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Evaluation of two transformation protocols for *Crambe hispanica* subsp. *abyssinica*.

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Evaluation of two transformation protocols for *Crambe hispanica* subsp. *abyssinica*.

Evaluering av två transformations protokoll för *Crambe hispanica* subsp. *abyssinica*.

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Abstract

Background Crambe is an oil-crop suitable for industrial purposes and breeding aiming at producing novel oil-qualities may be achieved by gene-transformation. Understanding the synthesis of fatty acids and an efficient transformation protocol is of pivotal role in this context.

Two transformation protocols (*Agrobacterium* mediated according Bang-Quang Huang and floral dip according Clough and Bent 1998) for crambe are evaluated, and a RNAi construct is produced aiming to down-regulate CaDGAT1.

Results None of the evaluated transformation protocols produce any successfully transformed plants during this evaluation. CaDGAT1 is partially sequenced and a RNAi construct for CaDGAT is produced, while the functionality of the construct is un-validated.

Conclusions Alternative *Agrobacterium* mediated transformation protocols published by Li et al. 2010 have managed to transform crambe successfully but a functional floral dip protocol may be even more cost effective. more studies on floral dip must be carried out to evaluate this however.

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Contents

Abbreviations

ACP Acyl carrier protein

CaACET *Crepis alpina* Δ^{12} acetylenase

CaDGAT1 crambe DGAT1

DGAT Diacylglycerol diacyltransferase

dsRNA Double-stranded RNA

ER Endoplasmic reticulum

FA Fatty acid

FAD fatty acyl desaturases

FAE fatty acid elongase

FAR Fatty acyl-CoA reductase

Fat Acyl-ACP Thioesterase

GPAT Glyserol-3-phosphate acyltransferase

KAS 3-ketoacyl-ACP synthase

LB Lysogeny broth

LPAAT *lyso*-phosphatidic acyltransferase

miRNA micro RNA

miRNP miRNA-containing effector complex

MLCFA Medium long-chained Fatty acid

MS salt Murashige & Skoog salt mixture

PCR Polymerase chain reaction

PDAT Phospholipid:diacylglycerol acyltransferase

RISC RNAinduced silencing complexes

RNAi RNA interference

siRNA Short interfering RNA

TAE Tris-acetate-EDTA

TAG Triacylglycerol

T-DNA Transfer DNA

VLCFA Very-long-chain fatty acid

WS Wax synthase, fatty acyl-CoA:fatty alcohol acyltransferase

1 Introduction

The need for renewable resources in an oil based economy will most probably become of increasing importance. Crambe may provide unique oil-qualities which currently are derived from mineral oil. The distinct morphological characteristic of the crambe compared to other Brassicaeae taxa used for food production in modern agriculture can be used to prevent mixture between seed lots intended for industrial use and food production.

1.1 Botany

Crambe hispanica subsp. *abyssinica* (Hochst. ex R.E. Fr.) Prina (hereinafter abbreviated crambe) belonging to the Brassicaeae family is a branched annual herb with erect growth habit reaching 1.5-2 meters in height (Prina, 2009). The lower leaves are petiolate and pinnatley lobed, while upper leaves are shortly petiolate or sessile. The terminal lobe of the lower leaves is ovate or kidney shaped and much larger than the lateral lobes. The upper leaves are ovate or rhombic. The margin of the leaves is irregularly toothed. The inflorescence is arranged as a raceme, often branched, containing 20-50 small flowers. The flowers are bisexual and tetrameric with 6 stamens and a superior ovary. The petals are white and 2.5-6 mm long (van der Vossen and Mkamilo, 2007). The fruit of crambe is a two-segmented silique where the cylindrical underdeveloped proximal joint is sterile and the distal joint is spherical and contains a single seed. The spherical seed with a smooth or slightly reticulate testa is surrounded by a distinctive funiculus (Prina, 2009).

1.2 Agricultural traits

The crop management and crude fat yield per hectare of crambe is rather similar to that of spring rape. Cultivation time varied between 113-149 days during field trials carried out at four different locations in southern Sweden during 2007 and 2008. Available cultivars are rather primitive, prone to seed scattering which in combination with a fast ripening progress makes harvest so far rather challenging. The crude fat yield varied between 705-883 kg/ha for a variety of different crambe genotypes to be compared to 859 kg/ha for the spring rape included in the field trials as a standard. Pollen beetle (*Meligethes aeneus*) infestation in the spring rape was a big problem during the trial while crambe seemed less affected resulting in less yield losses due to pollen beetle damages (Nilsson et al., 2008).

The pollen beetle uses flower colour as a visual cue to identify host plants. Yellow colour is much more attractive to pollen beetles compared to white

(Blight and Smart, 1999). Danish trials with rape indicate that rape with yellow petals experienced 3-4 times higher infestation rate of pollen beetle compared to those with white petals (Tybirk, 2012).

The seeds are orthodox (Kew, 2013) and scattered seeds may survive for 10 years in the soil, but volunteer plants can be easily handled in a crop rotation system. The pollen of crambe is incompatible with other economically important crops grown and the risk of gene flow to wild relatives is regarded as low in North Europe (Carlsson et al., 2007). The ripe seeds have an average protein content of 29% and an oil content of 42% (Kew, 2013) of which up to 57% is 22:1 erucic acid in conventional cultivars (Carlsson et al., 2007).

1.3 Utilisation

Oilseed production has steadily increased globally the last years from 314 million metric tons in 2000/2001 to 442 million metric tons in 2011/2012 (USDA, 2013). More than 300 types of fatty acids (FA) have been identified in seed storage lipids (Millar et al., 2000). Beside their importance as food and feed FAs can be used for a wide range of industrial purposes e.g. biofuel, cosmetics, detergents, lubricants, paints and soaps. In many cases the metabolic pathway has already been identified enabling genetic modification to introduce the production of unique oil qualities. There is an increasing interest for these products with increasing oil prices and a shift towards renewable resources (Thelen and Ohlrogge, 2002). Genetically modified crambe could have a great potential for the production of new oil qualities aiming at industrial processes (Nilsson et al., 2008).

1.4 Plant lipid synthesis

Fatty acid synthesis in plants occurs in the plastids. FAs are derived from Acetyl-CoA and Malonyl-CoA transferred to an acyl carrier protein (Malonyl-ACP) which are condensed in a cyclic manner by a group of enzymes, 3-ketoacyl-ACP synthases (KAS I-III) (Ohlrogge et al., 1995). During each cycle four reactions occur; condensation, reduction, dehydration and reduction (Li-Beisson et al., 2010).

This cyclic condensation produces saturated fatty acids while more than 75% to the FAs usually found in plants are unsaturated. An enzyme unique to the plant kingdom, stearoyl-ACP Δ^9 desaturase, introduce a double bond and is responsible for the major part of the unsaturated FAs found in plants (Ohlrogge et al., 1995).

The cyclic elongation is terminated when the acyl-ACP is hydrolysed by an acyl-ACP thioesterase (FatA & FatB), releasing the FA, see Figure 1 on page 3. The majority of plant FA are 16-18 carbons long (Ohlrogge et al., 1995).

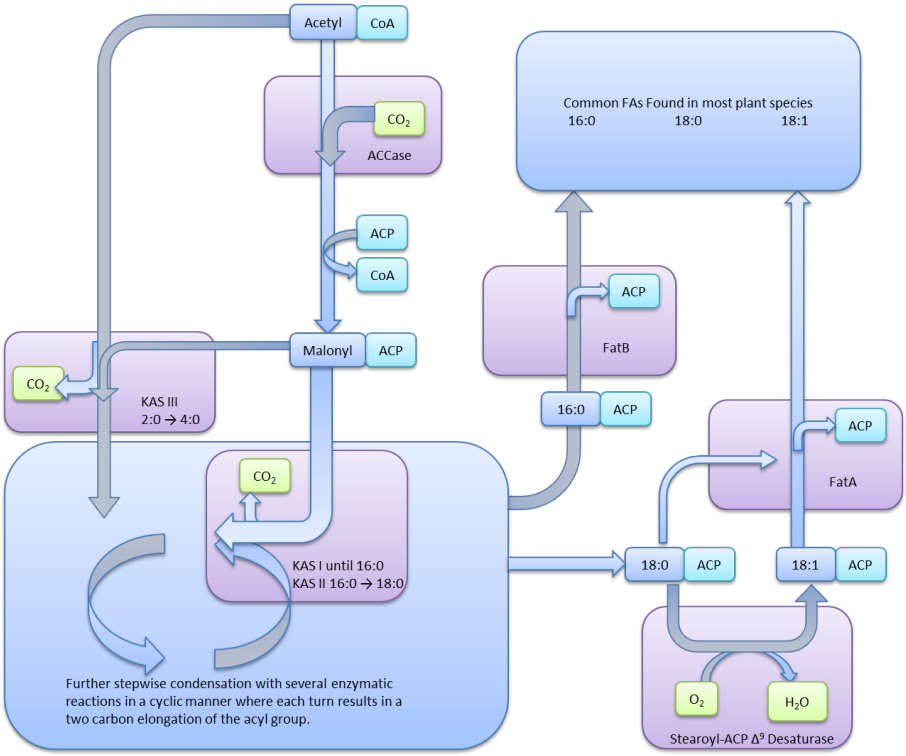

Figure 1: Simplified schematic drawing of the FA synthesis.

