

Evaluation of transgenic lines of field pepperweed (*Lepidium campestre* L.)

Utvärdering av transgena linjer av fältkrassing (*Lepidium campestre* L.)

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Kurstitel: Examensarbete inom hortonomprogrammet

Kurskod: EX0544

Omfattning: 30 hp

Nivå: E

Fördjupning: A2E

Program/utbildning: Hortonomprogrammet

Ämne: Biologi

Serienamn: Självständigt arbete vid LTJ-fakulteten, SLU

Utgivningsort: Alnarp

Utgivningsmånad och -år: juni 2013

Elektronisk publicering: <http://stud.epsilon.slu.se>

Omslagsbild: Anna Lehrman - mogen fröställning från fältkrassing

Nyckelord: field pepperweed, transgenic, *WRII*, oil content, PCR, Southern blot, vernalization.

Ansvarig institution:

SLU, Sveriges lantbruksuniversitet

Fakulteten för Landskapsplanering, trädgårds- och jordbruksvetenskap

Institutionen för växtförädling

Preface and Acknowledgements

Professor Li-Hua Zhu at the Department of Plant Breeding and Biotechnology at SLU, Alnarp, initiated this master project.

I would like to thank my co-supervisor Emelie Ivarson for all her help, inspiration and joy. You really made all the hours of hard work to a happy journey. I would also want to thank Biomedical Analyst Annelie Ahlman for her great help and support concerning work in the laboratory and Professor Li-Hua Zhu and Professor Sten Stymne for trouble-shooting discussions and their great engagement in the project. A big thank to PhD Ida Lager and Research Assistant Pia Ohlsson for always putting a smile on my face.

I would also want to say a big thank you to my friends for support and advice, not forgetting my family who always believed in me and inspired me from childhood.

Last but certainly not least, I want to thank my God and Savior, Jesus Christ. Thanks to Him, I can study and admire His amazing creation.

And God said, Let the earth put forth grass, herbs yielding seed, and fruit-trees bearing fruit after their kind, wherein is the seed thereof, upon the earth: and it was so. And the earth brought forth grass, herbs yielding seed after their kind, and trees bearing fruit, wherein is the seed thereof, after their kind: and God saw that it was good.

Genesis 1:11-12

And he humbled thee, and suffered thee to hunger, and fed thee with manna, which thou knewest not, neither did thy fathers know; that he might make thee know that man doth not live by bread only, but by everything that proceedeth out of the mouth of Jehovah doth man live.

Deuteronomy 8:3

Jesus said unto them. I am the bread of life: he that cometh to me shall not hunger, and he that believeth on me shall never thirst.

John 6:35

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Abstract

The worldwide industrial demand of energy, often in form of fossil fuel, is continuously growing and at the same time these resources are coming to an end (Hamamre, 2013). The interest and focus in renewable sources are then more important than ever and plants are suggested to be a good alternative for production of raw material for industrial purposes (Nilsson *et al.*, 1998). But the world is also facing an increasing world population which also increase the nutritional needs, plants therefore need to be suitable for both industrial and nutritional purposes. To achieve this, common plants need to be improved but it also need new potential crops to be developed and introduced (Carlsson *et al.*, 2009). Modern technology like gene engineering is an important tool to achieve these challenges (Carlsson *et al.*, 2011).

This work focused on analyzes and evaluation of different transgenic lines of field pepperweed (*Lepidium campestre* L.). Field pepperweed has traditionally been seen as a weed in the agriculture sector but is in these days less common and most frequent along railway stations and ruderal areas (Lagerberg, 1958). The field pepperweed were bred with 3 different goals to (i) increase the rather low seed oil content, which is around 20% (Nilsson *et al.*, 1998), (ii) reduce the pod shatter sensitivity and (iii) optimize the fatty acid profile of the seed oil. Different transgenic lines were evaluated through DNA analysis (PCR, Southern blot) and oil analysis. The DNA analysis showed that the new traits could both be detected through PCR and Southern blot. The oil analysis also showed that the oil content could be increased through overexpression of both *WR11* and hemoglobin like genes with up to 25 % compared to the non-transgenic lines which resulted in a total oil content of over 27 % (27.34 %).

To optimize the breeding procedure different tests were arranged due to easier distinguish transgenic plants from non-transgenic plants through both finding suitable kanamycin concentration and through detection with red fluorescent protein.

Sammanfattning

Det världsvida industriella behovet av energi och oftast i form av fossila bränslen ökar stadigt samtidigt som de begränsande resurserna börjar sina (Hamamre, 2013). Intresse och fokus på förnyelsebara alternativ är därför viktigare än tidigare och växter anses vara bra alternativ för produktion av råmaterial för industriella ändamål (Nilsson *et al.*, 1998). Men världen möter också en växande population vilket också ökar efterfrågan på föda. Växter måste därför vara anpassade för både industriella och näringsändamål. För att uppnå detta måste vanliga grödor förbättras samt nya grödor utvecklas introduceras (Carlsson *et al.*, 2009). Modern teknologi som genmodifiering är viktiga redskap för att klara dessa utmaningar (Carlsson *et al.*, 2011).

Detta arbete fokuserade på att analysera och utvärdera olika transgena linjer av fältkrassing (*Lepidium campestre* L.). Fältkrassing har traditionellt setts som ogräs i jordbrukssammanhang men är nuförtiden mer sällsynt och förekommer vanligen vid järnvägsstationer och ruderatmarker. Fältkrassing förädlades med tre olika mål (i) öka den relativt låga oljehalten, som är ungefär 20 % (Nilsson *et al.*, 1998), (ii) reducera skördeförluster via dråsning samt (iii) optimera fettsyrasammansättningen i fröolja. Olika transgena linjer var sedan utvärderade genom DNA-analyser (PCR, southern blot) samt oljeanalyser. DNA-analyserna visade att de nya egenskaperna kunde både upptäckas med hjälp av PCR och southern blot. Oljeanalyserna visade även att oljehalten kunde ökas både genom överuttryck av *WRII* samt hemoglobinliknande gener med upp till 25 % jämfört med icke-transgena linjer vilket resulterade i en total oljehalt på över 27 % (27,34 %).

För att optimera förädlingsdelarna utfördes även olika test för att utskilja transgena plantor från icke-transgena plantor genom att hitta lämplig kanamycin-koncentration samt urskiljning med hjälp av röd fluorescens protein.

Introduction

World oil consumption

To fulfill the industrial demands of energy the consumption of fossil oil is continuously growing. Fossil oil in shape of petroleum is later used in a wide product range from lubricants and fuels to plastics and special chemicals (Vanhercke *et al.*, 2013). The increasing demand is facing higher prices due to the fact that the resources are approaching the end (Hamamre, 2013). Furthermore, the emissions from use are suggested to have a severe impact on the environment and peopling (Le Quèrè *et al.*, 2009). The interest in plant-derived renewable sources has becoming of interest due to these factors (Hamamre, 2013).

