Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Fakulteten för landskapsarkitektur, trädgårds- och växtproduktionsvetenskap**

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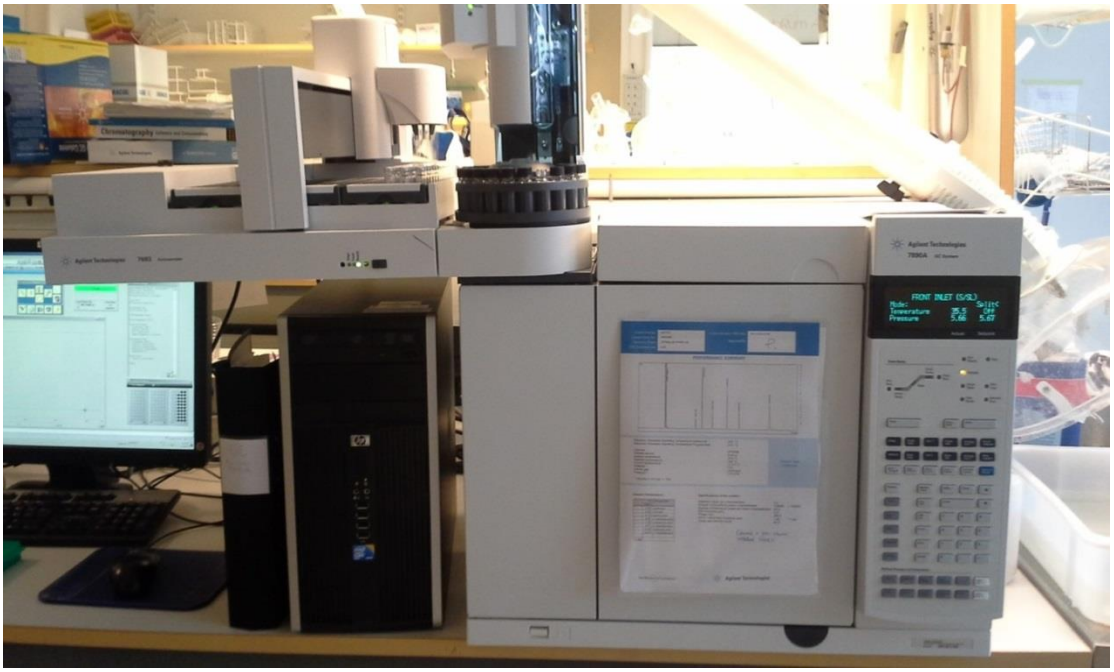
*Författare Philip Berg*



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# Analysis of oil content in the transgenic lines and wild-type of Lepidium campestre

Oljeanalys i transgena linjer och vildtypen av Lepidium campestre

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# Content

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## Abstract

The world's population is increasing and will be reaching up to 9 billion by 2050. More food needs to be produced in order to satisfy the growing demand in the future. Meanwhile there are increased focuses on the food we produce, which are healthier and have less negative impact on the environment. There is an urgent need for alternative oil sources. One of such alternative sources could be plant oils, and therefore the need of domesticating new oil crops is becoming obvious due to the limited numbers of the oil crops available in commercial production. At SLU in Alnarp, the screening work for identification of suitable oil producing wild Brassicaceae species for domestication began in the late 80's by the late Professor Arnulf Merker, using conventional plant breeding approaches. Then, Field cress (*Lepidium campestre*) was selected for domestication based on its agronomic and other desirable characters. Today, the rapid development of modern plant breeding methods, such as marker assisted selection, genome wide association studies, genomic selection and genetic engineering have provided us with an unprecedented opportunity for rapid domestication of wild plant species. In an ongoing PhD project, Emelie Ivarson is developing a potential future oil and catch crop - field cress using genetic transformation. One of the purposes of this PhD project is to increase the oil content by means of genetic modification. One strategy for increasing the oil content is to overexpress the haemoglobin gene (*VHb*) isolated from the bacterium *Vitreoscilla*. In this Bachelor project, we evaluated the oil content of seeds of transgenic lines of field cress transformed with the *VHb* gene by Emelie Ivarson and compared it with that of the wild-type (Control). The oil was extracted according to standard method used in the lab and gas chromatography was used for analyzing the oil content. Though this study did not show any statistically significant differences in oil content between the transgenic lines and the control, the highest and lowest values were recorded in the transgenic lines. Additional studies are required to fully understand the effect of *VHb* gene on seed oil content.