A big part of the FA produced in the plastid will eventually constitute the hydrophobic part of the polar membrane lipids – glycerolipids while some will form triacylglycerols (TAG) (Ohlrogge et al., 1995).

There are several fatty acyl desaturases (FAD) located in the plastid (FAD4, 5, 6, 7 & 8) which have the ability to introduce double bonds to the synthetized FAs commonly found in plants (Li-Beisson et al., 2010).

A major part of the synthetisied FA are cleaved from the ACP and exported to the cytoplasm where they are estrified to CoA. These FA-CoAs are then available for further modification and use in the endoplasmic reticulum (ER) (Millar et al., 2000). In addition to the FADs located in the plastid, two FADs are located in the ER, FAD2 and FAD3. FAD2 convert 18:1 to 18:2 while FAD3 produce 18:3 from 18:2 (Li-Beisson et al., 2010).

There are several categories of unusual FAs that are modified either in the plastid or in the ER some are shortly described below.

1.4.1 Unusual monounsaturated fatty acids

Some plants e.g. *Coriandrum sativum* have in addition to the previously stearoyl-ACP Δ^9 desaturase and other FADs closely related plastidial desaturases which can introduce double bonds at other unusual positions (Millar et al., 2000).

1.4.2 Medium long-chained FAs (MLCFA)

Some plants accumulate MLCFA (8-14 carbons long) e.g. *Cuphea hookeriana* accumulate high levels of shorter FA (8:0 and 10:0) in the seeds. A FatB enzyme (ch FatB2) has been identified which terminates the elongation of FA during the cyclic elongation in the plastid at an earlier stage resulting in MLCFA (Dehesh et al., 1996).

1.4.3 Very-long-chain FAs (VLCFA)

VLCFAs are FAs longer than 18 carbons. The elongation is initiated by an enzyme fatty acid elongase 1 (FAE1) which is bound to the membrane of ER. FAE1 condensates an acyl-CoA and malonyl-CoA which is the first step in the synthesis of VLCFA. Three other enzymes are necessary in the synthesis of VLCFA but are not unique to the VLCFA synthesis and does not play a regulatory role (Katavic et al., 2004). VLCFA are a found in the seed oil of many taxa in different plant families including Brassicaceae. Crambe seed oil has an unusually high content of 22:1 erucic acid compared to other taxa belonging to brassicaceae. Erucic acid is an important raw material for industrial purposes and there are more than 1000 potential patents for industrial applications. Erucic acid and the derivates erucamide and behenic acid can be used in production of e.g. plastic films, lubricants, cosmetics, pharmaceuticals, detergents and in coatings. The proportion of erucic acid in any seed oils is currently limiting the competiveness of plant oil when compared to petrol based products due to cost involved during purification (Mietkiewska et al., 2007).

1.4.4 Hydroxy FA

The seed oil of *Ricinus communis* contains almost 90% of hydroxy FA, ricinoleic acid(18:1-OH) that have many properties that are useful to the industry (Smith et al., 2003). Ricinoleic acid is used for e.g. lubricants, paints,

cosmetics, surfactants, foams and nylon-11. Castor oil is frequently used by the industry but the production has several drawbacks. *Ricinus communis* have poor agronomic traits, contains allergens and the plant is highly toxic due to the ricin content (Grushcow and Smith, 2006). The enzyme oleate Δ^{12}-hydroxylase introduces an alcohol group and is highly homologous to the oleate 12-desaturase (FAD2) which is localised in the ER (Smith et al., 2003).

1.4.5 Epoxy FA

Epoxy fatty acids can be found in the seed oil from several taxa belonging to the family Asteraceae and Euphorbiaceae. A common epoxy FA, Vernolic acid (12-epoxy-18:1 Δ^9) which have an epoxy group introduced between $\Delta^{12}$and Δ^{13} have a great potential for industrial purposes in paints and as a precursor of nylon-11 and nylon-12. In *Crepis palaestina* and *Veronia galamensis* both belonging to Asteraceae a Δ^{12}-oleic acid desaturase (FAD2) like enzyme introduce the epoxy group while a structurally unrelated P450-type enzyme has the same function in *Euphorbia lagascae*. Both groups of enzymes introduce the epoxy group at the Δ^{12} double bond of 18:2 resulting in vernolic acid (Cahoon et al., 2002).

1.4.6 Acetylenic FA

CaACET (*Crepis alpina* Δ^{12} acetylenase) is a bifunctional enzyme that is able to both desaturate oleate to linoleate as well as further acetylate linoleate to crepenynate (Reed et al., 2003). More specifically, oleate may be desaturated into either 18:2 12(E) or 18:2 12(Z) in a ratio of approximately 3:1 by CaACET. CaACET is however only able to produce crepenynate from the 18:2 12(Z) isomer (Carlsson et al., 2004). Carlsson et al. thoroughly studied the FA composition of transgenic *A. thaliana* carrying CaACET and two *Crepsis* species and thus a construct from this project was used in this study as a reporter gene during the different transformation protocol evaluations.

1.4.7 Glycerolipids

There are two separate pathways glycerolipids can be synthetised, the prokaryotic and the eukaryotic pathway. The prokaryotic pathway occurs in the plastids and uses Acyl-ACP as substrate. The glycerolipids produced in the prokaryotic pathway have 16:0 at sn-2 and most frequently 18:1 at sn-1. Five to 40% of the membrane lipids are produced by the prokaryotic pathway in angiosperms (Ohlrogge et al., 1995). The eukaryotic pathway occurs in the ER and uses FA-CoA as substrate to produce glycerolipids. The glycerolipids

synthesised in the ER exclusively contain C 18 at sn-2 instead of 16:0 (Li-Beisson et al., 2010).

Even though there is a large diversity of FA found in plants, few are found in the membrane lipids indicating that it's possible to alter the FA composition without disturbing plant physiology (Thelen and Ohlrogge, 2002).

1.4.8 TAG synthesis

Beside membrane lipids many FAs end up esterified to TAG which unlike membrane lipids do not have a structural role but instead as a storage form for carbon (Ohlrogge et al., 1995). TAGs are synthetisied in the ER and are major energy storage and carbon source in oil seeds (Zhang et al., 2009). Three acyltransferases (Glyserol-3-phosphate acyltransferases (GPAT), *lyso*-phosphatidic acyltransferase (LPAAT) and diacylglycerol diacyltransferase (DGAT)) and a phosphohydrolase (*lyso*-phosphatidate phosphohydrolase) use the substrates fatty acyl-CoA and Glycerol-3-phosphate to form TAG in a process known as the Kennedy pathway (Jako et al., 2001).

There have been identified two distinct groups of DGAT; DGAT1 which was first identified from mouse while the first DGAT2 was first found in *Morteriella ramanniana* (Zhang et al., 2009). For many years DGAT was regarded as the only enzyme performing the third and final acyl transfer reaction during TAG synthesis but in 2000 Dahlqvist et al. identified an enzyme, phospholipid:diacylglycerol acyltransferase (PDAT) capable of producing TAG in absence of fatty acyl-CoA (Dahlqvist et al., 2000). In a study by Zang et al. in 2009 *A. thaliana* DGAT1 null mutants displayed a decrease in oil content by 20% to 40%, indicating other TAG synthesis pathways were present. Expressing a PDAT RNAi construct in the DGAT1 RNAi mutant further reduced the oil content by 70% to 80%, this however greatly affected the plats ability to produce viable pollen. Enhanced expression of DGAT in wild-type *Arabidopsis* increased the oil content in the seeds by 30% to 40% (Jako et al., 2001).

1.4.9 Wax esters as storage lipids

As previously described oil seeds plants produce TAG as their major energy storage but there is a unique exception among plants, Jojoba (*Simmondsia chinensis* (Link) C.K. Schneid.) which store up to 60% of long-chain wax esters instead of TAG in their seeds (Thelen and Ohlrogge, 2002). The wax esters produced by Jojoba consists of very long unsaturated FA and fatty alcohols, primarily FA 20:1 and fatty alcohol 22:1. Three groups of enzymes; FAE, Fatty acyl-CoA reductase (FAR) and fatty acyl-CoA:fatty

alcohol acyltransferase (wax synthase, WS) are involved in the wax synthesis in developing Jojoba seeds. FAR present in Jojoba which reduce fatty acyl-CoA to fatty alcohol have a preference for very long fatty acyl-CoA and FAE elongates 18:1 fatty acyl-CoA to products preferred by jojoba FAR. WS produce wax esters from the FA moiety from fatty acyl-CoA and the fatty alcohols produced by FAR. WS in Jojoba can produce wax esters with a wide range of fatty acyl-CoA and fatty alcohols and the primary wax ester found in Jojoba is more likely to be the result product of the FAR substrate preferences rather than the substrate preferences of WS. Transgenic *Arabidopsis* with expressed jojoba cDNA of WS and FAR in combination of FAE from *Lunaria annua* have successfully produced a high content of wax esters, up to 70% by weight of the oil present in single seeds (Lardizabal et al., 2000). A transgenic wax ester producing crambe could potentially have a market of 2 million tons in Europe alone (Nilsson et al., 2008).