Nilsson *et al.*, (1998) suggests that plants with improved oil quality can be a good alternative for production of raw material for industrial use. Plant oils can be seen as the main substitute for petroleum and is already today used to provide an alternative for liquid transport fuel such as biodiesel (Vanhercke *et al.*, 2013). Despite the advantage with plant oils it has a drawback; it cannot be produced in sufficient amount to fulfill the global demand (Vanhercke *et al.*, 2013). Out of the world plant oil production, 10-15% is used as fuel (Stymne, 2005). Modern technology through genetic engineering can however largely increase plant oil production to meet future food demand and give a significant surplus for industrial purpose (Carlsson *et al.*, 2011). In this sentence Carlsson *et al.*, (2011) suggested that the global plant oil production could within two decades replace 40 % of fossil oil used in petrochemistry, without affecting the production of plant oil for human consumption. Rapeseed (*Brassica napus* L.) is an important oilseed crop for human consumption but also in Europe an important source for biodiesel production (Wang *et al.*, 2009). Plants for oil production is still mostly focused on food consumption and is dominated by rapeseed (canola), oil palm, sunflower, soybean and cottonseed. The oil from these food oils is mostly based on only five fatty acids C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 Δ^9 (oleic acid), C18:2 $\Delta^9, 12$ (linoleic acid) and C18:3 $\Delta^9, 12, 15$ (alpha-linolenic acid) (Vanhercke *et al.*, 2013). To increase plant oil production, common oilseed plants, like rapeseed, need to be improved and new ones need to be developed. Alteration of already cultivated oilseed crops as well as development of new ones can be made by help of genetic engineering (Carlsson *et al.*, 2009). An ongoing project aims to introduce field pepperweed (*Lepidium campestre* L.) as a novel oilseed crop with help of genetic engineering.

Field pepperweed

Lepidium campestre (field pepperweed) is a cruciferous diploid species (Nilsson *et al.*, 1998) belonging to the *Lepidium* species which is present all over the world. *Lepidium* is known for growing on mountainous areas and in areas with high salinity but some species like *Lepidium*

campestre has become more frequent in agricultural landscape where it is sometimes considered as a weed. *Lepidium campestre* is, like the Danish name "Kung Salomons Lysestage" (King Solomon's candlestick) shaped much as a candlestick with a straight stem and branched flowering branches.

Field pepperweed is known for its characteristic spoon shaped pods (Lagerberg, 1958). The plant has great winter hardiness and biennial life cycle (Merker *et al.*, 2010). According to field studies from 2004-2007 in the south of Sweden, *Lepidium campestre* can contribute a rather high seed yield per hectare (4045-6454 kg/ha) (Merker *et al.*, 2010).

Lepidium campestre has many favorable characteristics, but some traits need to be altered in order to make it an optimal future oil crop. This program aims to (i) increase the rather low seed oil content, which is around 20% (Nilsson *et al.*, 1998), (ii) reduce the pod shatter sensitivity and (iii) optimize the fatty acid profile of the seed oil, since the current fatty acid profile is unhealthy and unsuitable for human consumption and usage in food industry. The oil has high level, 25%, of erucic acid (22:1) and high level, 35%, of poly unsaturated fatty acids (18:3).

Genes of interest

3 Different genes were used to increase the oil content of the seed. WRINKLED1 (*WRI1*) and the hemoglobin like genes *AHb2* and *BHb2*. *WRI1* is an important protein in *Arabidopsis* controlling the oil accumulation in maturing seeds (Baud *et al.*, 2009).

***WRI1*-gene**

The 48,4 kDa long protein is a member of one of the largest transcription factor families, the APETALA2/ethylene- responsive element binding proteins (Riechmann *et al.*, 2000; Liu *et al.*, 2010). *WRI1* can control seed maturation towards fatty acid metabolism through being a direct target of LEAFY COTELYDON2 (*LEC2*). The protein also positively regulates part of the fatty acid synthesis through enhanced expression of the late part glycolysis genes (Baud *et al.*, 2009). Mutation of *WRI1* in *Arabidopsis thaliana* creates a wrinkle-shaped appearance of the seed coat, reflected by the name of the transcription factor. A mutation does not only result in a wrinkled seed coat, but also in an 80 % decrease of the seed oil content, as a result of the deficiency in the glycolytic pathway (Focks & Benning, 1998). On other hand, an overexpression of *WRI1* not only results in normal seed shape but also an increase of the seed oil content, in comparison to the wild type (Liu *et al.*, 2010).

Hemoglobin-genes

Two different hemoglobin-like genes, *AHb2* and *BHb2* were used to alter the total oil content. Recent studies have reported an increase of the total oil content in *Arabidopsis thaliana* when

overexpressing *AHb2* (Spyrakis *et al.*, 2011). Hemoglobins are related in the aspect that they all carry an iron ion in their heme group where it binds oxygen (Bülow *et al.*, 1999). According to Vigeolas *et al.*, (2011), an overexpression of *AHb2* could lead to an increase of the total oil content, and mainly the polyunsaturated fatty acids. Higher oxygen availability in developing seeds as well as an increase in the adenylate energy state and sucrose content of the seeds are considered to be the reasons for this.

***FAD2*-gene**

This breeding program also focused on improving the oil composition of field pepperweed. The goal was to increase the oleic acid (18:1 Δ^9) and decrease the linoleic acid (18:2 $\Delta^9,12$). High content of polysaturated fatty acid such as linoleic acid leads to shortened storage time of food products and a decrease in optimum flavor due to the low frying and oxidative stability (Pham *et al.*, 2012). Oleic acid is a member of the monounsaturated fatty acids and has a significant industrial potential (Yang *et al.*, 2012). Compared to polyunsaturated fatty acids, oleic acid has a better resistance to oxidation, and is therefore suitable in industrial products such as biodiesel or biolubricants (Yang *et al.*, 2012). The oleic acid is also important for industrial use since it through chemical processing easily can be cleaved at the double bond site, generating monomers which are highly demanded by the chemical industry when producing nylons (polyamides) (Vanhercke *et al.*, 2013).

After studying mutant lines of *Brassica napus* genes controlling the composition and amount of fatty acids have been discovered (Pham *et al.*, 2012). The enzyme $\Delta 12$ desaturase has been found responsible for converting 18:1 into 18:2 by adding a double bond to the carbon chain and the $\Delta 12$ desaturase gene has also been found code for *FAD2* (Sivaraman *et al.*, 2004). Studies have shown that the fatty acid desaturase gene *FAD2* was responsible for the production of up to 90 % of the polyunsaturated fatty acids in non-photosynthetic tissues such as developing seeds of oilseed crops (Pham *et al.*, 2012). Modification of the oil quality to develop a complete oleic acid producing line is of great interest. A recent trial has succeeded in develop a line of safflower containing 95 % oleic acid with only 2 % linoleic acid without any effect on agronomic traits. This was achieved through RNAi-mediated gene silencing against the seed-expressed *FAD2* genes and *FATB* (fatty acid thioesterase) (Vanhercke *et al.*, 2013).

This research aims to use RNAi-mediated gene silencing to decrease the activity of *FAD2* and by that increase the content of oleic acid (18:1).

***FAE1*-gene**

Fatty acids, with a carbon chain longer than 18 carbon atoms, are called very long chain fatty acids

(VLCA) and are common in seed oils and plant waxes in many plant families (Mietkiewska *et al.*, 2007). Erucic acid (*cis*-13 docosenoic acid, 22:1) is a common VLCA with interesting industrial purposes (Vanhercke *et al.*, 2013). The fatty acid is an important feedstock to more than 1000 patented industrial applications and is most frequently used as slipping agent or antiblock in the production of plastic films and as surface-active additive in coatings (Puyaubert *et al.*, 2005). Regular consumption of large amounts of erucic acid can adversely affect heart tissues, why a decrease of erucic acid in oils used for human consumption is essential (Park *et al.*, 2012). Reports have shown that the key enzyme for the erucic acid synthesis, β -ketoacyl-CoA synthase, is encoded by the fatty acid elongase (*FAE*) gene. By antisense down regulation of the gene expression the production of erucic acid can be reduced (Zebarjadi *et al.*, 2006). During this breeding program the aim is to use RNAi-mediated gene silencing to decrease the expression of *FAE* and thereby that decrease the content of erucic acid.

WS:WE-genes

The increased use of plant lipids for industrial purposes has also lead to an increase of specialized industrial use of a number of plant produced wax esters. Wax esters are very long carbon chains (average C40 to C60) with a fatty acid esterified to a fatty alcohol (Heilmann *et al.*, 2012). Plants mostly produce wax esters (WE) as components of their surface lipid layer (cuticula). The WEs protect the plant efficient from pathogen entry due to their stabile state and protect against desiccation because of their hydrophobicity and high resistance to hydrolytic degradation (Vanhercke *et al.*, 2013).