# Introduction

## *Lepidium campestre* (field cress)

*L. campestre* belongs to the Brassicaceae family (Ivarson et al., 2013). It is a biennial species that has the potential to become a catch crop. Field cress is more cold-hardy than canola, todays major oil crop growing in southern Sweden, and is thus more suitable for growing under cold Swedish climate conditions. It can lead to opportunities for growing oil crops on more acreage in Sweden, specifically higher up in the northern country (Ivarson et al., 2013).

During the late 80s and early 90s, investigations were made in order to select wild oil species suitable for domestication. After several field trials, four species were finally selected including *L. campestre* and three species in the genus *Barbarea* based on the following traits: plant height, hardiness, bloom, self-fertility, germination, seed yield and seed size (Eriksson, 2009).

In the mid 90's, three of those four crops were selected for further studies. These studies tested for both oil content and lipid composition (Eriksson, 2009). After approaching the 21st century and further investigations on nutritional values, *L. campestre* showed to be the most promising one. The facts that *L. campestre* is diploid and that it branches at the top also give it agronomic advantages and it was thus selected for further domestication studies (Eriksson, 2009).

Field cress has several serious problems. The oil content in the seeds is low and the oil composition is not suitable for edible oil. To improve the oil quality it is necessary to increase the oleic acid content which is a monounsaturated omega- 9 fatty acid and healthy oil. The third major problem with field cress is pod shattering (The sheath falls off prematurely), which causes high loss of seed yield. All the traits mentioned above are the targets addressed in the large interdisciplinary research project Mistra-Biotech and these problems will be solved by both traditional and modern breeding methods (Mistra Biotech *Annual Report 2012*).

## Fatty Acid, lipid, fat and oil

Fatty acid is a carboxylic acid (-COOH) bound to a long hydrocarbon tail. The tail may contain one or more double bonds between the carbon atoms. Saturated fatty acid has no double bonds and the carbon atoms of the tail hold two hydrogen atoms, meaning that no more hydrogen can bind to the carbon atoms in the tail (Bowsher et al., 2008).

Monounsaturated fatty acids have one double bond, while polyunsaturated ones have two or more double bonds. When there is a double bond there is no place for the second hydrogen atom to bind on the carbon atoms with the double bonding. They are therefore unsaturated since the carbon atoms don't hold its maximum amount of hydrogen atoms (Bowsher et al., 2008).

Fatty acids usually consist of an even number of carbon atoms in the tail since the carry enzyme acetyl-CoA delivers 2 carbon atoms a time during the fatty acid chain elongation (Bowsher et al., 2008).

The definition of a lipid is chemical substance that does not dissolve in water but in nonpolar organic solvents such as chloroform. These characteristics are due to the structure of the lipid. There is a water-soluble and polar head attached to one or more nonpolar and insoluble hydrocarbon tail/-s. They are then divided into two groups depending on the product formed when they react with a strong base- saponifiable and non- saponifiable. It is due to the ester bonds between the glycerol and the acyl molecule that allow the saponification (Bowsher et al., 2008).

What distinguishes a fat from oil is that fats are solid and oils are liquid at room temperature. This depends greatly on their amount of saturation. Fats are often more saturated fatty acids while oils often are more unsaturated. Both fats and oils are made up of lipids, triacylglycerol (TAG) used in the storage of carbon and energy in the plant. TAG is an example of a saponifiable lipid. In the cell TAG can form storage structures, round oil bodies (oleosomes). This is due to their unique structure with a hydrophobic part and a hydrophilic part that makes them form round shapes where the hydrophobic tails pulls into the middle of the oleosomen and the hydrophilic head pulling out towards the cytosol. Oleosins is a protein which coats the outer surface. This prevents oleosomes from merging with each other and helps stabilizing the structure (Browse et al., 2010).

## *Vitreoscilla*-Hemoglobin

*Vitreoscilla*-hemoglobin was the first observed hemoglobin to be detected in a bacterium in 1986. Before this it was believed that hemoglobin exists only in eukaryotic cells. Thus it belongs to the large globin family. Only after sequencing of the protein could it be determined that it was a hemoglobin protein and not a cytochrome-o as first believed. It is this sequencing that led to a successful cloning of the gene (Stark et al., 2011).

