Genetically modified tobacco (*Nicotiana tabacum*) plants for an increased production of wax esters

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Abstract

Wax esters (WE) are naturally occurring lipids consisting of a fatty acid bound to a fatty alcohol with an ester bond. As an alternative to petrochemicals, plant-derived WE:s are of considerable commercial interest as lubricants, but have in recent years also received great attention for their potential as a starting material for bio-fuel production.

It was recently shown that WE:s can be overproduced in stably transformed tobacco plants carrying a gene fusion between two genes encoding a fatty acid reductase (FAR) and wax ester synthase (PES), thus forming a single WE-synthesizing enzyme (Aslan 2015). Chloroplast- directed overexpression of the fusion enzyme led to an 8-fold induction of WE levels (0.15 % by dry weight) in transgenic plants (Aslan *et al.* 2015). However, this work also revealed negative growth effects, likely from high levels of the intermediate metabolite fatty alcohol, possibly inhibiting higher levels of WE production.

The present study was undertaken to investigate the possibility of increasing the WE levels in transgenic plants even further. Through different genetic approaches, a small number of transgenic tobacco lines overexpressing both FAR and PES were demonstrated to have a higher survival rate and a new phenotype compared to previous transformants. One of these lines, resulting from a cross of separately transformed FAR- and PES lines (FARxPES) was shown to have an increased WE level compared to the wild-type. These results will now be a basis for further investigations on WE production in transgenic plants.

Keywords: Wax esters, metabolic engineering, nicotiana tabacum, fatty acid reductase, phytyl ester synthase

Table of contents

Abbreviations

ACCase	acetyl-CoA carboxylase
ACP	acyl carrying protein
CoA	coenzym A
ECR	Enoyl-CoA
FA	Fatty acid
FAE	Fatty acid elongation
FAR	Fatty acyl-CoA reductase
HCD	β-hydroxyacyl-CoA dehydratase
KAS I, II, III	β-ketoacyl-ACP
KCR	β-ketoacyl-CoA reductase
KCS	β-ketoacyl-CoA synthase
MS	Murashige-Skoog
MT	Metric tonnes
PES1 & 2	Phytyl ester synthase 1 and 2
SAD	Stearyoyl-ACP desaturase
TAG	triacylglycerol
VIGS	Virus induced gene silencing
VLCFA	Very long chain fatty acid
WE	Wax ester
WS	Wax synthase
WT	Wild type

1. Introduction

The need for sustainable and renewable sources of material and chemical compounds for industrial purposes has never been greater. The amount of fossil oil available to humans is ever decreasing and the green-house gas emissions that they create are a major concern for our global climate. Plant-derived oils, waxes and fatty acids (FAs) have a function and structure that are similar to that of fossil oil, which gives them the potential to replace part of the fossil oil used in industries today. By genetically modifying plants it is possible to produce fatty acids and biochemical compounds with very specific compositions depending on what the end-purpose is. Vegetable oils will first and foremost be used for human consumption, so in order to have even remotely enough vegetable oil for industrial use, the production would have to increase to about two times the amount of what is currently produced. With an increasing population who demands certain comforts in life, this amount will most likely be even higher in the future. Despite the enormous challenge of replacing large enough quantities of petrochemicals, the demand for renewable industrial feedstocks could partly be met by converting crops with a high biomass production into accumulating oils and waxes in their biomass (Dyer *et al.* 2008; Vanhercke *et al.* 2013; Carlsson *et al.* 2011).

There are four main oil crops that produce about 79% of all the vegetable oil in the world (Dyer *et al.* 2008). This small variety of crops create a limitation for how often a crop can be produced on a certain piece of land and also where they can be produced geographically. Genetic and metabolic engineering can help to increase the yield and the quality of these crops as to produce more from the same amount of land, but it can also introduce new crops to the market that normally would not be considered oil crops. Having a broader variety of oil crops to choose from would open up new opportunities for increasing the oil production globally (Aslan 2015).

Commercial tobacco (*Nicotiana tabacum*) is a close relative to *Nicotiana benthamiana,* which is commonly used as a model plant within molecular and plant biology and they are both known for producing large amounts of biomass (Aslan 2015). As an example, 6.1 million metric tonnes of commercial tobacco was produced in 2018 to make cigarettes with (*Global tobacco production 2018*). In tobacco leaves you can find between 2.1%-4.4% oil by dry weight (Koiwai *et al.*

1983) even without genetic modification, so there is potential to theoretically produce a large amount of oil in tobacco.

Vegetable oils are nutritionally important to humans and animals and are often used in the food industry to make margarine, frying oils and salad oils. The molecules that make up vegetable oils are rich in energy and are composed of triacylglycerols (TAGs). Because of the high density of energy in TAGs they are very useful for making biodiesel, where the FAs of the TAGs are transesterified, and also in other industrial formulations that are bio-based (Lu *et al.* 2011).