For industrial use the WE serves as good lubricants due to their chemical stability at high as well as low temperatures. Wax esters have traditionally been extracted from whaling industry (spermaceti oil) but can also be refined from petrochemicals (Vanhercke *et al.*, 2013). Due to dwindling resources focus is more turned to plant derived WE. Jojoba (*Simmondsia chinensis*) has the natural ability to produce WE as seed oil, but the plant, being a desert shrub, is not suitable for large-scale cultivation in humid areas. Jojoba is unique in being able to produce straight-chain WE within its seed, and these WE are valuable ingredients in personal care products and cosmetics (Al-Hamamre 2013; Vanhercke *et al.*, 2012). The WE production in jojoba seeds has contributed much knowledge utilized in metabolic engineering of domesticated high-yield oilseed crops (Wältermann *et al.*, 2007).

The WE production is mainly controlled by two enzymes, the wax synthase (*WS*) and the fatty acyl-CoA reductase (*FAR*) (Heilmann *et al.*, 2012). According to recent studies wax ester can be produced in *Arabidopsis thaliana* by combining and expressing both *FAR* and *WS* from jojoba (Lardizabal *et al.*, 2000). The oleic acid (18:1) is the major substrate for fatty acid species in the

wax synthesis. The oleic acid is transported from the plastids and converted to a CoA ester. The oleic acid is then transformed in a membrane-associated fatty acyl-CoA elongase (*FAE*) system to 20:1, 22:1 and 24:1 acyl-CoAs which are the preliminary stages of wax synthesis. The long-chain acyl-CoA is later reduced to an alcohol by *FAR* and is formed to wax ester by *WS* (Metz *et al.*, 2000). To optimize the production of WE, an overexpression of *FAEI* is preferable (Lardizabal *et al.*, 2000).

***IND*-genes**

Plants use different strategies to disperse their seeds in order to ensure the survival of the next generation. These strategies are often based on different formation of specific tissues which help the fruits to efficiently release and spread the seeds at maturity (Girin *et al.*, 2010). Although these strategies are beneficial for plants living in the wild, irregular pod shattering is a large problem for oilseed production and causes up to 11-25% annual yield losses in oilseed rape production (Price *et al.*, 1996). To minimize yield losses, genes controlling seed dispersal have been studied (Girin *et al.*, 2011; Mühlhausen *et al.*, 2013). Recently the INDEHISCENT gene (*IND*) was found to be a key regulator of the separation layer in valve margin development as well as the lignified layer. An *IND* mutant with no expression from the *IND* gene fails to open on maturity (Girin *et al.*, 2011). Local degradation of auxin at the valve margin induces fruit opening in wild-type fruits. The *IND* gene has been found controlling directly the regulation of auxin transport and also induces the expression of *GA4* to start producing the hormone gibberellin which promotes fruit maturity and seed dispersal (Girin *et al.* 2011; Arnaud *et al.* 2010). To minimize pod shattering and accordingly yield losses, this breeding program used RNAi silencing of the Bra.A-*IND* gene. An identified mutation in the Bra.A-*IND* gene disrupt the gene function because of a premature stop codon in the beginning of the bHLH domain which results in dramatically reduce of pod shattering (Girin *et al.*, 2010).

Marker genes

During the breeding program the marker gene neomycin phosphotransferase II (*nptII*) was used to select transgenic lines. The marker gene encodes resistance against kanamycin (aminoglycosides) and the resistance is probably based on active transport of kanamycin into the vacuole (Libiakova *et al.*, 2001). The gene is used in commercially cultivated crops and has been thoroughly studied for safety (Rosellini, 2011).

As an alternative to *nptII* as marker and reporter gene, a red fluorescent protein from *Discoma sp.* was used in the breeding program. The gene is expressed in the plant cytosol, the endoplasmic reticulum and the vacuole, resulting in a red light when being exposed to green light. The marker gene does not affect the seed quality and germination ability, but only gives rise to a red

fluorescent phenotype (Jach *et al.*, 2001).

Project aim

This master project is based on an ongoing doctoral project aiming to introduce a new oilcrop by use of genetic transformation. The aim of this project was to analyze and evaluate the already obtained transgenic lines, with the goal to optimize the evaluation procedure. To be fully aware of the whole transformation procedure, from explant to the resulting transgenic lines, a transformation event was carried out during this project. During the project, I also tried to investigate different opportunities to make the breeding procedure more time effective. Higher concentrations of antibiotics to distinguish non-transgenic plants in an early stage was tested.

Material and methods

Plant material

The transgenic lines and the putative transgenic lines originated from transformations performed on one collection of *Lepidium campestre* L. 'NO 94-7', collected by late prof. Arnulf Merker. The transgenic lines were categorized in:

- Lines for increased oil content (*WR11*)
- Lines for increased oil content (*AHb2*, *BHb2*)
- Lines for wax ester production (*FAE1:WS:FAR*)
- The putative transgenic lines were categorized in:
- Lines for high oleic acid content (*FAD2:FAE1*)
- Lines for reduced pod shattering (*Bra.A-IND*)

Seed germination

Seeds were surface-sterilized using 10% calcium hypochlorite (CaCl_2O_2) with Tween20 and shaken for 20 minutes. Highly contaminated seeds were first put in a tube with 70% ethanol for 5 minutes. Seeds were then thoroughly rinsed with sterile water. Fifty surface-sterilized seeds were planted in a container with germination medium. The germination medium contained half strength of MS (Murashige & Skoog, 1962), 10 g l^{-1} sucrose and 7 g l^{-1} Bacto Agar at pH 5.7.

Germination of seeds for next generation

The transgenic lines which had shown promising results in analyses were to be used further in the project. Seeds from the first generation were sown and allowed to produce second generation seeds which could be analyzed further. Since not all seeds produced from a transgenic plant are transgenic, the shoots needed to be confirmed transgenic in order to keep them. To speed up the

procedure and reduce the work load, the seeds were sown on germination medium with kanamycin selection (100 mg l^{-1}). The seeds that developed cotyledons with a normal appearance were assumed to be transgenic, and planted in the greenhouse. The shoots were allowed to grow for approximately two weeks before plant material was collected. DNA was extracted and a PCR analysis was run to confirm that the shoots were transgenic.

Culture conditions

All in vitro cultures were maintained in a growth chamber with a 16 h day length at $33 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and temperature 21/18°C (day/night).

Plants were established in greenhouse with 16h day length at $200\text{-}250 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and temperature 23/13°C (day/night).

Transformation

Agrobacterium tumefaciens strain AGL-1 with the binary vector “Watergate” containing *neomycin phosphotransferase (nptII)* gene under the IND promoter from *Arabidopsis thaliana* and the IND RNAi sequence derived from *Lepidium campestre* was used for transformation.

Hypocotyls from 6 days old seedlings were used as explants. The explants were pre-cultured on a filter paper on a callus induction medium containing full strength of MS with MES, 30 g l^{-1} sucrose, $0,5 \text{ g l}^{-1}$ 2,4-D and Gelrite $2,5 \text{ g l}^{-1}$ at pH 5,8 for 2 days before the infection of *Agrobacterium*. Filter paper was used to facilitate the transfer of the explants from the callus induction medium to the bacterial suspension (Li *et al.*, 2010). The *Agrobacterium* for transformation was cultured in liquid LB medium with appropriate antibiotics over night. The bacteria was then centrifuged at 3,500 rpm for 15 minutes and the pellet was suspended in liquid MS20 medium (full MS + MES, 20 g l^{-1} sucrose, pH 5,2) to a final concentration of 0,85 at OD_{600} . The explants were submerged in the bacteria suspension, dry blotted on filter paper and transferred to a filter paper on co-culture medium for 4 days in light. The co-culture medium contained full MS + MES, 30 g l^{-1} sucrose, $1,1 \text{ mg l}^{-1}$ TDZ and $2,5 \text{ g l}^{-1}$ Gelrite at pH 5,8. After the co-culture, the explants were washed in liquid MS20 medium, quickly dried on filter paper and placed on selection medium with 15 mg l^{-1} kanamycin and 150 mg l^{-1} ticarcillin. The explants were then transferred after two weeks to the second subculture with 25 mg l^{-1} kanamycin. The explant were transferred to fresh selection medium every second week and cultured in climate chamber.