VHb is a soluble protein with the function of being able to bind and deliver oxygen at low concentrations. Its main functions are to transport oxygen to the terminal respiratory oxidase during hypoxia and to direct the transcription of other genes. The transfer of oxygen to the last protein in the electron transport increases the adenosine triphosphate (ATP) production during oxygen stress. It has since been found, by microarray, that VHb controls the expression of over 300 genes with both negative and positive regulation, making its expression on the metabolism difficult to grasp. The expression of *VHb* is regulated by the level of oxygen, but also by other transcription factors (Stark et al., 2011).

These features help plants when submerged, to better protect themselves against oxygen deficiency. But VHb also helps in the assistants of eliminating free nitrogen radicals. With the presence of VHb in a cell the redox state may be altered leading to regulation of the carbon and nitrogen metabolism in *E. coli* (Stark et al., 2011).

## *Agrobacterium* transformation

*Agrobacterium tumefaciens* (*At*) is one type of soil bacteria being able to alter the plant genome sequence and cause tumor formation, called also crown gall, in the infected plant. The *Agrobacterium* carries a circular plasmid known as the tumor-inducing (Ti) plasmid (Taiz, L. & Madlung A. 2010). On the Ti plasmid there are genes coding for the production of opines and the phytohormones auxin and cytokinin, which are located on the transfer DNA (T-DNA) on the Ti-plasmid and are the cause of tumor formation (Taiz, L. & Madlung A. 2010; Fosket, D. & Kieber J. (2010).

There is also a set of virulence (Vir) genes (A-G) on the Ti-plasmid outside of the T-DNA region responsible for initiation and completion of the T-DNA transfer (Taiz, L. & Madlung A. 2010).

Opines are nonprotein amino acids and can only be used as carbon and nitrogen nutrition and metabolic energy for *At* in the host plant (Taiz, L. & Madlung A. 2010; Fosket, D. & Kieber J. 2010).

*At* is resistant to most antimicrobial agents that wounded plant cells exude. When *At* comes in contact with the wounded cells/tissues, antimicrobial agents, specifically the phenolic

compounds, such as acetosyringone produced in response to wounding will trigger the expression of *Vir* A and G. This will then lead to initiation of the infection pathways. The circular Ti plasmid is then cut by the virulence proteins forming a ds-DNA complex with help of Vir proteins, such as D2 protein, a carry protein. After this path is made *At* fools the plant cell very elegantly by first attaching its flagellum to the leucine rich repeat receptor. In turn this starts the MKK4/5 signaling pathway which has the end product phosphorylated VIP1. Phosphorylated VIP1 usually enters the nucleus where it works as a transcription factor for the pathogenesis related bacterial resistance genes. Vir E2 proteins covers the Ti ds-DNA and Vir D2 protein. This will make the phosphorylated VIP1 bind in to the complex and allow it to enter the nucleus. Vir F protein also enters the nucleus removing the Vir E2 protein as well as the phosphorylated VIP1 from the Ti ds-DNA and Vir D2 protein. Vir F will also degrade the phosphorylated VIP1 and by so inhibiting the transcription of bacterial resistance genes. The T-DNA is then by means still not fully understood incorporated into the plant cell genome (Taiz, L. & Madlung A. 2010).

By exchanging the phytohormones and the opine genes on the T-DNA with genes of interest, the plasmid can be used for plant transformation for improving plant properties. The ready to use plasmid is then moved back to *Agrobacterium* for plant transformation. There are different transformation methods depending on species (Taiz, L. & Madlung A. 2010).

# Material and method

## Material

Sieves, glass tube, automatic pipette, glass pipette, Ultraturrax, vortex, HAc (acetic acid), $CHCl_3$/MeOH (Chloroform Methanol 1:2), $H_2O$ (Milipore Water), $CHCl_3$ (Chloroform), Hexane, heating block, 2% $H_2SO_4$ (sulphuric acid) in 98% absolute methanol, $8{,}9e^{-4}$M 17:0-Me standard (Methylated 17:0 fatty acid), GC vial, Agilent Gas Chromatography.

The plant material used was collected in Öland by the late professor Arnulf Merker and seeds were then multiplied in greenhouse. The wild type Lepidium was *L. campestre* 'NO-94-7', which was also used in the transformation. The transformed lines are the first generation with the integration of the *VHb* gene and the transgenic lines were previously obtained in the lab using the transformation method described by Ivarson et al., (2013). Both the wild type and transgenic lines were grown in the biotron to be sure they were growing under the same controlled climate chambers.

