

Fatty acid analysis of *Arabidopsis thaliana* seeds transformed with class 2 non-symbiotic hemoglobins

Introduction

As the prices for oil increases and the world gets ever more environmentally conscious, biodiesel becomes a more and more attractive alternative to regular diesel. Plant oils can be turned into biodiesel by converting their triacylglyceride (TAG) molecules into fatty acid esters. TAG molecules consist of three fatty acid chains esterified to glycerol and are converted to biodiesel by esterification with a primary alcohol, usually methanol (1).

Biodiesel has a low net greenhouse emission and is produced from a renewable resource. The use of biodiesel also reduces the emission of carbon-monoxide and particulate matter. Biodiesel has its drawbacks, it has an increased emission of NO_x and its cold flow properties are not suitable for colder climates (1). Its biggest drawback though is the vast need for plant oils (2). These plant oils are often used for food and the production of biodiesel will compete with food production for its raw material, increasing the prices. Increasing the production of plant oils could therefore prove a great economical advantage. This can be achieved by switching to plants with a high areal yield of plant oil or even using drought resistant crops like *Jatropha curcas* that can grow in dry places not suitable for other plants, expanding available crop land. Another approach is to increase the oil yield from the plants using plant breeding and genetic engineering. This requires a good understanding of how the plants convert the carbohydrates it produces through photosynthesis into TAG. These efforts typically focus on increasing the production of the fatty acids, the production of the glycerol backbone, or increasing the activities of the enzymes used in the assembly of the TAG molecules. (1)

The production of biodiesel has non-the less increased dramatically the last years from a worldwide production of 179 million barrels in 2007 to 309 million barrels in 2009 and 404 million barrels in 2011 according to the US energy information agency (3).

In a previous study, Vigeolas et al. (4) used microsensors to study the effects of increased oxygen levels on rapeseeds and rapeseed siliques. The study shows that rapeseed metabolism is restricted by the low oxygen concentration inside the seeds and that the metabolism can be increased by elevating the external oxygen levels. Three approaches were suggested for increasing the oxygen concentrations in plant seeds to increase their metabolism, expression of oxygen binding proteins, changes in silique gas permeability and increase of the photosynthesis.

Another study by Vigeolas et al (5) showed that overexpressing non-symbiotic Hb2 (AtHb2) in the seeds of *Arabidopsis* increases the fatty acid content, energy levels, and reduces the effects of anaerobic metabolism when the external oxygen levels are lowered. The energy levels (ATP/ADP) increased

threefold and sucrose levels increased twofold, indicating increased supply of energy for biosynthetic activities, such as fatty acid synthesis. The increase in fatty acids was mainly due to an increase of the unsaturated fatty acids C18:2 (number of carbons:number of double bonds) and C18:3. The levels of anaerobic intermediaries were also studied. The levels were measured at normal oxygen levels (21%) and at very low oxygen levels (4%). The results showed that the plants overexpressing AtHb2 in their seeds had a higher energy level and produced less intermediaries of anaerobic metabolism than wild type plant seeds, indicating that AtHb2 increased the oxygen availability to the seeds.

Arabidopsis thaliana is a plant member of the Brassicaceae family which also includes agriculturally important plants such as rapeseed. *Arabidopsis* has become the model system of choice for research in plant biology because of several traits such as short generation time, small size, prolific seed production through self pollination, a small genome, and the development of efficient transformation procedures. (6).

The aim of this study was to explore the possibility of using non-symbiotic hemoglobins to increase the fatty acid production in *Arabidopsis* seeds. The hemoglobins used in this study were two class 2 non-symbiotic hemoglobins, one from *Arabidopsis thaliana* (AtHb2) and the other from *Beta vulgaris* (BvHb2) and also a hemoglobin from *Vitreoscilla stercoraria* (Vgb). Seeds expressing these hemoglobins as well as wild type lines were grown under controlled conditions and the mature seeds were analyzed for fatty acid content and composition.

Theoretical Background

Hemoglobins

Hemoproteins are proteins containing a heme prosthetic group. The heme prosthetic group consists of a reduced iron atom (Fe^{2+}) centered in a porphyrin ring. The heme iron acts as an electron transfer component and/or as a chelating agent for small biologically important ligands (7). The most well known of these proteins are probably the hemoglobins. Among these, the ones in our red blood cells that bind oxygen in our lungs and transport it out into our bodies. Hemoglobins can be found in all three domains of life, eukaryotes, bacteria and archaea (8). Hemoglobins can be either hexa- or penta-coordinated. This means that the iron atom at the center of the porphyrin ring is bound with four of its six coordination sites to the porphyrin ring and either one or two of the remaining coordination sites attached to histidine sidechains of the protein (9).