DNA extraction for PCR

Plant material derived from first generation seeds of *WR11-AHb2-*, *BHb2-* lines and plant material from regenerated shoots of *FAD2:FAE1* RNAi-lines, was collected in an Eppendorf-tube with a metal ball. The tubes were immediately frozen in liquid N_2 before extraction or stored in a -80°C

freezer. The plant material was homogenized with a shaker (MM 400 Retsch®). After the homogenization, 500 µl CTAB buffer (Cetyl Trimethyl Ammonium Bromide) containing 1 % 2-mercaptoethanol preheated to 65°C was added. The samples were then immediately shaken and incubated 65°C for 10 minutes. After the incubation 500 µl chloroform/isoamylalcohol (24:1) was added, and the samples were vortexed and centrifuged for 3 minutes at 12 000 rpm. The top phase was then transferred to new Eppendorf-tubes and an equal volume of pre-chilled isopropanol was added. The tubes were gently mixed and then centrifuged at 12 000 rpm for 3 minutes before the supernatant was poured off. Thereafter, 500 µl WASH buffer (76 % Ethanol, 10 mM sterile Ammonium acetate and sterile water) was added and the samples were incubated for two minutes, before they were gently inverted and centrifuged at 12 000 rpm for 3 minutes. The supernatant was then poured off and the pellet was dried in a laminar flow hood. The dry pellet was dissolved in TE buffer and 0.1µg/µl RNase, and incubated in 37°C for 1 h. The DNA concentration was measured on a NanoDrop® (ND-1000 Spectrophotometer) and, in cases needed, diluted.

PCR

A master mix was prepared, containing H₂O, 1 x buffer, 2 mM dNTP, 0,2 µM forward primer, 0,2 µM reverse primer and 0,25 u/10 µl enzyme. The PCR (Polymerase Chain Reaction) was run with optimal annealing temperature due to gene of interest (65°C *nptII*, 58°C *WR11*, 54°C *BHb2*, 54°C *AHb2*, 55°C *FAD2/FAE1*) for 30 cycles. The PCR products were run on a 1 % Agarose gel and visualized under UV light.

DNA extraction for Southern blot

Approximately 4 grams of plant tissue from first generation of lines with wax ester production (*FAE1:WS:FAR*) was collected for DNA extraction and frozen in liquid N₂. The frozen plant tissue was homogenized by hand and quickly transferred to pre-chilled 50 ml tubes before 16 ml preheated (65°C) 2% CTAB buffer containing 5% 2-mercaptoethanol was added. The samples were incubated at 65°C for 1 h with regular shaking. An equal volume of chloroform/isoamylalcohol (24:1) was added, the tubes were shaken and centrifuged for 10 minutes at 3000 rpm. The procedure was repeated to purify the samples from impurities. Thereafter the top phase was transferred to new tubes and precipitated with 2/3 volume of pre-chilled isopropanol. The samples were then gently mixed and centrifuged for 10 minutes at 5000 rpm. The supernatant was poured off and the DNA pellet was washed twice with WASH buffer (76 % Ethanol, 10 mM sterile Ammonium acetate and sterile water) and centrifuged for 5 minutes at 5000 rpm. The pellet was air dried in a laminar flow hood and suspended in TE-buffer. RNase was added to a final concentration of 100 µg ml⁻¹, and the samples were incubated at 37°C over night.

Southern blot

Southern blot analysis was performed to detect the copy number of the transgenes. Approximately 20 µg genomic DNA was digested with the restriction enzyme *Bgl*III. The Southern blot hybridization was based on the non-radioactive DIG system from Roche (Van Miltenburg *et al.*, 1995) and the *nptII* probe was synthesized using the same primer set as for PCR according to Zhu *et al.*, (2008).

Transgenic seed identification

Transgenic seeds containing a red fluorescent protein from *Discoma sp* (DsRed) were identified with a DFP-1™ Dual Fluorescent Protein Flashlight (NightSea). Seeds were later sorted in three categories (I) Transgenic, (II) medium transgenic, (III) non-transgenic. The groups were based on a color spectra where the transgenic seeds were bright colored under the light and the non-transgenic were dark colored. The medium group was in between the bright and the dark. The seeds were later used for oil analysis.

Pooled seed analysis of wax esters

For analysis of wax esters 10 pooled first generation seeds from confirmed transgenic lines were selected and placed in a glass tube and 3.75 ml MeOH:CHCl₃ (2:1) and 1 ml 0.15M HAc were added. The seeds were then homogenized with an IKA® T18 basic (ULTRA TURRAX®). Every tube was homogenized 20s x 5 to ensure that the seeds were well homogenized. Thereafter 1.25 ml of CHCl₃ and 1.25 ml of H₂O were added, the tubes were mixed well and the samples were immediately transferred to new tubes with screw caps.

The tubes were centrifuged at 3000 rpm for 3 minutes. The bottom phase (chloroform phase) was transferred to a new screw cap tube with a glass pipette and immediately dried under a N₂ stream. When all liquid was evaporated, 200 µl CHCl₃ was added.

30 µl of the solution was put on a thin layer chromatography (TLC) silica plate. A small amount of the samples were placed in the right corner for staining. The TLC plates were run in a tank containing hexane:DEE (diethyl ether): HAc (100 %) (90:10:1 ml) to separate the WEs from the triacylglycerols (TAG) The solution was allowed to migrate until 1 cm of the plate was left. The plates were thereafter carefully covered with double-layered aluminum foil except the extra sample point for staining. A new tank with iodine was used for staining the plates for a couple of minutes. The triacylglycerol (TAG) and wax ester (WE) areas were immediately marked with extra space to ensure that all TAG and WE were included (upper line: WE, lower line: TAG) and collected in separate screw cap tubes.

After collecting TAG and WE 200 µl methanol was added and the samples were evaporated under N₂ stream to get rid of water completely. 2 ml methylation solution (2 % H₂SO₄ in MeOH) was added to the samples and incubated at 90°C for 50 minutes.

After the tubes had cooled down, 200 nmol 17:0 artificial fatty acid standard (80 μ l for TAG and 20 μ l for WE) was added together with 0.6 ml of hexane and 2 ml H₂O. The tubes were then vortexed and centrifuged for 3 minutes at 3000 rpm.

Thereafter the upper organic layer (hexane phase) was pipetted (approx. 300 μ l) into an Agilent vial with an insert and then covered with a Shrink cap. The samples were then analyzed with a 7890A GC system (Agilent Technologies).

