOF* genes by FRUITFULL during *Arabidopsis* fruit development. *Science* 289: 436-438.

Ferrándiz C. 2002. Regulation of fruit dehiscence in *Arabidopsis*. *J Exp Bot* 53(377): 2031-2038.

Friends of the Earth. 2009. Resisting GMOs. http://www.foei.org/en/what-we-do/gmos (2009-08-27).

Galun E. 2005. RNA silencing in plants. *In Vitro Cell Dev Biol – Plant* 41: 113-123.

Grabowska A, Filipecki M. 2004. Infiltration with *Agrobacterium* – the method for stable transformation avoiding tissue culture. *Acta Physiol Plant* 26(4): 451-458.

Greenpeace. 2009. Say no to genetic engineering. http://www.greenpeace.org/international/campaigns/genetic-engineering (2009-08-27).

Hadfi K, Batschauer A. 1994. *Agrobacterium*-mediated transformation of white mustard (*Sinapis alba* L.) and regeneration of transgenic plants. *Plant Cell Rep* 13: 130-134.

Halder AC. 2007. Induction of mutations in *Lepidium campestre*. *MSc thesis*, Dept of Crop Science, Swedish University of Agricultural Sciences, Alnarp, Sweden.

Hu FY, Tao DY, Sacks E, Fu BY, Xu P, Li J, Yang Y, McNally K, Khush GS, Paterson AH. 2003. Convergent evolution of perenniality in rice and sorghum. *PNAS* 100(7): 4050-4054.

IAEA. 2009. Plant breeding and genetics. http://www.naweb.iaea.org/nafa/pbg/index.html (2009-09-02).

Jaffe G. 2004. Regulating transgenic crops: a comparative analysis of different regulatory processes. *Transgenic Res* 13: 5-19.

Jaworski J, Cahoon EB. 2003. Industrial oils from transgenic plants. *Curr Op in Plant Biol* 6: 178-184.

Jensen ES. 1991. Nitrogen accumulation and residual effects on nitrogen catch crops. *Acta Agric Scand* 41: 333-344.

Johnsson H, Larsson M, Lindsjö A, Mårtensson K, Persson K, Torstensson G. 2008. Läckage av näringsämnen från svensk åkermark. *Naturvårdsverket*, rapport 5823.

Joubés J, Raffaele S, Bourdenx B, Garcia C, Laroche-Traineu J, Moreau P, Domergue F, Lessire R. 2008. The VLCFA elongase gene family in Arabidopsis thaliana: phylogenetic analysis, 3D modelling and expression profiling. *Plant Mol Biol* 67: 547-566.

Kerschen A, Napoli CA, Jorgensen RA, Muller AE. 2004. Effectiveness of RNA interference in transgenic plants. *FEBS letters* 566: 223-228.

Kladivko EJ. 2001. Tillage systems and soil ecology. *Soil and Tillage Research* 61(1-2): 61-76.

Känkänen H, Eriksson C. 2007. Effects of undersown crops on soil mineral N and grain yield of spring barley. *Europ J Agronomy* 27: 25-34.

Li X, Wang XD, Zhao X, Dutt Y. 2004. Improvement of cotton fiber quality by transforming the *acsA* and *acsB* genes into *Gossypium hirsutum* L. by means of vacuum infiltration. *Plant Cell Rep* 22: 691-697.

Li C, Zhou A, Sang T. 2006. Rice domestication by reducing shattering. *Science* 311: 1936-1939.

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF. 2000., SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404: 766-770

Liljegren SJ, Roeder AHK, Kempin SA, Gremski K, Østergaard L, Guimil S, Reyes DK, Yanofsky MF. 2004., Control of fruit patterning in Arabidopsis by INDEHISCENT. *Cell* 116: 843-853

Liu Q, Singh SP, Green AG. 2002. High-stearic and high-oleic cottonseed oils produced by hairpin RNA-mediated post-transcriptional gene silencing. *Plant Physiol* 129: 1732-1743.

Los DA, Murata N. 1998. Structure and expression of fatty acid desaturases. *Biochim et Biophys Acta* 1394: 3-15.

Merker A, Nilsson P. 1995. Some oil crop properties in wild *Barbarea* and *Lepidium* species. *Swedish J Agric Res* 25: 173-178.

Merker A, Eriksson D, Bertholdsson N-O. 2009. Barley yield increases with under-sown *Lepidium campestre*. *Acta Agric Scand Section B – Soil and Plant Sci*, published online 13 July.

Mikolajczak KL, Miwa TK, Earle FR, Wolff IA. 1961. Search for new industrial oils. V. Oils of Cruciferae. *The J of the Am Oil Chem Soc* 38: 678-681.

Millar AA, Kunst L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal* 12(1): 121-131.

Mullison WR. 1982.. A review of the plant physiological effects of the phenoxy herbicides. *Down to Earth* 38: 12-15.

Mummenhoff K, Polster A, Muhlhausen A, Theissen G. 2009. *Lepidium* as a model system for studying the evolution of fruit development in Brassicaceae. *J Exp Bot* 60(5): 1503-1513.

Murphy DJ. 1996. Engineering oil production in rapeseed and other oil crops. *Tibtech* 14: 206-213.

Nilsson P, Johansson S-Å, Merker A. 1998. Variation in seed oil composition of species from the genera *Barbarea* and *Lepidium*. *Acta Agric Scand Section B – Soil and Plant Sci* 48: 159-164.

Nishida I, Murata N. 1996. Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol Biol* 47: 541-568.

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J. 1994. *Arabidopsis FAD*2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell* 6: 147-158.

Østergaard L, Kempin, SA, Bies D, Klee HJ, Yanofsky MF. 2006. Pod shatter-resistant *Brassica* fruit produced by ectopic expression of the *FRUITFULL* gene. *Plant Biotech J* 4: 45-51.

Østergaard L. 2009. Don´t "leaf" now. The making of a fruit. *Current Opinion in Plant Biology* 12: 36-41.

Pande D, Malik S, Bora M, Srivastava PS. 2002.. A rapid protocol for *in vitro* micropropagation of *Lepidium sativum* Linn. and enhancement in the yield of lepidine. *In Vitro Cell Dev Biol – Plant* 38: 451-455.

Park D. 2005., LaNe RAGE: a new tool for genomic DNA flanking sequence determination, *Electronic J of Biotech* 8(2): 218-225

Persson C. 2003. Höstrybsen klarar hårda vintrar. *Svensk frötidning* 7: 8-10.

Pioneer Hi-Bred International, Inc. 2009. http://www.pioneer.com/CMRoot/Pioneer/research/pipeline/spec_sheets/HO.pdf (2009-08-27).

Poulsen GB. 1996. Genetic transformation of *Brassica*. *Plant Breeding* 115: 209-225.

Princen LH. 1979. New crop developments for industrial oils. *J of the Amer Oil Chem Soc* 56: 845-848.

Pyper W. 2003. More plastic from plants. *ECOS* 116: 35.

Qing CM, Fan L, Lei Y, Bouchez D, Tourneur C, Yan L, Robaglia C. 2000. Transformation of pakchoi (*Brassica rapa* L. ssp. *Chinensis*.) by *Agrobacterium* infiltration. *Mol Breeding* 6: 67-72.

Rajani S, Sundaresan V. 2001. The *Arabidopsis* myc/bHLH gene *ALCATRAZ* enables cell separation in fruit dehiscence. *Current Biology* 11: 1914-1922.

Roeder AHK, Ferrándiz C, Yanofsky MF. 2003. The role of the REPLUMLESS homeodomain protein in patterning the *Arabidopsis* fruit. *Current Biology* 13: 1630-1635.

Rohini VK, Rao KS. 2000. Transformation of peanut (*Arachis hypogaea* L.): a non-tissue culture based approach for generating transgenic plants. *Plant Science* 150: 41-49.

Rossak M, Smith M, Kunst L. 2001. Expression of the *FAE*1 gene and *FAE*1 promoter activity in developing seeds of *Arabidopsis thaliana*. *Plant Mol Biol* 46: 717-725.

SCB. 2009. Yearbook of Agricultural Statistics Sweden. http://www.scb.se/Pages/PublishingCalendarViewInfo____259923.aspx?PublObjId=10670 (2009-08-27).

Sharma KK, Bhatnagar-Mathur P, Thorpe TA. 2005. Genetic transformation technology: status and problems. *In vitro Cell Dev Biol – Plant* 41: 102-112.

Simpson BB, Ogorzaly MC. 2001. *Economic botany*. Third edition, McGraw-Hill Book Co, Singapore. ISBN 0-07-118188-1.

Singh SP, Zhou X, Liu Q, Stymne S, Green AG. 2005. Metabolic engineering of new fatty acids in plants. *Curr Op in Plant Biol* 8: 197-203.

Somerville CR, Bonetta D. 2001. Plants as factories for technical materials. *Plant Physiol* 125: 168-171.

Spence J, Vercher Y, Gates P, Harris N. 1996. "Pod shatter" in *Arabidopsis thaliana*, *Brassica napus* and *B. juncea*. *J of Microscopy* 181: 195-203.

Stoutjesdijk PA, Singh SP, Liu Q, Hurlstone CJ, Waterhouse PA, Green AG. 2002. hpRNA-mediated targeting of the Arabidopsis *FAD*2 gene gives highly efficient and stable silencing. *Plant Physiol* 129: 1723-1731.

Stymne S, Appelqvist L-B. 1978. The biosynthesis of linoleate from oleoyl-CoA via oleoyl-phosphatidylcholine in microsomes from developing safflower seeds. *Eur J Biochem* 90: 233-229.

Stymne S. 2005. Nya vegetabiliska oljor för tekniskt bruk med genteknik. *Frö och Oljeväxtodlarna*, Försöks- och forskningsrapport.

Summers JE, Bruce DM, Vancanneyt G, Redig P, Werner CP, Morgan C, Child RD. 2003. Pod shatter resistance in the resynthesized *Brassica napus* line DK142. *J Agric Sci* 140: 43-52.

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Rathore KS. 2002. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant Mol Biol* 50(3): 463-479.

Thelen JJ, Ohlrogge JB. 2002. Metabolic engineering of fatty acid biosynthesis in plants. *Metabolic Engineering* 4: 12-21.

Thrupp LA. 2002. Linking agricultural biodiversity and food security: the valuable role of agrobiodiversity for sustainable agriculture. *Int Affairs* 76(2): 283-297.

Ullah I, Rashid H, Khan RM. 2004.. Establishment of tissue culture protocol in Brassica (*B. napus* L.). *Pakistan J of Biol Sci* 7(2): 277-278.

Vancanneyt G, Redig P, Child R, Yanofsky M, Botterman J. 2002. Podshatter resistance: Exploitation of Arabidopsis genes to develop a productivity trait in oilseed rape. *13th International Conference on Arabidopsis Research*, Seville, Spain, 28 June – 2 July.

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Pérez-Grau L, Kinney AJ, Hitz WD, Russell Booth Jr J, Schweiger B, Stecca KL, Allen SM, Blackwell M, Reiter RS, Carlsson TJ, Russell SH, Feldmann KA, Pierce J, Browse J. 1993. Cloning of higher plant ω-3 fatty acid desaturases. *Plant Physiol* 103: 467-476.

Acknowledgements

I would first and foremost like to express my deepest gratitudes to my supervisor and the "father" of the Lepidium project; Prof. Arnulf Merker. I am grateful that you gave me the opportunity to work with this project. Let´s hope that we will see Lepidium on the fields all over Skåne in a not too distant future!

I would also like to thank my assistant supervisors; Profs. Sten Stymne and Margareta Welander, for interesting discussions and support whenever needed.

My gratitudes to the laboratory assistants at the department; Helén Lindgren, Anneli Ahlman and Ann-Sofie Fält. You have always been very kind and helpful.

A special thanks to Peter Olsson, for giving me a map to the cloning-and-bioinformatics jungle.

I would like to thank Dr. Yu Hao at the Temasek Life Sciences Laboratories in Singapore. Visiting your laboratory gave me inspiration and further motivation to pursue a career in scientific research.

A special thanks also to Sten Stymne and Erland Liljeroth for your much appreciated support in all the tedious preparations for my thesis defence.

And to everyone else at the department, thank you for making Alnarp a decent place to work. Keep up the good work!

My wonderful wife Nélida, thank you for putting me on the right track back there in 2004. You and Rebecka are what matters most in my life.

This work was financially supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS). Travel grants were received twice from SLU-FUR and once from the The Royal Swedish Academy of Agriculture and Forestry (KSLA). Contributions to laboratory consumables were received twice from the Royal Physiografic Society in Lund.

Popularised summary in Swedish

Ett synnerligen allvarligt miljöproblem i det svenska jordbruket är läckaget av kväve och fosfor från bruksjordarna till grundvatten, sjöar, åar och hav. Detta läckage leder till övergödning i vattensystemen med algblomning, igenväxning och rubbning av ekosystemen som följd. Sedan mitten av 1990-talet har läckaget av kväve och fosfor minskat något, men ligger fortfarande på en helt oacceptabel nivå. En av orsakerna till detta miljöproblem är att odlingen domineras av ettåriga grödor som lämnar marken bar under vinterhalvåret. En av lösningarna är följaktligen att odla fleråriga grödor eller även fånggrödor som förhindrar att näringsämnena i jorden lakas ur.

Ett miljöproblem av ett helt annat slag är det beroende av fossil olja som hela det globala samhället har byggt upp. Fossil olja är en ändlig resurs som vid användning har mycket stor påverkan på miljön genom utsläpp av bland annat kol-, svavel- och kväveföreningar samt sotpartiklar. Växtoljor är den förnyelsebara och relativt miljöneutrala resurs som har störst potential att ersätta fossil olja. Hittills har dock växtoljor varit betydligt dyrare än fossil olja, men med stigande världsmarknadspriser på den senare råvaran samt framsteg i forskningen på hur växter syntetiserar vegetabilisk olja så håller situationen snabbt på att förskjutas till fördel för växtoljan. Redan används omkring 10-15% av all vegetabilisk olja till olika tekniska ändamål.

Med dessa två miljöproblem i fokus har ett projekt inletts för domesticering av en ny, flerårig oljegröda. Efter att ha testat och utvärderat en rad olika vilda växter under 1990-talet så valdes fältkrassingen (*Lepidium campestre*) ut för det fortsatta förädlingsarbetet. Denna tvååriga växt tillhör familjen korsblommiga växter och återfinns sparsamt i södra och mellersta Sverige. Den valdes ut framförallt på grund av sitt för odling lämpliga växtsätt, sin utmärkta vinterhärdighet och sin höga fröskörd. Att den dessutom är självbefruktare och diploid (har två kromosomuppsättningar) gör det enkelt att hantera nedärvda anlag i ett förädlingsprogram. Idén är att så in fältkrassingen som en fånggröda i en vårstråsäd, som till exempel vårkorn. Efter skörd av stråsäden och en övervintringsperiod ska fältkrassingen ge skörd följande sommar.

Där är dock vissa saker som behöver förbättras hos fältkrassingen. För det första har den en tendens att dråsa, det vill säga släppa ut fröna. Det är en naturlig mekanism som hjälper en växt att sprida sig, men är ett elände för bonden som då förlorar en del av skörden. För det andra är fröoljekvalitén bra – men kan bli bättre. En vegetabilisk olja är sammansatt av olika fettsyror och det är typerna och halterna av fettsyror som ger oljan dess specifika kvalitet. För tekniskt bruk är det ekonomiskt fördelaktigt att ha en så hög halt som möjligt av en enda fettsyra i fröoljan för att undvika uppreningskostnader, och detta är alltså något som bör förbättras i fältkrassingen. För det tredje måste mängden olja i fröna höjas. Fältkrassingen innehåller bara omkring 20% olja i fröna vilket, trots den höga fröskörden, ger en ganska låg slutlig oljeskörd. Slutligen ska helst den tvååriga fältkrassingen göras flerårig för att förlänga fördelarna med minskat näringsläckage och minskad jordbearbetning. Detta kan åstadkommas genom korsningar med fleråriga, nära släktingar till fältkrassingen.

Denna avhandling fokuserar på problemen med frödråsning och fröoljekvalitet, samt utvärderar fältkrassingens egenskaper som fånggröda i fältförsök. Frödråsning och fröoljekvalitet är egenskaper som genetiskt sett är relativt enkla att förändra. Det finns flera sätt att angripa problemen. Ett sätt är att använda mutagena kemikalier för att inducera slumpmässiga mutationer i genomet och därefter screena tusentals plantor för att identifiera de som har fått mutationer i just de gener som styr frödråsning eller fröoljeproduktion. Ett annat sätt är att klona dessa gener och sedan transformera växten med en typ av genkonstruktion som gör att genens funktion slås ut. Jag har valt att jobba med den senare metoden eftersom den är mer kontrollerad och ger upphov till färre bieffekter, samt att den inte kräver så många plantor att jobba med.