The first hemoglobin found in plants was the symbiotic hemoglobin leghemoglobin (10). Leghemoglobin is found in the root nodules of some leguminous plants where it is a crucial part of symbiotic nitrogen fixation, buffering the free oxygen available to avoid the deactivation of oxygen-labile nitrogenase while still keeping the oxygen flux high enough for respiration (11). Several additional hemoglobins have since been found in plants and they have been divided into three different categories: symbiotic, non-symbiotic (nsHb) and truncated (trHb) (10). The non-symbiotic hemoglobins (nsHbs) are further divided into two classes, class 1 and class 2 based on oxygen affinity and sequence similarity (12). All nsHbs have

at least a partial hexacoordination of the heme-group. They bind one of the histidine groups tightly but the second one reversibly, allowing the binding of exogenous ligands (9).

Class 1 nsHbs are induced when plants experience hypoxic conditions, improving energy state and decreasing reduction levels by scavenging nitric oxide produced by the low oxygen conditions, providing essential oxidative capability when the oxygen levels fall to low for aerobic respiration (13). Class 1 nsHbs have a low hexacoordination equilibrium constant, giving them an extremely high avidity for oxygen. When the protein binds oxygen there is a conformational change, stopping the oxygen from quickly dissociating. This oxygen is then used to oxygenate nitric oxide into nitrite, leaving the hemoglobin in an oxidized ferric state, which can then be reduced by NADH to increase the recycling of NADH to NAD^+ (9) (14).

Class 2 nsHbs are not as well studied as the class 1 nsHbs. It is believed to play a role as an oxygen carrier, and its oxygen binding characteristics have been studied in detail by Spyraakis et al. (15), showing characteristics comparable to leghemoglobin (9). They have tighter hexacoordination than class 1 nsHbs which gives them lower oxygen affinities, making them less effective at NO scavenging but makes them more likely to function with sensing low oxygen levels, oxygen storage and diffusion (9). The results gathered by Vigeolas et al (5) also suggest that nsHb2 act as oxygen transport proteins.

TAG production pathway in plants

The pathways used for the production of triacylglycerol (TAG) in plants can be seen in Figure 1. The biosynthesis can be easily divided into two different parts depending on their localization in the cells. De novo synthesis of fatty acids occurs in the plastid. The fatty acids are then exported to the cytoplasm where they can be further elongated or desaturated before being incorporated into TAG molecules.

The first step in the lipid synthesis is carried out by Acetyl-coenzyme A (CoA) carboxylase (ACCase), catalyzing the first committed step, the formation of malonyl-CoA from acetyl-CoA. The malonyl group is then transferred to an acyl carrier protein (ACP) and used for the sequential elongation of the lipids.

Fatty acids are synthesized by a multi subunit complex called fatty acid synthase (FAS). FAS uses four reactions to build the fatty acids (condensation, reduction, dehydration and reduction) (16). Each reaction is catalysed by separate enzymes and the first enzyme, the condensation enzyme, determines substrate specificity (17).

Ketoacyl-ACP synthase III is the first condensation enzyme in the de novo synthesis of fatty acids. It catalyses the first elongation step using acetyl-CoA as a primer and adds two carbons using malonyl-ACP, creating a four carbon lipid. The four carbon lipid is then further elongated by Ketoacyl-ACP synthase I using additional malonyl-ACP, extending the lipid by two carbons for each malonyl-ACP used up to 16 carbons. The fatty acids are then extended to the full 18 carbon lipids by acyl-ACP thioesterase II. The fatty acids are released from the FAS complex by hydrolysis by an acyl-ACP thioesterase. The free fatty acids are activated to CoA esters by long-chain acyl CoA synthases on the outer membrane before they are exported towards the endoplasmic reticulum (ER) (18).

De novo TAG assembly occurs at the ER where acetyl-CoA units from the plastids are added to the glycerol 3-phosphate backbone through acylation reactions. The first two acylations are to the sn-1 and sn-2 positions of glycerol 3-phosphate and are carried out by glycerol 3-phosphate acyltransferase and lysophosphatic acid acyltransferase yielding phosphatidate. Phosphatidate is then converted to diacylglycerol (DAG) by phosphatic acid phosphohydrolase (18).

DAG is an important branch point. From here the DAG can be acylated by diacylglycerol acyltransferase using acyl-CoA as substrate, yielding TAG. Another option for generating TAG is phospholipid:diacylglycerol transferase. This enzyme transfers one acyl group from phosphatidylcholine (PC) to DAG, generating TAG and lyso-PC. There are two more pathways between DAG and PC. CDP-Choline:diacylglycerol cholinephosphotransferase transfers the choline head group from cytidyne diphosphate-choline(CDP-choline) generating PC and cytidyne mono-phosphate(CMP), and phosphatidylcholine diacylglycerol cholinephosphotransferase transfer the choline group from PC to DAG, this is used to switch PC that has been desaturated with de novo DAG (19).