# Method

## Harvesting

Seeds were harvested and threshed by hand and separated from other tissue by means of a sieve.

## Extraction

Ten seeds were weighed with three decimal precision on an analytical scale and placed in a glass tube. After, it was added 1ml 0.15M HAc and 3.75ml $CHCl_3$/MeOH to glass tubes with samples, then homogenized in 100 seconds with five intervals. Thereafter, the sample hade added 1.25ml $CHCl_3$, 0.9 mL $H_2O$ and vortex for 30 seconds. After each homogenization the Ultraturrax was washed five times with absolute ethanol. Samples were centrifuged for 3 min by 3000rpm. Then the $CHCl_3$ phase (bottom) was transferred to a new glass tube. Pipetting 200µl of the $CHCl_3$ phase to a new glass tube and evaporate under nitrogen. Then 100µl Hexane, 2ml $H_2SO_4$ and 100µl 17:0 -Me standards were added to the sample, and closed with a lid. The samples were methylated in a heating block at 95°C for 60 minutes. Samples let cool and 1ml $H_2O$, 0.75 ml of hexane was added. The samples were vortexed and centrifuged for two minutes with 2000rpm. Finally pipetted 100µl of the top phase to the GC vial and transferred to the GC for analysis.

## Gas chromatography (GC)

GC is an analytic method for volatile organic compounds. It is based on that an organic substance which is to be investigated is dissolved in the appropriate solvent. It may in special cases be a solid. By injecting its volatile substance in the injection chamber, where it is vaporized and carried by the mobile phase (for example, hydrogen, nitrogen, helium or argon) into the column. In the column each compound is transported at different speeds or rates. This is due to its volatility and how well it interacts with the solid phase (thin surface inside the column). Usually, the column has a fixed temperature so the compounds volatiles smoothly but specific program may occur in some analyses. When samples come out of the column, it has been separated. They will then come to a detector (there are different kinds of detectors with different ways of measuring compounds) where those various compounds, depending on amount of substance and the time in the column, give different peaks with different integral in the chromatogram (Atkins & Jones 2010).

## Calculation of the oil content

On the chromatogram it shows different peaks (See Figure 1). The content of the fatty acid determines the integral of the peak. The known amount of 17:0-Me standard is used as an indicator to calculate the concentration of the other fatty acids. 17:0 is a fatty acid that does not occur normally in plant oils due to the fact that cells can only create even numbers such as 16, 18, and 20 and so on.