En nackdel är dock att det inte finns någon metod för att transformera fältkrassingen. Detta var alltså det jag tog itu med först. En rad olika metoder har utvecklats för andra växter och jag hämtade inspiration framförallt inom samma växtfamilj (korsblommiga), som till exempel raps och växtforskarnas 'labbråtta' backtrav. Man använder oftast *Agrobacterium tumefaciens*, en jordbakterie som har en naturlig mekanism för att föra över gener till växter, tillsammans med ett protokoll för att regenerera nya skott i sterila plastskålar. Det var inga problem att utveckla protokollet för att regenerera skott från fältkrassingen. Däremot var själva transformeringen mer besvärlig. Jag provade även en annan metod som utvecklats för backtrav och innebär att man doppar en blommande planta i bakterielösning och sedan skördar fröna. Därmed kringgår man det arbetskrävande regenereringsprotokollet. Inte heller denna metod lyckades dock.

När det gäller generna som styr frödråsning och fröoljekvalitet så har jag, med stor hjälp av tidigare forskning på backtrav, lyckats klona samtliga enligt min föresats. *INDEHISCENT* är namnet på en central faktor i ett nätverk av gener som styr frödråsning i korsblommiga växter, och jag har bekräftat funktionen av fältkrassingens *INDEHISCENT* genom experiment med backtrav. Två gener, *FATTY ACID ELONGASE* 1 och *FATTY ACID DESATURASE* 2, som var för sig styr produktionen av erukasyra med sin långa kolkedja samt den fleromättade linolsyran, har jag på samma sätt klonat från fältkrassing och analyserat i backtrav. När väl transformeringsmetoden fungerar för fältkrassing så kommer vi att kunna använda dessa gener för att ta fram en dråsfast fältkrassing med en lämplig kvalitet på fröoljan. Som lämplig kvalitet kan man till exempel tänka sig en hög halt av oljesyra (vilket blir resultatet av att inhibera produktionen av erukasyra och linolsyra) som kan användas till inblandning i biodiesel. En hög halt av erukasyra skulle å andra sidan innebära en lämplig smörjolja och en hög halt av fleromättade syror skulle ge en lämplig olja att blanda i lack och färger.

Ett treårigt fältförsök med fältkrassing insådd i vårkorn har utförts i Lönnstorp utanför Lomma. Resultaten från dessa visade att fältkrassingen har en mycket hög potential för fröskörd, med i genomsnitt nära fem ton per hektar. Detta är betydligt mer än höstrapsens dryga tre ton. En mycket intressant sak som framkom i fältförsöken är även att det förefaller som om fältkrassingen, som insådd fånggröda, har viss positiv effekt på skörden av huvudgrödan, i det här fallet korn. Detta är ovanligt för fånggrödor, som normalt har en negativ effekt och i bästa fall inte påverkar huvudgrödan alls. Fler studier krävs för att undersöka vad den positiva effekten beror på.

Denna avhandling har lagt grunden till ett framtida förädlingsprogram för att utveckla fältkrassingen till en perenn fånggröda som kan ge en lämplig teknisk olja. Detta projekt är av största intresse då det representerar ett historiskt skifte både i jordbrukssystemet och i det industriella systemet. Ensidig odling av ettåriga grödor har allvarliga negativa miljöeffekter och det är absolut nödvändigt att utveckla fleråriga alternativ. Om möjligt än mer akut är behovet av att utveckla förnyelsebara och miljöneutrala alternativ till den fossila oljan.

I

Propagation of Ornamental Plants
Vol. 9, № 2, 2009: 78-83

AN EFFICIENT ADVENTITIOUS SHOOT REGENERATION PROTOCOL FOR *LEPIDIUM CAMPESTRE* (L.) R. BR.

Dennis Eriksson* and Arnulf Merker

Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences,
P.O. Box 101, 230 53 Alnarp, Sweden,
*Fax: + 46 40 415519, *E-mail: Dennis.Eriksson@ltj.slu.se

Abstract

Lepidium campestre (L.) R. Br. of the Brassicaceae family is being developed as a novel oil crop as well as ornamental garden plant. The goal of this study was to develop an efficient shoot regeneration protocol for this species. Inducing callus formation with 2,4-D was an absolute requirement for shoot regeneration to occur. A temporary desiccation treatment was also shown to further enhance the shoot regeneration frequency. However, the composition of plant growth regulators in the shoot induction medium seems to have little effect. The optimized shoot regeneration protocol includes culturing of 5-day old cotyledons for 3 days on 1.0 mg l^{-1} 2,4-D, shoot regeneration on MS medium containing 0.5 mg l^{-1} zeatin and a temporary desiccation treatment for 2 days, applied after 2 weeks on shoot induction medium. Using this protocol indirect adventitious shoot regeneration could be obtained at a frequency of 68.3% with 2.4 shoots per explant. The five studied accessions of *L. campestre* showed varied shoot regeneration frequencies, and accessions Öland and Budapest demonstrated the highest shoot regeneration responses.

Key words: adventitious shoots, cotyledons, 2,4-D, *Lepidium campestre* (L.) R. Br., temporary desiccation.

INTRODUCTION

Lepidium campestre (L.) R. Br. (wild cress) is a biennial plant species of the Brassicaceae family with great potential as a new oil crop for temperate as well as colder climate. It has a suitable agronomic plant type, excellent winter hardiness, high seed yield and a good oil quality (Merker and Nilsson 1995).

L. campestre also has potential for decorating flowerbeds and garden hedges. Standing at 0.6 to 0.7 m and branching only in the upper part of the plant, it is known in Sweden as King Solomon´s candlestick (*Kung Salomos ljusstake*). It is highly appreciated for its small, white flowers that appear in abundance in July and August. Also after flowering, the peduncules with the flat, round fruits are highly decorative. The winter hardiness of *L. campestre* enables its use as a garden plant even at the more northern latitudes.

There are many reasons for introducing *L. campestre* as a new oil crop. One is to offer alternatives to mineral oil for various industrial and chemo technical applications as well as fuel. Another reason is the environmental problem with leaching of nutrients, especially nitrogen. The biennial *L. campestre* could be used as a catch crop which covers the fields during the winter and accumulates nitrogen (Merker and Nilsson 1995, Andersson et al. 1999).

One problem with *L. campestre*, however, is the shattering of the seeds, a common problem also seen in rapeseed and other Brassicaceae species (Jenkins et al. 1996). This may cause problems with harvesting, when *L. campestre* is cultivated as a crop in the field, or with weeding, when it is grown in flowerbeds as an ornamental plant. The genetic regulation of seed shattering has been studied extensively in *Arabidopsis thaliana* (Liljegren et al. 2004, Ibid, 2000, Rajani and Sundaresan 2001, Gu et al. 1998, Roeder et al. 2003).

In our attempt to control the seed shattening in *L. campestre* we try to modify the genes controlling seed shattening. Establishment of a shoot regeneration protocol is an important part of that work.

Regeneration protocols have been developed for a number of species from various genera in the Brassicaceae family, including *Brassica napus* (Ullah et al. 2004), *B. oleraceae* (Christey and Earle 1991), *Arabidopsis thaliana* (Feldman and Marks 1986), *Sinapis alba* (Hadfi and Batschauer 1994) and *Lepidium sativum* (Pande et al. 2002).

The aim of this study was to develop a shoot regeneration protocol for *L. campestre*.

Received: December 16, 2008 *Accepted: March 10, 2009*

MATERIALS AND METHODS

Plant material

Seeds of *Lepidium campestre* were previously collected by Merker at four different locations in Sweden as well as one location in Hungary, representing the five different accessions tested. The locations, after which the accessions are named, were Arild, Öland, Bergianska botanic garden in Stockholm, Budapest (Hungary) and Stjärnelund. If nothing else is stated, the accession Öland was used in the experiments.

Seed germination and growth chamber conditions

Seeds were surface sterilized by vigorous shaking in 70% ethanol for 1 min, 3% calcium hypochlorite for 15 min and thorough rinsing with sterile water, before placing on germination medium containing half-strength MS salts (Murashige and Skoog 1962). Seeds were germinated at 23°C/18°C (light/dark) using a 16-h photoperiod with a photosynthetic photon flux density of 33 μmol m^{-2} s^{-1}.

Culture media and adventitious shoot regeneration

The constituents of the basal medium were MS salts and vitamins with 3% sucrose. The pH was adjusted to 5.7 before addition of 2.5% Gelrite and autoclaving. The callus induction medium (CIM) was MS basal medium supplemented with 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) added before autoclaving. The shoot induction media (SIM) were basal medium (MS) supplemented with different combinations of zeatin (0 to 3 mg l^{-1}), 6-benzylaminopurine (BAP, 1 mg l^{-1}), indole-3-acetic acid (IAA, 0.5 mg l^{-1}) and α-naphtaleneacetic acid (NAA, 0.25 mg l^{-1}), which were filter sterilized and added after autoclaving. Cotyledons (app. 2 to 5 mm long) were excised after 5 days on germination medium, pricked 8 to 10 times with a scalpel and transferred to CIM or SIM. All the explants were transferred to fresh medium every two weeks. Shoots were counted after 8 weeks on SIM. Explants were kept in darkness until the first appearance of shoots, which normally occurred after approximately 5 to 6 weeks. Thereafter the entire treatment batch was put in light (with the same conditions as for seed germination).

Desiccation treatment

After 2 weeks on SIM, the explants were removed from the medium and put on empty Petri dishes for 0 to 3 days and thereafter transferred back to fresh SIM.

Statistical analysis

In every regeneration experiment 60 explants on 6 Petri dishes were used, except in the first experiment where 40 explants on 4 Petri dishes were used. Each experiment was repeated twice. Regeneration data are presented as means of two independent experiments with the standard error. Experimental data were analyzed by SAS software using ANOVA and Tukey´s HSD.

RESULTS AND DISCUSSION

A series of initial experiments, of which data are not shown, were performed to identify important factors for adventitious shoot regeneration. One of these experiments indicated that cotyledons are the most regenerative explant type in this plant species. Hypocotyls completely failed to regenerate any shoots whereas true leaves had a considerably lower regeneration frequency. It was also determined that younger explants tended to have a higher shoot regeneration frequency. Thus the cotyledons were excised as early as possible. Cotyledons younger than five days were very small and extremely difficult to handle. Therefore five-day old explants were used in all the experiments presented in this article. One thing which was concluded early was that inducing cell division and callus formation on the strong auxin 2,4-D was essential to achieve adventitious shoot regeneration. Without this 2,4-D subculture we did not obtain any shoot regeneration at all, as seen in Table 1. Later on it was also noted that wounding of the tissue with a scalpel before the subculture on 2,4-D seemed to further enhance the adventitious shoot regeneration response, probably because of the increase in callus formation on the cut surfaces.

The surface sterilization procedure of the seeds was very effective as was concluded from the almost complete absence of contaminated explants in all experiments. However, this is probably also due to the fact that germinating *L. campestre* seeds develop a kind of protective, antiseptic gel surrounding the entire seeds when they absorb water from the germination medium.

After the initial experiments we decided to more carefully evaluate the following factors: callus induction with 2,4-D, plant growth regulators (PGR), and a temporary desiccation treatment. Finally, the five available accessions were compared using the optimized adventitious shoot regeneration protocol.

Callus induction with 2,4-D

The first experiment demonstrated the absolute requirement of a 2,4-D subculture for subsequent adventitious shoot regeneration to occur at all, as cotyledons that were cultured on SIM without prior subculture on CIM completely failed to regenerate any shoots (Table 1). There was a small but statistically significant tendency of increased adventitious shoot regeneration frequency when the length of CIM culture was extended from 6 days to 9 days, with an average of 48.8% and 60.0%, respectively (Table 1). However, the regeneration after 9 days of subculture on 2,4-D was not significantly better than after 3 days. There

Table 1. Effect of 2,4-D subculture and plant growth regulators on adventitious shoot regeneration on cotyledon cultures of *L. campestre*.

Subculture on 1.0 mg l^{-1} 2,4-D	Plant Growth Regulator (mg l^{-1})				Mean % explants forming shoots ± SE	Mean number of shoots / regenerating explant ± SE
	Zeatin	BAP	IAA	NAA		
0 days	1.0				0.0 ± 0.0 d	0.0 ± 0.0 d
0 days	1.0		0.5		0.0 ± 0.0 d	0.0 ± 0.0 d
0 days	1.0			0.25	0.0 ± 0.0 d	0.0 ± 0.0 d
0 days		1.0			0.0 ± 0.0 d	0.0 ± 0.0 d
0 days		1.0	0.5		0.0 ± 0.0 d	0.0 ± 0.0 d
0 days		1.0		0.25	0.0 ± 0.0 d	0.0 ± 0.0 d
3 days	1.0				52.5 ± 4.9 bc	2.0 ± 0.3 ab
3 days	1.0		0.5		46.3 ± 6.3 c	1.9 ± 0.2 b
3 days	1.0			0.25	52.5 ± 4.1 bc	2.3 ± 0.2 a
3 days		1.0			48.8 ± 7.2 bc	1.9 ± 0.2 b
3 days		1.0	0.5		60.0 ± 3.8 ab	1.8 ± 0.2 bc
3 days		1.0		0.25	55.0 ± 7.3 bc	1.8 ± 0.2 bc
6 days	1.0				60.0 ± 4.6 ab	2.2 ± 0.2 ab
6 days	1.0		0.5		46.3 ± 6.3 c	2.1 ± 0.3 ab
6 days	1.0			0.25	46.3 ± 6.5 c	2.5 ± 0.3 a
6 days		1.0			48.8 ± 4.8 c	1.7 ± 0.2 bc
6 days		1.0	0.5		46.3 ± 5.0 c	1.5 ± 0.1 c
6 days		1.0		0.25	45.0 ± 6.8 c	2.1 ± 0.2 ab
9 days	1.0				70.0 ± 6.3 a	1.9 ± 0.1 b
9 days	1.0		0.5		57.5 ± 4.1 bc	2.4 ± 0.2 a
9 days	1.0			0.25	51.3 ± 4.8 bc	2.0 ± 0.2 ab
9 days		1.0			55.0 ± 7.1 bc	2.5 ± 0.3 a
9 days		1.0	0.5		65.0 ± 7.1 a	2.1 ± 0.2 ab
9 days		1.0		0.25	61.3 ± 4.8 ab	1.7 ± 0.2 bc

Means in the same column followed by different letters are significantly different at $p \leq 0.05$.

were no statistically significant differences between the subculture regarding the average number of shoots per regenerating explant (Table 1).

The nearly equal adventitious shoot regeneration response already after 3 days motivated an attempt to minimize the 2,4-D subculture since this strong auxin is also known to cause mutations at high exposure (Mullison 1982). Another reason to minimize the exposure to 2,4-D is the relatively long half-life of this auxin. Remaining 2,4-D in the plant tissue after transfer of the cotyledons to SIM might be the reason why it takes as much as 5 to 6 weeks for the shoots to appear (see Materials and Methods).

2,4-D is a strong auxin which is often used to induce callus formation and somatic embryogenesis in a number of plant species, including carrot (Lee et al. 2001), rice (Gairi and Rashid 2004), quinoa (Eisa et al. 2005) and cauliflower (Chakrabarty et al. 2002). In our study however, we did not see any tendency to somatic embryogenesis as adventitious shoots rather appeared from the calluses through an organogenic mode of regeneration (Fig. 1A,B).

Defining shoot induction medium

In the first experiment, designed to simultaneously evaluate the 2,4-D subculture and various PGRs, we saw no statistically significant difference between any of the SIM, neither for adventitious shoot regeneration frequency nor number of shoots per regenerating explant (Table 1).

A wide range of zeatin from 0 to 3 mg l^{-1} was tried in the following experiment. No statistically significant difference in adventitious shoot regeneration frequency was found between any of the zeatin concentrations in the SIM, with frequencies ranging from 40.0% on 2 mg l^{-1} zeatin to 55.8% on 0.5 mg l^{-1} zeatin. Even the medium which contained no PGR at all showed a shoot regeneration frequency of 51.7% (Fig. 2). There was also no statistically significant difference between the variants of the medium for the number of shoots per regenerating explant, with values ranging from 2.0 to 2.2 (data not shown). In subsequent experiments, a SIM containing 0.5 mg l^{-1} zeatin was used.