While in the acyl-CoA pool, the fatty acids can be elongated further by another fatty acid synthase complex very similar to the one in the plastids. This FAS can be used to elongate the fatty acids for longer fatty acids in TAG or for other uses such as waxes (17). The fatty acids can also be directly incorporated into the PC pool by the PC:acyl-CoA acyl editing cycle. The PC acyl editing cycle involves rapid deacylation of PC, generating lyso-PC and releasing the FA. Reacylation of Lyso-PC with a different acyl-CoA completes the cycle (18).

The PC pool is an important intermediary in the generation of TAG; here the fatty acids can be further desaturated to fit the cells needs. Most of the newly synthesized DAG will become PC and go through acyl editing before being converted back to DAG and then finally TAG (19).

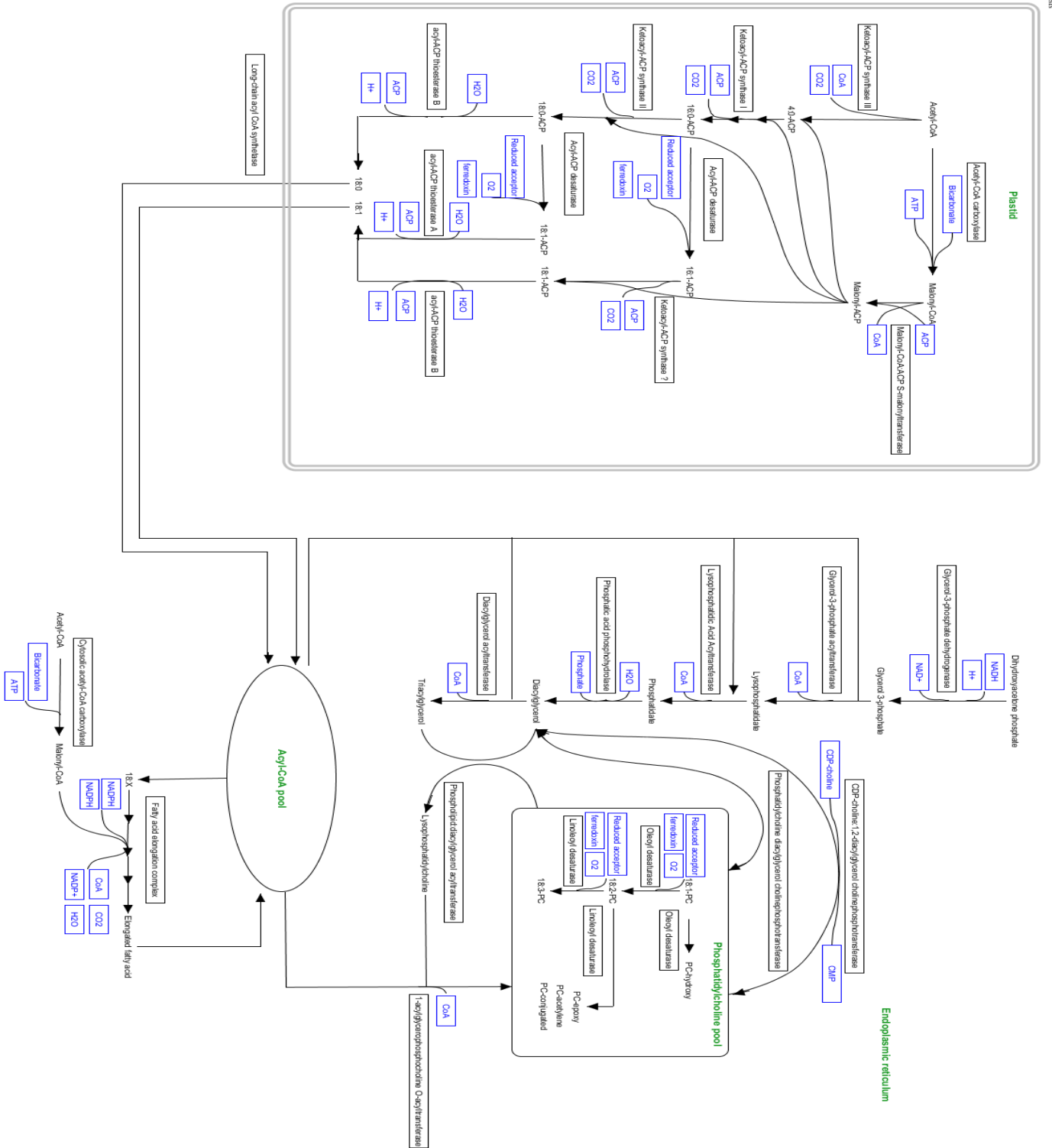


Figure 1: Metabolic pathway of fatty acid synthesis.