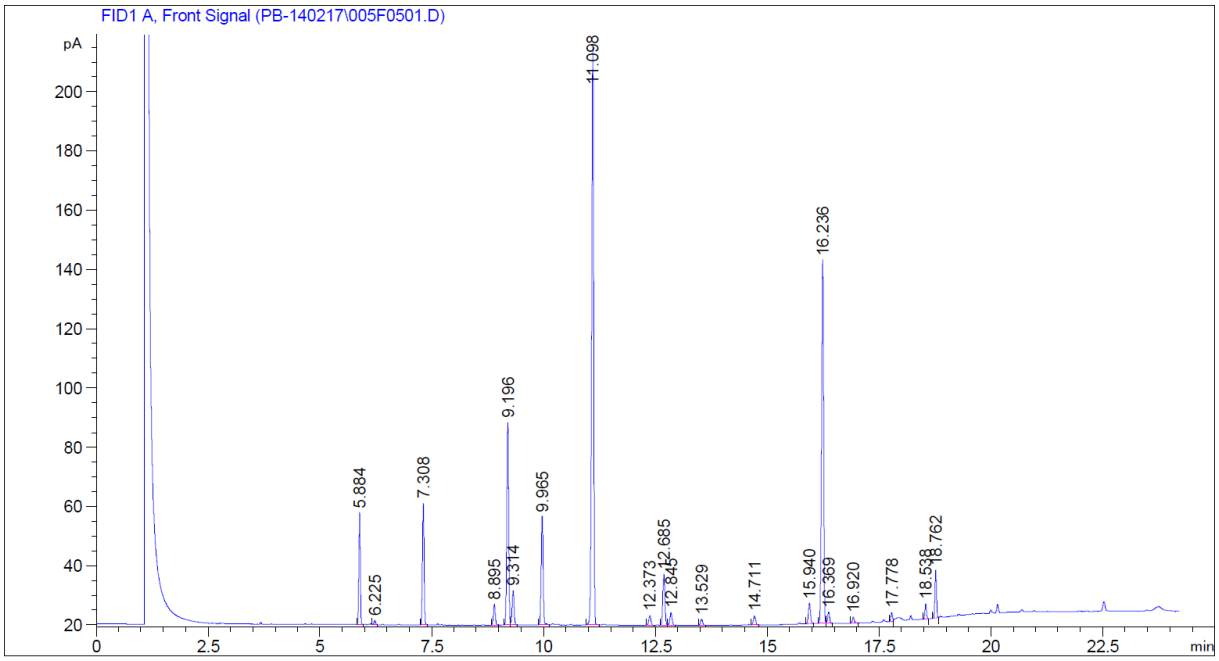


**Figure 1. Example of a chromatogram of the seed oil composition in field cress. Each peak represent one type of fatty acid.**

Definitions of algebraic abbreviations:

$FA^i$ = Fatty Acid chosen for examination; Tot = Total; The amount of $CHCl_3$ used for analysis (Share $CHCl_3$) = 200µl; $CHCl_{3Tot}$ = total volume of the $CHCl_3$ phase in the extraction; %FA and %17:0 read on the chromatogram.

The formula to calculate different values are as follows:

Formula 1:

$$\left( \frac{\%FA \left( \frac{g/mol_{FA^i}}{g/mol_{17:0}} \right)}{\%17{:}0} \right) * 89 = x\ nano\ mol\ FA^i$$

Explanation formula 1:

%FA and %17:0 are taken out of the chromatogram. After multiplication of the ratio of molecular weight with %FA it is given a 1:1 ratio in the percentage mole content as %FA change equivalent to the difference in molecular weight. It is then multiplied by 89 which is the number of moles of 17:0 added before methylation. This is done for each $FA^i$ chosen for examination.

Formula 2:

$$x * 0{,}001 * g/mol_{FA} = \mu g\ FA$$

Explanation formula 2:

Then multiplying by 0.001 giving the value in micromoles instead of nanomoles, multiplying by the molecular weight and the weight is given.

Formula 3:

$$\mu g\ FA^i * \frac{CHCl_{3_{Tot}}}{Share\ CHCl_3} = \mu g\ FA^i{}_{Tot}$$

Explanation formula 3:

The relationship between how much larger the whole $CHCl_3$ phase is than the 200µl which was pipetted over in the extraction.

Formula 4:

$$\left(\frac{\left(\frac{\mu g\ FA^i}{g/mol\ FA}\right)}{3}\right) * g/mol\ glycerol = \mu g\ glycerol^j{}_{tot}$$

Explanation formula 4:

The assumption that all fatty acids is in TAG structures are made. Division with the molecular weight of the fatty acid gives moles; divide by three because there are three fatty acids per glycerol head in TAG structure. Multiplying the molecular weight of the glycerol gives the total weight of glycerol heads in the sample of the selected fatty acid.

Formula 5:

$$\frac{\sum \mu g\ FA^i{}_{Tot} + \mu g\ glycerol^j{}_{Tot}}{Seed\ weight} * 100 = \%Triacylglycerol$$

Explanation formula 5:

The sum of the total weight of all the fatty acids and the total weight of all glycerol heads is divided by the weight of the seeds and multiplied by 100. This gives %TAG of the average seed in the sample.

## Result & Discussion

Table 1 shows that none of the transgenic lines has statistically significant different oil content than the wild-type.