1.5 Transformation

Numerous economically important and scientific model crops have been genetically modified since 1984, when tobacco for the first time was successfully transformed with novel genes. In 1997 more than 120 plant species from 35 families had been transformed (Birch, 1997). A high percentage of the grown acreage of important crops as corn, soybeans, cotton, canola, potatoes, and tomatoes was transgenic in some countries by 2003 (Gelvin, 2003).

Several protocols for plant transformation, both direct and indirect, have been developed during the last decades. The direct protocols mainly rely on bombardment with inert micro particles coated with DNA or protoplast transformation, while the indirect protocols are based on *Agrobacterium tumefaciens* or *A. rhizogenes* mediated transformation (Rakoczy-Trojanowska, 2002).

1.5.1 *Agrobacterium tumefaciens*

A. tumefaciens is a gram negative, soil-borne, tumour inducing plant pathogen. The host-range includes a wide range of dicotyledonous genera and to a smaller extent some monocotyledonous genera (McCullen and Binns, 2006).

The induced tumours commonly known as crown galls will continue to grow in absence of the initiating *A. tumefaciens*. Pathogen-free tumours observed for more than 10 years, continued to grow autonomously in culture medium as if being "permanently altered cells" (Braun, 1958).

This demonstrates that the *Agrobacterium* has transformed the plant cells. Virulent strains of *Agrobacterium* contain a tumour inducing plasmid

(Ti plasmid), where a part is delimited by 25bp border repeats (left border, Lb, right border, Rb) (McCullen and Binns, 2006). This delimited piece or the T-DNA of plasmidial DNA, is incorporated into the genomic DNA of the plant cells, and carries several genes. This results in the synthesis of plant hormones as well as opines, once the genes are incorporated to the plant genome and expressed. The plant hormones induce the tumour growth while opines are an important source of nutrients for the bacteria (Tzfira and Citovsky, 2006).

The T-DNA can be replaced with other genes of interest by cloning and subsequently become successfully incorporated into the plant genome Valentine (2003). By replacing the tumour inducing T-DNA genes with genes of interest, transformed plant cells can be regenerated into fertile plants carrying the engineered DNA to their progeny (McCullen and Binns, 2006).

Cell exudates from wounded plants such as acetosyringone and other phenolic compounds and sugars not only indicate a pathogenic opportunity to the bacteria but also induce the virulence genes (vir) necessary for plant cell transformation (Valentine, 2003). Beside the vir genes located in the Ti-plasmid a number of bacterial chromosomal genes as well as various host proteins, are necessary for the *Agrobacterium*-mediated transformation of plant cells.

The host range differ between different *Agrobacterium* strains (Anderson et al., 1979) more specifically it is determined by genes in the vir region (Leroux et al., 1987). In natural occurring *A. tumefaciens* strains the virulence genes and the T-DNA reside on the same plasmid while many disarmed strains used for scientific purposes carry a small autonomous binary plasmid containing the T-DNA instead (Tzfira and Citovsky, 2006).

The T-DNA binary vector system with the vir helper plasmid and the binary vector containing the T-DNA with dedicated restriction sites described by Hoekema et al. in 1983 made *Agrobacterium*-mediated transformation much more convenient to use. Up to approximately 200 kbp long T-DNA large enough to include tens of genes has been incorporated into plants using this technique even though such events are rare (Gelvin, 2003).

1.5.2 *Agrobacterium*-mediated transformation protocols including tissue culture

Most plant transformation employs tissue culture, which is an efficient tool for transformation, regeneration and selection (Birch, 1997). In order to achieve successful *Agrobacterium*-mediated transformation with tissue culture a compatible *Agrobacterium* strain accompanied with a suitable binary vector and vir helper plasmid must be selected, in combination with a potent

tissue culture protocol (Tzfira and Citovsky, 2006). The regeneration media constituents as well as temperature and photoperiod, must be carefully adjusted to the genotype considered (Razdan, 2003). Some of the parameters defining a potent tissue culture media that has to be taken into consideration are described below:

Inorganic elements At least 12 different minerals are essential for plants. Nitrogen, phosphorous, sulphur, calcium, potassium and magnesium are by plants in concentrations greater than 0.5 mmol $\times$ l^{-1} and are referred to as macro nutrients. While iron, manganese, copper, zinc, boron and molybdenum are referred to as micro nutrients and are needed in concentrations less than 0.5 mmol $\times$ l^{-1} by plants (Razdan, 2003).

In 1962 Toshio Murashige and Folke Skoog published a now regarded classic scientific article where they published a medium used for rapid growth and bio assays with tobacco cultures. The medium consist of 14 different salts including the majority of essential macro and micro plant nutrients as well as organic constituents (Murashige and Skoog, 1962).

The inorganic salt composition usually referred to as basal Murashige & Skoog salt mixture or MS salt has proven to be very suitable and is nowadays the now the most used formulation for in vitro cultures (Thorpe, 2007) including many genera within Brassicaceae e.g. *Brassica oleracea* L. var. *botrytis* L. (Yu et al., 2010) *Brassica napus* L. (Kartha et al., 1974) and *Lepidium sativum* L. (Pande et al., 2002).

Organic supplements Vitamins are endogenously synthesised in plants but *in vitro* cultures may produce suboptimal quantities and supplementing vitamins often have a positive effect on growth (Abrahamian and Kantharajah, 2011).

Thiamine B_1, nicotinic acid B_3, pyrioxin B_6 are vitamins found among the organic constituents in Murashige and Skoog medium (Murashige and Skoog, 1962). Thiamine is the most common vitamin additive and generally regarded as essential in tissue cultures (Al-Khayri, 2001).

Carbon and energy source Few in vitro cultures can be regarded as photoautothrophic and sugar is often added in culture media both as carbon source and energy source to compensate. Sucrose is the most widely used carbon and energy source which during autoclaving is partly converted to glucose and fructose.

Sugars are in addition, the major osmotic component of tissue culture mediums (George et al., 2008).

Growth regulators Plant growth regulators and hormone-like substances or phytohormones are necessary for growth, differentiation and organogenesis in tissue culture. There are five classes of naturally occurring phytohormones; auxins, abscisic acid, cytokinins, ethylene and gibberellins. Auxins and cytokinins and the interaction between these groups of hormones are usually regarded as most important in tissue cultures (Gaspar et al., 1996).

The most frequently used auxins in tissue cultures are IAA, IBA and NAA while the most common cytokinins are BAP, 2ip, kinetin, TDZ and zeatin (Bhojwani and Razdan, 1996). This does not mean that that the other groups of plant hormones should be ignored (Gaspar et al., 1996).

During the 1950ies Skoog and Miller discovered that the ratio between auxins and cytokinins influenced the morphogenesis of roots and shoots during tissue culture experiments (Eckardt, 2003). High ratio of cytokinins to auxin produced shoots while the opposite produced roots (Barton, 2010).

pH regulation pH has to be regulated in order to provide optimum ion uptake which occurs between pH5.5-6 (George et al., 2008).

Antibiotics Antibiotics are often not included in pure tissue cultures, since they decreases the growth, while frequently included in transformation protocols as a selection agent for transformed cells (Razdan, 2003).

Somaclonal variation The aim with transformation is often to alter the gene expression in the plant to introduce a desired trait or in other mean enhance the properties of the plant. Undesirable changes may occur frequently during the transformation process.

It might be difficult to conclude whether these changes are the result of the gene insertion or somaclonal variation during the tissue culture. There is a vast variation of altered traits due to somaclonal variation including male sterility, increased carotene content altered growth habit, *fusarium* resistance, susceptibility to soft rot. These altered traits are often due to chromosomal aberrations, DNA methylation changes, transposable elements, point mutations and epigenetic variation.

There are currently many proposals for the underlying mechanisms responsible for these alterations e.g. culture type and culture age (Veilleux and Johnson, 1998).

In addition to the parameters already described for tissue culture, somaclonal variation should be avoided or minimised.