Results

Gas chromatography was used to measure the amount of each fatty acid in mature seeds from wild-type *A. thaliana* and the three constructs. Ten T3 BvHb2 lines, ten T3 AtHb2 and eight T3 Vgb lines with three replicates from each line as well as wild type seeds were analyzed. It should be noted that the results are preliminary as the data is the result of an initial screening. A list of the identifiers for each line and which hemoglobin they express can be found in table 1.

Table 1: Identifiers for each line

BvHb2	AtHb2	Vgb
19-5-4	15-3-3	15-1-4
10-3-5	M3-4-8	2-1-5
8-1-10	14-3-3	3-3-10
22-2-4	5-1-5	11-1-6
18-3-5	2-1-1	11-1-1
18-3-7	4-2-9	2-1-10
18-3-10	14-3-8	5-2-5
18-3-6	7-2-3	5-2-7
1-4-3	13-1-6	
M2-2-10	15-3-4	

During the cultivation of the *Arabidopsis* plants only one line stood out from the rest, 7-2-3 showed a delay in growth during the first weeks, growing significantly slower than the rest (Fig 2) and producing slightly smaller plants with less seeds at the end.



Figure 2 Growth delay of line 7-2-3 compared to 5-2-5

Dry weight

The total dry weight of the seeds showed that there were nine lines with a statistically significant change from the wild type. Three of these had an increase and six a decrease (Fig 3). There is one line with increased seed dry weight from each construct. One of the lines with reduced seed weight is from the BvHb2 construct, two are from the AtHb2 construct and three are from the Vgb construct.

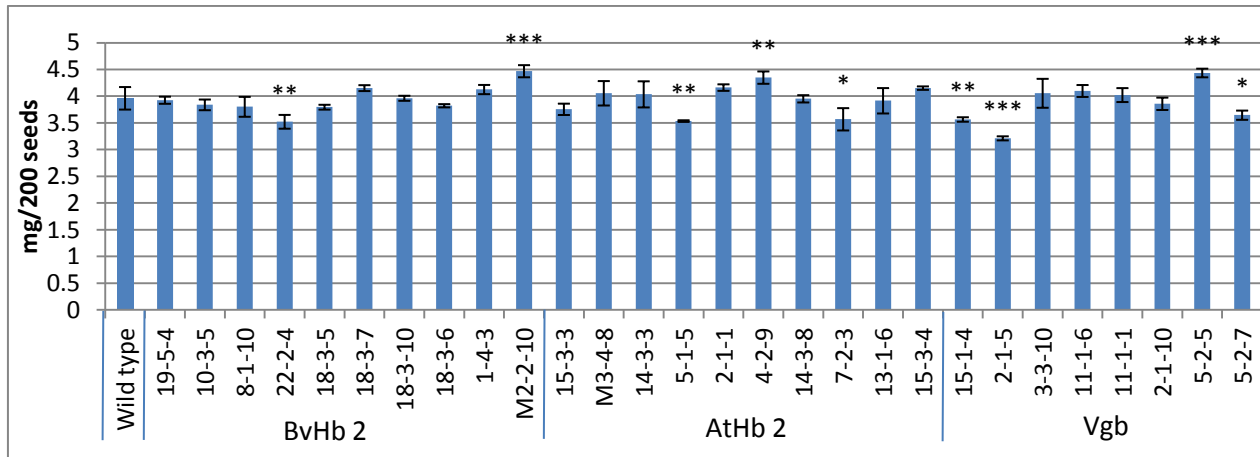


Figure 3: Seed weight (mg) of 200 seeds from each line. Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). n=3.

Total fatty acid content

There are only three lines with a statistically significant change in the total amount of fatty acids in the seeds, all of which have decreased (Fig 4). Two of these lines are from the AtHb2 construct and one from the Vgb construct. There are two lines with increased fatty acid content, 15-3-4 and 5-2-5 that have p-values just above the 5% significance level. The variation between the samples from each line is quite high here, indicating that more care must be taken during the sample preparation and more replicates should be done in the future.