1.1. Plant lipids

Plants have lipids in the form of fatty acids, fats, waxes, mono/di/triacylglycerols, sterols, sterol-containing metabolites, fat-soluble vitamins and phospholipids. They are essential to the plants and mainly acts as an energy storage where TAGs are very common, or as structural parts of biological membranes where phospholipids and sterols are more common (Aslan 2015). Despite the diversity of lipids, this article will focus on wax esters and fatty acids as those are the molecules of interest here.

1.1.1. Replacing petrochemicals

A big part of the industrial feedstock is currently based on non-renewable resources derived from petroleum, a source that is finite and that contributes to green-house gas emissions and thus global warming. Plant oils provide a renewable option to replace part of the fossil raw materials used in industries. There has been a clear focus on making biodiesel from plant oils, but unfortunately it is not possible to replace the enormous amount of fossil fuel that humans currently use, with biodiesel (Vanhercke *et al.* 2013; Biermann *et al.* 2011). There is, on the other hand, a possibility to replace up to 40% of the petrochemicals used in industries. The petrochemicals are molecules based on carbon, which is also the case when it comes to plant-based chemicals derived mainly from plant oils and the lipids that they consist of. In order to have enough vegetable oil to replace the 40% of petrochemicals and to still have a sufficient amount of food and feed, the global oil crop production would have to increase to more than 400 MT per year (Carlsson *et al.* 2011). This means a doubling of the current production, which in 2018 was 204 MT (*World vegetable oil production 2020*).

To be able to double the global plant oil production it will take all the novel plant biotechnology available today, and it would be used to first raise the level of purity in the plant oils so that single fatty acids can be extracted, secondly to have plants producing less common fatty acids with a specific end-purpose, and thirdly to make sure that the crops that we already have can increase their oil content and to

introduce new oil crops that can accumulate oil in their biomass (Carlsson *et al.* 2011). By understanding how the plants allocate carbon for starch, there is a possibility of changing that mechanism into allocating carbon for fatty acids instead (Nishida 2004). Metabolic engineering will most likely be the frontrunner when it comes to achieving these changes (Vanhercke *et al.* 2013).

1.1.2. Industrial applications

Most vegetable food oils commonly have these five fatty acids: palmitic (C16:0), stearic (C18:0), oleic ($C18:1^{\Delta 9}$), linoleic ($C18:2^{\Delta 9, 12}$) and α-linolenic acid ($C18:3^{\Delta 9, 12, 15}$). Depending on the length of the FA and how saturated/unsaturated it is, the fysical and chemical properties differ. Around 20% of the plant food oils produced today are already being used for non-food applications (Vanhercke *et al.* 2013; Carlsson *et al.* 2011). Laurate acid (C12:0) is a medium chain fatty acid that is found in palm kernel oil and coconut oil and it is one of the main FAs used for producing soap, detergents and similar products for personal care as it has excellent surfactant properties (Vanhercke *et al.* 2013). From soybean and linseed oil highly unsaturated FAs can be extracted and used in inks and surface coatings as drying agents, or be further processed into epoxygenated oils for making industrial glues and resins. All of the vegetable oils mentioned here are multipurpose, but there are some less common oils such as tung oil, castor oils and high-erucic rapeseed oil that can be used for more specific purposes because of their unusual FAs (Biermann *et al.* 2011; Vanhercke *et al.* 2013).

Genes for metabolism and synthesis of FAs with epoxy and hydroxyl residues have been identified and these molecules are well suited for production of plasticizers, lubricants and nylon precursors. This opens up the possibility to give oil crops a new function (Lu *et al.* 2011). Producing specialty FAs in in highly productive crops instead of their original source could effectively lower the cost of these components (Vanhercke *et al.* 2013).

Part from FAs there is also a great surge for plant-derived waxes, such as wax esters (WE), in different industries. Typical characteristics of wax esters are anti-rust, -foam and -wear and it reduces friction in lubricants (Dyer *et al.* 2008).

1.1.3. Wax esters

Wax esters (WE) are a combination of a long-chain fatty acids (C14-C36) and a long-chain fatty alcohol (C16-C30) linked with an ester bond, and can be found in a lot of plants as a surface lipid in the leaves, where it protects the plant from infections, dehydration, insects and UV-light (Jetter & Kunst 2008) This protection comes from their ability to resist hydrolytic degradation, their hydrophobicity and their solid state. The same abilities is what makes WE interesting for industrial use (Vanhercke *et al.* 2013).

There was a time when WE was extracted from the spermaceti organs of sperm whales as their specific wax esters had excellent properties for making cosmetics, lubricants and medical products. The unsustainable hunt for sperm whales has been banned worldwide and instead the industry has been looking at jojoba (*Simmondsia chinensis*) plants in order to source wax esters. In jojoba seeds, as much as 60% of the dry weight can be WE and the molecule form a straight chain unlike the typical WE that is usually folded. (Aslan *et al.* 2015b; Vanhercke *et al.* 2013). Unfortunately, jojoba is a desert shrub with a low yield that makes it expensive to produce for anything but luxury products, instead there is the possibility of genetically modifying a more suitable plant into producing jojoba wax esters (Aslan *et al.* 2015b).