The lack of a clear response in our experiments with various combinations of PGRs in the SIM is

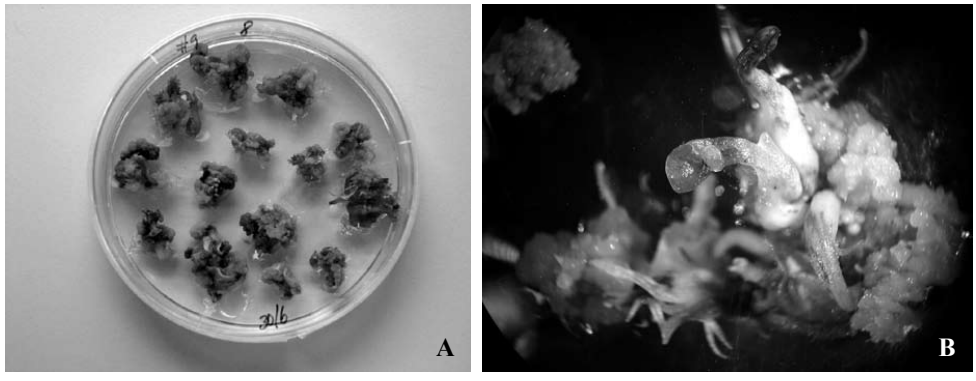


Fig. 1. *In vitro* shoot regeneration of *Lepidium campestre*. A, B) Regenerating shoots from callogenic cotyledons after 8 weeks on shoot induction medium containing 0.5 mg l^{-1} zeatin.

intriguing. Regeneration protocols for species within the Brassicaceae family most often use a combination of cytokinins (especially BAP, zeatin or kinetin) and auxins (especially NAA, IAA or 2,4-D) to induce shoot regeneration (Ullah et al. 2004, Christey and Earle 1991, Feldman and Marks 1986, Hadfi and Batschauer 1994, Pande et al. 2002). Normally the cytokinins that stimulate cell division are particularly important in this respect. Even in another *Lepidium* species, the *L. sativum*, there is a clear effect on the callogenic response when applying various concentrations of BAP and NAA (Pande et al. 2002). One explanation to our result might be that there is residual 2,4-D in the calluses from the subculture and this overshadows the effect of the cytokinins and auxins in the SIM. Another possible explanation might be that *L. campestre* develops very rigid and high levels of endogenous PGRs in the callus tissue which then overcomes the effect of the externally applied PGRs. It would certainly be interesting to analyse the levels of endogenous PGRs in various tissues, including callus, of *L. campestre*.

Desiccation treatment

The desiccation treatment, where the explants after two weeks on SIM simply were put on empty Petri dishes, had a very clear and statistically significant effect on the adventitious shoot regeneration frequency. The average frequency had its maximum of 68.3% after 2 days of desiccation, but after 3 days the average frequency decreased again to 52.5% (Fig. 3). Apparently the tissues were damaged severely by a prolonged period of desiccation. After 3 days the explants were extremely dry and shrivelled and some of them failed to recover when put back on SIM. There was no statistically significant difference between the treatments for the number of shoots per regenerating explant, with values ranging from 1.8 without desiccation to 2.4 after 2 days (data not shown). In the optimized protocol a desiccation treatment of 2 days was therefore applied.

This stress-induced response has previously been shown in carrot (*Daucus carota*) where production of somatic embryos from callus cultures was increased up

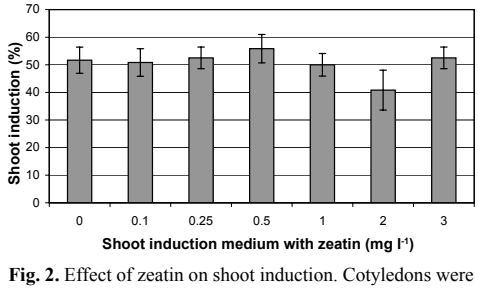


Fig. 2. Effect of zeatin on shoot induction. Cotyledons were cultured for 3 days on MS medium containing 1.0 mg l^{-1} 2,4-D and thereafter transferred to SIM. Vertical bars show standard error.

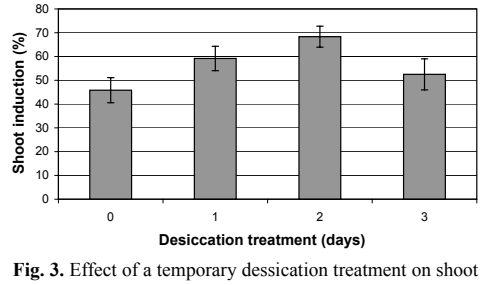


Fig. 3. Effect of a temporary dessication treatment on shoot induction. Cotyledons were cultured for 3 days on MS medium containing 1.0 mg l^{-1} 2,4-D and thereafter transferred to SIM (MS medium containing 0.5 mg l^{-1} zeatin). The desiccation treatment was applied after 2 weeks on SIM. Vertical bars show standard error.

to 20-fold after a similar desiccation treatment. Further experiments with carrot indicated that this stress-induced increase in somatic embryogenesis was due both to the withdrawal of nutrients, and especially sucrose, as well as to the decrease in relative humidity (Lee et al. 2001). However, increased somatic embryogenesis in carrot has been shown also on high levels of sucrose (Kamada et al. 1989). In *L. campestre* the increase in shoot production was not as dramatic as in the carrot study by Lee et al. (2001), with an average increase of 20-30% compared to treatments without desiccation. As far as we know though, such a desiccation treatment has not previously been shown to stimulate adventitious shoot organogenesis in a similar fashion as it has been shown for somatic embryogenesis.

What is not completely clear is if the stress response in our study is caused mainly by the loss of water or by the withdrawal of nutrients, or a combination of both. Another alternative explanation, not discussed in the study of Lee et al. (2001), for the adventitious shoot regeneration response might also be that the endogenous concentration of sugar in the cells increases as the tissue loses water when removed from the medium. However, this remains to be tested.

Accession

Finally, we evaluated the adventitious shoot regeneration response in the five available accessions of *L. campestre*, using the optimized adventitious shoot regeneration protocol. There was a statistically significant difference between some of the accessions, with Öland and Budapest having the highest shoot regeneration frequency of 60.8% and 64.2%, respectively, and Arild and Bergianska having the lowest of 41.7% and 46.7%, respectively (Fig. 4). There was no statistically significant difference between any of the accessions for the number of shoots per regenerating explant, with values ranging from 1.8 for Bergianska to 2.3 for Budapest (data not shown).

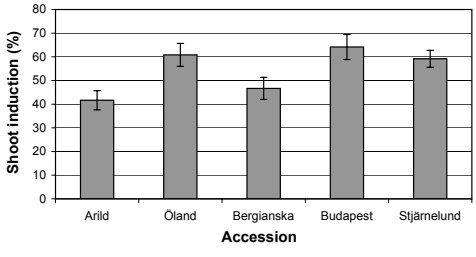


Fig. 4. Comparing the shoot regeneration response of five different accessions of *L. campestre*. Cotyledons were cultured for 3 days on MS medium with 1.0 mg l^{-1} 2,4-D and thereafter transferred to SIM (MS medium containing 0.5 mg l^{-1} zeatin). A desiccation treatment of 2 days was applied after 2 weeks on SIM. Vertical bars show standard error.

Acknowledgement: We would like to thank FORMAS for financing the *Lepidium campestre* project. We are also grateful to Beata Dedicova and Margareta Welander for valuable comments on the manuscript and Jan-Eric Englund for help with the statistical calculations.

REFERENCES

Andersson A. A. M., Merker A., Nilsson P., Sorensen H., Åhman P. (1999). Chemical composition of the potential new oilseed crops *Barbarea vulgaris*, *Barbarea verna* and *Lepidium campestre*. Journal of the Science of Food and Agriculture, 79: 179-186.

Chakrabarty R., Viswakarma N., Bhat S. R., Kirti P. B., Singh B. D., Chopra V. L. (2002). *Agrobacterium*-mediated transformation of cauliflower: Optimization of protocol and development of Bt-transgenic cauliflower. Journal of Biosciences, 27(5): 495-502.

Christey M. C., Earle E. D. (1991). Regeneration of *Brassica oleracea* from peduncle explants. Horticultural Science, 26: 1069-1072.

Eisa S., Koyro H. W., Kogel K. H., Imani J. (2005). Induction of somatic embryogenesis in cultured cells of *Chenopodium quinoa*. Plant Cell, Tissue and Organ Culture, 81: 243-246.

Feldman K. A., Marks M. D. (1986). Rapid and efficient regeneration of plants from explants of *Arabidopsis thaliana*. Plant Science, 47: 63-69.

Gairi A., Rashid A. (2003). TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2,4-D. Plant Cell, Tissue and Organ Culture, 76: 29-33.

Gu Q., Ferrándiz C., Yanofsky M. F., Martienssen R. (1998). The *FRUITFULL* MADS-box gene mediates cell differentiation during *Arabidopsis* fruit development. Development, 125 (8): 1509-1517.

Hadfi K., Batschauer A. (1994). *Agrobacterium*-mediated transformation of white mustard (*Sinapis alba* L.) and regeneration of transgenic plants. Plant Cell Reports, 13: 130-134.

Jenkins E. S., Paul W., Coupe S. A., Bell S. J., Davies E. C., Roberts J. A. (1996). Characterization of an mRNA encoding a polygalacturonase expressed during pod development in oilseed rape (*Brassica napus* L.). Journal of Experimental Botany, 47: 111-115.

Kamada H., Kobayashi K., Kiyosue T., Harada H. (1989). Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. In Vitro Cellular and Developmental Biology-Plant, 25: 1163-1166.

Lee E. K., Cho D. Y., Soh W. Y. (2001). Enhanced production and germination of somatic embryos by temporary desiccation in tissue cultures of *Daucus*

carota. Plant Cell Reports, 20: 408-415.

Liljegren S. J., Ditta G. S., Eshed Y., Savidge B., Bowman J. L., Yanofsky M. F. (2000). *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. Nature, 404: 766-770.

Liljegren S. J., Roeder A. H. K., Kempin S. A., Gremski K., Ostergaard L., Guimil S., Reyes D. K., Yanofsky M. F. (2004). Control of fruit patterning in *Arabidopsis* by INDEHISCENT. Cell, 116: 843-853.

Merker A., Nilsson P. (1995) Some oil crop properties in wild *Barbarea* and *Lepidium* species. Swedish Journal of Agricultural Research, 25: 173-178.

Mullison W. R. (1982). A review of the plant physiological effects of the phenoxy herbicides. Down to Earth, 38: 12-15.

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Pande D., Malik S., Bora M., Srivastava P. S. (2002). A rapid protocol for *in vitro* micropropagation of *Lepidium sativum* Linn. and enhancement in the yield of lepidine. In Vitro Cellular and Developmental Biology- Plant, 38: 451-455.

Rajani S., Sundaresan V. (2001). The *Arabidopsis* myc/bHLH gene *ALCATRAZ* enables cell separation in fruit dehiscence. Current Biology, 11 (24): 1914-1922.

Roeder A. H., Ferrándiz C., Yanofsky M. F. (2003). The role of the REPLUMLESS homeodomain protein in patterning the *Arabidopsis* fruit. Current Biology, 13 (18): 1630-1635.

Ullah I., Rashid H., Khan R. M. (2004). Establishment of tissue culture protocol in Brassica (*B. napus* L.). Pakistan Journal of Biological Sciences, 7 (2): 277-278.

II

Identification of a gene involved in pod shatter in the novel oil crop *Lepidium campestre* L.

Dennis Eriksson* **and Arnulf Merker**
Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Alnarp, Sweden
(*Corresponding author)

Summary

Our aim is to domesticate *Lepidium campestre* L. of the Brassicaceae family as a novel oilseed crop. A major problem for cultivation is the dispersal of the seeds through pod shatter. Analysis of *L. campestre* fruits demonstrated a cellular structure in the fruit valves as well as a dehiscence zone similar to that of *Arabidopsis thaliana* and *Brassica napus*. Taking advantage of the knowledge generated in *A. thaliana*, we targeted the network of transcription factors regulating pod shatter in order to produce pod shatter-resistant *L. campestre*. We have cloned the orthologous gene of one of the main transcription factors, INDEHISCENT, and confirmed its function in *A. thaliana* through functional complementation of the *ind* mutant as well as generation of pod shatter-resistant *A. thaliana* plants with an RNAi construct. This study shows that basic research on the genetic regulation of fruit development in *A. thaliana* can readily be applied to crop plants in the Brassicaceae family in order to reduce seed loss.

Keywords: *L. campestre* L., *A. thaliana*, pod shatter, valve margin, dehiscence zone, *INDEHISCENT*

Introduction

An environmental problem of modern agriculture in the temperate regions is the leaching of nutrients from arable land into ground and surface water. A contributing factor to this is the growing of annual crops that leaves the ground uncovered in the winter season (Jensen, 1991). A shift in the agricultural system towards perennial crops may be a way to alleviate the problem. An environmental problem of a different kind that also may find part of its solution in agriculture is the dependence on fossil oil for fuel and for different industrial applications. In addition to being a limited resource, fossil oil is also the source of major pollution, not the least to the atmosphere (Merker *et al.*, 2009). Environmentally friendly and renewable oils produced by plants have the potential to replace fossil oil in many applications. Already, 10-15% of all vegetable oil produced in the world is consumed as fuel or in the oleochemical industry (Stymne, 2005).

A domestication programme has been initiated with the goal of introducing *Lepidium campestre* (field cress) as a perennial oilseed crop to meet these two objectives. *L. campestre* is a biennial species of the Brassicaceae family with close perennial relatives. It has a suitable agronomic stature, excellent winter hardiness for cultivation in the Scandinavian countries, a high seed yield and a suitable oil quality (Merker and Nilsson, 1995). The main obstacles in order to adapt *L. campestre* to the proposed cropping system is: 1) to reduce the sensitivity to pod shatter and 2) to modify the fatty acid profile of the seed oil in order to generate *L. campestre* crop accessions with different oil qualities for different industrial applications. The work presented here focuses on the problem with pod shatter. Pod shatter is a common cause of production loss in many Brassicaceae crops, with losses ranging between 10% and 50% in oilseed rape (*Brassica napus*) (Summers *et al.*, 2003). In field trials with *L. campestre*, we have observed severe seed dispersal due to the opening of the pods (Merker *et al.*, 2009) and daily

monitoring of the crop for a timely harvest is necessary to reach an optimal balance between seed maturity and risk of pod shatter.

The genetic regulation of pod shatter has been studied thoroughly in *Arabidopsis thaliana* and a model involving several transcription factors has been proposed (Dinneny and Yanofsky, 2004; Østergaard, 2009). With *A. thaliana* being a member of the Brassicaceae family and the mechanism of seed dispersal being apparently similar to other species within this family, it is possible that the cell differentiation in the fruit leading to pod shatter and its genetic regulation is conserved between various species.

The aim of this study was therefore first to describe the *L. campestre* fruit, with focus on the cell layers in the fruit valves responsible for pod shatter, to identify similarities and differences to other species such as *A. thaliana* and *B. napus* having a similar mechanism for seed dispersal. Secondly, we cloned the orthologous *INDEHISCENT* (*IND*) gene from *L. campestre* and confirmed its function in *A. thaliana*. The results obtained will facilitate the development of pod shatter-resistant *L. campestre* through transformation (Eriksson and Merker, 2009)or TILLING (Colbert *et al.*, 2001).

Results and Discussion

Dehiscence zone in the L. campestre fruit

Several members of the Brassicaceae family develop fruits that are composed of two fused carpels. The carpel valves (fruit walls) are separated by a central lamella called septum. The replum, to which the valves attach, is the outer margin encircling the septum. The dehiscence zone (DZ) develops along this valve-replum margin and runs along the entire length of the silique/silicle (Spence *et al.*, 1996). There are mainly three cell layers involved in the opening of the fruit: a valve layer (endocarp *b*, en*b*) and a valve margin layer, that both become lignified, and a separation layer that disintegrates due to the activity of hydrolytic enzymes (Ferrándiz, 2002). The modification of these three cell layers takes place mainly during post fertilisation stages 17 and 18 according to the division of fruit developmental stages proposed by Ferrándiz *et al.* (1999).

The *L. campestre* fruit (fig 1a-e) is a silicle with two fused, round carpel valves enclosing the two seeds that are attached to the septum. Microscopy analysis of the valve margin revealed a cellular structure similar to that of *A. thaliana* and *B. napus* siliques described by Spence *et al.* (1996). The carpel valves consist of seven cell layers: the outer epidermis, four intermediate layers of mesophyll tissue and two endoderm layers (en*a* and en*b*) (fig 2a). Staining with safranin-o of *L. campestre* fruits at post fertilisation stage 18 demonstrated the lignification of the en*b* layer as well as the valve margin layer in the DZ (fig 2b). The differentiation of the en*a* layer resembles that of *B. napus* fruits. The en*a* cell layer in *A. thaliana* disintegrates completely as the fruit matures, whereas a thickened primary wall lining the fruit locule remains in *B. napus* as the en*a* cells collapse (Spence *et al.*, 1996). These remaining cell walls of en*a* are seen also in the maturing fruits of *L. campestre* (fig 2a). Interestingly, it appears as if also the en*a* of *L. campestre* becomes lignified (fig 2b). This is something not observed in neither *B. napus* nor *B. juncea* (Spence *et al.*, 1996).