1.5.3 *Agrobacterium*-mediated transformation based on infiltration

In 1993 Bechtold et al. managed to produce genetically transformed *A. thaliana* seed by *in planta* transformation based on infiltration. Inflorescences were submerged in a pH calibrated solution containing agrobacterium MS salt and vitamins, BAP and sucrose (Clough and Bent, 1998). Vacuum was applied to facilitate the infiltration of inflorescences where meiotic cell division occurs. Up to 95% of the infiltrated plants produce transgenic seeds and 0.5% to 4% of the harvested seeds are transformed during optimal conditions for *Arabidopsis thaliana* with this protocol (Rakoczy-Trojanowska, 2002). The progeny is often hemizygote indicating that a successful transformation occurs after the differentiation of anther and ovary cells.

In 1998 Clough and Bent evaluated the Bechtold et al. infiltration protocol for *A. thaliana* and simplified it in several ways. They concluded that sufficient transformation rate could be achieved by adding a surfactant (Silwet L-77) to the solution which replaced the need for vacuum infiltration. Further, they excluded the MS salt and vitamins, BAP, and pH calibration of the solution with good results. They also identified an optimal developmental stage of the plant where the plants produced most transgenic seeds. This protocol is reliable and simple avoids the cumbersome in vitro tissue culture, minimising the somaclonal variation and most progeny is non-chimeric (Clough and Bent, 1998).

There are however limitations for plants to be transformed according to this protocol in practice. To work efficiently the plant should fulfil several criteria, it should be small, have a short generation cycle and be a prolific seed producer (a single *Arabidopsis* plant can produce 4000-8000 seeds (Bressan et al., 2001) where each silique usually contains 40-60 seeds(Meyerowitz and Somerville, 1994)). These criteria's for infiltration transformation are not fulfilled by any economically important plant (Birch, 1997).

1.6 RNA interference

Plants possess a mechanism of gene silencing induced by double-stranded RNA (dsRNA), commonly referred to as RNA interference (RNAi) (Meister and Tuschl, 2004). RNAi is known to defend plants from viruses, regulate gene expression and protect the genome from transposons (Baulcombe, 2004). The precursors of dsRNA initiating this post-transcriptional gene regulation may origin from different sources (Meister and Tuschl, 2004). dsRNA is cleaved by dicer enzymes into fragments with 21-26 bp and referred to as short interfering RNA (siRNA) or micro RNA (miRNA) (Baulcombe,

2004). dsRNA from viruses and transposons give rise to siRNA while endogenous transcripts with 20-50 bp complementary inverted repeats give rise to miRNA. Dicer enzymes contains RNase III and dsRNA-binding domains and excises dsRNA into miRNA or siRNA fragments(Meister and Tuschl, 2004).

In *A. thaliana* there are different effector- or silencing-complexes where single strands of these RNA fragments are assembled which contribute to RNAi. RNA-induced transcriptional silencing (RITS) complexes (Meister and Tuschl, 2004) which guide regional condensation of chromatin into hetrochomatin, a transcriptionally silent chromatin structure (Bailis and Forsburg, 2002). RNA induced silencing complexes (RISC) and miRNA-containing effector complex (miRNP) targets and slices complementary mRNA or result in translational repression (Meister and Tuschl, 2004). Plants miRNA mainly regulates transcripts by cleavage and seldom by translational repression (Brodersen and Voinnet, 2009).

Transformation of plants with a RNAi construct containing complementary inverted repeats from the gene of interest will fold back and result in a hairpin double-stranded mRNA, and effectively down-regulate the gene (Meister and Tuschl, 2004).

1.7 Objectives

Two indirect transformation protocols utilising *Agrobacterium tumefaciens* is evaluated for transformation of crambe: One protocol provided by Bang-quan Huang was evaluated, where excised hypocotyls is immersed in a bacterial suspension containing *A. tumefaciens*, followed by tissue culture where putative transgenic plantlets are grown on selective media. A second protocol is also evaluated based on infiltration according to Clough and Bent 1998, where the progeny is screened for putative transformants.

If any of the protocols are successful they should be compared to the existing transformation protocol established by Li et al. 2010

The aim of this study is to evaluate if the two separate transformation protocols are able to successfully incorporate tDNA into the genome of crambe and subsequently successfully silence a native previously un-sequenced gene by an RNAi construct.

For practical reasons and due to time limitations transformation protocol evaluations are carried out with an already finished construct while the RNAi construct is prepared in parallel.

2 Method

2.1 Evaluation of transformation protocol for crambe including tissue culture according to Huang's protocol

An expression cassette provided from Anders Carlsson containing *Crepis alpina* Δ^{12} acetylenase gene, CaACET (Carlsson et al., 2004) driven by a 35s promoter was cut from the cloning vector pART7 (Gleave, 1992) with NotI and cloned into the binary vector pART27 (Gleave, 1992), 35s-CaACET-pART27. The Binary vector was transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993), using electroporation. Confirmation of insert was carried out by digestion of the plasmid with NotI.

Pods containing seeds of crambe cv. 'Meyer' were provided from the Nordic Genebank, accession number NGB 2989.1. Seedpods were surface sterilised by half strength commercial bleach for 15 minutes followed by a brief 70% ethanol wash. The seeds were left to imbibe water for two days at a temperature of +8°C before germinated. The germination of 200 seeds was carried out in 90mm Petri dishes containing medium I[1].

Several hundreds 2-5 mm long segments of hypocotyls appearing healthy were excised from emerging shoots after four days under sterile conditions and were transferred to Petri dishes with medium II and incubated for 24 hours. Lysogeny broth (LB) medium (Bertani, 1951) with 5.0 mg $\times$ l^{-1} kanamycin was inoculated with transformed EHA105 and harvested at an OD600 of 0.2. The bacteria was re-suspended in full strength MS salts and 100 μg $\times$ l^{-1} acetosyringone. The hypocotyl segments were immersed in the bacterial suspension for 5 minutes before transferred to medium III. After 24 hours of co-cultivation the hypocotyl segments were washed three times with sterile water including 500mg $\times$ l^{-1} cefotaxime and thereafter allocated to medium IV.

The germination and the regeneration occurred in a climate chamber with 25°C and a photoperiod of 15 hour light and 9 hour darkness. When shoots had regenerated from the induced callus DNA was extracted as described in appendix from six shoots and was analysed. Primers annealing to 35S promoter and CaACET was used in a PCR (Saiki et al., 1985) to screen the putatively transformed shoots. Further, leaf blades from all six shoots were analysed for their fatty acid composition by acidic methylation using 2% H_2SO_4 in MeOHand GC as described by Thomæus et al. 2001.

[1]The different media compositions are found in Table 1 on page 14

	Medium composition			
Component	Medium I	Medium II	Medium III	Medium IV
Benzylaminopurine	-	3.0	3.0	3.0
Thidiazuron	-	8.0	8.0	8.0
1-naphthalene acetic acid	-	0.2	0.2	0.2
Zeatin	-	2.0	2.0	2.0
Acetosyringone	-	-	0.1	-
Cefotaxime	-	-	-	500
Carbenicillin	-	-	-	50
Kanamycin	-	-	-	5.0
$AgNO_3$	-	-	-	2.5

Table 1: All values are presented in mg $\times$ l^{-1}. The medium were solidified with 0.25% (V/W) gelerite, contain full strength MS salts including vitamins, 30 g$\times l^{-1}$ sucrose. pH calibrated to 5.8-6.0.

2.2 Southern blot (Southern, 1975) modified according Sambrook and Russel, 2001

Genomic DNA, 10 μg from the six regenerated crambe shoots were digested overnight by EcoRI and separated on a 0.7% agarose gel together with the 35s-CaACET-pART27 construct as a control. The agarose embedded DNA was depurinated for a short period of time and rinsed in millipore water. The gel was soaked in alkaline transfer buffer prior to the southern blot was assembled. The DNA fragments were blotted onto a positively charged nylon transfer membrane overnight. The membrane was subsequently treated with a neutralisation buffer.

A PCR product (411 bp) of the NTPII gene was amplified from the 35s-CaACET-pART27 construct and labelled with P32 to be used as a hybridisation probe. A sub sample of the PCR product was digested by RsaI to confirm that a restriction site was present at predicted location. The remaining part of the PCR product was size determined on an agarose gel and the excised fragment was extracted with QIAquick gel extraction kit according to the provided protocol. The probe was labelled by mixing with Klenow fragment exonuclease minus provided with the template, hexanucleotides dNTP including dNTP where dCTP had radioactive ^{32}P incorporated. The mixture was incubated for 30 minutes at 37°C. The radioactive probe was eluted with Micro Bio-Spin 30 chromatography column (Bio-Rad laboratories, Inc).

The blotted membrane was soaked briefly in 6X SCC and then placed

in a pre-heated roller bottle containing pre-hybridisation buffer in a 60°C incubator. The pre-hybridisation buffer was replaced after one hour and replaced with fresh pre-hybridisation buffer to which the prepared probe was added. Hybridisation was carried on in the 60°C incubator overnight. Hybridisation was followed with washing of the membrane in with washing buffer I for 5 minutes, II for 15 minutes and III for 30 minutes in at 60°C. Finally the membrane was briefly washed with wash buffer IV and enclosed in a plastic sheet. An image was developed of the membrane with Packard Instant Imager Electronic Autoradiography System. Further details on the buffers can be found in appendix.

