Using Genetic Transformation to Produce Enhanced Levels of Erucic Acid and Novel Wax Esters in *Crambe abyssinica*

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Content

Chapter 1: General introduction

1.1 *Crambe abyssinica* (crambe), general characteristics

The oil (mostly as triacylglycerol (TAG)) stored in plant seeds is not only a very crucial resource for seedling growth (Gurr et al., 1974; Carlsson, 2009; Chapman and Ohlrogge, 2012), but also an essential renewable resource for humans. Oil seed crop cultivation is an important part of global agriculture. The vegetable oil produced by oil seed crops, is being used in diets (as nutrition, flavor, additive and for frying), as well as feedstock for modern chemical industrial products like lubricants, detergents, cosmetics and pharmacology. To meet the growing demand of vegetable oil, it is pivotal to enhance the yield and quality of oil seed crops. For this reason, breeders keep making efforts to improve the traditional oil seed crops, such as soybean, rapeseed, oil palm and sunflower. On the other hand, there is also a lot of breeding research conducted on new oil seed crops, trying to use novel interesting plants for the production of specific types of oil.

Figure 1.1 Crambe morphology: A, Morphological description of a crambe plant (**a**/stem and branch; **b**/leaf; **c**/flower; **d**/petal & calyx; **e**/pistil & calyx; **f**/petal; **g**/stamens & pistil; **h**/stamens; **i**/pistil & ovary; **k**/developing seedpod; **l**/pod containing a seed; **m**/seed); **B,** Flowers and developing seedpods of crambe; **C:** A field of crambe plants about to be harvested.

The oil seed plant crambe, which is also known as Abyssinian mustard, Abyssinian kale, colewort or datran, is a new crop cultivated usually to produce high erucic acid plant oil desired by the chemical industry (Bruun and Matchett, 1963; White and Higgins, 1966). This crop is an erect, annual herb with large pinnately lobed leaves similar to mustard leaves (Figure 1.1 A and B). The plant height normally varies between one to two meters, depending on the season and the plant density. It shows an indeterminate flowering habit and may continue to set seeds even late in the season. Although crambe is mainly self-pollinated, still outcrossing was reported to occur at a rate of 4 to 7% (Meier and Lessman, 1973). Typically, the flowering period is approximately three weeks (White and Higgins, 1966). The flowers are numerous, small and produce small brownish and round seedpods of 5 mm in diameter. The seedpod consists of two parts: a terminal spherical capsule and a much smaller cylindrical lower segment (Figure 1.1 A, B and C). Each capsule-like seedpod contains a single spherical seed, which are greenish brown and 0.8-2.6 mm in diameter. As a plant preferring moderate rainfall, crambe can be field-cultivated in warm-temperate regions and does not possess a high level of frost resistance. Its center of origin is the Mediterranean region (White, 1975), and it is also prevalent across Asia and Western Europe, having been grown experimentally and commercially in a wide climate range (Wang et al., 2000).

Crambe abyssinica is an allohexaploid (2n=6x=90), which is a member of the genus *Crambe* (*Brassicaceae* family) (Rudloff and Wang, 2011). Genus *Crambe* has a disjunctive distribution among four major geographical regions: Macronesian (12 species), Mediterranean (four species)