Pooled seed oil analysis

For seed oil analysis, 10 first generation seeds from confirmed transgenic lines (*WR11*, *BHb₂* and *AHb₂*) were picked randomly with three replicates, samples were weighed and placed in glass tubes together with 1 ml 0.15M HAc and 3.75 ml CHCl₃/MeOH. The seeds were then homogenized with an IKA[®] T18 basic (ULTRA TURRAX[®]). Every tube was homogenized 20s x 5 to ensure that the seeds were well homogenized. The rod was washed by running it in ethanol (5 tubes in a row) between the samples to avoid contamination. Thereafter 1.25 ml of CHCl₃ and 0.9 ml H₂O was added, the tubes were mixed well for 30 s and the samples were then immediately transferred to a new tube with screw cap. The samples were centrifuged for 2 minutes at 3000 rpm. The lower phase (chloroform phase) was removed to a test tube and from that tube 200 μ l was transferred to a methylation tube and dried under nitrogen. Thereafter 100 μ l hexane and 100 nmols of 17:0-Me standard were added to the residue. The tubes were methylated for 1h in 95°C with 2 ml H₂SO₄. After the samples had cooled down, 1 ml H₂O and 0.75 ml of hexane were added, the samples were shaken and centrifuged for 2 minutes at 2000 rpm. 200 μ l of the upper phase (hexane phase) was then transferred into Agilent vials with an inner vial and analyzed with a 7890A GC system (Agilent Technologies).

Selection test- Germination

The test was initiated to find which concentration was the most suitable for selection. From the very beginning, 50 mg l⁻¹ was selected to distinguish the non-transgenic seeds, but too long time was needed before the transgenic seeds could be distinguished from the non-transgenic ones.

Non-transgenic seeds from field pepperweed (*Lepidium campestre* L. ‘NO 94-7’) were surface sterilized as mentioned above (Seed germination). The surface sterilized seeds were sown on germination medium with different kanamycin concentrations (0 mg l⁻¹, 50 mg l⁻¹, 100 mg l⁻¹, 150mg l⁻¹ and 200 mg l⁻¹), with 50 seeds per treatment and two replicates. The plant development was conducted for 6 weeks.

Selection test- Plant growth

The test started by germination of 300 non-transgenic seeds from field pepperweed (*Lepidium*

campestris L. ‘NO 94-7’) on humidified filter paper, surface sterilized as mentioned above (Seed germination. The roots were excised from the hypocotyls (shoots), and 50 shoots per treatment were placed on shoot induction medium (full strength of MS+MES, 30 g l⁻¹ sucrose, 2,5 g l⁻¹ GELRITE, 1,1 mg l⁻¹ TDZ at pH 5,7.) with different concentrations of kanamycin (0 mg l⁻¹, 30 mg l⁻¹, 40 mg l⁻¹, 50 mg l⁻¹, 70 mg l⁻¹). The test was conducted for 6 weeks.

Results & Discussion

Lines for increased oil content (*WR11*)

The occurrences of both *nptII* and *WR11* in second generation shoots were analyzed through PCR analysis. The result from the gel electrophoresis (**Figure 1.**) showed that most of the shoots were transgenic and could be used for further evaluations.

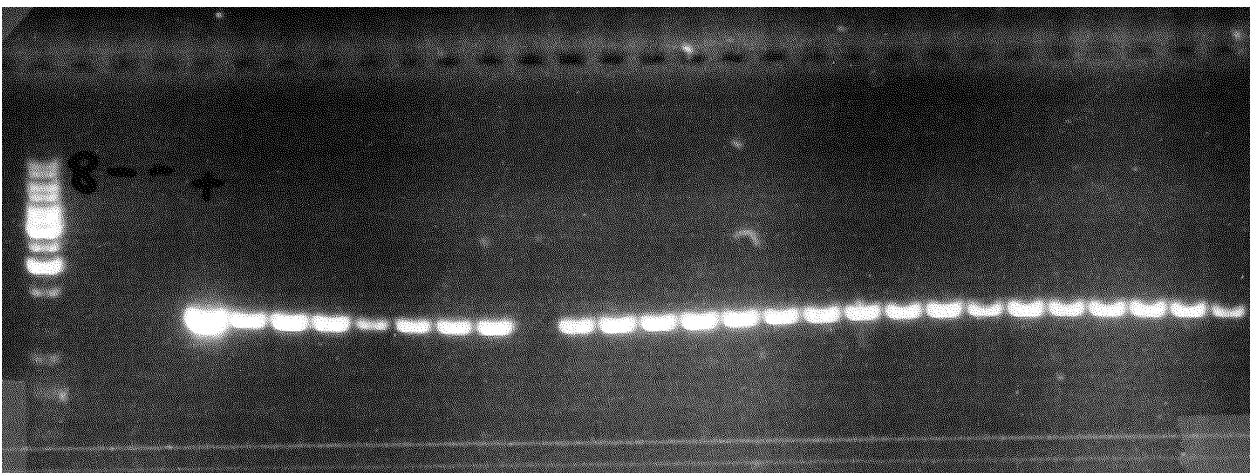


Figure 1. Gel electrophoresis of *nptII* one ladder and 28 samples. The first sample (from the left) is a water sample, the second and third samples are non-transgenic controls, the fourth sample is a positive control. Sample 5-28 are transgenic shoots from the second generation. All transgenic shoots except number 8 were confirmed transgenic, containing the *nptII* gene.

When analyzing the oil, several lines showed higher oil content compared to the non-transgenic control (NO94-7), with some lines reaching over 25 % (**Figure 2.**)

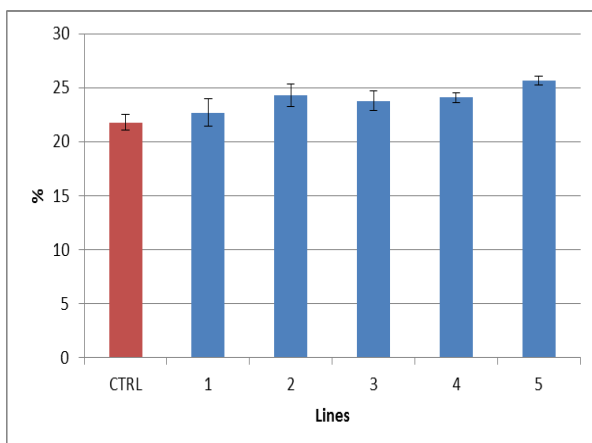


Figure 2. Diagram showing increased oil content in transgenic lines (number 1-5) in comparison with the non-transgenic control. When calculating the oil content from the total lipid extraction, the membrane lipids were considered as a negligible fraction.

The oil analyses showed that by overexpressing the *WRI1* gene in field pepperweed, an increase of almost 18% in oil content could be achieved. The oil content of the non-transgenic control was 21.79 % and the line with the highest oil content reached 25.69 %. This increase had already been observed in *Arabidopsis thaliana* (10-40% increase) due to overexpression of the *WRI1* gene (Liu *et al.*, 2010). In homozygous lines of the second generation, the oil content could be expected to be even higher.

An overexpression of the *WRI1* gene can also lead to other beneficial improvements. According to Cernac *et al.*, (2006) an over expression of the *WRI1* gene lead to improved seed germination.

According to Liu *et al.*, (2010), an over expression of the *WRI1*-gene also leads to an increase in seed mass. As illustrated in **Figure 3.**, a correlation between increased oil content and increased seed mass could not be proved. This may be a result of the heterozygous lines or other factors controlling seed development (Taiz & Zeiger, 2010). Seed mass was derived from the pooled seed samples where 10 seeds were weighed on an analytical scale.