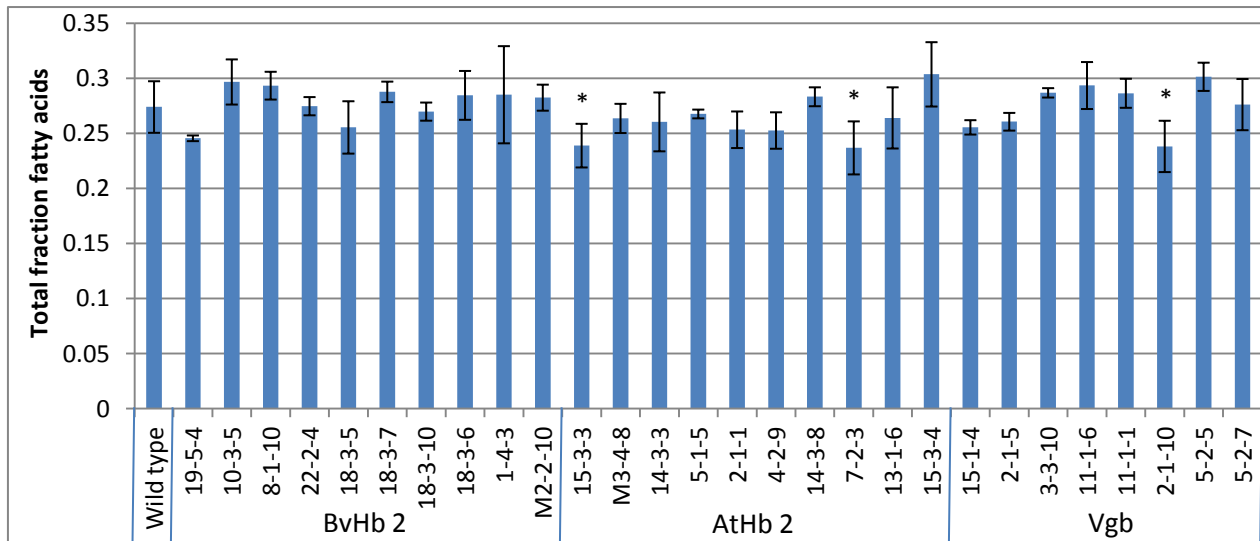


Figure 4: Total fraction fatty acids for each line. Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). n=3.

Plastidic fatty acid desaturation

It has previously been reported that the AtHb2 gene increases the level of unsaturated fatty acids in *Arabidopsis* (5). Looking at the plastidial desaturation of stearic acid by examining if there has been a change in the ration of 18:1/18:0, two lines from BvHb2, two lines from AtHb2, and two lines from Vgb show a decrease. There is only one line from Vgb showing an increase in C18:1/C18:0 levels (Fig 5).

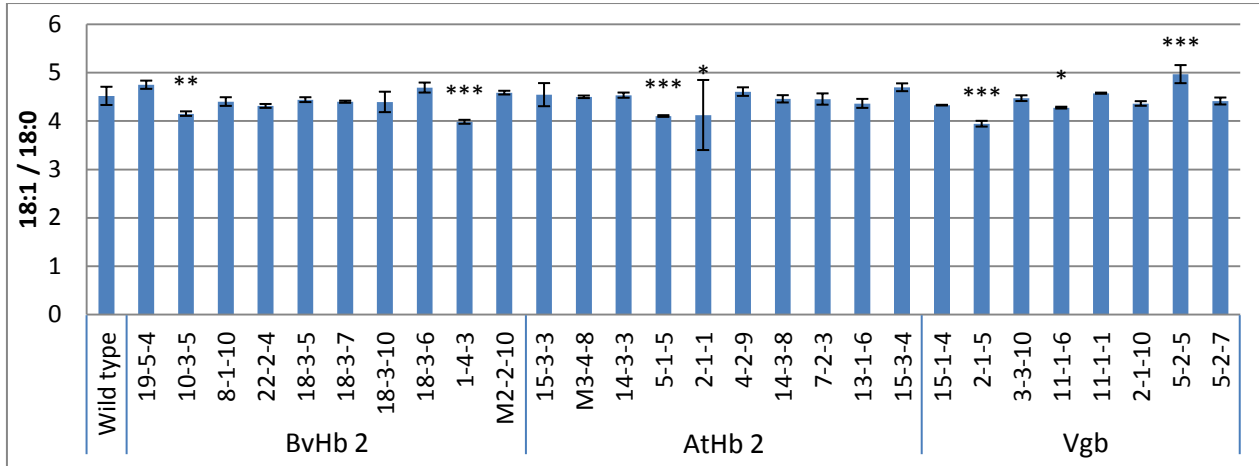


Figure 5: Amount of oleic acid (18:1) divided by the amount of stearic acid (18:0). Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). n=3

Cytoplasmic fatty acid desaturation

Examining the desaturations of C18 fatty acids in the PC-pool by looking at the ratio of the poly unsaturated 18 carbon fatty acids (18:2 and 18:3) compared to 18:1, there are five lines showing a decrease, two BvHb2, three AtHb2. Five lines show an increase, one BvHb2, three AtHb2 and one Vgb (Fig 6).