1.2. Biosynthesis of fatty acids, fatty alcohols and wax esters in plants

1.2.1. Fatty acid biosynthesis

The biosynthesis of fatty acids (FAs) takes place in every cell in plants, primarily in the plastids. It is a primary metabolic pathway as it is essential for growth, so if the fatty acid biosynthesis is inhibited the cell will die. In plants the FAs most commonly have 16 or 18 carbons structured in chains, and with up to three double bonds in *cis* formation. In structural glycerolipids (found in most plant membranes), more than 90% of the acyl chains are made up of only five FAs (16:0, 16:3, 18:1, 18:2 and 18:3) (Ohlrogge & Browse 1995; Nishida 2004; Maeo *et al.* 2009).

It is unusual to find FAs in their free form as they are nearly always esterified on their carboxyl group. When two FAs are esterified to glycerol the result is a glycerolipid which is most often used in the bilayers of membranes. If a third FA is esterified to the glycerol backbone a triacylglycerol (TAG) is created, which works as energy storage material in seeds. Fatty acids are also found in cuticular lipids such as wax esters (where FAs are esterified to fatty alcohols), cutin (cross-linked hydroxy fatty acids) or they end up being reduced to alcohols and aldehydes, which is a part of the complex cuticular lipid matrix (Ohlrogge & Browse 1995).

There are three main substrates required for FA synthesis, namely acetyl-coenzyme A (CoA), ATP and NAD(P)H. Depending on the origin of the plastid there are several different pathways that supply these substrates. The cytosolic glycolysis is said to provide pyruvate and phosphoenolpyruvate that are converted into acetyl-CoA in the plastids, and in some plant species malate can also be converted into acetyl-CoA via pyruvate (Nishida 2004). To elongate the FA chain, carbon is taken from malonyl-CoA which in turn is synthesized from acetyl-CoA. The transformation from acetyl-Coa to malonyl-CoA requires ATP and is

performed by the enzyme acetyl-CoA carboxylase (ACCase), thus ACCase is an important regulatory enzyme when it comes to synthesizing fatty acids. Fatty acid synthase II (FASII) is another combination of enzymes in the plastids. FASII uses acyl-carrier proteins (ACP) to catalyse the reactions needed for chain extension of FAs. The length of the FA chain is determined by three condensing enzymes called β-ketoacyl-ACP (KASI, II and III) (Nishida 2004; Aslan 2015; Ohlrogge & Browse 1995; Ohlrogge & Jaworski 1997). KASIII catalyses the first reaction where acetyl-CoA is condensed with malonyl-ACP to a C4:0-ACP product, acyl-ACP. The next step is catalysed by KASI which condensates the chain from C4:0-ACP all the way up to C16:0-ACP. KASII catalyses the last elongation of the FA from C16:0-ACP to C18:0-ACP. A double bond is added by Stearoyl-ACP desaturase (SAD) to position 9 before the FA chain is finished off by removing, or hydrolysing, the ACP from the acyl chain (Aslan 2015).

Fatty Acid Elongation (FAE) system

Very long chain fatty acids (VLCFAs) can have up to 34 carbons and are the first precursors when biosynthesizing wax. The VLCFAs are C18:0 fatty acids that have been extended with blocks of two carbon acetyl-CoA in several cycles. There are four enzymatic reactions involved in the FAE system that occur in consecutive order by β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase (KCR), β-hydroxyacyl-CoA dehydratase (HCD) and enoyl-CoA reductase (ECR) as they have certain chain lengths that they specialize on (Wang *et al.* 2015; Samuels *et al.* 2008). When the VLCFAs are done they can enter either the alcohol-forming pathway or the alkane-forming pathway, in which various cuticular waxes will be the result (Aslan 2015; Wang *et al.* 2015).

1.2.2. Wax ester biosynthesis

Alcohol-forming pathway

The biosynthesis of wax esters has been well studied and, compared to other lipid classes, is a fairly simple process (Vanhercke *et al.* 2013; Lardizabal *et al.* 2000). In the formation of wax esters a long-chain fatty alcohol is esterified to a long-chain fatty acid with the action of a fatty acid reductase (FAR) and a wax synthase (WS), two enzymes that have been described in many different organisms. When it comes to plant derived wax esters, jojoba (Simmonsia chinensis) has been the frontrunner when it comes to providing genetic information about FAR and WS because of their high level of wax esters in their seeds. As much as 60% of the seeds dry weight can be wax esters (Lardizabal *et al.* 2000; Metz *et al.* 2000; Aslan *et al.* 2015b).