2.3 Floral dip

The 35s-CaACET-pART27 construct was cloned into the *Agrobacterium* strains GV3101 and GV2260 in addition to the pre-existing EHA105. Nine plants of crambe cv. 'Meyer' with inflorescence containing flowers of different developmental stages were equally divided and treated separately with the three *A. tumefaciens* strains according to Clough and Bent 1998. Each plant was dipped once more in a similar manner the following week.

Mature seeds were harvested and sown in peat. The emerging seedlings were given unique numbers and a leaf sample was excised from one of the cotyledon from every plant. Leaf samples were pooled in groups of ten, keeping record of which plants belonged to which pool. A DNA extraction was carried out for each pool of plants as an initial screening for transformants. Individual plants belonging to a group DNA indicating positive for NTPII in the initial screen were rescreened by PCR individual by individual.

Mature seeds were harvested and sown in peat. Ten leaf samples were excised from emerging cotyledons and pooled for each DNA extraction. The extracted DNA was screened for presence of NTP II by PCR.

2.4 Partial CaDGAT1 sequencing

Total RNA was extracted from developing seeds of crambe cv. 'Galactica' and served as template for cDNA further used for sequencing of CaDGAT1. Seeds of different developmental stages were pooled to avoid temporal gene expression variation. Invitrogen Concert™ Plant RNA Reagent was used following the small scale RNA isolation protocol provided for the RNA isolation. To ensure the quality of the total RNA, only those samples with no or little signs of degradation when separated on an agarose gel were further used as template for cDNA synthesis.

The total RNA was subsequently used to produce single stranded cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Oligo(dt)18 primer was used to exclusively produce cDNA of mRNA origin.

To amplify a PCR fragment of CaDGAT fragments four primers were designed. DGAT sequences from *A thaliana* AF051849 (*A. Thaliana* DAGAT complete cds) and *Brassica napus* AF251794 (*B. napus* putative DAGAT complete cds) were aligned in Vector NTI AlignX to identify homologous regions. The both sequences showed 86% nucleotide consensus in the alignment with several long stretches completely homologous and four of those regions were chosen for primers. The primers were designed in a nested manner as seen in Figure 2, positioned in AF051849 – *A. thaliana* DGAT1 mRNA.

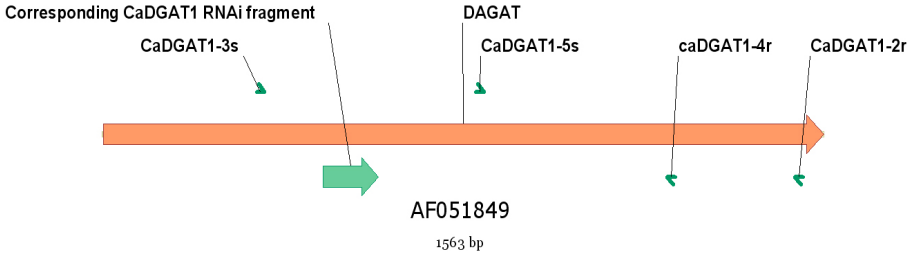

Figure 2: *Arabidopsis thaliana* DGAT1 mRNA and annealing sites for designed primers.

Primers were ordered from Sigma-Aldrich with the following sequences:

CaDGAT1-3s CCAGCTCATCGGAGGG

CaDGAT1-5s ATAAGGCCAATCCTGAAG

CaDGAT1-4r TGAACAGGCATATTCCAC

CaDGAT1-2r GAAGCACACACARCGGTT

Amplification was done using Taq polymerase (Sigma), the two primers CaDGAT1-3s and CaDGAT1-2r and the crambe cDNA as template. As an initial confirmation of the identity of the amplified fragment (expected size 1186 bp from comparison with the *A. thaliana* DGAT1) the PCR product was analysed on a 1.2% TAE agarose gel.

The GeneJet ™ PCR cloning kit (Fermentas) was used to clone the fragment in the cloning vector pJET1 that subsequently was transformed into *E. coli* DH5α. Since Taq polymerase that adds A-overhang to the DNA (Clark, 1988) was used for the PCR, the amplified fragment was blunted prior the ligation into the Eco32I pre-cut pJET1 vector (DQ317600, see Figure 3.). Ligation was performed with T4 DNA ligase.

The ligated pJET1 vector containing the CaDGAT1-3s/CaDGAT1-2r fragment was electroporated into electro competent *E. coli* DH5δ bacteria. The bacteria were incubated on solidified LB medium with ampenicillin ($100\mu g \times ml^{-1}$ LB) as selection medium overnight at a temperature of 37°C.

Colony PCR was used to quickly screen the visible colonies for presence of the insert using the CaDGAT1-3s and CaDGAT1-2r primers. A few colonies that were found positive for the insert were inoculated in an overnight culture (LB medium with ampenicillin ($100\mu g \times ml^{-1}$ LB). Plasmid DNA was isolated from the cultures PCR positive colony using QIAprep spin Miniprep Kit (QIAGEN). A sub sample of the plasmid DNA was digested with SspI to further confirm whether the plasmid contained the fragment of interest or not.The putative CaDGAT1 insert was sequenced with two separate ¼ BigDye Terminator reactions ver. 3.1, with pJET1 forward sequencing primer and pJET1 reverse sequencing Primer respectively and prepared according to Applied BioSystems Dilution of BigDye Terminators Application Note.

2.5 CaDGAT1 RNAi construction

A 120 bp region was chosen from the sequenced fragment, corresponding to be situated from 478 to 598 in *A. thaliana* DGAT1 (AF051849, figure 2).

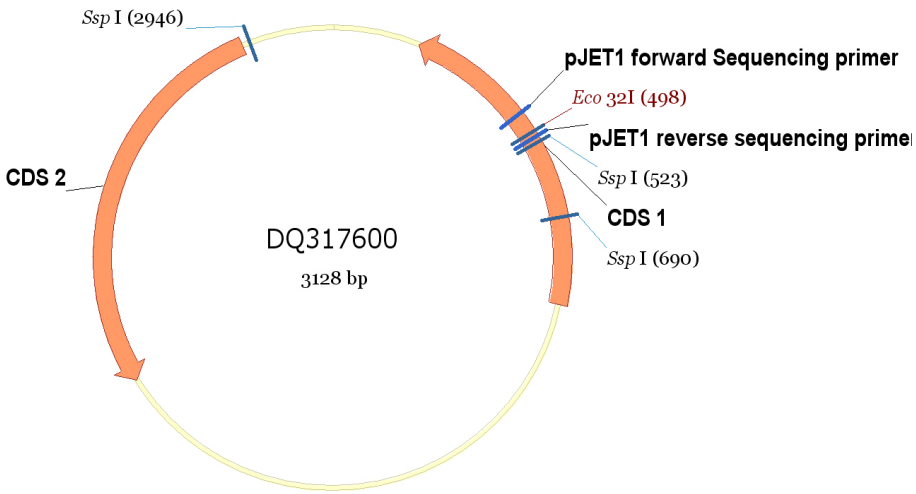

Figure 3: Schematic drawing of pJET1 cloning vector.

An important feature of the selected sequence is that it must not contain any NotI restriction sites, due to subsequent interference in the RNAi construction. The pHannibal vector, figure 4, (Wesley et al., 2001) contains two multiple restriction sites separated by an intron which results in highly efficient RNAi constructs. Two primer pairs were designed to amplify the 120 bp RNAi fragment as well introducing restriction sites (to a total length of 136 bp, including 2 extra base pairs for more efficient digestion by the restriction enzymes) at the borders to facilitate directional cloning into pHANNIBAL. Sense RNAi Primer sequences, bold letters indicate the restriction sites in the primer sequence:

CaDGAT1Bsu15I-RNAi GC**ATCGAT**TGGTTGATCAGAACTGA

CaDGAT1XbaI-RNAi GC**TCTAGA**TACCAATTTCTCGACGG

Anti-sense RNAi primer sequences:

CaDGAT1EcoRI-RNAi GC**GAATTC**TGGTTGATCAGAACTGA

CaDGAT1xhoI-RNAi GC**CTCGAG**TACCAATTTCTCGACGG

Both sense and antisense RNAi fragments were amplified with Taq PCR from the Crambe cDNA template. The product was cleaned with Gene-elute™ PCR cleanup kit (Sigma), to remove the enzyme, dNTP and oligonucleotides, and subsequently precipitated and re-suspended in a suitable amount of deionised water. Further, the products were restricted over night with Bsu15I,