Cloning of LcIND

Over the last ten years, research on *A. thaliana* has identified a number of transcription factors involved in the regulation of pod shatter. Liljegren *et al.* (2000) showed through double mutant analysis that the closely related *SHATTERPROOF* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) MADS-box genes redundantly control valve margin development. The same research group also showed that the *FRUITFULL* (*FUL*) MADS-box gene, necessary for valve differentiation, is a negative regulator of *SHP* expression (Ferrándiz *et al.*, 2000). Similarly, the REPLUMLESS (RPL) homeodomain protein was shown to specify replum development by negative regulation of *SHP* (Roeder *et al.* 2003). Rajani and Sundaresan (2001) then described the myc/bHLH transcription factor ALCATRAZ (ALC) and its function to specify the separation layer. Finally, Liljegren *et al.* (2004) identified the INDEHISCENT

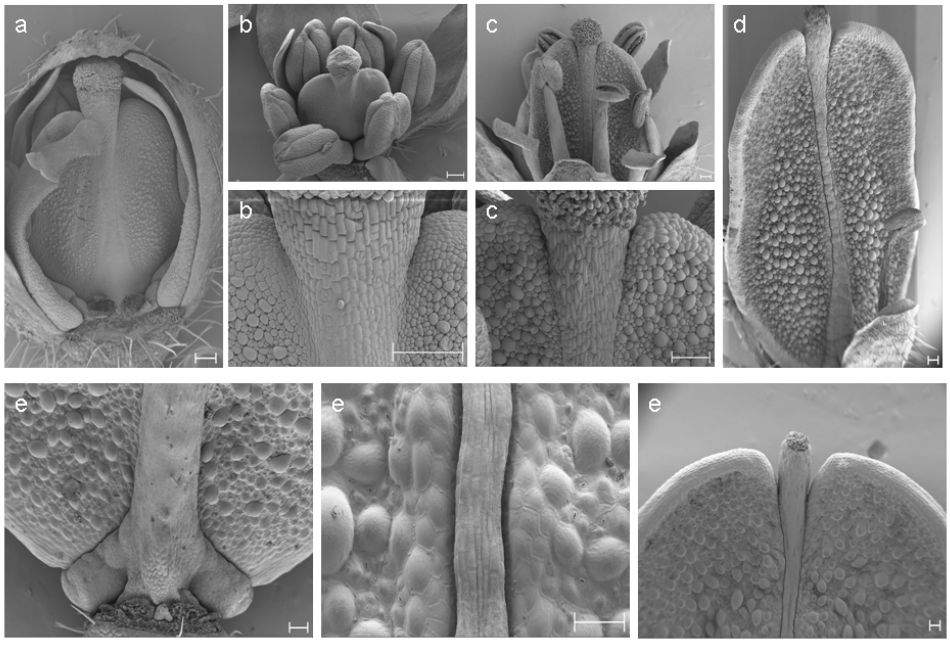


Figure 1a-e. SEM pictures of *L. campestre* fruits at various developmental stages according to Ferrándiz *et al*. (1999). a) stage 12, b) stage 13, c) stage 15, d) stage 16, e) stage 18. Scale bar = 100 µm.

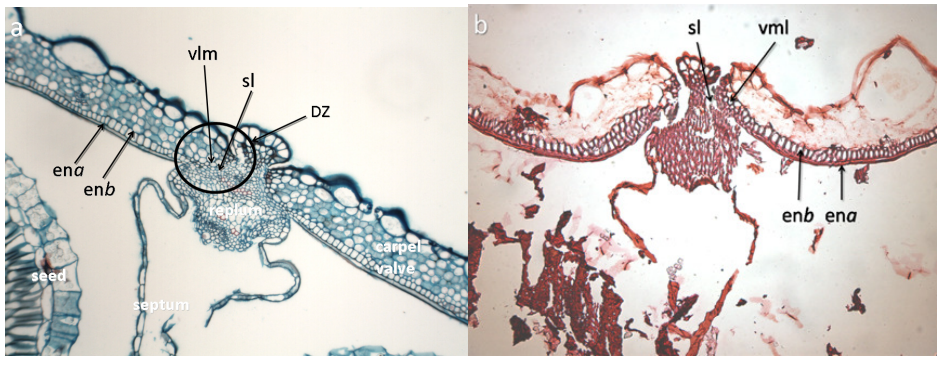


Figure 2a,b. Cross sections of *L. campestre* fruits. a) Post fertilisation stage 16-17A, showing the distinct endocarp a (ena) and endocarp b (enb) layers in the valves as well as the valve margin layer (vml) and the separation layer (sl) in the dehiscence zone (DZ). The cross section was stained with the general stain alcian-blue and the lignin-specific red stain safranin-o. There is a tendency to lignin formation in the vascular bundle of the replum, however, the enb and the vml are not yet lignified. b) Post fertilisation stage 18-19. The cross section was stained with safranin-o. There is lignification of the cell walls of the slightly elongated enb cells and of the valve margin layer all the way up to where it meets the exocarp, as well as lignification of the remaining ena layer. The separation layer has already disintegrated to some extent.

(IND) bHLH protein involved in specification of all the three cell layers required for pod shatter.

These discoveries have led to the establishment of a model for genetic regulation of pod shatter (fig 3), where SHP1/2 promote the expression of *ALC* in the separation layer and of *IND* in the entire valve margin. FUL and RPL negatively regulates the *SHP/IND/ALC* cluster to prevent their expression in the valves and replum, respectively, and thereby limiting their expression to the valve margin (Dinneny and Yanofsky, 2004; Østergaard, 2009). The usefulness of this model for applied crop research has been demonstrated. With the assumption that the genetic regulatory network controlling pod shatter is conserved between *Arabidopsis* and *Brassica*, Østergaard *et al.* (2006) expressed the *A. thaliana FUL* gene constitutively from a 35S promoter in *B. juncea* to produce pod shatter-resistant fruits. In *B. napus*, down regulation of each of the two *IND* orthologues (*Bn1IND* and *Bn2IND*) found in this species caused a complete prevention of pod shatter (Vancanneyt *et al.*, 2002).

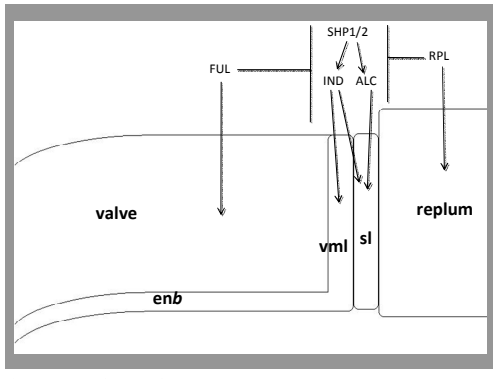


Figure 3. Model for genetic regulation of pod shatter *in A. thaliana*, with interactions as discussed in the text. ena, endocarp a; enb, endocarp b; vml, valve margin layer; sl, separation layer.

Considering the similarity of *L. campestre* to *Arabidopsis* and *Brassica* in valve margin structure and the presence of a DZ in *L. campestre*, it is likely that the genetic regulation of pod shatter is conserved also in this species. With the aim of generating pod shatter-resistant *L. campestre*, we therefore attempted to clone the orthologous *LcIND* gene in order to down regulate its function through RNA interference (RNAi), a technique commonly used for various applications in basic research and crop improvement (Galun, 2005).

From the sequences of the *A. thaliana IND* (*AtIND*, NCBI accession number NM_116229) and *B. napus IND* genes, we identified regions with close homology and used these to design degenerate primers. With these primers we amplified a 290 bp fragment from *L. campestre* genomic DNA. From this fragment we used the genome walking method LaNe RAGE (Park, 2005) to amplify fragments reaching to positions +1109 and -103 from a putative start codon, and subsequent sequence analysis identified a putative *LcIND* ORF of 531 nt, showing 88% homology to the *AtIND* (fig 4). RACE analysis confirmed a 43 nt 5´-UTR and a 324 nt 3´-UTR. Further PCR analysis on *L. campestre* genomic DNA revealed that the *LcIND* lacks introns. The *LcIND* sequence has been submitted to the NCBI genbank with accession number FJ907544. RT-PCR analysis of *L. campestre* wildtype (WT) fruits at the approximate developmental stages 13 (at anthesis), 16 (small, green fruits with sepals and petals starting to fall) and 17 (mature, full-size but still green fruits) as well as of tissue from leaves and roots confirmed that *LcIND* is expressed mainly in all the examined stages of developing fruits and not in the other examined parts of the plant (fig 5).

```
LcIND           MMEPQPEVQPHDLLMDWNKANNDLIITQDHPFLHDP-HLILDPTPETLIHLEEADEEDED 59
AtIND           MMEPQP--E-HHLLMDWNKAN-DLLTQEHAAFLNDPHHLMLDPPPETLIHLDEDEE--YD 53
                ******    *.********* **:  :. .**:** **:***.*******:* :*   *

LcIND           EDMDAMKEMQYMIAAMQPVDIDPATVPKPNRRNVRISDDPQTVVARRRRERISEKIRILK 119
AtIND           EDMDAMKEMQYMIAVMQPVDIDPATVPKPNRRNVRISDDPQTVVARRRRERISEKIRILK 113
                **************.************************************************

LcIND           RIVPGGAKMDTASMLDEAIRYTKFLKRQVRILQPHSHLGPPTSTSDPYLCYYHNSQLS 177
AtIND           RIVPGGAKMDTASMLDEAIRYTKFLKRQVRILQPHSQIGAPMANPS-YLCYYHNSQPS 170
                ************************************::*.* :... ********* *
```

Figure 4. ClustalW alignment of LcIND and AtIND amino acid sequences. Region within the box was targeted for RNAi. Regions in blue were used for design of degenerate primers. Region in yellow/green is basic helix-loop-helix (bHLH).

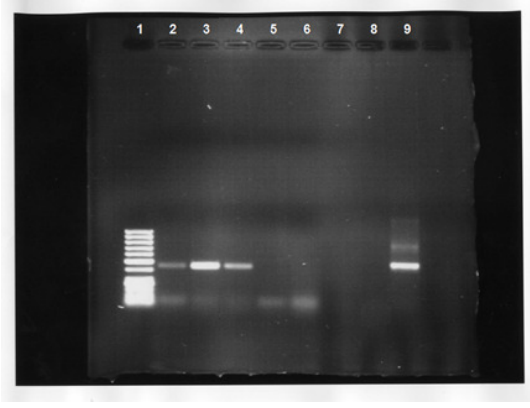


Figure 5. RT-PCR with *LcIND* primers on cDNA from *L. campestre* wildtype tissues. 1) DNA ladder GeneRuler 50 Bp (Fermentas), 2) fruits at post fertilisation stage 13, 3) fruits at post fertilisation stage 16, 4) fruits at post fertilisation stage 17, 5) leaves, 6) roots, 7) negative control without the reverse transcriptase to assess for genomic DNA contamination, 8) negative control without template to assess for reagent contamination, 9) positive control with *GAPDH* RNA and *GAPDH*-specific primers to assess for proper reverse transcription.

Functional complementation of Arabidopsis ind mutant

The high (88%) sequence homology between the putative *LcIND* and the *AtIND* indicates that *LcIND* may be able to perform its function in an *A. thaliana* genetic background. This is a reasonable assumption, since the *A. thaliana FUL* has been shown to work in a *B. juncea* background (Østergaard *et al.*, 2006). Also, the *Bn1IND* and *Bn2IND* from *B. napus* have both been used in RNAi constructs to down regulate the IND function and produce pod shatter-resistant *A. thaliana* (Vancanneyt *et al.*, 2002).

To confirm the function of the putative *LcIND*, we therefore designed a construct, named indr, to complement the IND function in an *A. thaliana* loss-of-funtion *ind* mutant (*Atind*) exhibiting a complete loss of pod shatter. The full-length *LcIND* ORF was cloned into the Gateway overexpression vector pK2GW7 and used to transform *Atind*. 15 independent transformed indr T2 lines were generated. Phenotypic observations confirmed that six of them (40%) had completely restored the pod shatter sensitive-phenotype. The other nine lines still retained the pod shatter-resistant phenotype to varying degrees. One line (29-1) developed very short fruits (<1/2 of normal length) and produced little seeds. Fruit samples from three of the pod shatter sensitive lines (3-1, 18-1 and 30-8) were collected and the tissue was fixed for microscopy analysis. Whereas the *Atind* lacks a clearly defined separation layer in the DZ, staining and light microscopy (LM) of the indr lines showed the recovery of such a separation layer (fig 6a,b). The indr lines more resembled the WT in this respect (fig 6b,c). Scanning electron microscopy (SEM) also showed the lack of a distinct DZ in the *Atind* but the complete restoration of such a DZ in the examined indr lines (fig 7a,b). These results show that the putative LcIND is able to restore the pod shatter-sensitive phenotype in the pod shatter-resistant *Atind* mutant, and they indicate that LcIND performs the same function to regulate pod shatter in *L. campestre* as the AtIND in *A. thaliana*.

Down regulation of Arabidopsis IND

To examine if the putative *LcIND* also can be used to down regulate the IND function, we designed an RNAi construct, named indk, for transformation of WT *A. thaliana*. We targeted a 242 nt region with relatively high homology between the *LcIND* and the *AtIND*, avoiding the conserved bHLH region (fig 4) since this may lead to unspecific targets in the bHLH family of transcription factors being down regulated. 19 independent transformed indk T2 lines were generated. Phenotypic observations showed, however, that only two lines (2-6 and 15-3) displayed a strong pod shatter-resistant phenotype. The other transformed lines still retained a pod shatter-sensitive (WT) phenotype to some extent. Microscopy analysis of fruit samples from the two shatter-resistant lines showed that they to some extent resemble the *Atind* fruit, regarding the failure to disintegrate the separation layer (fig 6a,d).

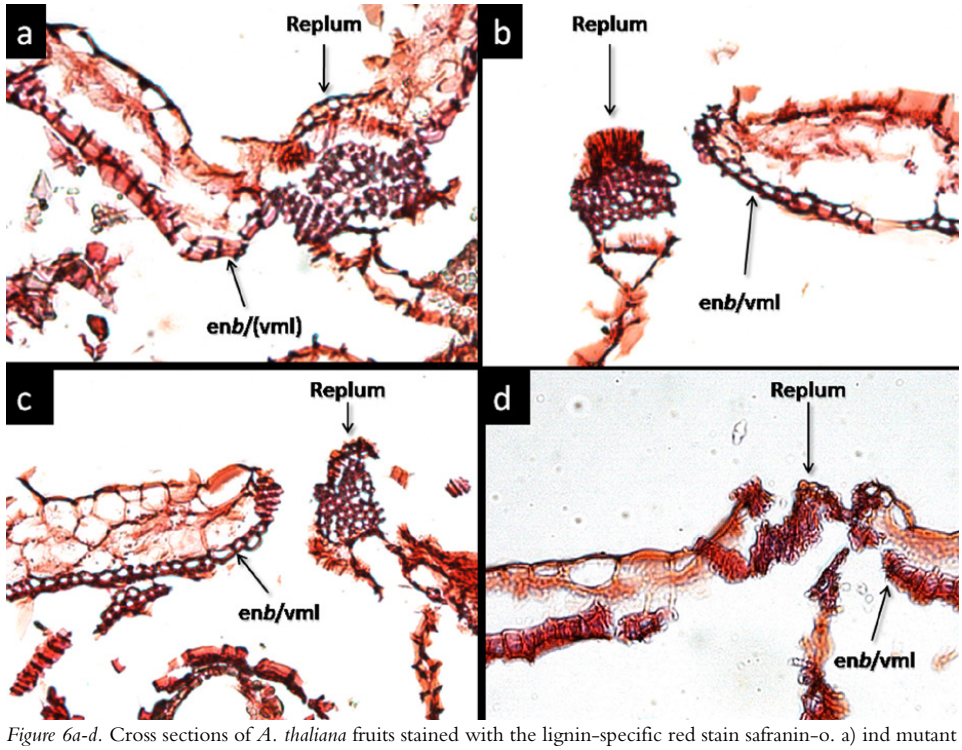


Figure 6a-d. Cross sections of *A. thaliana* fruits stained with the lignin-specific red stain safranin-o. a) ind mutant lacking a separation layer (sl) as well as a lignified valve margin layer (vml). The valve remains attached to the replum, b) indr (line 3-1) showing a complete recovery of the sl and the vml, allowing the valves to separate from the replum, c) wildtype showing the lignification of the vml and separation of the valve from the replum, d) indk (line 2-6) lacking a defined sl, however, there is the tendency to lignification of the vml and the valves are not as firmly attached to the replum as in the ind mutant.