De novo fatty acid synthesis is the first step in wax ester biosynthesis as both FAR and WS require activated fatty acids, this is also the case in other lipid biosynthetic pathways (Miklaszewska *et al.* 2018; Aslan *et al.* 2015a). The next

step involves a reduction of an activated fatty acid to a fatty alcohol by the FAR enzyme. Depending on the origin of the FAR enzyme, different chain lengths of the fatty acid might be preferred, something that is provided by the FAE system with VLCFAs or directly from *de novo* fatty acid biosynthesis with C16 and/or C18 (Lardizabal *et al.* 2000; Aslan *et al.* 2015a; Miklaszewska *et al.* 2018). In the final reaction, the fatty alcohol is esterified to a fatty acid by a WS enzyme with a wax ester as the result, and again the exact composition can vary depending on what acyl-ACP substrates that are available and also depending on the specific FAR and WS enzymes. The last step can in plants also be carried out by a phytyl ester synthase (PES) which is localized in the chloroplasts (Vanhercke *et al.* 2013; Aslan *et al.* 2015a; Aslan *et al.* 2014).

Wax esters have successfully been produced in transformed tobacco (*Nicotiana benthamiana)* plants through metabolic engineering of leaf chloroplasts. In order to produce wax esters in the leaf chloroplasts of *N. benthamiana*, a couple of different enzymes were used to transform the metabolic pathway in the chloroplasts, but it was a combination of a FAR gene from *Marinobacter aquaeolei* (MaFAR) and a PES gene from *Arabidopsis thaliana* (AtPES2) that produced the highest amount of wax esters (Aslan *et al.* 2014). Transformed tobacco plants with a fusion-gene consisting of both FAR and PES showed an eight-fold increase of wax esters compared to wild type plants of tobacco, this is equal to 0.15% of wax esters in the dry weight biomass (Aslan *et al.* 2015b).

1.3. Tobacco as a model crop for increasing energy density

The *Nicotiana* genus is part of the *Solanaceae* family, the same family as for example potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) (Aslan 2015). The leaves of tobacco naturally contain 2.1%-4.4% oil by dry weight, with metabolic engineering this could be increased to an amount of commercial interest (Koiwai *et al.* 1983; Aslan 2015, Aslan *et al.* 2015b)

Nicotiana benthamiana

One of the most extensively used plants for biotechnology and research is Nicotiana benthamiana, which is a type of tobacco. It has become famous because of its susceptibility to a diverse range of plant viruses and other pathogens such as bacteria and fungi, something that makes it the perfect candidate for pathogen-host research. A large part of *N. benthamiana'*s genome has been described, another aspect that makes it good for genetic modification and research concerning protein localization, -interaction, -expression and -purification, often through the use of

virus-induced gene silencing (VIGS) because of how prone it is to viral infections (Goodin *et al.* 2008; Bombarely *et al.* 2012).

Nicotiana tabacum

Commercial tobacco (*Nicotiana tabacum*) is a close relative to *N. benthamiana* and is most commonly used for making cigarettes, but it is also a model plant with a lot genetic information available for further research (Bindler *et al.* 2011; Ding *et al.* 2016; Aslan 2015). This tobacco species has an exceptionally high production of biomass, as much as 70 metric tonnes fresh weight per hectare per year, and that together with the genetic information makes it interesting for metabolic engineering with the purpose of creating a high energy crop that can be used for biofuel or chemical feedstocks (Sheen 1983; Vanhercke *et al.* 2013; Aslan 2015).

2. Aims of the study

This thesis is part of an on-going project that started in 2014 with the work by Aslan *et al.* (2015, and references therein). Folke Sitbon[1], co-writer on these articles, have continued this work by producing crosses between the most interesting lines of plants and also added the biosynthetic genes to *Nicotiana tabacum* in order to try a more robust host for the wax ester production.

In the previous studies it was shown that wax esters (WEs) can be overproduced in tobacco plants that were stably transformed with a single gene fusion encoding both a fatty acid reductase (FAR) and wax ester synthase (PES, also known as phytyl ester synthase). Fused together the encoded proteins form a single WE-synthesizing enzyme that is active in the chloroplasts. The overexpression of the enzyme-fusion led to an 8-fold increase of WE levels (0.15% by dry weight) in transgenic plants, but it was also in some cases associated with stunted growth and low survival. The growth problem was most likely due to a high activity of FAR in relation to the PES enzyme, thus creating high levels of the intermediate metabolite, fatty alcohol, with cell damage as a likely consequence (Aslan et al 2015).

The objective for this study was to identify plants with an adequate balance between the formation of fatty alcohols by FAR and the esterification of fatty alcohols by PES to form wax esters. To do so, plants were generated from seeds of previously transformed *Nicotiana benthamiana* and *N. tabacum* in order to later make a selection of interesting phenotypes:

- Third generation *N. benthamiana* plants were generated to confirm the presence of WE:s according to previous studies where FAR and PES have been fused as a single protein
- Plants of offspring from crosses between single transformants of FAR and PES were generated, as the enzymes are assumed to function more efficiently when expressed separately than when fused. The same type of crosses was generated with *N. tabacum*. Without analysing the genetic information in the crosses it is not possible to know for certain that the plants have both of the enzymes, as the genes for the enzymes carried the same marker (resistance towards the antibiotic kanamycin). It is however

[1] Folke Sitbon, Professor in Plant Biology, SLU, May 2020

possible to select plants that most likely will have both of the genes as plants with only FAR have a high lethality, plants with only the PES-gene tend to resemble the wild type plant and plants with both of the genes are likely to be different to some extent, e.g. to have a generally smaller phenotype compared to the wild type.