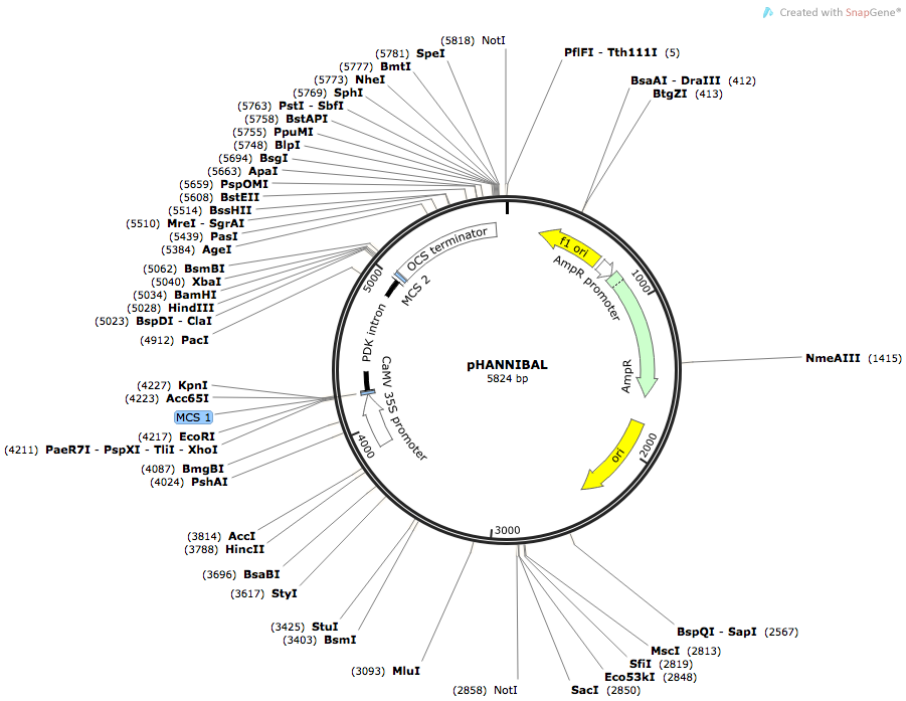

Figure 4: Schematic drawing of pHANNIBAL vector (SnapGene, 2015).

XbaI for the sense fragment and EcoRI, XhoI for the anti-sense fragment. The products were once again cleaned with the Gene-elute™ PCR cleanup kit (Sigma). The pHANNIBAL plasmid was cut with EcoRI and XhoI restriction enzymes and ligated with the prepared anti-sense fragment using T4 Ligase (Fermentas) and transformed into electro-competent *E. coli* XL1-blue. The transformed bacteria were incubated overnight on LB plates containing 100 μg ampicillin $\times$ml^{-1} LB. Several colonies were screened with colony PCR, and two colonies positive for insert, were selected for inoculation in LB overnight. A plasmid preparation of the pHANNIBAL vector with the anti-sense fragment incorporated was isolated from the cultures with QIAprep spin Miniprep Kit (QIAGEN). A sub-sample of the plasmid DNA was re-cut with EcoRI and XhoI restriction fragments for confirmation, the remaining part was restricted with Bsu15I and XbaI to be ligated with the sense fragment in a similar manner, and again, electroporated into E. coli XL1. Ten colonies were screened, five from each confirmed anti-sense harbouring bacteria colony by colony PCR for confirmation of successful incorporation of the two fragments.

3 Results

3.1 Evaluation of transformation protocol for crambe including tissue culture according to Bangquan Huang's protocol

Six differentiated shoots were rescued from the tissue culture selection media including kanamycin (5.0 mg $\times$ l^{-1}) after co-cultivation with *A. tumefasciens* strain EHA105 harbouring the binary vector construct 35s-CaACET-pART27 construct including NTPII which facilitates kanamycin resistance. DNA was amplified using primers annealing to the 35S promoter and CaACET. All the PRC products of the amplified DNA from the shoots indicated positive transformation and the size of the product correlated well with the amplified fragment from the control, which originated from the 35s-CaACET-pART27 construct, when separated on an agarose gel as seen in Figure 5. FAs were extracted from leaf blades sampled from the regenerated shoots and gas chromatograms of these were analysed by trained personnel. There were no obvious alterations of the FA compositions when compared with gas chromatograms of normal seedlings of NGB2989.1 which should have been the case if CaACET was expressed and functional.

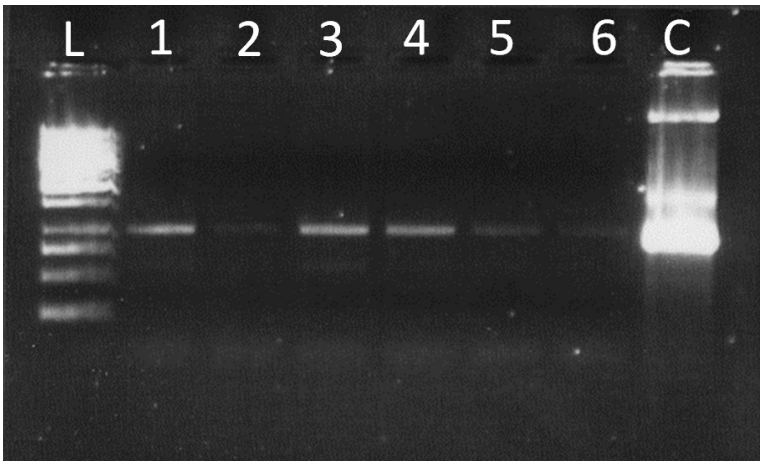

Figure 5: Agarose gel with PCR fragments separated from six regenerated shoots (1-6), Ladder (L) and 35s-CaACET pART27.

3.2 Southern blot

The final analysis carried out to determine whether the regenerated shoot were successfully transformed or not was a Southern blot. Genomic DNA from all shoots was used in the Southern blot analysis. There was no significant hybridisation between the radioactive NTP II probe and the genomic DNA while there was a strong response from the positive NTP II control as can be seen in Figure 6.

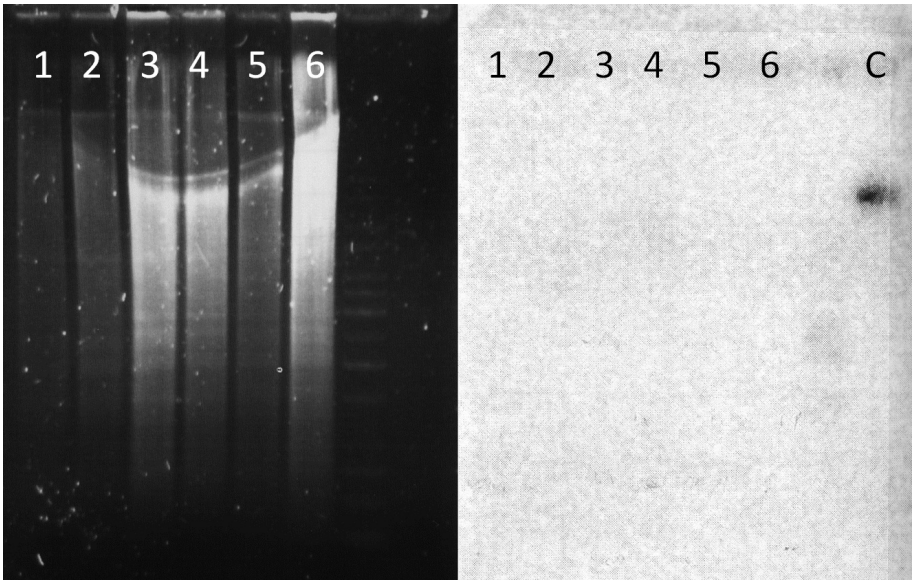

Figure 6: To the left: Genomic DNA from the six shoots (1-6) separated on agarose gel.
To the right: developed image of the southern blot NTP II control (C).

3.3 Screening of floral dip transformants

480 individual seedlings (180 EHA105, 150 GV3101 and 150 GV2260) in total were screened for NTP II and all were negative.

At one occasion, a weak fragment could be observed from a pooled sample, but DNA from the ten individual plants represented in the pooled sample were all negative when screened, and thus the pooled sample was regarded as contaminated.

3.4 Sequencing

3.4.1 Evaluation of the CaDGAT1 primers

Both primer pairs, CaDGAT1-3s/ CaDGAT1-2r and CaDGAT1-5s/ CaDGAT1-4r produced distinct products with crambe cDNA as template. Comparing the products amplified with CaDGAT1-3s/ CaDGAT1-r2 from cDNA template and crambe DNA reveals at least one intron between the two primers.

Figure 7 also clearly demonstrates that there is no need for a nested PCR procedure since the bands are clearly distinct and no visible smear occur. Comparing CaDGAT1-5s/ CaDGAT1-4r templates may indicate that any of the two primers might gap an intron previously observed since no clear band can be found in lane six.