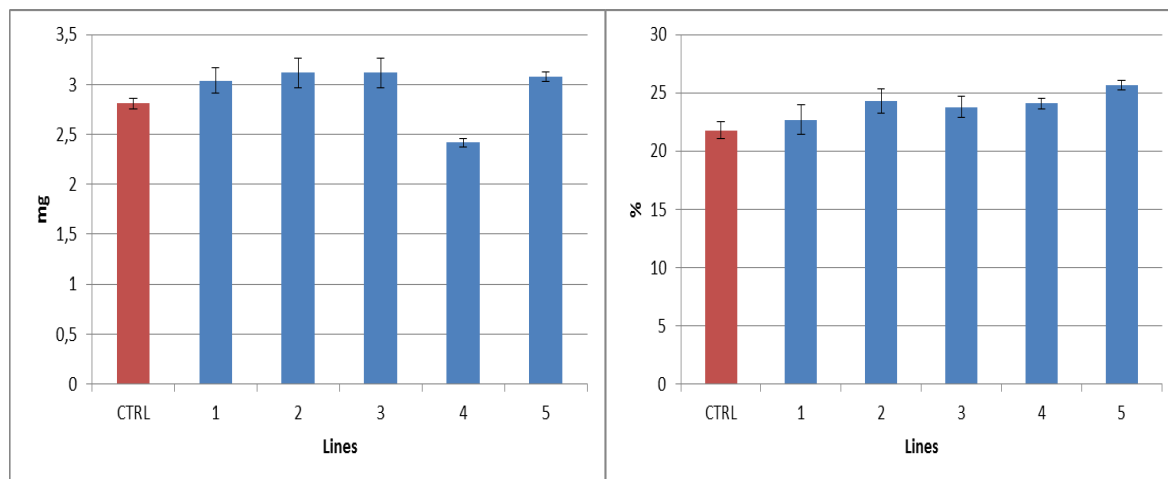


Figure 3. The seed mass and oil content of the transgenic *WRI1*-lines. **Left:** seed mass (mg) **Right:** Oil content (%).

The data was combined to better analyze the correlation between seed mass and oil content (**Figure 4.**). The illustration clearly showed that no clear connection between oil content and seed weight could be observed.

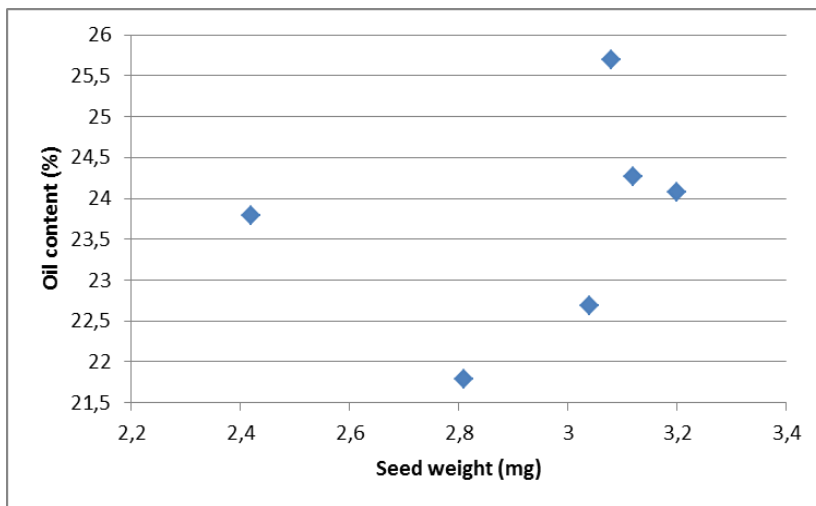


Figure 4. A picture showing that there is no correlation between seed weight (mg) and oil content (%) in transgenic *WR11*-lines.

The lines with highest oil content were analyzed by Southern blot analysis, and the lines with low copy numbers were chosen for further evaluation in second generation.

Lines for increased oil content (*AHb2*, *BHb2*)

Putative transgenic shoots derived from the first generation seeds with *AHb2* and *BHb2* were confirmed by PCR analysis and gel electrophoresis, presented in **Figure 5**.

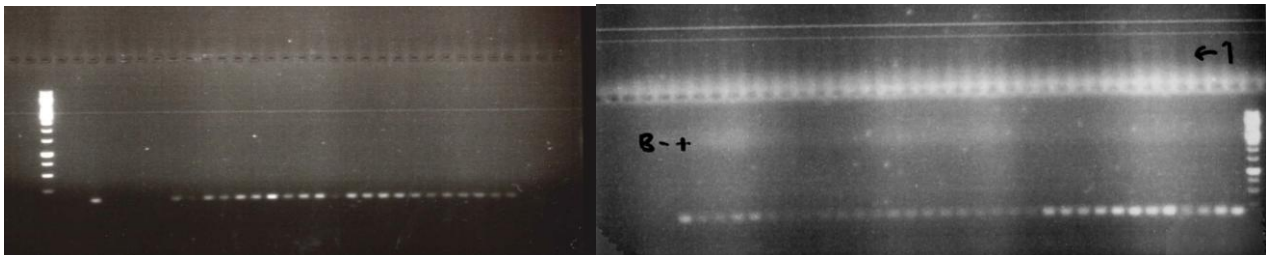


Figure 5. Gel electrophoresis of *BHb2* (left panel) and *AHb2* (right panel). Left figure: a ladder followed by a water blank, a negative control, a positive control and 27 samples. 20 samples were confirmed transgenic, containing the *BHb2* gene. Right figure: A water blank followed by a negative control, a positive control, 32 samples and a ladder. All samples were confirmed transgenic, containing the *AHb2* gene.

The oil from confirmed transgenic lines was analyzed by GC analysis. The result is presented in **Figure 6**. and shows a clear increase in oil content of the transgenic lines containing hemoglobin genes.

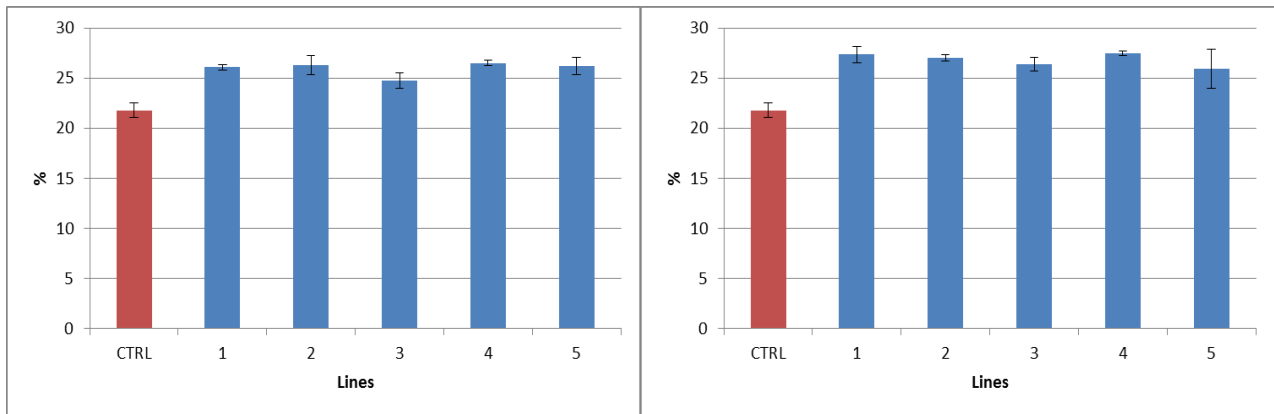


Figure 6. The oil content of transgenic hemoglobin-lines. Left: Oil content of non-transgenic control (CTRL) and transgenic lines with the *AHb2* gene (1-5). Right: Oil content of non-transgenic control (CTRL) and transgenic lines with the *BHb2* gene (1-5).

The oil analyses showed that the oil content of transgenic hemoglobin-lines was up to 27.34 %, compared to the non-transgenic line (NO94-7) with an oil content of 21.79 %, giving a total increase of nearly 25.5 %. The increase might be a result of stimulation of triacylglycerol synthesis, caused by higher energy state and higher sucrose content of the seeds contributed by the hemoglobin-genes (Vigeolas *et al.*, 2011). This might however affect the plant germination negative, since high sucrose content could inhibit germination (Dekkers *et al.*, 2004). According to Vigeolas *et al.*, (2011), transgenic *AHb2*-lines of *Arabidopsis* resulted in an increase of oil content by up to 40 %. Field pepperweed may have hard to perform such as high increase. A further increase is however expected when analyzing seeds from homozygous lines of the second generation.