**Tabel 1. Showing oil content in the transgenic lines and wild-type (control line) of *Lepidium campestre*. Lines that share the same letter do not differ significantly by Tukey's grouping using the software Minitab. Control line marked with red.**

| Line | Average % TAG | Min % TAG | Max % TAG | Line | Average % TAG | Min % TAG | Max % TAG |
|---|---|---|---|---|---|---|---|
| T56P18 | 27,187[a] | 21,05 | 31,89 | T55P1 | 21,330[ab] | 19,28 | 24,76 |
| T54E5 | 26,118[ab] | 22,78 | 29,46 | T55E3 | 21,017[ab] | 17,58 | 24,45 |
| T55E10 | 25,696[ab] | 24,010 | 26,854 | T55A14 | 20,836[ab] | 19,15 | 22,53 |
| T55P11 | 25,270[ab] | 22,98 | 27,56 | T54A9 | 20,626[ab] | 19,638 | 21,932 |
| T56P33 | 24,385[ab] | 23,255 | 25,218 | T55P3 | 20,334[ab] | 19,117 | 21,161 |
| T54A5 | 24,199[ab] | 20,71 | 29,77 | T55P6 | 19,947[ab] | 19,213 | 21,359 |
| T55A38 | 23,429[ab] | 21,989 | 24,640 | T54E9 | 19,874[ab] | 18,589 | 21,607 |
| T55P31 | 23,162[ab] | 21,16 | 26,87 | T56A15 | 19,731[ab] | 17,63 | 21,73 |
| T55P10 | 22,853[ab] | 19,24 | 25,27 | T56E1 | 19,669[ab] | 18,774 | 20,563 |
| T56P9 | 22,842[ab] | 20,65 | 25,04 | T54E2 | 19,566[ab] | 17,70 | 22,06 |
| T56P5 | 22,638[ab] | 21,40 | 24,66 | T55P4 | 19,519[ab] | 19,203 | 19,834 |
| T56P29 | 22,624[ab] | 22,210 | 23,147 | T54E3 | 19,123[ab] | 18,616 | 19,629 |
| T54E6 | 22,277[ab] | 21,212 | 23,247 | T56A3 | 19,048[ab] | 16,721 | 21,275 |
| Control | 22,252[ab] | 14,348 | 27,667 | T54A34 | 19,046[ab] | 17,994 | 19,756 |
| T54A8 | 21,926[ab] | 21,020 | 23,058 | T56P2 | 18,927[ab] | 17,486 | 19,870 |
| T56A14 | 21,743[ab] | 18,41 | 27,84 | T56P19 | 18,746[ab] | 16,812 | 19,860 |
| T56P4 | 21,416[ab] | 17,76 | 23,34 | T54A32 | 18,505[ab] | 15,62 | 21,39 |
| T54A38 | 21,399[ab] | 16,98 | 27,22 | T56A16 | 18,268[ab] | 17,531 | 19,451 |
| | | | | T55P24 | 16,020[b] | 7,10 | 20,75 |

However, it would need more replicates for the control line as it shows a high standard deviation as can be seen in Figure 2. With the line T56P18 (marked as blue in Figure 2 and 3) an outlier is seen in Figure 4, suggesting further analysis with more replicates may be needed.