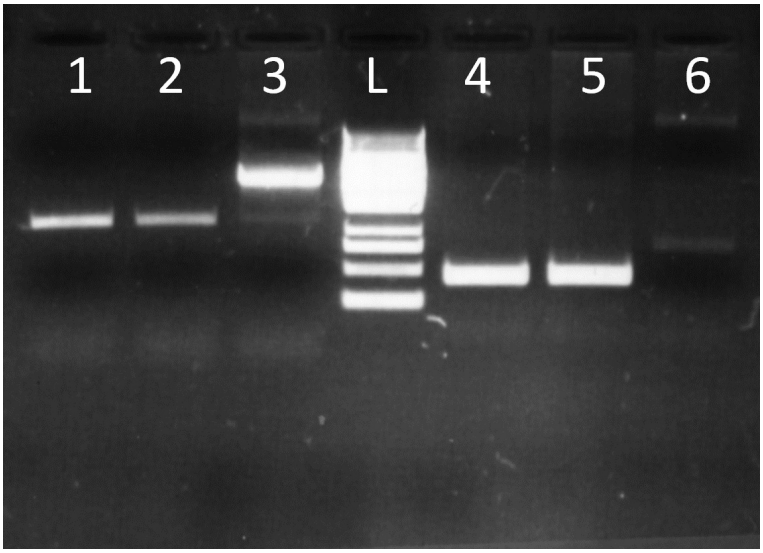

Figure 7: PCR products separated on an agarose gel from: CaDGAT1-3s/ CaDGAT1-2r primers -Crambe cDNA (1-2), CaDGAT1-3s/ CaDGAT1-2r - Crambe DNA (3), ladder (L), CaDGAT1-5s/ CaDGAT1-4r - Crambe cDNA (4-5), CaDGAT1-5s/ CaDGAT1-4r - Crambe DNA (6).

3.4.2 Sequences

The returned sequences were analysed and trimmed manually and only those parts which were regarded as reasonably reliable were further studied. The trimmed sequences aligned with the *Arabidopsis* DGAT1 sequence as seen in Figure 8. A survey with BLASTX 2.2.17 and the two sequences returned

the expected value to be $8e^{-67}$ for the forward sequence and $2e^{-98}$ for the reverse sequence compared to *Arabidopsis* DGAT1 (AF0551849) (Altschul et al., 1997).

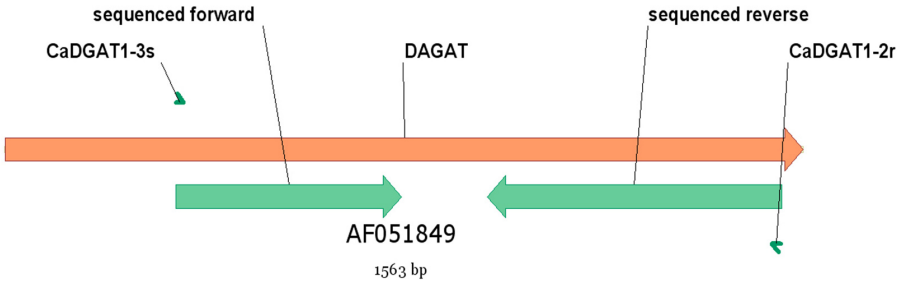

Figure 8: Schematic drawing of Crambe CaDGAT sequences (green arrows).

3.5 CaDGAT1 RNAi construct

Two colonies containing the anti-sense fragments were identified during the first colony PCR and were used in the subsequent step of incorporating the sense fragment. Ten colonies (five from each anti-sense harbouring colony) were screened for both sense and anti-sense fragment presence by colony PCR, indicating a fully incorporated CaDGAT1 RNAi construct, see Figure 9.

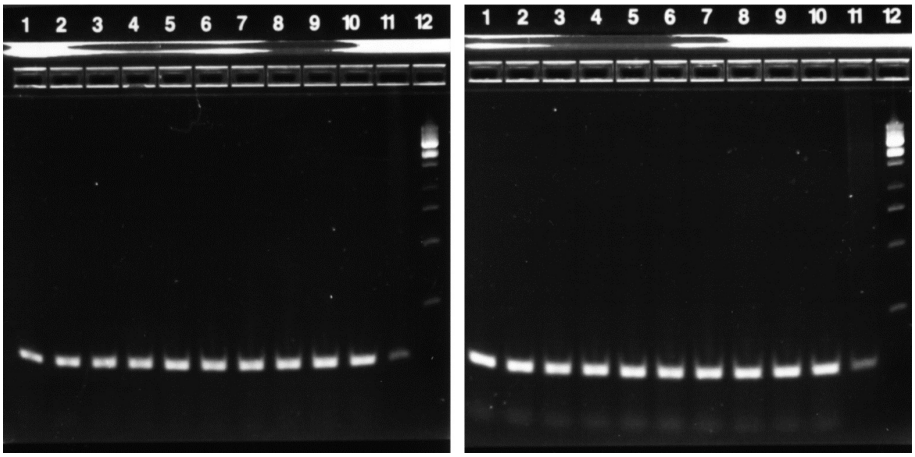

Figure 9: To the left: anti-sense colony PCR (1-10), control (11), ladder (12). To the right: sense colony PCR (1-10), control (11), ladder (12).

4 Discussion

Cultivars must probably be further developed aiming at a more uniform maturity and less seed scattering before large scale production is possible.

The reduced losses of yield due to pollen beetle damages observed during trials could be explained by several reasons e.g. later flowering date, chemical exudates but could also simply be due petal colour as described by e.g. Blight and Smart 1999; Tybirk 2012 . Whatever reason behind the reduced damages it indicates that it's probably possible to reduce the need for applied pesticides compared to yellow flowering spring rape, which is very positive.

The currently available untransformed cultivars may already be of interest to produce oil with a high content of erucic acid even though it most probably could be increased even further both by conventional breeding and by genetic engineering. Genetically modified crambe as a crop platform would enable a range of oil qualities and waxes with a wide range of properties at competitive prices. For this to be realised, establishment of successful transformation protocol on sustainable and efficient crop platforms is a necessity.

4.1 Transformation protocol for crambe including tissue culture according to Huang's protocol

Shoots from the transformation protocol has been thoroughly examined both by PCR, leaf fatty acid analysis and finally Southern blot. Looking at the initial PCR screen of regenerated shoots, it's easy to draw the conclusion that the regenerated shoots were transformed. Contrary to the PCR screening, the leaf fatty acid analysis failed to indicate any acetylenic fatty acid. If the gene was expressed properly there should have been some levels of the non-native fatty acid. Further, the southern blot did not show any presence of the NTP II gene incorporated into the genomic DNA. The most likely scenario is that no successful transformation event has occurred in this study. The PCR only indicate that DNA of the construct is present, not the origin of the DNA, whether it's remnants of plasmidial- or successfully transformed crambe genomic-DNA.

The vast majority of the seeds were infected by unidentified pathogenic fungi and/or bacteria. Even though hypocotyl segments were exclusively excised from seemingly healthy seedlings, infections were a constant problem throughout the *in vitro* shoot regeneration. The six regenerated shoots eventually perished as well, due to these infections and not the kanamycin present in the selection medium. Noteworthy, three of the shoots had a developing root system. The number of regenerated shoots must be regarded as very low regardless of the negative effects of the present pathogens. In 2010 the

first successful transformation protocol for crambe was published where a regeneration rate of 43.7% was produced using TDZ (10 μM) and NAA (2.7 μM) probably a far improvement compared to the protocol evaluated in this study (Li et al., 2010). This regeneration rate was still regarded as rather low and Li et al. 2011 managed to increase the regeneration rate to over 95%.

It is evident that the transformation protocol evaluated has to be optimised in order to be used as a tool for plant transformation of crambe. The seed-borne pathogens must be eradicated to avoid contamination during the transformation. Proper threshing and seed cleaning where the pod is removed and debris is separated from the seeds could remove much of the source of infection. The cleaned seeds could then be sterilised as described in the method earlier, targeting at the seed surface instead of the pod surface if pathogens still would present a problem. Alternatively, seeds could be surface sterilised with a 15% $CaCl_2O_2$ solution for 25 minutes (Li et al., 2010). Production of seeds under more controlled conditions could also decrease the amount of pathogens present.

Healthy seeds of crambe are fully capable to germinate without MS salts and vitamins (data not published) and could probably be excluded.

As earlier mentioned six untransformed shoots were regenerated some even producing roots even though 5.0 mg$\times$l^{-1} kanamycin was used as a selection agent, thus this level of selection agent is not sufficient.Li et al. 2010 found that a fivefold concentration of kanamycin (25 mg$\times$l^{-1}) was suitable as a selection agent during a toxicity test of crambe. Further, more than 90% of the surviving regenerated shoots were indeed transformed after 8 weeks, while un-transformed shoots perished after 40-50 days.

The *Agrobacterium* strain EHA 105 used in this study which had an successful transformation rate of 0.6% compared to the strain AGL-1 (2.1%), the most efficient strain included in the study of Li et al. 2010.