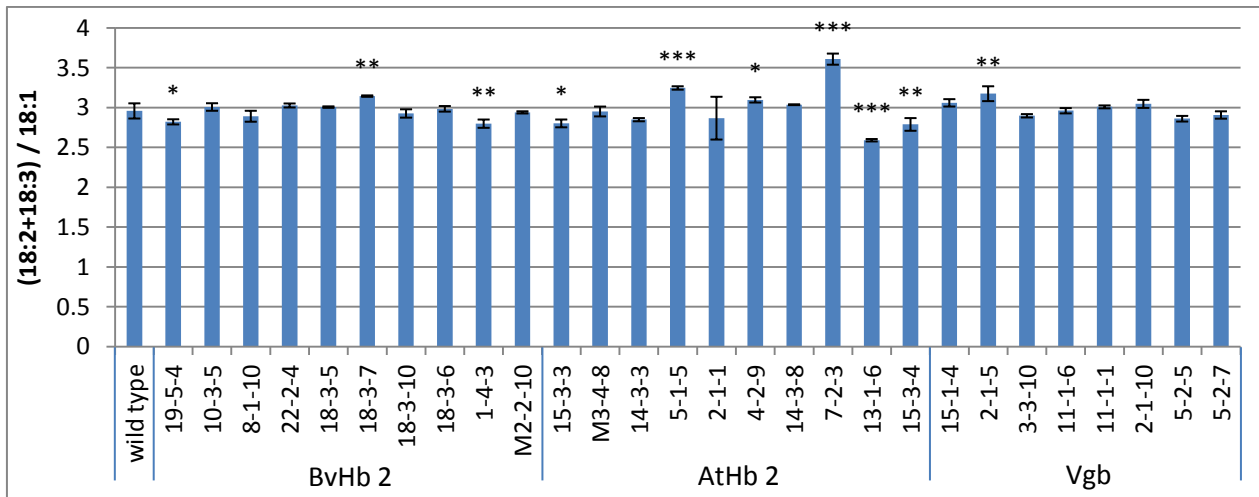


Figure 6: Linoleic acid (C18:2) and linolenic acid (C18:3) divided by oleic (18:1) acid. Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). n=3

At the second desaturation step in the PC-pool where the fatty acids are desaturated a second time from 18:2 to 18:3, many lines show a change; 16 decreases in total. Seven were from the BvHb2 lines, seven from AtHb2 lines and two Vgb. There were also 6 increases, two lines from each construct (Fig 7).

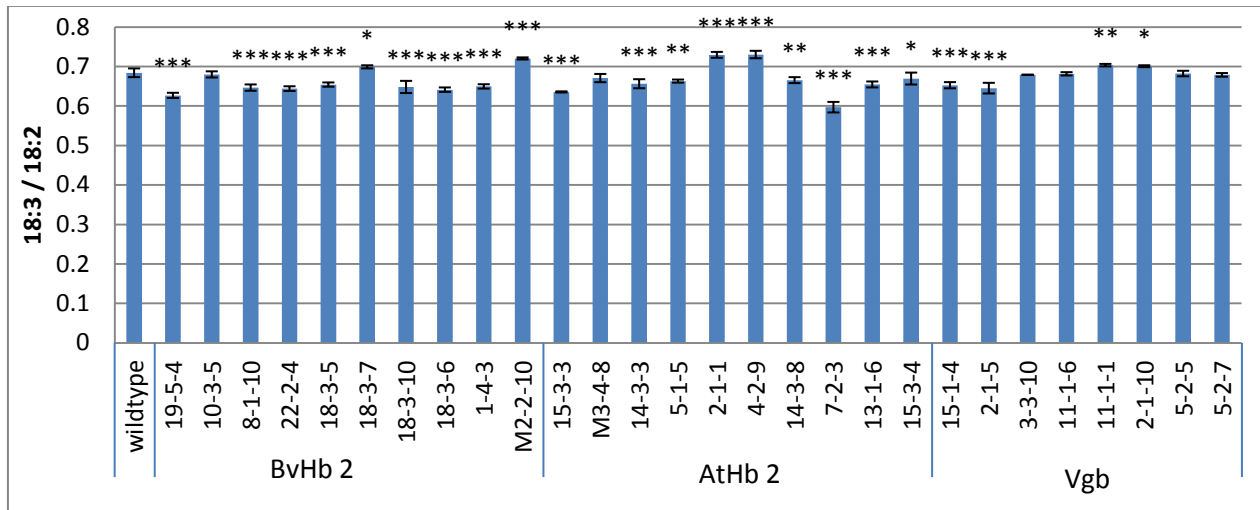


Figure 7: Linolenic acid (18:3) divided by linoleic acid (18:2). Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ ***). n=3

Cytoplasmic fatty acid elongation

To see if the cytoplasmic elongation has been affected, we look if there has been a change in the amount of C20- and C22- compared to the C18-fatty acids that they originate from (fig 8). There have been 6 decreases; two BvHb2 lines, three AtHb2 lines, and one Vgb line. One AtHb2 line shows an increase.