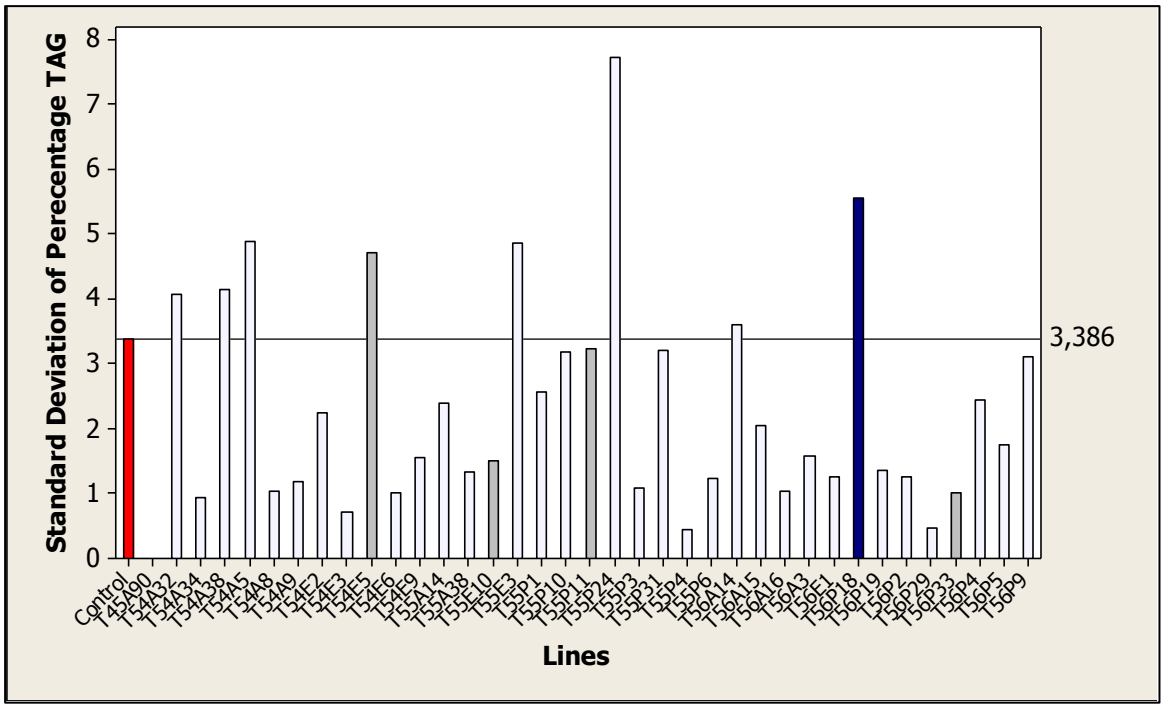


Figure 2. Control marked with red with a support Line drawn at the controls standard deviation. Blue bar for the highest average %TAG, grey denotes the seconded to fifth with highest average %TAG.

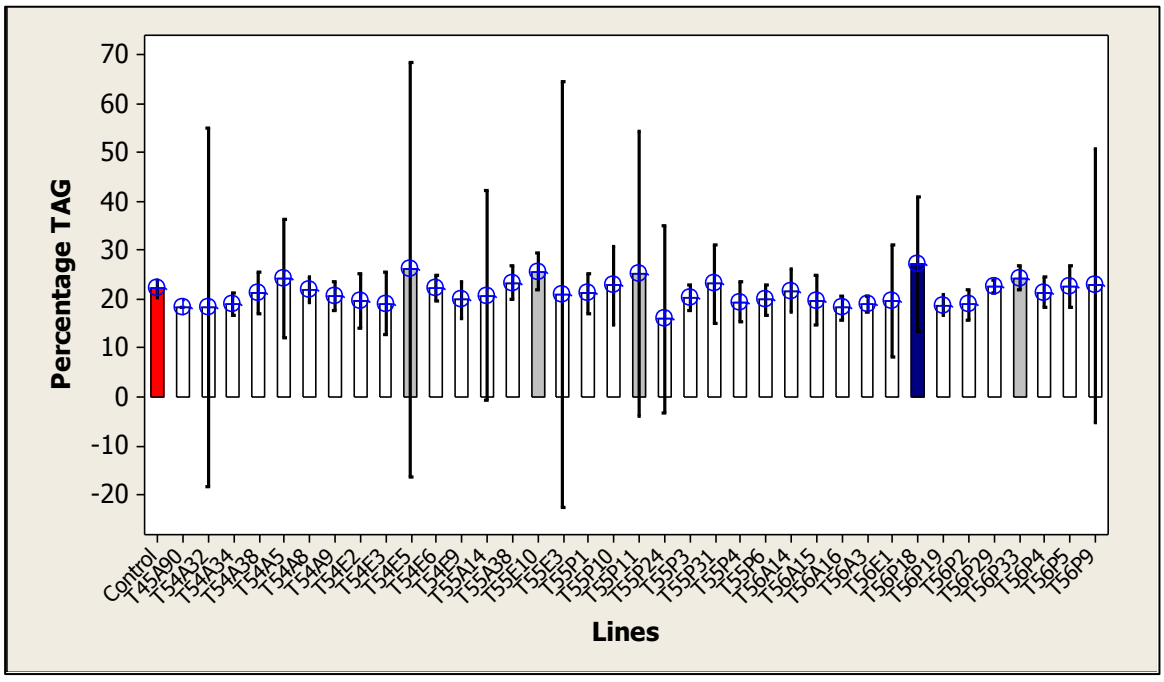


Figure 3. Bar chart with average oil content plotted and a 95 % confidence interval. Blue bar for the line with highest average% TAG, grey for those four after that. Red denotes control.