4.2 Floral dip

An established protocol for floral dip transformation would greatly simplify the transformation process and decrease the cost per transformation event. Successful transformation protocol does exist for *Arabidopsis* and a few other species, and with acceptable transformation rates. As *Arabidopsis*, crambe is also a Brassicaceae and due to similar structural flower organisation could be accessible by *Agrobacterium* transferring DNA to the nucleus in crambe cells.

However, this needs setting up trials to test different parameters of importance for the transferring process and based on the existing protocols for *Arabidopsis* optimising them for crambe. The racemes of crambe contained

flowers of different developmental stages but microscopic studies of developing flowers may reveal what stage a successful transformation is most likely to occur. Further, the bacteria suspension seemed to have no visual negative effect on the maturing plants, which indicate that the same kind of chemical compounds could be used if desired.

4.3 Sequencing of CaDGAT1

The CaDGAT1gene most likely contain at least one intron in the amplified region which results in the different fragment size observed in Figure 7 on page 22 when comparing crambe dna (1 & 2) with crambe cDNA (3). It might be situated at one of the annealing sites of either the primer CaDGAT1-5s or CaDGAT1-4r since no distinct fragment is amplified using these primers on Crambe DNA while a clear band is seen when cDNA is used as template. This can be explained by several reasons e.g. failed PCR at this particular event or that one of the two primers are gapping the intron. To confirm the intron locations further PCR experiments or secuensing of both crambe DNA and cDNA is recommended.

A setup where CaDGAT1-4r/CaDGAT1-3s and CaDGAT1-5s/CaDGAT1-2r is used on crambe DNA instead of CaDGAT1-3s/CaDGAT1-2r and CaDGAT1-5s/CaDGAT1-4r (as seen in Figure 7 on page 22) should yield fragments of the sizes 908 bp and 711 bp respectively without any intron (see Figure 2 on page 16 for primer annealing positions on *Arabidopsis* DGAT1). The likely scenarios would be:

1. One of the fragments is significantly bigger than expected. This would indicate that at least one intron is situated in the region between CaDGAT1-3s – CaDGAT1-5s or CaDGAT1-4r - CaDGAT1-2r.

2. Both fragments are equally bigger than expected. This could be the result if one intron is situated between CaDGAT1-5s – CaDGAT1-4r.

3. Both fragments are bigger than expected, but the increase is of different magnitude. At least two inserts occur in the CaDGAT1 fragment analysed.

4. One of the primer pairs fail to amplify a fragment. This could happen if the case is as previously mentioned, either CaDGAT1-5s orCaDGAT1-4r gap the an intron.

The chance that the fourth scenario is true is rather small and a quick estimation; simply dividing the primer length with the total length of Arabidopsis DGAT1 is around 0.12%. Another approach would be to sequence the

CaDGAT1-3s/ CaDGAT1-2r CaDGAT1 fragment of DNA origin and compare with the cDNA fragment already sequenced. The sequenced part of CaDGAT1 corresponds to approximately 65% of the total length.

New primers designed to anneal closer to the lacking gap between the sequenced parts could be used for to yield the full stretch between CaDGAT1-3s - CaDGAT1-2r.

4.4 RNAi construct of CaDGAT1

The CaDGAT1 RNAi construct should be sequenced before proceeding further. The function of the construct will first be fully confirmed once expressed in crambe. It could however, prove to be really beneficial for future applications, if functional. With this RNAi construct it would be possible to down-regulate the production of TAG by 20-40% if crambe behaves in a similar manner as *A. thaliana* as described by Jako et al. 2001. For a genetically modified crambe with incorporated FAE, FAR and WS this could increase the proportion of wax esters compared to TAG. An alternative PDAT1 RNAi could probably fulfil the same purpose as PDAT and DGAT to some extent have overlapping functions (Zhang et al., 2009; Xu et al., 2012). Incorporating RNAi constructs for both PDAT and DGAT would decrease the TAG production even more drastically but as discovered by Zhang et al. 2009 the incorporation results in poor pollen quality and thus not advisable.

4.5 Current status of genetically modified crambe

Transgenic crambe with *Limnanthes douglasii* LPAAT, *Brassica napus* FAE1 and FAD2-RNAi incorporated into the genome has successfully been able produce 73% erucic acid to be compared to 60% in wild type crambe (Li et al., 2012). Crambe with incorporated FAR, WS and FAE into their genome has been able to produce seeds containing with up to 60% wax esters but these are not capable to germinate, while Two-gene crambe lines containing jojoba FAR and WS seem to have good seed setting and are able to produce 25% wax esters (Anonymous, 2013).

The transgenic ultra-high erucic acid crambe and wax ester producing crambe will tested in field trials during the summer of 2014 by SLU (Anonymous, 2013).

With an efficient transformation protocol established, new challenges arise. Some constructs will work as planned as in the case with ultra-high erucic acid crambe. Others will have to be adapted as the wax ester producing crambe. But there is no doubt that there will be progress within the field of new tailor made oil qualities from crop plants.

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Appendix

Genomic DNA extraction from plant material

1. Place the plant material in a 1.5 ml Eppendorf tube.
2. Add 300 μl of extraction buffer and grind the material with a piston.
3. Add 300 μl phenol/chloroform/isoamyl (25/24/1 V/V/V) and mix thoroughly.
4. Centrifuge 2 minutes and transfer the top phase to a new Eppendorf tube.
5. Add 300 μl chloroform and mix by flicking the tube gently.
6. Centrifuge 2 minutes and transfer the top phase to a new Eppendorf tube.
7. Add twice the volume of 99% ethanol (-20°C) and wait for 15 minutes.
8. Centrifuge 10 minutes at full speed and remove the supernatant.
9. Wash the pellet two times in 75% ethanol and then centrifuge for 1-2 minutes.
10. Remove the supernatant and let all ethanol evaporate, and resuspend the pellet in 30 μl water containing RNAase.

Southern Blot Analysis reagents

Depurination

Concentration	Component
0.2 N	HCl

Alkaline transfer buffer

Concentration	Component
0.4 N	NaOH
1.0 M	NaCl

calibrated to pH 7.2

Neutralisation buffer

Concentration	Component
0.5 M	tris-Cl
1.0 M	NaCl

calibrated to pH 7.2

Prehybridisation and wash buffers

20× SSC

Amount	Component
175.3 g×l^{-1}	NaCl
88.2 g×l^{-1}	Sodium citrate·2 H_2O

calibrated to pH 7.0

20% SDS

Amount	Component
200 g×l^{-1}	SDS

calibrated to pH 7.2

100× Denhart's Reagent

Amount	Component
20 g×l^{-1}	Bovine serum albumin
20 g×l^{-1}	Polyvinylpyrrolidone
20 g×l^{-1}	Ficoll400

0.5 M EDTA

Amount	Component
186.1 g×l^{-1}	EDTA·2 H_2O

calibrated to pH 8.0

Prehybridisation buffer

Amount	Component
25 ml	20× SSC
2 ml	20% SDS
5 ml	100× Dendhart's reagent
10 mg	Sonicated Salmon sperm DNA
2 ml	0.5 M EDTA

Add H_2O to a final volume of 100 ml

	Wash buffers			
Component	I	II	III	IV
SCC	2×	2×	0.1×	0.1×
SDS	0.5%	0.1%	0.1%	-

Sonicated Salmon sperm DNA protocol according Sambrook and Russell 2001, modified by Xiaofeng Yan and Simon Jeppson, DNA sonication based on Knight and Adami 2003.

Reagents

Amount	Component
90 mg	Salmon sperm
9 ml	DNAase-free sterile water
4 ml	Water with RNAase
310 μl	3 M Sodium Acetate pH 5.2
9 ml	phenol/chloroform/isoamyl (25/24/1 V/V/V)
9 ml	chloroform
16 ml	99% ethanol (-20°C)
32 ml	75% ethanol (20°C)

1. Dissolve 90 mg Salmon sperm DNA in 9 ml water in a 15 ml falcon tube over night and vortex to avoid clogging.
2. Add 310 μl 3 M Sodium acetate pH 5.2 to final concentration of 0.1 M.
3. Transfer the solution to 12 1.5 ml Eppendorf tubes, 750 μl each.
4. Add 750 μl phenol/chloroform/isoamyl (25/24/1 V/V/V), mix well and spin at 13 000 g for two minutes.
5. Transfer the supernatant to 12 new 1.5 Eppendorf tubes and add 750 μl chloroform, mix well and spin for an additional 2 minutes.
6. Collect the supernatants to a 15 ml falcontube.
7. Sonicate (5 microns) at varying depth for 20-30 seconds.
8. Allocate the sonicated Salomon sperm DNA to 16 new 1.5 ml eppendorf tubes (500 μl each).
9. Add 1000 μl 99% ethanol (-20°C) to each tube and precipitate for 15 minutes.
10. Spin at maximum speed for 10 minutes and decant the supernatant.
11. Wash the pellets twice with 1000 μl 75% ethanol (20°C), decant and let the remaining ethanol evaporate.
12. Re-spuspend the pellets in 100 μl water with RNAase