- *N. tabacum* plants with a two-gene construct (one gene encoding for FAR and one gene encoding for PES on a single stretch of T-DNA) were generated as a different way of balancing the activity of FAR and PES. However, having both genes on the same stretch of DNA makes the sequence very long and possibly unstable, something that might affect the success of the transformation.
- Sequentially transformed *N. tabacum* plants were generated as a third way of creating a better balance between FAR and PES. Kanamycin resistant PES-transformants were sequentially transformed with a FAR-gene with a hygomycin resistance added as a marker. Using both kanamycin and hygomycin in the medium used for germinating the seeds ensure that both PES and FAR are present in the plants. The activity of each enzyme in this type of transformation is unknown.

In order to identify plants with a higher amount of WE:s, three different analyses are relevant to do:

- Visual screening where the phenotypes are compared to those seen in previous studies.
- PCR analysis for checking presence of transformed genes and their expressions.
- WE levels analysed from tissue samples.

The time for this Independent Project only allowed visual screening and collection of tissue samples for further analyses.

3. Materials and methods

3.1. Plant materials

Seeds from previously transformed *Nicotiana benthamiana* (Table 1) and *N. Tabacum* cv W38 (Table 2) were sterilized and then germinated in Petri dishes on Murashige-Skoog (MS) medium (Duchefa, RV, Haarlem, The Netherlands) with the addition of 3% sucrose as a supplement, 0.3% of Gel-rite (Sigma AB, Malmö, Sweden) to solidify the medium and kanamycin and/or hygromycin to select against non-transformants. The Petri dishes were kept in growth rooms for two weeks with a 16 h photoperiod under fluorescent lamps providing 80 μmol photons m^{-2} s^{-1} and a temperature of 22 °C during the day and 17 °C at night. Antibiotic-resistant seedlings from different transgenic lines were transferred to soil in pots and placed in a green house where they received sterilised water with 1% N.P.K. on a regular basis from an automated system in the greenhouse.

All transformants with a two-gene construct, fusion-gene transformants and single transformants have been generated with a gene to tolerate the antibiotic kanamycin in order to efficiently select only transformants on growth medium. For sequentially transformed plants an additional tolerance for hygromycin has been added to make sure that both genes are present.

The third generation (T3) of fusion-gene transformants, see Table 1, have previously been shown to contain 0.15% of wax esters calculated from dry weight of the plant, this corresponds to nearly an eight-fold increase compared to the levels found in the wild type plants (Aslan *et al.* 2015b).

The *N. benthamiana* lines and crosses shown in Table 1 were made with transformants that only had a mild expression of the genes. Plants with a higher expression of the genes, especially FAR, tended to die and so it was only possible to do crosses with transformants that have a mild expression of the genes. Crosses in Table 2 were made with *N. tabacum* W38 plants with a stronger FAR phenotype, in order to allow a screen for phenotype rescue after a PES cross.

Table 1. Lines of different seed materials used from transformed Nicotiana benthamiana. Fusion-gene transformants were plants transformed with a fusion-gene where 35SMaFAR and 35SAtPES2 have been fused to a single protein having both enzyme activities produced. Crosses represent offspring from crosses made between single transformant MaFAR #5 and single transformant AtPES2 #2. Single transformants have either MaFAR or AtPES2. T2 is the second generation and T3 is the third generation.

Fusion-gene transformants	**Crosses, T3**	**Single transformants, T2**
2.10.1, T3	1:1	FAR3 (F3)
2.10.2, T3	1:2	FAR5 (F5)
6.1.1, T3	1:3	PES2
6.1.2, T3	1:4	PES3
2.10, T2	1:5	
6.1, T2	2:1	
10.2, T2	2:2	
	2:3	

Table2. Lines of different seed materials used from transformed Nicotiana tabacum cv W38. Two-gene transformants were transformed with a two-gene contruct containing the gene for both MaFAR and AtPES2. Sequential transformants are PES-transformants #5 that have been transformed with a FAR gene construct. Crosses are from crosses made between single transformant MaFAR and single transformant AtPES2. Single transformants have either MaFAR or AtPES2.

Two-gene transformants	**Sequential transformants**	**Crosses**	**Single transformants**
Double 1	FAR/PES5 #1	F3xP10R	FAR3 (F3)
Double 2	FAR/PES5 #3	F3xP1b	FAR8 (F8)
Double 3	FAR/PES5 #4	F3xP11	
Double 4	FAR/PES5 #5	F3xG19	
Double 5	FAR/PES5 #6	F3xP14	
Double 7	FAR/PES5 #7	F8xP10R	
	FAR/PES5 #8	F8xP1b	
		F8xP11	
		F8xG19	
		F8xP14	

3.2. Phenotype analyses

Different phenotypes were scored and counted from each genotype in the Petri dishes at two and three weeks after sowing the seeds. The result was then statistically analysed to confirm any differences between genotypes.