According to Vigeolas *et al.*, (2011), hemoglobin-genes are suggested to increase the level of unsaturated fatty acids in *Arabidopsis* which, according to **Figure 7.**, could not be seen in *Lepidium campestre*.

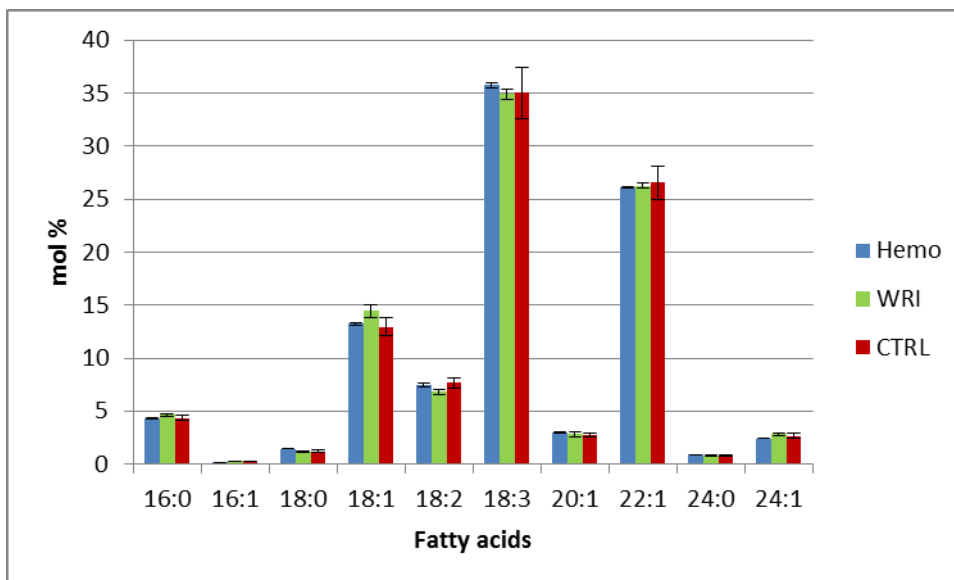


Figure 7. Fatty acid composition μg^{-1} oil of the transgenic lines compared to the control 'NO-94-7'.

An increase in oleic acid is interesting for further breeding, since oleic acid is a desired fatty acid.

Lines with high oil content containing high level of oleic acid (18:1) and lower level of erucic acid (22:1) compared with non-transgenic control might be beneficial for further breeding. The increase was however the most important since the oil composition lines (*FAD2:FAE1*) can be combined with the high oil content-lines (*WRI1* or hemoglobin) later in the breeding program.

For future aspects, a combination of the *WRI1*-line and *BHb2*-line might result in a line containing both the *WRI1*- and the *BHb2* gene generating even higher oil content, since the genes control different stages in the oil accumulation.

RNAi-lines for high oleic acid content (*FAD2:FAE1*)

The transgenic RNAi-lines of *FAD2:FAE1*-lines were analyzed through PCR analysis. The result (**Figure 8.**) showed that the majority of the lines were transgenic, containing both genes but in some lines only the *FAD2*-gene was confirmed. The primary goal was to select transgenic lines containing both *FAD2* and *FAE1* genes in order to measure the changes in oil composition. Similar studies have been made on *Arabidopsis thaliana* which showed interesting results in oil composition with significantly higher level of 18:1 compared to the wild type (Eriksson & Merker, 2009). On the other hand, lines only containing the *FAD2*-gene could still be interesting for the breeding program see the effect of *FAD2* silencing in field pepperweed. Studies on transgenic-lines of *Brassica juncea* (Indian mustard) with silenced *FAD*-gene through RNAi showed a significantly higher level of monounsaturated fatty acids and lower levels of polyunsaturated fatty acids (Sivaraman *et al.*, 2004).

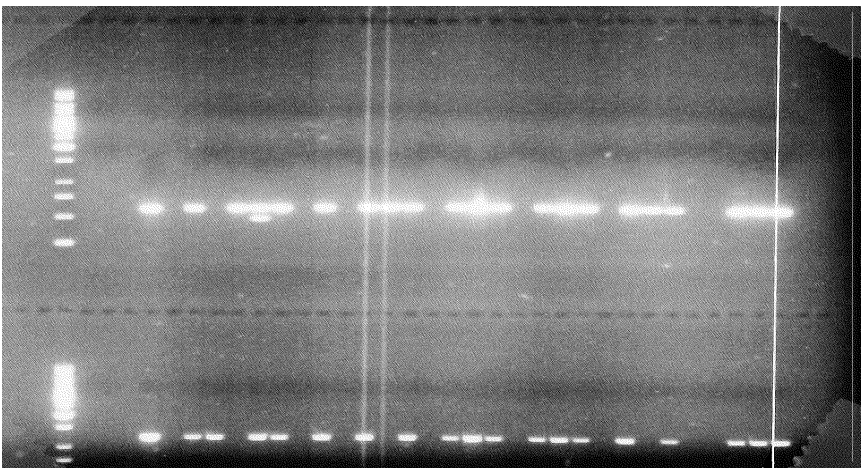


Figure 8. PCR-result. The samples are loaded in the same order in both rows, but shows the analysis of two different genes. The upper row is an analysis of the *FAD2* gene, and the lower row is an analysis of the *FAE1* gene. The upper row shows a ladder followed by a blank (water sample), two negative controls, a *FAD2* positive control and 28 transgenic lines (*FAD2* gene). The lower row shows a ladder followed by a blank (water sample), two negative controls, a *FAE1* positive control and 28 transgenic lines (*FAE1* gene).

Changing the oil composition and down regulating the *FAD2* gene may however not be free of consequences for plant vigor. According to O'Quin *et al.*, (2010), *FAD2* regulates the level of unsaturated fatty acids in order to protect the plant from cold temperature. The regulation also helps

the seed coat and embryo with cold acclimation (Angeli *et al.*, 2013). Further evaluations are therefore necessary in order to study the overwintering and germination capacity.

Lines for reduced pod shattering (*IND*)

The transformation with *IND* constructs and regeneration of shoots was successful. The shoots are still not analyzed but at the end of the master project some lines had already survived on selection medium for several weeks.

Techniques for further evaluations of the shattering behavior are yet not discovered. The pod shattering is a complex system and can be affected by many factors. Factors that influence the level of shattering in the field depend on weather conditions before and during harvesting. Plant population density in the field can also affect the pod shattering during windy conditions among pods and other canopy components. Also, disease and insect-pest damages can result in accelerated ripening and pod shattering (Rameeh, 2013).

Since many factors affect pod shattering in the field, a laboratory test can be a good measurement of pod shattering resistance which many reports already have designed (Kadkol *et al.*, 1984; Morgan *et al.*, 2000; Rameeh, 2013). Breeding for reduced pod shattering is a big challenge. The *IND* gene should not be completely silenced, since the pods should be able to open during threshing of the seeds.