However, there is still the tendency to lignification of the valve margin layer. SEM showed the lack of a distinct DZ in the two indk lines even though there is still a tendency to a valve-replum boundary, which is almost completely absent from the *ind* mutant (fig 7a,d). In any case, the valves in the two indk lines completely failed to detach from the replum, which they do in WT *A. thaliana* (fig 7c,d). Our results on down regulation of *IND* are in line with previous reports. Liljegren *et al.* (2004) reported the characterisation of various *A. thaliana ind* mutants. One of the stronger mutants (which is the same mutant that I have used in the functional complementation experiment presented here) showed no lignification in the valve margin, whereas one of the weaker, but nevertheless pod shatter-resistant, mutants still had a margin lignification similar to WT. The RNAi method for down regulation of gene expression commonly fails to yield a complete knock-out of gene function (Stoutjesdijk *et al.*, 2002). Because of this, it is not surprising that the pod shatter-resistant indk lines generated in these experiments quite resemble a weak *ind* mutant. Also, the reason why as few as two out of 19 indk transformed lines showed a phenotype similar to an *ind* mutant may be the design of the indk construct and the region targeted for RNAi. It is not uncommon to reach a quite low level of success in an RNAi experiment and often several constructs have to be designed and evalutated.

These results nevertheless show that the *LcIND* can be used to down regulate the IND function in an *A. thaliana* background. It is therefore highly likely that the targeting of *LcIND* in *L. campestre* will generate pod shatter-resistant plants.

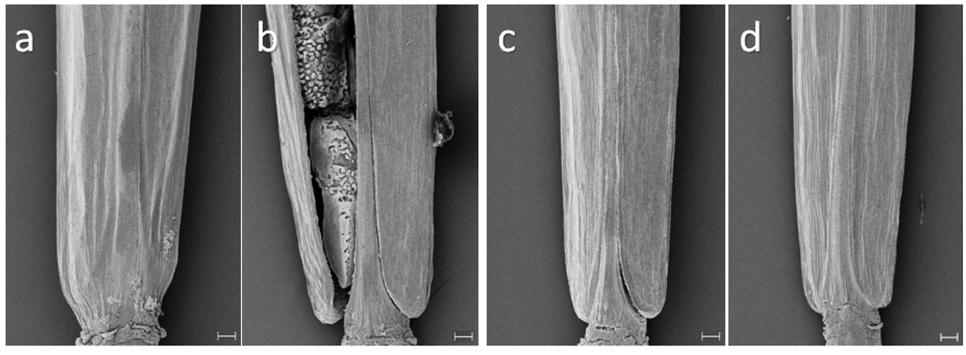


Figure 7a-d. A. thaliana fruits at post fertilisation stage 18-19. a) *ind* mutant showing a lack of a distinct dehiscence zone (DZ) and a strongly pod shatter-resistant phenotype, b) indr line 3-1 (ind mutant transformed with the indr construct) showing a recovery of the pod shatter-sensitive phenotype, c) wildtype (WT) with one valve starting to detach from the replum, d) indk line 15-3 (WT transformed with the indk construct) showing only a tendency to develop a DZ and displaying a pod shatter-resistant phenotype. Scale bar = 100 μm.

Concluding remarks

The aim of our project is to develop pod shatter-resistant *L. campestre* as the seed dispersal is one of the main problems with using this plant species as a commercial oilseed crop. Our approach is to make use of the knowledge generated in *A. thaliana* on the genetic regulation of seed dispersal and to target the transcriptional network regulating pod dehiscence. In this paper we have presented an anatomical and histological study of the *L. campestre* fruit with focus on the valve margin. We have also cloned the orthologous *LcIND*, which is one of the main genes in the mentioned transcriptional network. However, the down regulation of its function through RNAi is so far hindered by the lack of an efficient transformation protocol for *L. campestre*. The method of floral dipping, commonly used for transformation of *A. thaliana* and some other species (Grabowska and Filipecki, 2004), has unfortunately not yet yielded any result for *L. campestre* in our laboratory. We are therefore also working on the establishment of a transformation protocol using in vitro tissue culture and so far an efficient shoot regeneration protocol has been established (Eriksson and Merker, 2009).

Experimental procedures

Plant material

L. campestre accession NO94-7 collected in Öland, Sweden, was used in these experiments. WT *A. thaliana* were of ecotype columbia. *A. thaliana ind* mutant (ecotype landsberg erecta) was received from Yanofsky lab, University of California, San Diego, USA.

Cloning of LcIND

From DNA sequences for *A. thaliana IND* (accession number NM_116229) and *B. napus IND* (…..) retrieved from NCBI, we designed the following degenerate primers: dpIND-F (5´-GCGGAATTCATRGATSMGATGAAGRAGAT-3´) and dpIND-R (5´-GCGCAAGCTTCCAAKCTGAGWGTGAGGCTGA-3´). PCR on *L. campestre* genomic DNA generated a 290 bp fragment. From this fragment we used the genome walking method LaNe RAGE (Park 2005) to amplify fragments reaching to positions +1109 and -103 from a putative start codon, covering the entire putative *LcIND* ORF of 531 nt. The entire *LcIND* from start to stop codon was

then amplified from genomic DNA and sequenced three times independently using Advantage 2 Polymerase Mix (Clontech) and primers IND-F (5´-AAAGAAAGGTGTGTGCGACAA-3´) and IND-R (5´-TATGGTTGGCTTAGGGCTTG-3´). Total RNA was extracted from *L. campestre* fruits using Plant RNA Isolation Reagent (Invitrogen) and 5´- and 3´-RACE were performed using SMART RACE cDNA Amplification Kit (Clontech). All the DNA sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed by ABI 3100 Genetic Analyzer.

PCR analysis

Total RNA for RT-PCR expression analysis was extracted from *L. campestre* leaves, roots and fruits using Plant RNA Isolation Reagent (Invitrogen). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas). Transgenic *A. thaliana* T2 lines were confirmed by PCR analysis of leaf tissue using primers spanning part of the 35S promoter together with the *IND* fragment. Down regulation of *IND* was confirmed by RT-PCR on total RNA extracted from fruit samples collected at stage 16-17.

Vector construction

The indr construct for functional complementation of the *A. thaliana ind* mutant was made using the Gateway Technology with Clonase II (with *pDONR™221*, Invitrogen) according to manufacturer´s protocol and inserting the entire *LcIND* ORF into the pK2GW7 Gateway overexpression vector (Plant Systems Biology (PBS), Ghent University, Belgium). This vector contains a *npt*II gene in the T-DNA for plant selection on kanamycin. The indk construct for down regulation by RNA interference (RNAi) of *IND* in wildtype *A. thaliana* was made using the same Gateway Technology and inserting a 242 bp fragment (fig 4) amplified by the primers indk-F (5´-ACCTCATGATCTCCTCATGG-3´) and indk-R (5´-GGTTAGGCTTAGGGACAGTGG-3´) into the pB7GWIWG2(II) Gateway hairpin RNA expression vector (PBS). This vector contains a *bar* gene in the T-DNA for plant selection on DL-phosphinotricin (BASTA).

A. thaliana transformations

32 parental lines each of *A. thaliana ind* mutant and WT were raised and cut once five days before the first dipping. *Agrobacterium tumefaciens* strain EHA105 (C58) containing either one of the two vector constructs (indr or indk) was cultured in 250 ml of Luria-Bertani (LB) medium containing 50 mg/L of both rifampicin and spectinomycin, for *Agrobacterium* chromosomal selection and vector selection, respectively. Dipping was performed according to the protocol of Bechtold *et al.* (1993). *A. thaliana ind* mutant lines were dipped with construct indr and *A. thaliana* WT lines were dipped with construct indk. Seeds from indr-transformed plants were selected on 50 mg/L kanamycin and seeds from indk-transformed plants were selected on 10 mg/L BASTA. Of indr-transformed and indk-transformed plants, 15 and 19 independent T2 lines, respectively, were raised for subsequent analysis.

Histochemical staining

Wildtype, mutant and transgenic fruits were fixed overnight at room temperature (RT) in 10 mM Na-phosphate buffer with 4% paraformaldehyde at pH 7.2. After dehydration through an ethanol series into xylene, the tissues were embedded in paraffin. Samples were sectioned to 10 µm and mounted on slides. Sections were dewaxed in a reverse xylene-ethanol series, air dried and then stained with alcian blue and/or safranin-o in acetate buffer. Slides were then rinsed in water, air dried, mounted in Biomount (BB International) and photographed with a Leica DC300.

Scanning electron microscopy

Fruits were fixed overnight at RT in 0.1 M Na-phosphate buffer with 2% paraformaldehyde and 2.5% glutaraldehyde at pH 7.2. After critical point drying, the tissue was coated with gold/palladium (3:2) and examined in a LEO 435VP microscope using an acceleration voltage of 10.0 kV.

Acknowledgements

We would like to thank the Swedish Research Council Formas for financing the *L. campestre* project. We would also like to thank Peter Olsson for technical advice on the cloning and Kerstin Brismar and Salla Marttila for technical assistance with the microscopical analysis. We are thankful to Gary Ditta at the University of California, San Diego, for kindly providing the *A. thaliana ind* mutant and to Gunilla Swärdh at the Uppsala Transgenic Arabidopsis Facility for *A. thaliana* transformations.

References

Bechtold N, Ellis J, Pelletier G. 1993. In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. *CR Acad Sci Paris Life Sci* 316: 1194-1199.

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S. 2001. High-throughput screening for induced point mutations. *Plant Physiol* 126: 480-484.

Dinneny JR and Yanofsky MF. 2004. Drawing lines and borders: how the dehiscent fruit of *Arabidopsis* is patterned. *BioEssays* 27: 42-49.

Eriksson D, Merker A. 2009. An efficient adventitious shoot regeneration protocol for *Lepidium campestre* (L.) R. BR. *Prop Ornam Plants* 9(2): 78-83.

Ferrándiz C, Pelaz S, Yanofsky MF. 1999. Control of carpel and fruit development in Arabidopsis. *Annu Rev Biochem* 68: 321-354.

Ferrándiz C, Liljegren SJ, Yanofsky MF. 2000. Negative regulation of the *SHATTERPROOF* genes by FRUITFULL during *Arabidopsis* fruit development. *Science* 289: 436-438.

Ferrándiz C. 2002. Regulation of fruit dehiscence in *Arabidopsis*. *J Exp Bot* 53(377): 2031-2038.

Galun E. 2005. RNA silencing in plants. *In Vitro Cell Dev Biol – Plant* 41: 113-123.

Grabowska A, Filipecki M. 2004. Infiltration with *Agrobacterium* – the method for stable transformation avoiding tissue culture. *Acta Physiol Plant* 26(4): 451-458.

Jensen ES. 1991. Nitrogen accumulation and residual effects on nitrogen catch crops. *Acta Agric Scand* 41: 333-344.

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF. 2000., SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404: 766-770

Liljegren SJ, Roeder AHK, Kempin SA, Gremski K, Østergaard L, Guimil S, Reyes DK, Yanofsky MF. 2004., Control of fruit patterning in Arabidopsis by INDEHISCENT. *Cell* 116: 843-853

Merker A, Nilsson P. 1995. Some oil crop properties in wild *Barbarea* and *Lepidium* species. *Swedish J Agric Res* 25: 173-178.

Merker A, Eriksson D, Bertholdsson N-O. 2009. Barley yield increases with under-sown *Lepidium campestre*. *Acta Agric Scand Section B – Soil and Plant Sci*, published online 13 July.

Østergaard L, Kempin, SA, Bies D, Klee HJ, Yanofsky MF. 2006. Pod shatter-resistant *Brassica* fruit produced by ectopic expression of the *FRUITFULL* gene. *Plant Biotech J* 4: 45-51.

Østergaard L. 2009. Don´t "leaf" now. The making of a fruit. *Current Opinion in Plant Biology* 12: 36-41.

Park D. 2005., LaNe RAGE: a new tool for genomic DNA flanking sequence determination, *Electronic J of Biotech* 8(2): 218-225

Rajani S, Sundaresan V. 2001. The *Arabidopsis* myc/bHLH gene *ALCATRAZ* enables cell separation in fruit dehiscence. *Current Biology* 11: 1914-1922.

Roeder AHK, Ferrándiz C, Yanofsky MF. 2003. The role of the REPLUMLESS homeodomain protein in patterning the *Arabidopsis* fruit. *Current Biology* 13: 1630-1635.

Spence J, Vercher Y, Gates P, Harris N. 1996. "Pod shatter" in *Arabidopsis thaliana*, *Brassica napus* and *B. juncea*. *J of Microscopy* 181: 195-203.

Stoutjesdijk PA, Singh SP, Liu Q, Hurlstone CJ, Waterhouse PA, Green AG. 2002. hpRNA-mediated targeting of the Arabidopsis *FAD2* gene gives highly efficient and stable silencing. *Plant Physiol* 129: 1723-1731.

Stymne S. 2005. Nya vegetabiliska oljor för tekniskt bruk med genteknik. *Frö och Oljeväxtodlarna*, Försöks- och forskningsrapport.

Summers JE, Bruce DM, Vancanneyt G, Redig P, Werner CP, Morgan C, Child RD. 2003. Pod shatter resistance in the resynthesized *Brassica napus* line DK142. *J Agric Sci* 140: 43-52.

Vancanneyt G, Redig P, Child R, Yanofsky M, Botterman J. 2002. Podshatter resistance: Exploitation of Arabidopsis genes to develop a productivity trait in oilseed rape. *13th International Conference on Arabidopsis Research*, Seville, Spain, 28 June – 2 July.

III

Lepidium campestre FAD2 and *FAE1* cDNAs complement the corresponding *fad2* and *fae1* mutations in transgenic *Arabidopsis* plants

Dennis Eriksson* **and Arnulf Merker**
Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Alnarp, Sweden
(*Corresponding author)

Summary

We aim at domesticating *Lepidium campestre* L. of the Brassicaceae family as a novel oilseed crop. The potential to modify the fatty acid profile of the seed oil to obtain a suitable industrial oil quality in this species is being explored. Using a degenerate primer approach, two cDNAs corresponding to a putative *FATTY ACID ELONGASE 1* (*FAE1*) and a putative microsomal *FATTY ACID DESATURASE 2* (*FAD2*), respectively, were obtained from a *L. campestre* RACE-ready cDNA library from developing fruits. The *LcFAE1* cDNA contains an open reading frame of 1521 nt encoding a protein of 507 amino acids and the *LcFAD2* cDNA contains an open reading frame of 1152 nt encoding a protein of 383 amino acids. These *L. campestre* cDNAs both show high homology to other plant *FAE1* and *FAD2* genes. Functional complementation with the *LcFAE1* and *LcFAD2* cDNAs in *Arabidopsis thaliana fae1* and *fad2* mutants, low in erucic acid and linoleic acid, respectively, restores the wildtype fatty acid profile in the seed oil. Thus development of *L. campestre* accessions with suitable technical seed oil qualities through suppression or overexpression of the cloned genes can be achieved providing that an efficient transformation protocol for this species is developed.

Keywords: *L. campestre* L., *A. thaliana, FAE1, FAD2*, functional complementation

Introduction

Our society depends on fossil oil for transport and for many industrial applications. There are, however, increasing concerns about the environmental impact of fossil oil as well as the need to develop renewable sources of fuel and industrial raw materials (Dyer *et al.*, 2008). Vegetable oil is the biological raw material that has the largest potential to replace fossil oil. Still, however, the production of vegetable oil is 2-3 times more expensive than fossil oil. Nevertheless, this situation is now rapidly changing with the increasing world market prices of fossil oil. For many industrial applications, vegetable oil can already compete with fossil oil and 10-15% of all vegetable oil produced in the world is consumed as fuel or in the oleochemical industry (Stymne, 2005).

The predicted future increase in demand for vegetable oils will most certainly require the domestication of potential wild oilseed plants, since the huge number of technical applications will require many different oil qualities. The principle "one crop, one trait" would avoid mixing of seeds destined for different applications, which is especially important when it comes to oils for human consumption vs. technical applications (Nilsson *et al.*, 1998).

A domestication programme has been initiated with the goal of introducing *Lepidium campestre* (field cress) as an oilseed crop. *L. campestre* is a biennial species of the Brassicaceae family. It has a suitable agronomic plant type, excellent winter hardiness for cultivation at more northern latitudes and a high seed yield (Merker and Nilsson, 1995). The main traits we need to modify in *L. campestre* in order to

make it a suitable oil crop is: 1) to modify the fatty acid profile of the seed oil in order to generate *L. campestre* crop accessions with different oil qualities for different industrial applications, 2) to increase the relatively low oil content of the seeds and 3) to reduce the sensitivity to pod shatter. The work presented here focuses on modifying the fatty acid profile of the seed oil.