Tissue samples were taken when the plants were 8 weeks old. The samples were frozen in liquid nitrogen and saved for later analysis of wax esters and free fatty alcohols. However, the result of this sampling will not be published in this project as the results will come long after it is finished.

Phenotypes of 8-12 weeks old plants were compared to phenotypes from Aslan (2015) and they were also checked for certain characteristics mentioned by Folke Sitbon[2] who have been involved in all projects concerning this subject.

[2] Folke Sitbon, Professor in Plant Biology, SLU, May 2020

4. Results and discussion

4.1. Plants transformed with a FAR:PES fusion-gene

Most of the *N. benthamiana* plants that had been transformed with a FAR:PES fusion-gene showed very little of the characteristics that could be expected from plants with a high production of wax esters. Compared to the phenotypes seen in Aslan (2015), the growth here was not as stunted and the leaves were not chlorotic, suggesting that the production of wax esters was low. This will need to be confirmed by the test results from the tissue sampling that was done 8 weeks from sowing. Generation T3 and T2 of *N. benthamiana* have previously been grown to the flowering stage and both generations have been shown to contain higher levels of wax esters (Aslan *et al.*, 2015b).

Some of the *N. benthamiana* plants died, see Figure 2, from unknown reasons at the same period of time and again the tissue sampling might give an explanation as to why. Plants with a high FAR activity tend to suffer from the high concentrations of free fatty alcohols that are produced, see Figure 3.

Figure 1. Second and third generation of Nicotiana benthamiana transformed with a fusion gene compared with the wild type plant on the far right. In order from left to right: 6.1.1, 6.1.2, 2.10.1, 2.10.2, 10.2, 6.1, Wild type.

Figure 2. Third generation of Nicotiana benthamiana transformed with a fusion gene compared with the wild type plant on the far right. In order from left to right: 6.1.1, 6.1.2, 2.10.1, 2.10.2, Wild type.

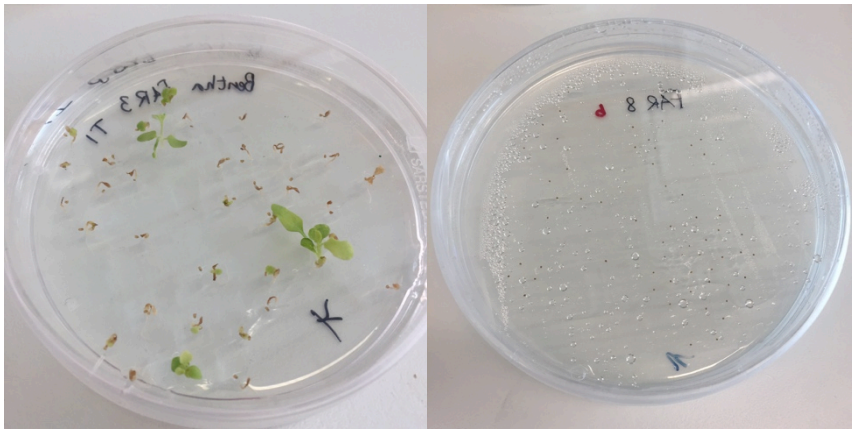


Figure 3. Single transformants FAR3 and FAR8 Nicotiana benthamiana three weeks from sowing. Both lines show that a higher activity of only FAR is lethal for the plant in most cases, the seed either germinates and dies shortly after or does not germinate at all.

4.2. Plants transformed with a two-gene construct

None of the *N. tabacum* transformed with a two-gene construct, containing both 35S:FAR and 35S:PES, showed any of the characteristics that could be expected from plants with a high production of wax esters. All of the *N. tabacum* transformed with this two-gene construct looked like the wild type through out the whole growth period, see Figure 4, which suggest that the expression of the two enzymes is very low.

Figure 4. Wild type N. Tabacum to the left and a typical N. Tabacum with a two-gene construct to the right. No visual difference can be seen which suggest low FAR and PES activity and thus a low content of WE:s.

4.3. Sequentially transformed *N. tabacum*

For the sequential transformation, a single 35S:PES.kana transformant was transformed a second time with a 35S:FAR.hyg gene construct. Out of the seven kanamycin+hygromycin-resistant lines obtained of this genotype, only one plant (FAR/PES5 #5) showed signs of excess amounts of free fatty alcohol, giving the plant a characteristic dark surface on the veins of older leaves (Figure 5). This was only visible once the plant was 6 weeks and older, which makes it difficult to tell when selecting phenotypes from plants only 2 weeks old for further development. A much larger amount of transformants would need to be raised and saved in order to find the ones of greater potential.