Lines with wax ester production (*FAEI:WS:FAR*)

Seeds from transgenic wax ester-lines (*FAEI:WS:FAR*) were analyzed through oil extraction with separation of TAG (triacylglycerol) and wax ester on TLC-plates and later analyzed with a GC. **Figure 9.** illustrates the separation of TAG and wax ester in different transgenic-lines on a TLC-plate stained with iodine. The result showed that the transgenic lines could produce wax esters, which can be seen in **Figure 9.**

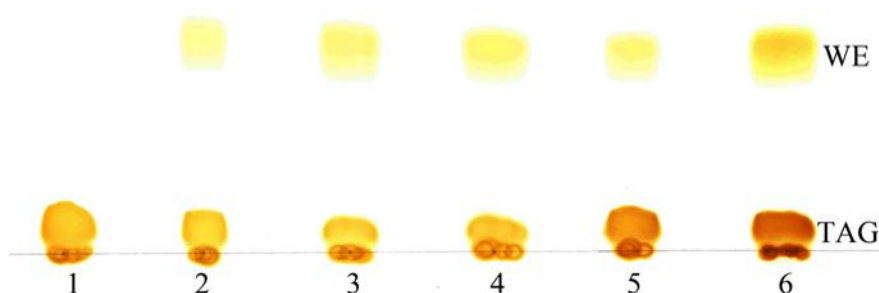
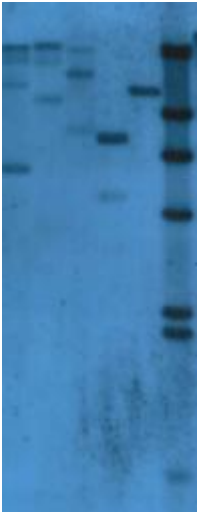


Figure 9. Separation of TAGs and WE s (wax esters) on a TLC-plate. Number 1. is a non-transgenic control ('NO94-7'). Number 2.-6 are transgenic lines. Photo: Li-Hua Zhu.

The wax ester content could not be calculated because of missing standards. The chromatograms gave however some indications of high-performance lines and 20 lines were selected for further evaluations.

These lines were later used for a Southern blot analysis to detect the copy number of the transgenes. During the Southern blot analysis the number of *nptIII* copies were analyzed and presented in **Figure 10**. The result showed that about 50 % of the chosen lines had a single copy of the *npt II* gene, and hopefully single set of the *FAEI*, *WS* and *WE*-genes which indicates that the high concentration of wax esters depends on the gene expression of the genes instead of the multiple copies.

Introducing wax ester producing genes may bring about undesirable effects on the plant, such as impaired germination ability.



L

Figure 10. The result from the southern blot analysis of five transgenic lines. L: ladder.

Selection test- Germination

The result from the test presented in **Figure 11**. showed clear differences between the different treatments. The goal was to find a suitable concentration to distinguish transgenic seeds from non-transgenic seeds in an early stage of the breeding process. If non-transgenic seeds could be distinguished in the germination stage it would save both labor and money, since only transgenic seeds would be analyzed by PCR, Southern blot and oil analyses which together are very time consuming.



Figure 11. Result from the selection medium test-Germination after 4 weeks. From left: Kanamycin 0 mg l⁻¹, 50 mg l⁻¹, 100 mg l⁻¹, 150mg l⁻¹ and 200 mg l⁻¹.

According to the test it showed that clear differences can be seen at 50 mg l⁻¹, but higher concentrations enabled distinguishing transgenic seeds from non-transgenic seeds at an earlier

stage. Plants from the higher concentrations 150 and 200 mg l⁻¹ showed clearly that non-transgenic seeds cannot grow on kanamycin and some plants were even dead when the test was ended.

Although differences can be seen at the lowest concentration (50 mg l⁻¹), the recommendation should instead be to use the concentration 100 mg l⁻¹ to ensure that only transgenic seeds are used further in the breeding program.

During the transformation step of the *WR11*-lines one line was grown on selection medium and did not look affected by the selection. However, the PCR analysis of *nptII* later showed that the line was non-transgenic. The line was despite the PCR result chosen for further analyses which later showed that the line both contained the selection gene and the *WR11*-gene. It is therefore important to use the selection medium for evaluation and not only base the selection on the PCR-result. Non-transgenic leaves can be developed in the early stage of transformation and could therefore be picked for DNA-extraction used in the PCR and incorrectly show that the line is non-transgenic.

Selection test- Plant growth

The result from the test presented in **Figure 12.** showed clear differences between the treatments. The goal was to find an optimal concentration to distinguish non-transgenic shoots from transgenic shoots during transformation. The result showed that non-transgenic shoots were very sensitive to kanamycin and were suffering already at the lowest concentration.



Figure 12. Result from the selection medium test. Plant growth after 6 weeks. From left: Kanamycin 0 mg l⁻¹, 30 mg l⁻¹, 40 mg l⁻¹, 50 mg l⁻¹ and 70 mg l⁻¹.

The test proved that non-transgenic shoots could be detected already at low concentrations.

Transgenic seed identification

Transgenic seeds containing a red fluorescent protein from *Discoma sp* (DsRed) were identified with a DFP-1™ Dual Fluorescent Protein Flashlight (NightSea). **Figure 13.** illustrates how transgenic seeds containing DsRed can be detected. This identification showed that there are suitable marker genes to use in breeding programs to avoid the use of antibiotics. The method is however time consuming and labor intensive, since every seed needs to be identified and sorted. The oil composition was later evaluated through a gas chromatograph (same procedure as the wax ester analysis) and it showed that there were differences in oil composition between the bright transgenic seeds and the dark non-transgenic seeds.

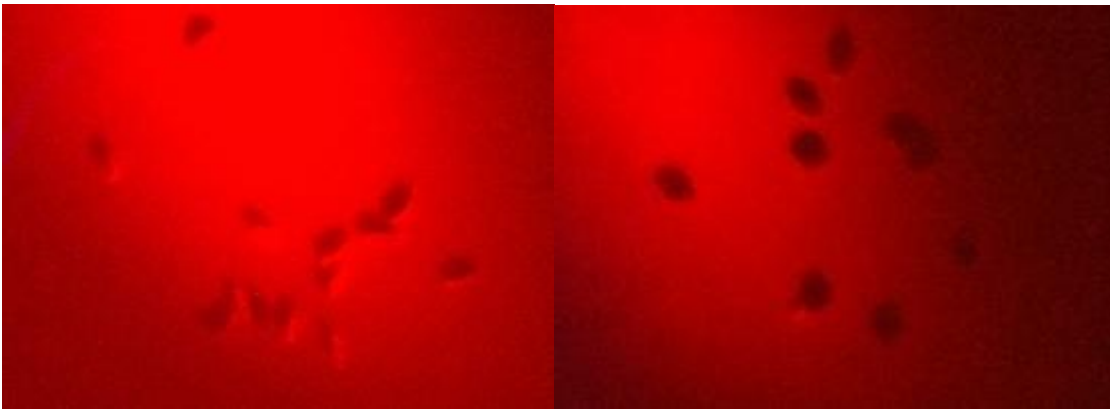


Figure 13. Transgenic seed detection with a DFP-1. Left: Transgenic seeds showing fluorescence. Right: Non-transgenic seeds without fluorescence.

Perspective

The aim to analyze and evaluate transgenic lines of field pepperweed will not end with this master project, but proceed faster and more efficient. Different tools have been investigated to better evaluate the transgenic lines such as DNA analysis, oil analysis and kanamycin studies.

The oil analyses from second generation of the *WR11*-lines and the hemoglobin-lines will be very interesting. A higher oil content in homozygous lines compared to the first generation heterozygous lines is expected. The lines will later be combined with the high oleic acid and low polyunsaturated fatty acids *FAD2:FAE1* RNAi-lines which hopefully will lead to a high performing line with suitable oil composition. Earlier studies have showed that the *WR11* gene is important seed germination and seedling development (Cernac *et al.*, 2006). It would therefore be interesting to see how an overexpression of the gene could affect the germination of field pepperweed and perhaps even improve the seed germination.

More studies need to be made to better understand how the genes affect other factors such as germination, flowering and pest- and disease susceptibility.

The less shattering transgenic lines are still only in the DNA analyzing stage and their ability to resist external impacts will later be evaluated.

The concentration of the selection medium (kanamycin) has been evaluated but more studies need to be made on how the kanamycin affect the plant growth with even lower concentrations than 30 mg l^{-1} .

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