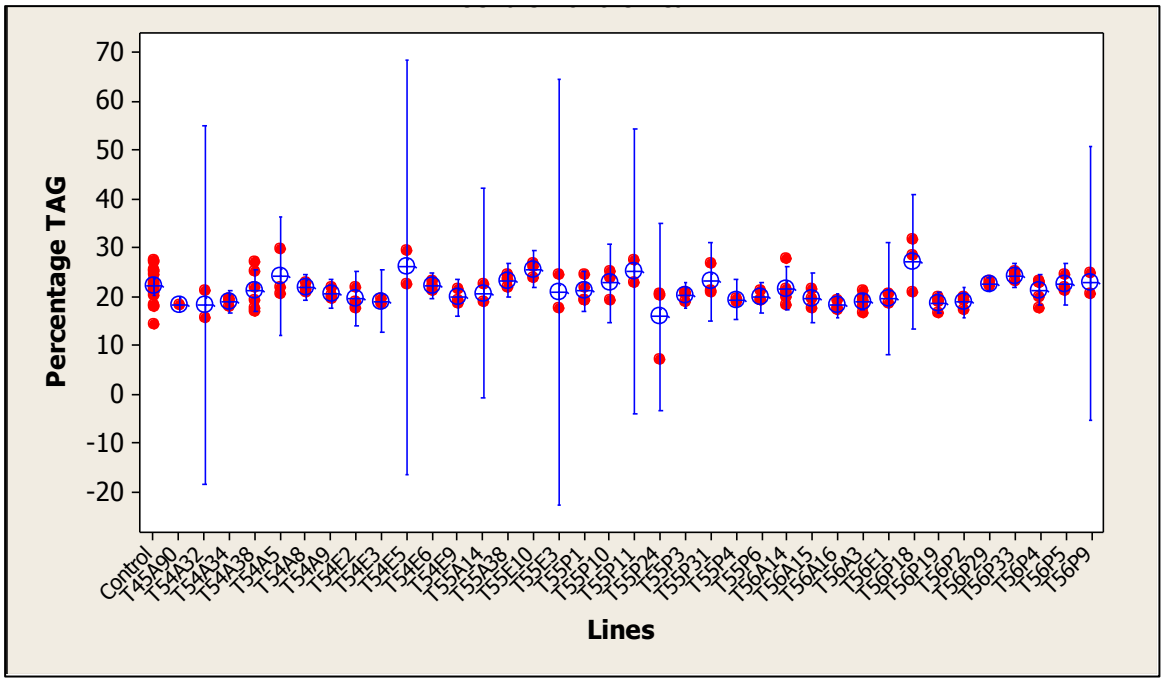


Figure 4. Individual value plot with a 95% confidence interval, helping to display outliers.

According to Stark et al., (2011) the presence of VHb in a cell alter the redox state, which leads to a simultaneous change in the metabolism. Though this study did not yield any statistical significance a tendency of increase in oil content could be observed some lines had low standard deviation (SE), while others had high SE, indicating a need for increasing the replicate number. Lines which are recommended to increase the number of replicates are marked with grey and blue in Figure 2 and 3. Those which seem more promising can be further bred with other transgenic varieties and wild-types that have attractive properties.

The wide spread of oil content seen in Table 1 may be due to the effect of VHb overexpression. It may also be due to nitrogen metabolism being up regulated instead of carbon metabolism. Most likely it is due to the transgenic lines being more heterozygous as they were of the first generation.

VHb is usually in a dimer stage but it has weak interconnection to its subunit. It has also been shown to exist in monomeric form under certain conditions, which may affect its function (Stark et al., 2011). The more complexity of eukaryotic cells compared to prokaryotic cells might lead to VHb forming more monomer structures or maybe different dimer structures which can affect the function of the protein due to its weak coupling with the subunit. Further studies are needed to find out the VHb effects on the oil content.

Examinations on the amount of VHb protein both in monomer and dimer states in the transgenic *L. campestre* in correlation to the amount of oil content may provide indication how the VHb protein functions.

Other more promising hemoglobin genes such as Arabidopsis hemoglobin-2 can be tested in field cress and this gene has shown a 40% increase in the oil content in Arabidopsis when overexpressed (Vigeolas et al., 2011).

## Conclusion

There is a variation in oil content both in the wild-type and in some transgenic lines. The highest and lowest oil content was in transgenic lines although no significant difference was found. The reason for not detecting a significant increase in oil content may be due to the heterozygous nature of the transgenic lines, variation in SE or other internal factors regulating expression and function of VHb since functions of VHb on metabolic pathways and the factors affecting VHb proteins' function are still unclear.

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