The carbon chains of fatty acids are initially synthesized in the plastids of the seeds. The monounsaturated oleic acid (C18:1) is then exported to the cytosol where further extension to very long chain fatty acids (VLCFA) or desaturation to polyunsaturated fatty acids (PUFA) occur on the endoplasmic reticulum (ER) (Buchanan *et al.*, 2006). The synthesis of VLCFAs with 20 (eicosenoic acid) or 22 (erucic acid) carbon atoms in the acyl chain is catalysed by an acyl-CoA elongase complex. This is an ER-bound enzymatic complex composed of four distinct enzymes: a condensing enzyme, two reductases and one dehydrase. The initial step in the elongation reaction is the condensation by the 3-ketoacyl-CoA synthase (KCS). This KCS enzyme is thought to determine the substrate and tissue specificity of fatty acid elongation whereas the three other enzymes in the elongase complex have broad substrate specificity and are found in all tissues exhibiting VLCFA biosynthesis (Joubés *et al.*, 2008). In *A. thaliana*, 21 KCS-like genes have been found, however only eight of them have so far been experimentally confirmed to be involved in VLCFA production (Joubés *et al.*, 2008). The gene encoding the seed-specific KCS18, considered to be the main enzyme responsible for VLCFA biosynthesis in the storage oil of Brassicaceae seeds (Rossak *et al.*, 1997), was cloned by transposon tagging already in 1995 by James *et al.* and named *FATTY ACID ELONGATION 1* (*FAE1*). *FAE1* is the name we will use in this report when we refer to the seed-specific *KCS18* analysed by Joubés *et al.* (2008). In the seed, FAE1 uses mainly oleic acid as substrate for elongation to eicosenoic acid (C20:1) and the same enzyme also catalyses the further elongation to erucic acid (C22:1) (Ghanevati and Jaworski, 2002). Further desaturation of oleic acid to the PUFA linoleic acid (C18:2) is catalysed by an acyl-lipid oleate desaturase enzyme, the FATTY ACID DESATURASE 2 (FAD2). The *FAD2* gene, cloned by Okuley *et al.* (1994), encodes an ER-bound enzyme. The signature of a fatty acid desaturase is the presence of three histidine clusters which bind iron ions to form the catalytic centre of the desaturase (Los and Murata, 1998).

With the aim of providing a platform for generating L. campestre cisgenic lines with altered fatty acid composition in the seed oil, we targeted the main enzymes responsible for synthesis of seed-specific VLCFAs and PUFAs. Taking advantage of the wealth of knowledge generated on biosynthesis of fatty acids in the seed oil of other Brassicaceae species, we cloned the orthologous *LcFAE1* and *LcFAD2* genes in *L. campestre* and showed the catalytic functions of their encoded proteins.

Results and Discussion

Cloning of L. campestre FAE1 and FAD2 cDNAs

From the *A. thaliana*, *B. napus* and *B. juncea* sequences for *FAE1* and *FAD2*, we identified regions with close homology and used these to design degenerate primers. With these primers we managed to amplify a 1324 bp putative *LcFAE1* cDNA fragment and a 1080 bp putative *LcFAD2* cDNA fragment from a *L. campestre* developing fruit RACE-ready cDNA library. From the obtained fragments we then continued with upstream and downstream RACE analysis to obtain the entire coding sequence (CDS) for both genes. We managed to obtain fragments covering the putative start and stop codons of both genes, however only the *LcFAE1* 3´-RACE appeared to yield a complete UTR. One reason for the failure to obtain full-length 5´-UTR and 3´-UTR for *LcFAD2* and 5´-UTR for *LcFAE1* could be minor disintegration of the RNA sample used for cDNA synthesis. Another reason could be incomplete extension by the reverse transcriptase during cDNA synthesis.

Subsequent sequence analysis nevertheless identified a 1521 nt putative *LcFAE1* CDS, showing 87%, 82% and 79% homology in the translated amino acid sequence to the AtFAE1, BnFAE1 and BjFAE1, respectively, and a 1152 nt putative *LcFAD2* CDS, showing 94%, 89% and 89% homology in the amino acid sequence to the AtFAD2, BnFAD2 and BjFAD2, respectively (fig 1a,b). The LcFAE1

Figure 1a. ClustalW alignment of LcFAE1, AtFAE1, BnFAE1 and BjFAE1 amino acid sequences. Regions in blue were used for design of degenerate primers. Region in yellow is the active-site Cys223 residue and regions in green are the active-site His302, -387, -391 and -420 residues.

Figure1b. ClustalW alignment of LcFAD2, AtFAD2, BnFAD2 and BjFAD2 amino acid sequences. Regions in blue were used for design of degenerate primers. Regions in yellow are the conserved His-boxes.

contains, at the same positions, the active-site amino acid residues His302, -387, -391 and -420 as well as Cys223 identified in AtFAE1 (fig 1a) (Ghanevati and Jaworski, 2001). The LcFAD2 contains the highly conserved Fe-binding histidine-boxes found in all known desaturases (fig 1b) (Los and Murata, 1998). Further PCR analysis on *L. campestre* genomic DNA revealed that both the *LcFAE1* and the *LcFAD2* lack introns in the open reading frame (ORF). This is in agreement with the orthologous genes found in *A. thaliana* (*FAE1*: Rossak *et al.*, 1997; *FAD2*: Okuley *et al.*, 1994). The *AtFAD2* contains one intron in the transcribed region, however, it is located 5´ to the ORF (Okuley *et al.*, 1994). The *LcFAE1* and *LcFAD2* cDNA sequences have been submitted to the NCBI genbank with accession numbers FJ907545 (*LcFAE1*) and FJ907546 (*LcFAD2*).

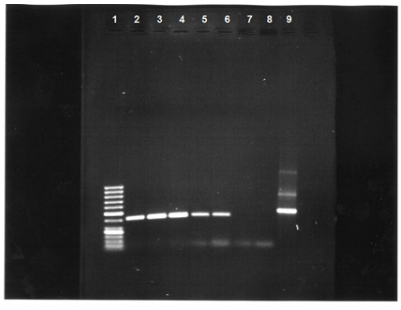


Figure 2. RT-PCR with *LcFAD2* primers on cDNA from *L. campestre* wildtype tissues. 1) DNA ladder GeneRuler 50 Bp (Fermentas), 2) fruits at post fertilisation stage 13, 3) fruits at post fertilisation stage 16, 4) fruits at post fertilisation stage 17, 5) leaves, 6) roots, 7) negative control without the reverse transcriptase to assess for genomic DNA contamination, 8) negative control without template to assess for reagent contamination, 9) positive control with GAPDH RNA and GAPDH-specific primers to assess for proper reverse transcription.

RT-PCR analysis of *L. campestre* wildtype (WT) fruits at the approximate developmental stages 13 (at anthesis), 16 (small, green fruits with sepals and petals starting to fall) and 17 (mature, full-size but still green fruits) as well as of tissue from leaves and roots confirmed that *LcFAD2* is expressed in each of the examined parts of the plant (fig 2). This result is in accordance with expression of *FAD2* in *A. thaliana* (Okuley *et al.*, 1994). For *LcFAE1*, we did not manage to detect any expression in any of the plant tissues examined. The reason for this peculiar failure is not known. The integrity of the RNA samples was maintained, as demonstrated by the RT-PCR experiment on *LcFAD2*.

The high (87% and 94%, respectively) amino acid sequence homology between the putative LcFAE1 and LcFAD2 and their orthologs in *A. thaliana* indicates that the *L. campestre* genes may be able to perform their function in an *A. thaliana* genetic background. To confirm the functions of the putative *LcFAE1* and *LcFAD2*, we therefore designed constructs, named fae1r and fad2r, to complement the *FAE1* and *FAD2* functions in *A. thaliana* loss-of-function *fae1* and *fad2* mutants (*Atfae1* and *Atfad2*) exhibiting a greatly reduced synthesis of eicosenioc acid (20:1) and of linoleic acid (18:2), respectively. The full-length *LcFAE1* CDS and *LcFAD2* CDS were each cloned into the Gateway overexpression vector pK2GW7 and used to transform their respective loss-of-function *A. thaliana* mutant. 20 independent transformed fae1r T2 lines and 23 independent transformed fad2r T2 lines were generated. Seeds from all of these lines were harvested at a mature stage and 100-150 seeds, with three independent plant repetitions, of each line were subject to gas liquid chromatography (GC) analysis to examine the fatty acid profile of the seed oil.

Expression of the L. campestre FAE1 cDNA restores oleate elongase activity in an A. thaliana fae1 mutant line

Of seeds from the 20 fae1r T2 lines analysed by GC, seven (4-1, 6-1, 9-1, 10-1, 16-1, 18-1 and 20-1) exhibited a statistically significant recovery of the elongated eicosenoic acid in the seed oil to some extent. However, only one line (6-1) contained wildtype level, at 16.9% of the total fatty acid content compared to the wildtype control with 15.6%. The other six lines contained between 2.9% and 5.6% eicosenoic acid, which was low but still significantly more than the *Atfae1* mutant control with 0.4% (table 1a). The remaining 13 lines were not significantly different from the *Atfae1* mutant and contained between 0.3% and 2.0% eicosenoic acid (data not shown).

A reduction in the level of eicosenoic acid in the *Atfae1* mutant leads to not only a higher level of oleic acid but also a concomitant increase in the level of linoleic acid, since there will be more oleic acid available as substrate for incorporation in phospholipids and further desaturation. Accordingly, the level of oleic and linoleic acid is higher in the *Atfae1* mutant compared to the corresponding levels in

wildtype *A. thaliana*. In the fae1r 6-1 line, both the level of oleic acid (13.9%) and linoleic acid (28.9%) were not significantly different from wildtype (13.2% and 28.1%, respectively). The other six lines with minor production of eicosenoic acid, however, all contained levels of linoleic acid similar to the mutant, and only 10-1 and 20-1 showed some reduction in the level of oleic acid compared to the mutant (fig 3a). No phenotypic abnormalities were observed in any of the examined plants.

Fae1r line 6-1 clearly demonstrates that the *LcFAE1* CDS is able to perform the function of VLCFA production in an *A. thaliana* genetic background. However, the reason why so few (one in 20) of the transformed lines for functional complementation of the loss-of-function mutant managed to recover the wildtype fatty acid profile may be the use of a 35S promoter for expression of the *LcFAE1*. This promoter is considered to be ubiquitously expressed, however an expression study by Sunilkumar *et al.* (2002) suggested that the 35S promoter is not expressed at an early stage of the embryogenesis. For the purpose of strong gene expression in the seeds it is better to use the napin storage protein promoter of *Brassica napus* (Ellerström *et al.*, 1996). However, for our purpose of confirming the function of the putative *LcFAE1* clone, the use of the 35S promoter has been sufficient.

A. thaliana line	wt	*fae1* mut	4-1	6-1	9-1	10-1	16-1	18-1	20-1
Level of C20:1	15.6 ± 1.1	0.4 ± 0.1	3.5 ± 0.5	16.9 ± 0.4	2.9 ± 0.7	3.5 ± 0.7	2.9 ± 0.4	3.3 ± 1.0	5.6 ± 1.3
P-value			0,00033	3E-07	0,00284	0,00161	0,00057	0,0079	0,00246

Table 1a. Level of eicosenoic acid (C20:1) in *A. thaliana* WT, *fae1* mutant and fae1r lines. Values are given in percentage of total seed fatty acid content ± standard deviation. fae1r lines were compared to the *fae1* mutant through Student´s t-test and were all found to be different at a significance level of 0.005.

A. thaliana line	wt	*fad2* mut	8-1	11-1	19-1	23-2	25-1	26-1	28-1	29-1	31-1	32-1
Level of C18:2	28.1 ± 0.4	4.1 ± 0.7	27.3 ± 3.2	26.3 ± 3.7	21.4 ± 1.5	20.1 ± 3.7	20.4 ± 2.0	13.9 ± 0.5	23.2 ± 1.5	19.3 ± 2.0	17.8 ± 0.2	16.2 ± 0.5
P-value			1E-04	3E-04	2E-05	8E-04	6E-05	1E-06	1E-05	8E-05	3E-08	5E-07

Table 1b. Level of C18:2 (linoleic acid) in *A. thaliana* WT, *fad2* mutant and fad2r lines. Values are given in percentage of total seed fatty acid content ± standard deviation. fad2r lines were compared to the *fad2* mutant through Student´s t-test and were all found to be different at a significance level of 0.005.

Expression of the L. campestre FAD2 cDNA restores oleate-Δ^{12} desaturase activity in an A. thaliana fad2 mutant line

Of seeds from the 23 fad2r T2 lines analysed by GC, ten (8-1, 11-1, 19-1, 23-2, 25-1, 26-1, 28-1, 29-1, 31-1 and 32-1) exhibited a statistically significant recovery of the polyunsaturated linoleic and linolenic acid in the seed oil to some extent, as well as a decrease in the level of monounsaturated oleic acid. However, only two lines (8-1 and 11-1) contained wildtype levels of linoleic acid, at 27.3% and 26.3%, respectively, of the total fatty acid content compared to the wildtype control with 28.1%. The other eight lines contained between 13.9% (line 26-1) and 23.2% (line 28-1) linoleic acid. This was still considerably more than the *Atfad2* mutant control with 4.1% linoleic acid (table 1b). The remaining 13 lines that were analysed were not significantly different from the *Atfad2* mutant and contained between 3.2% and 6.0% linoleic acid (data not shown).

A reduction in the level of linoleic acid leads to not only a lower level of linolenic acid and a higher level of oleic acid, but also a concomitant increase in the level of elongated eicosenoic acid since there will be more oleic acid available as substrate for further elongation. Accordingly, the level of oleic and eicosenoic acid is higher in the *Atfad2* mutant compared to the corresponding levels in wildtype *A. thaliana* (fig 3b). In the fad2r 8-1 and 11-1 lines, the levels of oleic acid (17.8% and 18.1%, respectively), linolenic acid (19.8% and 20.0%, respectively) and eicosenoic acid (13.2% and 16.4%, respectively) were not significantly different from wildtype (13.2%, 21.7% and 15.6%, respectively). All of the other eight lines with less-than-wildtype production of linoleic acid nevertheless contained wildtype levels of linolenic acid and considerably lower levels of oleic acid and eicosenoic acid

compared to the *Atfad2* mutant, ranging from 18.2% to 31.8% and from 16.9% to 20.6%, respectively. No phenotypic abnormalities were observed in any of the examined plants.

These results clearly demonstrate that the *LcFAD2* CDS is able to perform the function of linoleic acid production in an *A. thaliana* genetic background. The range of linoleic, oleic and eicosenoic acid levels seen in the ten lines which were significantly different from the *Atfad2* mutant is common and expected in this kind of functional complementation experiment. One of the reasons for obtaining such a range may be differences in expression levels of the introduced gene due to positional effects in the genome (Birch, 1997).

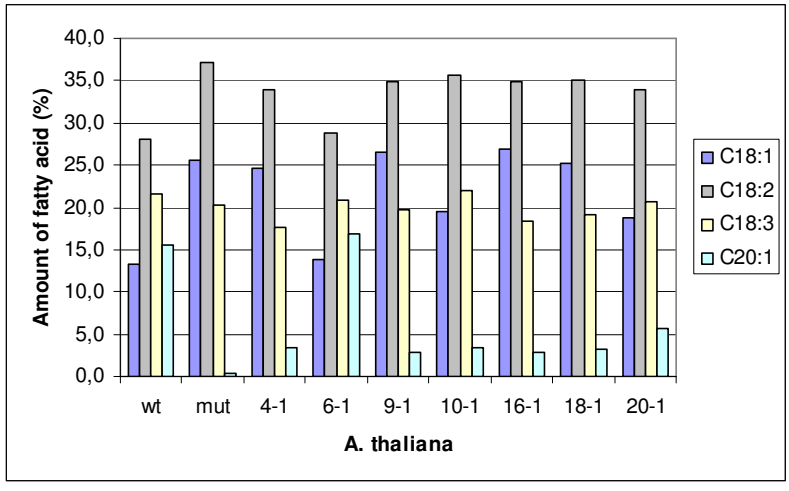


Figure 3a. Levels of C18:1, C18:2, C18:3 and C20:1 in *A. thaliana* wildtype (WT), *fae1* mutant and seven fae1r lines. All of the fae1r lines display C20:1 recovery to some extent, compared to the *fae1* mutant. However, only fae1r line 6-1 displays a fatty acid profile similar to WT.