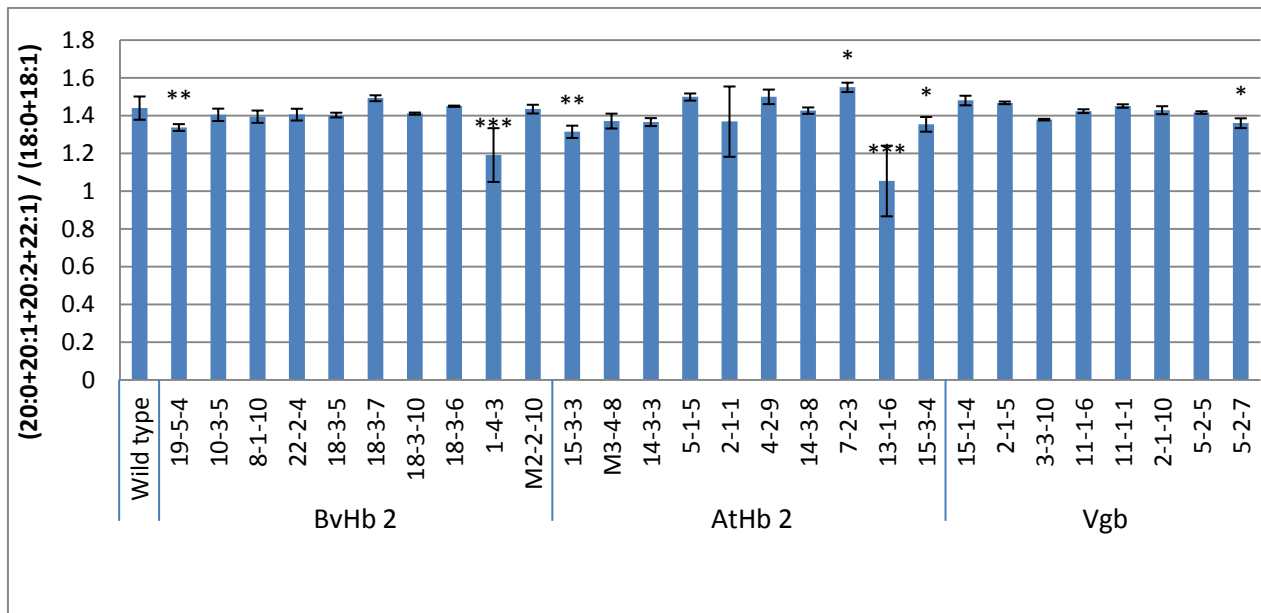


Figure 8: Arachidic (C20:0), gondoic acid (C20:1), eicosadienoic acid (C20:2) and docosenoic acid (C22:1) divided by stearic acid (18:0) and oleic acid (18:1). Asterisks denote statistically significant difference from wildtype at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ ***). n=3

Discussion

Biodiesel is a renewable resource that can help reduce the use of fossil fuels and climate change. The main thing holding biodiesel back is the vast quantities of plant oils needed for its production. Increasing the amount of oil produced could greatly increase the economical viability of biodiesel. One way of increasing the amount of oil produced is to increase the yield from the crops themselves. This research tries to investigate the possibility of using non-symbiotic hemoglobins to increase the oxygen transportation in the developing seeds, improving energy state and oil production. This research uses *Arabidopsis thaliana* as a model organism but the results gathered here should be adoptable to other oil-crops.

Total fatty acids

These results are preliminary and further evaluation of the material is necessary. They are nevertheless indicative for each line and should be used as a guideline for further investigations. Finding an effect that could be contributed to the extra hemoglobin proved very hard. In our study we used three replicates from each of the wild type lines because of the large amount of individual lines and limited time. This is not enough to get accurate results according to a study by Li et al. (20), so future studies should include additional replicates from each line to avoid false positive results from varying growth conditions and plant-to-plant variations. It is also very important to remember that no analysis of the hemoglobin expression in the seeds has been done, so we cannot be certain that the hemoglobin is being expressed.

A few lines show higher seed dry weight, and a few also show a decrease. These changes are relatively small (10-12%) with the exception of 2-1-5 which showed a decrease of 19%. These results are similar to the ones in the study by Vigeolas et al. (5). The lines that show a change in the seed dry weight does not show any correlating changes to their fatty acid profiles.