Figure 5. A high FAR activity often shows as dark nerves on older leaves, here shown on the sequential transformant FAR/PES5 #5.

4.4. Crosses between FAR and PES transformants

N. tabacum

Two FAR lines (#3 and #8) in *N. tabacum* W38 that showed a clear FAR-related phenotype were crossed with 5 phenotypically normal wild-type-like PES lines. When scored for survival as seedlings, there were two crosses that showed a significantly increased viability; in both cases with the P10R line. Thus, when the seeds of the F3xP10R and F8xP10R crosses germinated, there are very few that die from a high FAR activity compared to other crosses with F3 and F8. In most of the analysed crosses (8 out of 10), 35-40% die shortly after germinating because of the FAR activity, but in F3xP10R and F8xP10R only 2-9% die for the same reason, see Figure 6 and Table 3. The plants of F3xP10R and F8xP10R also have a distinguished phenotype appearing that none of the other crosses have, where they stay mostly small, have a much darker green colour and slightly droopy leaves, see Figure 7 and 8. There is a statistical difference (see Table 4) between the outcome of these two crosses and the rest of the crosses, that is most likely explained by the PES enzyme having a high enough activity to balance the activity and the FAR enzyme, thus creating less intermediate metabolites, fatty alcohol, that can be lethal to most of the plants. The PES-gene provided by P10R in particular seems to balance the production of fatty alcohol by FAR and the esterification of the fatty alcohol with fatty acids. If this is the case then the tissue sampling done when the plants were 8 weeks old should show a higher concentration of wax esters compared to the other crosses.

N. benthamiana

The *N. benthamiana* FARxPES crosses showed a stunted growth in general (see Figure 9 and 10), which is in line with the results from Aslan et al. (2015b). Some of the plants also showed signs of droopy leaves and a reduced level of chlorophyll as seen in Figure 10. None of the lines stood out from the rest but compared to the lines with a fusion-gene the crosses still show potential for a higher level of WEs, the results will need to be confirmed by results from the tissue sampling.

Indeed, an initial analysis showed a leaf WE level of 0,43 ± 0.04 µmol g^{-1} (FW) in one of these crosses (FAR5xPES2). This is ca 60% higher than the highest level in leaves (0,27 ± 0.01) µmol g-1 reported by Aslan et al. (2015) in fusion-gene transformants. This indicates that FARxPES crosses may be a successful way to increase WE levels in transgenic plants.

Table 3. Screened phenotypes of crosses and single transformants. Large green resembles wild type, small green shows stunted growth, green & brown shows some FAR activity, brown dead plants are due to a high FAR activity and white dead are due to a sensitivity to the selective medium.

Phenotype	Not germinated	Large green	Small green	Green & Brown	Brown, dead	White, dead	Unclear	Sum
Genotype								
F8	99%		1%					100%
F3	3%	36%	7%	7%	28%	19%		100%
F3xP10R a	4%	56%	18%	12%	4%	5%		100%
F3xP1b		28%	7%	14%	37%	15%		100%
F8xP10R c	5%	58%	12%	12%	2%	12%		100%
F8xP1b	2%	39%	3%	3%	35%	18%		100%
F3xP14		38%	4%	1%	38%	18%		100%
F3xP10R b		57%		25%	9%	9%		100%
FAR3		34%	3%	2%	45%	16%		100%
F3xP1		30%	1%	2%	43%	24%		100%
F3xG19	17%	26%	2%	2%	32%	20%		100%
F3xP11		39%	2%	6%	30%	24%		100%
F8xP10R a		39%	15%	28%	6%	11%	1%	100%
F8xP10R b		35%	6%	41%	8%	10%		100%
F8xP11 a		29%	4%	13%	32%	21%		100%
F8xG19	100%							100%
FAR8 a	100%							100%
F8xP14	1%	35%			44%	21%		100%
F8xP1		41%	1%		37%	22%		100%
FAR8 b	100%							100%
PES2 G19		77%				23%		100%
W38 NK k			100%					100%
FAR7				1%	98%	1%		100%
W38 NK kh	2%		98%					100%
F8xP11 b		51%			41%	7%		100%
W38 WT blank		100%						100%
FAR2		71%	6%	14%	6%	4%		100%

Table 4. Chi-square analysis of the combined phenotypes of FARxPES crosses after scoring at the seedling stage presented in Table 3. The table shows that F3xP10R and F8xP10R are similar to each other with respect to survival, but significantly different from the other genotypes.