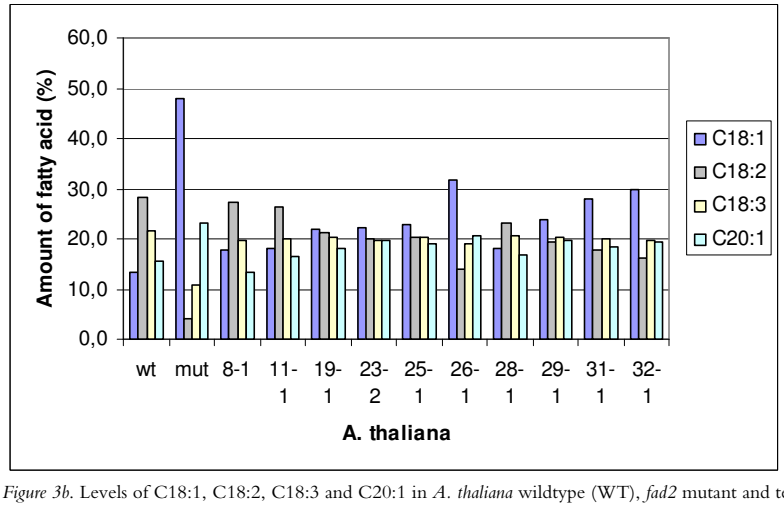


Figure 3b. Levels of C18:1, C18:2, C18:3 and C20:1 in *A. thaliana* wildtype (WT), *fad2* mutant and ten fad2r lines. All of the fad2r lines display C18:2 and C18:3 recovery and also a C18:1 reduction, compared to the *fad2* mutant.

Concluding remarks

The results presented in this report indicate that the putative LcFAE1 and LcFAD2 perform the same functions to regulate VLCFA and PUFA biosynthesis in *L. campestre* as the corresponding enzymes in *A. thaliana*. The next step is to down regulate their functions in *L. campestre* through the method of RNA interference (Galun, 2005). *L. campestre* has relatively high levels of erucic acid (23%) and linolenic acid (35%) in the seed oil with an amount of oleic acid of only 15% (Nilsson *et al.*, 1998). The reduction of both VLCFAs and PUFAs in the seed oil of *L. campestre* through simultaneous down regulation of *LcFAE1* and *LcFAD2* would most likely cause drastic increase in the amount of oleic acid. The *A. thaliana fae1/fad2* double mutant has 85% of oleic acid in its seed oil (Smith *et al.*, 2003). This would then make the *L. campestre* seed oil suitable for industrial applications requiring a high oxidative stability, such as biofuel (Rojas-Barros *et al.*, 2004). Another promising alternative is to down regulate only the *LcFAE1*. The high level of linolenic acid in *L. campestre* seeds indicates a very efficient enzymatic machinery for linolenic production. The reduction of VLCFAs would possibly redirect a portion of the oleic acid pool to PUFA production. Simultaneous overexpression of the *LcFAD2* is likely to cause an extremely high level of linolenic acid. This kind of oil would be excellent to use as an oxidising component of paints, varnishes and surface coatings (Jaworski and Cahoon, 2003). Down regulation of only the *LcFAD2* with simultaneous overexpression of *LcFAE1* and *Limnanthes douglasii LPAAT* would be a third alternative, potentially yielding an extremely high level of erucic acid, similar to what has been achieved in high erucic acid rapeseed (HEAR) [Nath *et al.*, 2009). A high level of the heat stable erucic acid would make the seed oil suitable as an industrial lubricant (Millar and Kunst, 1997) or in the production of erucamide, a slipping agent (Dyer *et al.*, 2008). All of these three alternatives would be interesting from a market point of view.

However, the down regulation of *LcFAE1* and/or *LcFAD2* is so far hindered by the lack of an efficient transformation protocol for *L. campestre*. The method of floral dipping, commonly used for transformation of *A. thaliana* and some other species (Grabowska and Filipecki, 2004), has unfortunately not yet yielded any result for *L. campestre* in our laboratory. We are therefore also working on the establishment of a transformation protocol using in vitro tissue culture and so far an efficient shoot regeneration protocol has been established (Eriksson and Merker, 2009).

Experimental procedures

Plant material

L. campestre accession NO94-7 collected in Öland, Sweden, was used in these experiments. All *A. thaliana* plants used in these experiments were of ecotype columbia. *A. thaliana fae1* and *fad2* mutants were purchased from the European Arabidopsis Stock Centre (*fae1*: NASC ID N6245, *fad2*: NASC ID N8041).

Cloning of LcFAE1 and LcFAD2

From ClustalW alignments of DNA sequences retrieved from NCBI for *FAE1* and *FAD2* from *A. thaliana* (NM_119617 and NM_112047, respectively), *B. napus* (EU543283 and FJ952144, respectively) and *B. juncea* (Y11007 and EF639848, respectively), we designed the following degenerate primers: dpFAE1-F (5′-AAGCTCCTTTACCRTTACGTCWTAACC-3′) and dpFAE1-R (5′-CGTTTTGGRCWYGAGTCTYTGACTT-3′) for amplification of a putative *LcFAE1* as well as dpFAD2-F (5′-CSGAAACCGACACCAYMAAGCG-3′) and dpFAD2-R (5′-CACCTTTCTTSTCACCTTSC-3′) for amplification of a putative *LcFAD2*. As template for degenerate PCR, we produced a RACE-ready *L. campestre* cDNA library with SMART RACE cDNA Amplification Kit (Clontech). Total RNA was extracted from *L. campestre* young fruits using Plant RNA Isolation Reagent (Invitrogen) and XX μg was used to produce the cDNA library. PCR generated a 1324 bp fragment for *LcFAE1* and a 1080 bp fragment for *LcFAD2*. Outward primers were then designed for 5′-RACE and 3′-RACE. Thereafter, the entire putative *LcFAE1* ORF (1521

nt) and *LcFAD2* ORF (1152 nt) were amplified from *L. campestre* genomic DNA and sequenced three times independently using Advantage 2 Polymerase Mix (Clontech) and primers FAE1-F1 (5´-GGACAAACTCAACAATGAGGTGGTC-3´) with FAE1-R1 (5´-AGAACAACACAACACCAAAGTATTC-3´), FAE1-F2 (5´-CGAAACTTACGGTCCAGAGG-3´) with FAE1-R2 (5´-AGCCCTAAGTTCTTCTCAAGCA-3´), FAD2-F1 (5´-CCTACGTCAGCTCCAGAAAC-3´) with FAD2-R1 (5´-CCTCTTCTGATAGTGAACTTTTTCC-3´) and FAD2-F2 (5´-CCTTCCTCCTCGTCCCTTAC-3´) with FAD2-R2 (5´-ACTGGTACAACAATAAGTTA-3´). All the DNA sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed by ABI 3100 Genetic Analyzer.

PCR analysis

Total RNA for RT-PCR expression analysis was extracted from *L. campestre* leaves, roots and fruits using Plant RNA Isolation Reagent (Invitrogen). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas). Transgenic *A. thaliana* T2 lines were confirmed by PCR analysis of leaf tissue using primers spanning part of the 35S promoter together with the *FAE1/FAD2* fragment.

Vector construction

The fae1r and fad2r constructs for functional complementation of the *A. thaliana fae1* mutant and *fad2* mutant, respectively, were made using the Gateway Technology with Clonase II (with *pDONR™221*, Invitrogen) according to manufacturer´s protocol and inserting either the entire *LcFAE1* CDS or the entire *LcFAD2* CDS into the pK2GW7 Gateway overexpression vector (Plant Systems Biology (PBS), Ghent University, Belgium). This vector contains a *npt*II gene in the T-DNA for plant selection on kanamycin.

A. thaliana transformations

32 parental lines of each *A. thaliana* mutant (*fae1* and *fad2*) were raised and cut once five days before the first dipping. *Agrobacterium tumefaciens* strain EHA105 (C58) containing either one of the two vector constructs (fae1r or fad2r) was cultured in 250 ml of Luria-Bertani (LB) medium containing 50 mg/L of both rifampicin and spectinomycin, for *Agrobacterium* chromosomal selection and vector selection, respectively. Dipping was performed according to the protocol of Bechtold *et al.* (1993). *A. thaliana fae1* mutant lines were dipped with construct fae1r and *A. thaliana fad2* mutant lines were dipped with construct fad2r. Transformed seeds were selected on 50 mg/L kanamycin. Of fae1r-transformed and fad2-transformed plants, 20 and 23 independent T2 lines, respectively, were raised for subsequent analysis.

GC analysis

100-150 seeds each from three plants from each line were harvested and GC analysed. Whole seeds were heated in 2% sulphuric acid in methanol for 45 min at 90°C. Fatty acid methyl esters were extracted into n-hexane and subsequently analysed with gas-liquid chromatography (Schimadzu, GC-17A, BergmanLabora, Sweden) using a WCOT fused silica 50 m/0.32 mm ID capillary column coated with CP-Wax 58-CB DF¼0.2 (Chrompack Inc., The Netherlands) The results are given as an average ±STD of the three samples.

Acknowledgements

We would like to thank the Swedish Research Council Formas for financing the *L. campestre* project. We would also like to thank Anders Carlsson and Sten Stymne for technical advice. We are thankful to NASC for kindly providing the *A. thaliana fae1* and *fad2* mutants and to Gunilla Swärdh at the Uppsala Transgenic Arabidopsis Facility for *A. thaliana* transformations.

References

Bechtold N, Ellis J, Pelletier G. 1993. In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. *CR Acad Sci Paris Life Sci* 316: 1194-1199.

Birch RG. 1997. Plant transformation: Problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol* 48: 297-326.

Buchanan BB, Gruissem W, Jones RL. 2006. *Biochemistry & molecular biology of plants*, sixth impression. American Society of Plant Biologists: Craft Print Int. Ltd., Singapore. ISBN 0-943088-39-9.

Dyer JM, Stymne S, Green AG, Carlsson AS. 2008. High-value oils from plants. *Plant J* 54: 640-655.

Ellerström M, Stålberg K, Ezcurra I, Rask L. 1996. Functional dissection of a napin gene promoter: identification of promoter elements required for embryo and endosperm-specific transcription. *Plant Mol Biol* 32(6): 1019-1027.

Eriksson D, Merker A. 2009. An efficient adventitious shoot regeneration protocol for *Lepidium campestre* (L.) R. BR. *Prop Ornam Plants* 9(2): 78-83.

Galun E. 2005. RNA silencing in plants. *In Vitro Cell Dev Biol – Plant* 41: 113-123.

Ghanevati M, Jaworski JG. 2001. Active-site residues of a plant membrane-bound fatty acid elongase β-ketoacyl-CoA synthase, FAE1 KCS. *Biochim et Biophys Acta* 1530: 77-85.

Ghanevati M, Jaworski JG. 2002. Engineering and mechanistic studies of the *Arabidopsis* FAE1 β-ketoacyl-CoA synthase, FAE1 KCS. *Eur J Biochem* 269: 3531-3539.

Grabowska A, Filipecki M. 2004. Infiltration with *Agrobacterium* – the method for stable transformation avoiding tissue culture. *Acta Physiol Plant* 26(4): 451-458.

James DW, Lim E, Keller J, Plooy I, Ralston E, Dooner HK. 1995. Directed tagging of the *Arabidopsis FATTY ACID ELONGATION* (*FAE1*) gene with the maize transposon activator. *The Plant Cell* 7: 309-319.

Jaworski J, Cahoon EB. 2003. Industrial oils from transgenic plants. *Curr Op in Plant Biol* 6: 178-184.

Joubés J, Raffaele S, Bourdenx B, Garcia C, Laroche-Traineu J, Moreau P, Domergue F, Lessire R. 2008. The VLCFA elongase gene family in Arabidopsis thaliana: phylogenetic analysis, 3D modelling and expression profiling. *Plant Mol Biol* 67: 547-566.

Los DA, Murata N. 1998. Structure and expression of fatty acid desaturases. *Biochim et Biophys Acta* 1394: 3-15.

Merker A, Nilsson P. 1995. Some oil crop properties in wild *Barbarea* and *Lepidium* species. *Swedish J Agric Res* 25: 173-178.

Millar AA, Kunst L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal* 12(1): 121-131.

Nath UK, Wilmer JA, Wallington EJ, Becker HC, Möllers C. 2009. Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with *Ld-LPAAT* + *Bn-fae*1 transgenes in rapeseed (*Brassica napus* L.). *Theor Appl Genet* 118: 765-773.

Nilsson P, Johansson S-Å, Merker A. 1998. Variation in seed oil composition of species from the genera *Barbarea* and *Lepidium*. *Acta Agric Scand Section B – Soil and Plant Sci* 48: 159-164.

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J. 1994. *Arabidopsis FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell* 6: 147-158.

Rojas-Barros P, de Haro A, Munoz J, Fernández-Martínez JM. 2004. Isolation of a natural mutant in castor with high oleic/low ricinoleic acid content in the oil. *Crop Science* 44: 76-80.

Rossak M, Smith M, Kunst L. 2001. Expression of the *FAE*1 gene and *FAE*1 promoter activity in developing seeds of *Arabidopsis thaliana*. *Plant Mol Biol* 46: 717-725.

Smith MA, Moon H, Chowrira G, Kunst L. 2003. Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. *Planta* 217: 507-516.

Stymne S. 2005. Nya vegetabiliska oljor för tekniskt bruk med genteknik. *Frö och Oljeväxtodlarna*, Försöks- och forskningsrapport.

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Rathore KS. 2002. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant Mol Biol* 50(3): 463-479.

IV

Acta Agriculturae Scandinavica Section B – Soil and Plant Science
2009, 1–5, iFirst article

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Barley yield increases with undersown *Lepidium campestre*

ARNULF MERKER, DENNIS ERIKSSON & NILS-OVE BERTHOLDSSON

Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Box 101, SE-230 53 Alnarp, Sweden

Abstract
The potential new oil crop *Lepidium campestre* (field cress) was undersown with long and short row distances in spring barley in three-year trials with eight replications. Plots with no undersowing were used as controls. The purpose was to assess the effect on barley-grain yield of the oil crop and its seed-yield potential. In the plots without undersowing, with long and short row distance of undersown *L. campestre* the barley-grain yields in the three years were, on average, 5069, 5130, and 5330 kg/ha, respectively. The trials showed a statistically significant positive effect on barley yield of undersowing with short row distance. The average seed yields of *L. campestre* in the following years were 4697 and 5000 kg/ha for plots with long and short row distance, respectively. The results are positive in relation to possibilities to domesticate this species as a new undersown oil crop for an energy-saving and environmentally friendly cropping system.

Keywords: *Cereal, cropping system, field cress, interaction, oil crop, undersowing, yield.*

Introduction

In plant production the cropping system to a large extent determines energy inputs and environmental effects. Present cropping systems with only annual species in, e.g., cereals, oil crops, and pulses are energy demanding through annual sowing and tilling. These systems also leach plant nutrients, which instead of benefiting crop growth pollute the ambient aquatic environment (Bergström & Brink, 1986; Gustafsson, 1987). Despite an extensive accumulation of research and knowledge in this field during the last few decades, no significant reduction of leaching of plant nutrients from Swedish agriculture has been recorded (Johnsson & Mårtensson, 2002). Production systems where spring cereals are undersown are more efficient in reducing leaching than are those with spring- or autumn-sown annuals (Jensen, 1991; Lemola et al., 2000; Känkänena & Eriksson, 2007). A production system with undersowing of an overwintering oil crop in a spring cereal would save energy by sowing two crops at the same time without tilling in between. Leaching of nutrients would also be reduced by absence of tilling and by accumulation of biomass of the oil crop after harvest of the cereal. The oil crop then overwinters and is harvested the next season. If it is perennial these positive effects are increased. The system will have many of the energy and environmental advantages of ley production.

Fossil fuels are today exploited to a degree where there is a serious concern that we will run out of these relatively cheap sources of energy relatively soon. Increasing concerns about carbon dioxide emissions and their effect on climatic change and high crop yields per unit area with relatively low world market prices have initiated the quest for alternatives. Production of fuel in the form of ethanol and bio-diesel and of industrial raw materials has the potential to cover substantial areas of arable land. Oil crops for production of special industrial oil qualities to substitute for petroleum oils (Princen, 1983; Dyer et al., 2008) are especially attractive. They are a renewable source of raw materials and reduce the direct use of fossil sources.

A project at the Swedish University of Agricultural Sciences (SLU) aiming at domesticating the wild biennial cress *Lepidium campestre* L. of the *Brassicaceae* family has its starting point in the problem

Correspondence: Prof. Arnulf Merker, Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Box 101, SE-230 53 Alnarp, Sweden. Tel.: +46 40 415527. Fax: +46 40 415519. E-mail: Arnulf.Merker@ltj.slu.se

(Received 19 December 2008; accepted 17 March 2009)

ISSN 0906-4710 print/ISSN 1651-1913 online
DOI: 10.1080/09064710902903747

complexes referred to above. It aims at developing an oil crop that is undersown in a spring cereal. It will be furnished with an industrial oil quality. This species has been selected among a number of earlier investigated candidates (Merker & Nilsson, 1995). It has several advantages in the proposed production system. It has a good agronomic plant type with branching only in the upper part of the upright stems. It is a spring-germinating biennial with closely related perennials. Its life cycle is hence pre-adapted to undersowing. It has a winter hardiness far better than that of the *Brassica* oil crops, a suitable seed size, a high seed yield, and resistance to the pollen beetle (Merker & Nilsson, 1995). It also has an oil quality with high content of linolenic acid, which is an industrial quality as such (Nilsson et al., 1998). Self-fertilisation and diploidy are other important advantages enabling simple pure-breeding of characters in a domestication project. Investigations on cultivation and effects on yield of different sowing densities and nitrogen supply were presented by Börjesdotter (1999a, 1999b).