There was some variation in the total fatty acid content of the seeds but no line with the significant increase that was seen in the study by Vigeolas et al. (5). Some of this might be masked by the high variation between samples from the same line and future investigations must take greater care in the extractions of the fatty acids from the seeds to minimize this.

Another possibility is that the differences in light intensities (160 vs $250 \mu\text{mol m}^{-2} \text{s}^{-1}$) during the plant growth play role in the results. Developing seeds of *Arabidopsis* have photosynthetic activity and light intensities have been shown to affect the development and oil content of the seeds (20).

Desaturation and elongation

The fatty acid profiles have seen some change in the lines but nothing consistent for each of the hemoglobins. The results vary, with some lines showing increases to fatty acid desaturation or elongation while others show a decrease, even lines expressing the same hemoglobin show different results. Most of these changes are relatively small though and could be the result of plant-to-plant variation. Attributing the results to the expression of the new hemoglobins is impossible without an expression analysis at this point.

It is worth noting that differences in light intensities did not change the fatty acid profile of wild type *Arabidopsis* in the study by Li et al. (20). The fraction of polyunsaturated fatty acids did however increase in the study by Vigeolas et al. where the seeds express *Arabidopsis* nsHb2.

Future analysis should also include expression analysis of the new hemoglobin to make sure that it is properly expressed in the seeds. This could also greatly affect the analysis of the results since it would be possible to correlate the expression of the hemoglobin to the changes in fatty acid content and profile.

For future work

From the results we have chosen 7 lines for further investigation. Two from Bv, four from At, one from Vgb.

1-4-3 (BvHb2). This line shows a decrease in both plastidial and cytoplasmic desaturation (fig 5 and 6) but the other parameters remain unchanged.

M2-2-10 (BvHb2). This line has increased seed dry weight, but the fatty acid profile is almost the same as wild type, showing only a small increase in the desaturation from 18:2 to 18:3(fig 7).

15-3-3 (AtHb2). This line shows no change in seed weight but a decrease in the percentage of fatty acids. It also shows a decrease in the desaturation of the fatty acids. These changes are contrary previous published results that use AtHb2 (5).

4-2-9 (AtHb2). This line shows an increased seed dry weight but no changes in the percentage fatty acids. Plastidial elongation and desaturation is unchanged. It shows an increase in the cytoplasmic desaturation from C18:2 to C18:3.

7-2-3 (AtHb2). This line shows reduced seed weight and percentage fatty acids. It has an increased percentage of C16:0 and reduced elongation from C16:0 to C18:0. The plastidial desaturation is however unchanged. There is an increase in the extra-plastidial desaturation, mainly the first step from C18:1 to C18:2, And a small increase in extra-plastidial elongation.

13-1-6 (AtHb2). This line shows no change in dry weight or percentage fatty acids. The plastidial elongation and desaturations are also unchanged. The extra-plastidial elongations and desaturation are both reduced.

5-2-5 (Vgb). This line shows an increase in seed dry weight and high levels of fatty acids. The percentage of fatty acid is not high enough to be statistically significant but very close to be. It has an increased ratio of C18:0 to C18:1 desaturation but no changes in the extraplastidial elongations or desaturations. This line seems to be one of the most promising ones.

Materials and methods

All *Arabidopsis* seeds, wild type and transformed were provided before-hand. Cloning and transformation procedures can be found in (21).

Arabidopsis thaliana ecotype Colombia plants were grown in a climate controlled room. 21°C day and 17°C night, 70% humidity with a photoperiod of 16-h day/ 8-h night at a light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Pooled seeds from different transgenic lines (BvHb2 and AtHb2) were analyzed for oil content with three replicates from each line according to standard oil content analysis protocol routinely used in SLU lab. The oil was analyzed by GC from Agilent with a 7890A GC system (Agilent Technologies).

200 seeds in triplicates from each plant line were used as samples for the GC analysis. Each sample was weighed then put in a glass tube. 1ml 0.15M HAc and 3.75 ml $\text{CHCl}_3/\text{MeOH}(2:1)$ were added before homogenization using a small rod Utraturrax. 1.25 ml CHCl_3 and 1.25 ml H_2O were added and the sample was then vortexed and centrifuged. 200 μl of the lower phase was then dried under nitrogen, 100 μl of hexane and 50 nmol 17:0-Me standard were then added. 2 ml H_2SO_4 was added and the tube was capped and put in a heating block at 95°C, 1h for methylation. 2 ml H_2O and 0.6ml hexane was added and the sample was vortexed before centrifuged. 200 μl of the top phase was taken as a sample for the GC.

A Student's t-test was performed to calculate if the changes in the lines expressing the extra hemoglobins (BvHb2, AtHb2 and Vgb) were of statistical significance. The lines showing a statistical significance levels of $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$ have been labeled with asterisks in the figures.

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