	F3xP10R	F8xP10R	F3xP1b	F8xP1b	F3xP11	F8xP11	F3	F8
F3xP10R	x	0.430	0.000	0.000	0.000	0.000	0.000	0.000
F8xP10R		x	0.000	0.000	0.000	0.000	0.000	0.000
F3xP1b			x	0.001	0.002	0.737	0.409	0.000
F8xP1b				x	0.198	0.010	0.193	0.000
F3xP11					x	0.158	0.195	0.000
F8xP11						x	0.590	0.000
F3							x	0.000

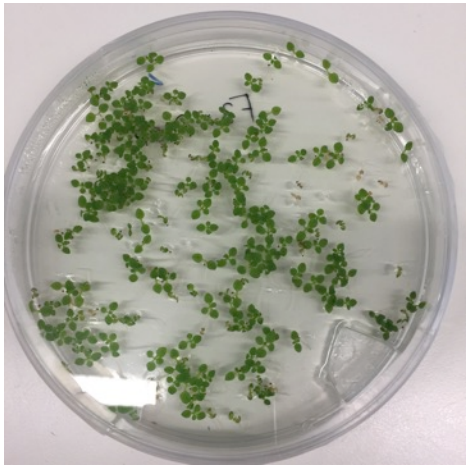
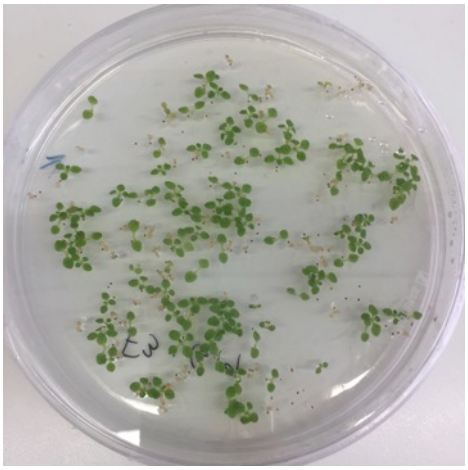
a)  b) 

Figure 6. A typical appearance of a) F3xP10R and F8xP10R with very few dead plants, and b) any other FAR3xPES or FAR8xPES cross, which show a high frequency of dead plants.

Figure 7. All phenotypes of F3xP10R (4 plants to the left) and F8xP10R (4 plants to the right), where the outer edges are close in size to the wild type tobacco.

Figure 8. A typical phenotype of F3xP10R and F8xP10R with a dark green color, small size and droopy leaves.

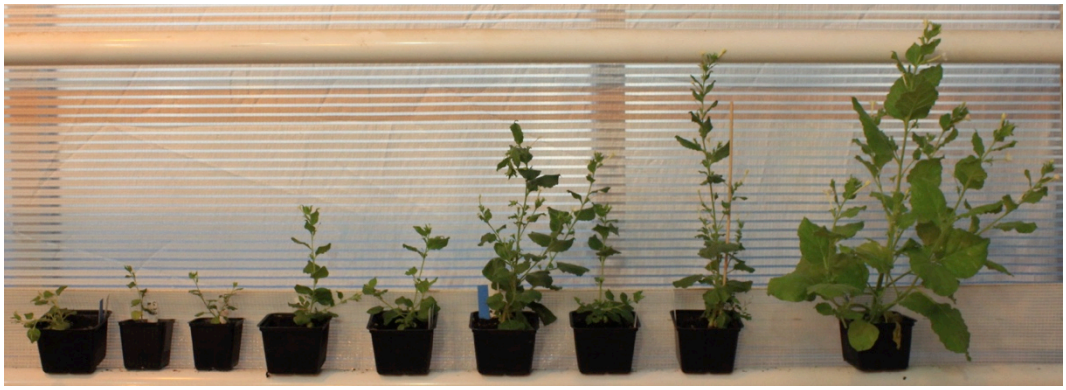


Figure 9. Nicotiana benthamiana crosses with a stunted growth compared with the wild type on the right. In order from left to right: 2:1, 2:2, 2:3, 1:1, 1:2, 1:3, 1:4, 1:5, WT.

Figure 10. Nicotiana benthamiana crosses with a stunted growth and droopy leaves. In order from left to right: 2:1, 2:1, 1:1, 1:1, 1:2, 1:3, 1:4, WT.

5. Conclusions

- Out of the four different approaches, the crosses derived from single FAR and PES transformants showed the greatest potential for complementation of the FAR phenotype, i.e. production of wax esters. After visual screening one phenotype in particular stood out for the crosses F3xP10R and F8xP10R of *N. tabacum*, with a stunted growth and high concentration of chlorophyll making them dark green. The PES enzyme provided by line P10R in combination with the single FAR transformants F3 and F8 resulted in increased survival as well as the striking phenotype, possibly being the signs of a balanced FAR and PES activity which produces wax esters.
- Preliminary analyses of WE levels in *N. benthamiana* crosses show an increase compared to previous studies done with fusion genes, indicating that this might be a viable way of increasing WE levels in transgenic plants.
- The sequentially transformed *N. tabacum* has potential for future research as one of the lines (FAR/PES5 #5) had signs of a higher FAR activity. However, a much larger amount of plants must be raised and saved in order to find the ones of interest, as the leaf phenotype possibly caused by FAR activity only showed after 6 weeks from sowing.
- The plants with either a fusion-gene or a two gene construct showed no signs of a higher wax ester production.

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