Shattering resistance and higher oil content are key properties in the efforts to make *L. campestre* a useful oil crop (Merker & Nilsson, 1995; Andersson et al., 1998). These are characters determined by few or single genes, while winter hardiness, yield, life cycle, and adaptation to the cropping system or the environment generally, are properties with complex inheritance. Hence from a plant-breeding point of view it should be easier to domesticate *L. campestre* for the new cropping system and end use than to adapt established oil crops (Merker & Nilsson, 1995). Content of glucosinolates and protein and other characters affecting feeding value need not to be addressed to start with, since a purely industrial crop is the goal.

In establishing *L. campestre* as a new undersown oil crop its seed yield and its effect on the yield of the spring cereal are crucial. The present study was designed to elucidate these two aspects. Three-year trials with barley plots with *L. campestre* undersown at two different row distances and without undersowing allowed the analysis of interactions between the factors year, barley yield, and undersowing at two row distances to be made. The seed-yield potential of *L. campestre* and its interactions with year and row distance were also analysed. The yield factors thousand-grain weight (TGW) and thousand-seed weight (TSW) were also studied in the trials.

Materials and methods

Trials were sown in 2004, 2005, and 2006 in sandy loam soil at the Lönnstorp experimental station at Global Positioning System (GPS) coordinates N 55°40′9″, E 13°6′10″ near Alnarp, Sweden. Sowing dates were 2004-04-14, 2005-04-13, and 2006-05-03. The spring barley variety Prestige was sown with a 16-drill Öyord machine in four rows of six plots of the size 187.5 cm × 300 cm at a density of 150 kg/ha. A block design was used with three treatments randomised in eight blocks. Each of the four rows of six plots hence contained two blocks. The three treatments were no undersowing (none), undersowing with 25-cm row distance (25 cm), and undersowing with 12.5-cm row distance (12.5 cm) of *L. campestre* accession NO94-6 collected in Arild, Sweden.

The undersowing with a plot size of 2.75 m^2 (137.5 cm × 200 cm with 9 rows 25 cm apart or 17 rows 12.5 cm apart) was made by hand by marking each row as a furrow at right angles to the barley rows by means of pressing into the ground a wooden frame of triangular rods. Furrows were covered after sowing. This gave a seed depth of 1–2 cm. The density of undersowing was 90 seeds per row.

The trials were given 100 kg N/ha for the barley before sowing and another 80 kg N/ha in early spring the following year for the *L. campestre*. Since herbicides could not be used in the trials, plots and paths between them were weeded by hand three times each season. This eliminated most weeds from the trials.

The trials were harvested with a combine harvester with a 1.6-m-wide header. This gave a harvested plot size of 4.8 m^2 and 2.75 m^2 for barley and *L. campestre*, respectively. Barley borders left after harvest were removed with the combine. All barley straw from the combine harvester was removed from the trial. Plot yields of the two species were weighed by the combine directly and a sample (700–1000 g) of each plot was taken and weighed manually. Samples were dried and the plot yields, TGW, and TSW were calculated on a 15%-moisture basis. Harvest dates were 2004-08-11, 2005-08-22, and 2006-08-12 for the barley and 2005-07-26, 2006-08-01, and 2007-07-18 for the *L. campestre*.

Besides yield the following parameters were recorded for each plot: stand of the barley (full stand = 100%), heading date of the barley, straw stiffness of the barley (standing upright = 100%), stand of the *L. campestre* (percentage of the total row length covered with plants) in the autumn and in the spring of the following year (full stand = 100%), date of initiation and termination of flowering of *L. campestre*, stalk stiffness of *L. campestre* (standing upright = 100%), and plant height of *L. campestre* in cm. TGW and TSW were determined on all dried plot samples.

Weather conditions were close to the average for the region in 2004, 2005, and 2007. 2006 was

deviating and was dry after sowing with some precipitation in late May and early June. Then a very dry summer followed in June and July. August and September had high precipitation. Weather conditions in 2006 affected agricultural yields negatively in the whole region.

The data were analysed as a split-plot experiment with the GLM procedure of the software Statgraphics Plus (Manugistics Inc.), with year as main factor and treatments as sub-factors. Year was tested against year (repetition) and treatment against the residual variance. Barley yield and TSW data were log transformed before analysis as the variances differed between years. A 2-way analysis of variance (ANOVA) was done on separate year data with treatment (undersowing or row distance) and replications as factors. The means were tested for significant differences at 95% with the Duncan test.

Results

Barley grain yield varied both with year ($P=0.0000$) and undersowing ($P=0.0038$). However, the treatment effects on grain yield differed between years, but within limits for nonsignificance ($P=0.0635$). The main difference was a stronger positive effect of the shorter row distance of undersowing in 2006 ($P=0.0949$) and also a positive effect of the longer row distance in 2005 ($P=0.1113$). Over the three years undersown *L. campestre* with 25-cm row distance had no significant effect on barley grain yield. With a row distance of 12.5 cm, however, it had $P=0.0038$. The effects of treatments on TGW were all small and not significant (Table I).

Seed yield of *L. campestre* the year after the barley crop was as high as 4848 kg/ha on average, but differed between years, with the highest yield in 2005 and lowest in 2006 ($P=0.0001$). There was no effect of different row distances over the three years ($P=0.0871$). However, in 2006 the 12.5-cm row distance out-yielded the 25-cm one ($P=0.0140$). This difference between years is also indicated by a significant year × row distance interaction ($P=0.0431$). The effect on TSW was different between years and row distance ($P=0.0001$ and $P=0.0071$, respectively). On average a short row distance resulted in smaller seeds. This effect was observed in all years, but was only significant for the high yielding year of 2005 ($P=0.0261$) (Table II).

No differences were observed in the barley plots between the three different treatments for stand of the plots, heading date, and straw stiffness. The stands were uniformly full (100%). Heading dates were 2004-06-24, 2005-06-23, and 2006-06-20 for the three years. The barley was standing upright (100%) in all plots over the three years.

The stands of the undersown *L. campestre* did not differ between row distances. In 2004/2005 there were good stands with an average score of 99%. In 2005/2006 the stands varied between 75 and 85% with an average for long and short row distances of 79 and 81%. In 2006/2007 the stands were again dense with averages of 98 and 99% for short and long row distances respectively. Stands in the spring were in all three years equal to those in the autumn.

The *L. campestre* plots started flowering in the first half of May (05-11, 05-07, and 05-12) and termi-

Table I. Barley grain yield and thousand-grain weight (TGW) without undersown *L. campestre*, with 25-cm row distance and with 12.5-cm row distance of undersown *L. campestre* 2004–2006. Different letters in each column denote significant differences according to the Duncan test at 95% significance level.

Undersowing	2004	2005	2006	Average
		Grain yield (kg × ha^{-1})		
None	5651a	5494a	4062a	5069a
25 cm	5625a	5703a	4063a	5130a
12.5 cm	5755a	5755a	4479a	5330b
Average	5677	5651	4201	5176
		P-values		
Year	–	–	–	0.0000
Undersowing	0.7917	0.1113	0.0949	0.0038
Y × U	–	–	–	0.0635
		TGW (g)		
None	43.4a	45.4a	49.2a	46.0a
25 cm	43.7a	46.5a	49.8a	46.7a
12.5 cm	43.4a	46.3a	51.0a	46.9a
Average	43.5	46.1	50.0	46.5
		P-values		
Year	–	–	–	0.0000
Undersowing	0.9441	0.2318	0.1800	0.1600
Y × U	–	–	–	0.5274

Table II. *L. campestre* seed yield and thousand-seed weight (TSW) with 25-cm and 12.5-cm row distance 2005–2007. Different letters in each column denote significant differences according to the Duncan test at 95% significance level.

Row distance:	2005	2006	2007	Average
		Seed yield (kg × ha^{-1})		
25 cm	6136a	3182a	4773a	4697a
12.5 cm	6454a	4045b	4500a	5000a
Average	6295	3614	4636	4848
		P-values		
Year	–	–	–	0.0001
Row distance	0.3609	0.0140	0.4758	0.0871
Y × R	–	–	–	0.0431
		TSW (g)		
25 cm	3.20a	2.76a	2.82a	2.93a
12.5 cm	3.01b	2.73a	2.74a	2.83b
Average	3.11	2.74	2.78	2.88
		P-values		
Year	–	–	–	0.0001
Row distance	0.0261	0.6451	0.0952	0.0071
Y × R	–	–	–	0.1976

nated flowering three weeks later. Flowering was between one and two days later for the plots with long row distances. All the *L. campestre* plots were standing upright in all three years. Plant height did not differ between row distances. Plant heights for the three years were 80, 75, and 75 cm, respectively. The *L. campestre* plots were harvested on 2005-07-26, 2006-08-01, and 2007-07-18 in the three years, respectively.

Discussion

The competition between the barley and the undersown species is crucial for the economic success and applicability of cropping systems with undersowing. Känkänen & Eriksson (2007) reported neutral or significantly negative effects on barley grain yield by undersowing of different legume and grass species. Spaner & Todd (2003), in barley trials with undersown forage mixtures, reported no effects of undersowing on barley-grain yields. In this light the present results are quite interesting. There seems to be a positive effect of undersowing of *L. campestre* on the barley yield. Different statistical treatments gave the same result. Although the present experiments give no direct explanation, the reasons can be sought in, e.g., water and nitrogen balance or in other indirect or direct interactions between the crops. The fact that the dry year 2006 showed the largest difference between plots without undersowing and plots with undersowing with short row distance could speak in favour of this possibility. Whatever the reasons are, it is interesting and promising for the cropping system with undersown *L. campestre* in barley that the two species function well together and that there may even be a yield advantage from the co-cultivation.

Another notable finding of this series of trials is the high seed yield of *L. campestre* with an average yield of over 4800 kg/ha. Border effects in the relatively small plots call for some caution in drawing conclusions from these results. On the other hand quite some shattering of seeds took place before and in connection with harvest. The measured seed-yield levels are anyhow surprisingly high, taking in account that we are dealing with a wild plant not affected by domestication and plant breeding. They are comparable with seed yields in southern Sweden of winter oilseed rape, *Brassica napus*. From trials in Uppsala, Merker & Nilsson (1995) reported yields up to 3700 kg/ha, and Börjesdotter (1999a, 1999b) recorded values of over 2000 kg/ha. The present much higher yields could be explained with, e.g., a different location of the trials, more plant nutrients available, and higher border effect in the smaller plots. The accession of *L. campestre* used in the present experiments was collected not far from the trial site and should hence have a good general adaptation at the trial site. The present trials, with eight replications per year during three years, safely reflect the very high seed-yield potential of the species.

The yield of *L. campestre* seeds was not significantly different between the two row distances over the three years. Looking at the years individually this difference was statistically significant in 2006. This can be explained by the stands not being full in that year. They were on average 80% full as a result of incomplete emergence. This is in line with results of Börjesdotter (1999a) who, in trials with *Barbarea* species and *L. Campestre*, found significant effects on seed yield of density of stands. Absence of plants in 20% of the total row length of the plots in 2006 had more severe effects in the plots with long row distance. The short row distance had a better ability to compensate for uneven emergence and stand. Taking in account also the effect of undersowing on the barley yield the short row distance is preferable in this cropping system, unless there are special reasons for a longer row distance, like, e.g., mechanical weeding.

The potential of using *L. campestre* as an effective oil crop is, however, influenced not only by the seed yield but also by the chemical composition of the seeds. Andersson et al. (1999) as well as Nilsson et al. (1998) in thorough analyses of seed composition of five accessions reported around 20% fat, 20% protein, and 40% dietary fibre. The high content of fibre is a result of a thick seed coat. With the present seed composition the species at a seed-yield level of 4 tons/ha produces the modest quantity of 800 kg oil/ha. This is comparable to the oil yield in southern Sweden of the relatively low yielding oil turnip rape, *Brassica rapa*. The isolation of mutants with a thin seed coat could improve this situation. It is known from the *Brassica* oil-crops that such mutants increase the oil content (Chen et al., 1988).

The numerical tendency of increasing TGW with undersowing is not statistically significant. The probable stimulation of barley grain yield by undersowing reported here is hence to be looked for mainly in other yield components. TSW of *L. campestre* plots with long row distance is statistically significantly higher than for short-row-distance plots. A compensation for fewer plants per plot by higher TSW seems to play a role, together with compensation also in other yield components.

The results presented here on possible positive effects of the undersowing on barley grain yield and the high seed yield of *L. campestre* seem promising for attempts to domesticate it as a new oil crop for a beneficial cropping system. In order to draw more

precise conclusions on the interactions between the two crops further experimental work is needed. Field trials drawing also water and nitrogen balance as well as weeds and insects into focus would be informative, as would different laboratory experiments on allelopathic effects.

Acknowledgements

Financial support for this study was received from The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas). Anders Engberg, Eskil Kempe, and Erik Rasmusson of the Lönnstorp experimental station are gratefully acknowledged for skilful handling of the trials, and Ann-Sofie Fält and Maria-Luisa Prieto-Linde for valuable technical assistance.

References

Andersson, A.A.M., Merker, A., Nilsson, P., Sörensen, H., & Åman, P. (1999). Chemical composition of the potential new oilseed crops *Barbarea vulgaris*, *Barbarea verna* and *Lepidium campestre*. *Journal of the Science of Food and Agriculture*, *79*, 179–186.

Bergström, L., & Brink, N. (1986). Effects of differentiated applications of fertilizer N on leaching losses and distribution of organic N in the soil. *Plant and Soil*, *93*, 333–345.

Börjesdotter, D. (1999a). Influence of plant density on stand development and seed yield of *Barbarea verna*, *Barbarea vulgaris* and *Lepidium campestre*. In: Börjesdotter, D. (1999). *Potential oil crops: Cultivation of Barbarea verna, Barbarea vulgaris and Lepidium campestre*. Doctoral dissertation at the Swedish University of Agricultural Sciences, Department of Ecology and Crop Production Science. ISSN 1401-6249, ISBN 91-576-5746-7.

Börjesdotter, D. (1999b). Effects of nitrogen supply and seed rate on plant stand and seed yield of *Barbarea verna*, *Barbarea vulgaris* and *Lepidium campestre*. In: Börjesdotter, D. (1999). *Potential oil crops: Cultivation of Barbarea verna, Barbarea vulgaris and Lepidium campestre*. Doctoral dissertation at the Swedish University of Agricultural Sciences, Department of Ecology and Crop Production Science. ISSN 1401-6249, ISBN 91-576-5746-7.

Chen, B.Y., Heneen, W.K., & Jönsson, R. (1988). Inheritance of seed colour in *Brassica campestris* L. and breeding for yellow-seeded. *B. napus L. Euphytica*, *59*, 157–163.

Dyer, J.M., Stymne, S., Green, A.G., & Carlsson, A.S. (2008). High value oils from plants. *Plant Journal*, *54*, 640–655.

Gustafsson, A. (1987). Nitrate leaching from arable land in Sweden under four cropping systems. *Swedish Journal of Agricultural Research*, *17*, 169–177.

Jensen, E.S. (1991). Nitrogen accumulation and residual effects of nitrogen catch crops. *Acta Agriculturae Scandinavica*, *41*, 333–344.

Johnsson, H., & Mårtensson, K. (2002). Kväveläckage från svensk åkermark. *Rapport* 5248, Naturvårdsverket.

Känkänen, H., & Eriksson, C. (2007). Effects of undersown crops on soil mineral N and grain yield of spring barley. *European Journal of Agronomy*, *27*, 25–34.

Lemola, R., Turtola, E., & Eriksson, C. (2000). Undersowing Italian ryegrass diminishes nitrogen leaching from spring barley. *Agricultural and Food Science in Finland*, *9*, 201–216.

Merker, A., & Nilsson, P. (1995). Some oil crop properties in wild Barbarea and Lepidium species. *Swedish Journal of Agricultural Research*, *25*, 173–178.

Nilsson, P., Johansson, S.Å., & Merker, A. (1998). Variation of oil composition of species from the genera Barbarea and Lepidium. *Acta Agriculturae Scandinavica, Section B. Soil and Plant Science*, *48*, 159–164.

Princen, L.H. (1983). New oilseed crops on the horizon. *Economic Botany*, *37*, 487–492.

Spaner, D., & Todd, A.G. (2003). The impact of underseeding forage mixtures on barley grain production in northern North America. *Canadian Journal of Plant Science*, *83*, 351–355.