that *C. abyssinica* belongs to, east African (three species), and Euro-Siberian-southwest Asian (ca 20 species) (Francisco-Ortega et al., 1999). *Crambe abyssinica* is morphologically similar to the other two species *C. hispanica* (n=30) and *C. glabrata* (n=15). A study based on morphological as well as molecular data sets including chloroplast DNA restriction sites and internal transcribed spacer regions analyzes, showed that *C. abyssinica* taxonomically should be organized as a subdivision under *C. hispanica* and with *C. glabrata* as a distinct species (Rudloff and Wang, 2011). So the full name of *C. abyssinica* is *Crambe hispanica* subsp. *abyssinica* Hochst. ex R.E.Fr. In fact, it is the only one cultivated within the *Crambe* genus because of the high erucic acid content in its seed oil. The oil content of crambe seed is about 35-40% per seed with pod (up to 60% when expressed per hulled seed) and the protein content is about 20-40% (Bruun and Matchett, 1963). The fatty acid composition is shown in Table 1.1. About 55% to more than 60% of total fatty acid is erucic acid (Lazzeri et al., 1994), which is 10-15% higher than in high erucic acid rapeseed (HEAR). A cloned *fatty acid elongase 1* (*FAE1*) gene from the genome of *C. abyssinica* (cv. 'Prophet') transformed and expressed in *Arabidopsis* could significantly elevate erucic acid content in the offspring seed (Mietkiewska et al., 2007). Asymmetric somatic hybridization between *Brassica napus* and *C. abyssinica* indicated that the seeds from the hybrids accumulated more erucic acid in their seed oil compared to the original *B. napus* (Don Palmer and Keller, 2011).

Table 1.1 Proportion of fatty acids in crambe oil

Fatty Acid		% of Total
Palmitic Acid	C16:0	1.8
Stearic Acid	C18:0	0.7
Oleic Acid	C18:1	17.2
Linoleic Acid	C18:2	8.7
Linolenic Acid	C18:3	5.2
Eicosanoic Acid	C20:1	3.4
Erucic Acid (*cis*-13-docosenoic)	C22:1	58-66
Brassidic Acid (*trans*-13-docosenoic)	C22:1	0.7
Tetracosanoic	C24:0	0.7
Nervonic	C24:1	1.6
Others		2.5

Crambe seed oil, due to its fatty acid composition, is not suited for making food or food additives. However, for the same reason crambe is an interesting crop for feedstock production for the chemical industry. The possible uses of crambe oil are listed in Table 1.2. Because of the high erucic acid content in crambe seed oil, its most significant value is for producing erucamide, which is in wide and permanent request all around the world. Erucamide is a derivative of erucic acid and added to plastics. The main purpose thereof is acting as lubricant to ease the production of plastic parts and to provide a thin layer of lubrication on the extruded plastic film surface, hereby reducing the coefficient of friction, acting as a 'slip-agent'. Some of the other derivatives of erucic acid like erucyl alcohol, behenic acid and behenyl alcohol are also widely used for producing wax esters, nylon, surfactants and pharmaceutical products.

Table 1.2 Oil product uses of crambe

Oil Product	Uses
Triglyceride (refined oil)	Pharmaceuticals, lubricants, heat transfer fluids, dielectric fluids, waxes and fish food coating agent.
Erucic acid/Erucamide	Antifriction coatings, plasticizers, anti-stats and corrosion inhibitors.
Behenic acid/Behenamide	Antifriction coatings, mold release agents, processing aids flow improvers and cosmetics.
Erucyl alcohol	Surfactants slip and coating agents.
Behenyl alcohol	Surfactants slip, coating agents and pharmaceutical products.
Wax ester	Lubricants cosmetics
Brassylic acid	Nylon, perfumes, plasticizers, synthetic lubricants and coatings, flavors.
Pelargonic acid	Perfumes, plasticizers, synthetic lubricants, coatings and flavors.
Fatty acids	Existing markets for C18-C22 acids and their amides.

1.2 The history of crambe breeding

Cultivation of crambe started in Europe, first in Russia in the 1930s (Mastebroek et al., 1994). After that, the cultivation extended from Russia to Sweden and Poland in the period leading up to the Second World War. Due to the war breeding in the crop almost ceased.

1.2.1 Breeding in USA

After the war in the 1960s, testing of 11 lines introduced into USA and Canada from Sweden and Russia did not show adequate crop yields. Breeding was therefore initiated at the agricultural experimental station of Purdue University (Maryland, USA) and three varieties were released in the 1970s, namely 'Prophet', 'Indy' and 'Meyer'. Through the continuation of the breeding work during the 1980s at the United States Department of Agriculture, Agricultural Research Service (USDA ARS) in Beltsville (Maryland, USA), 'BelEnzian', 'BelAnn' and C-22, C-29 and C-7 were released around 1985. Their yields and oil contents had improved compared to the earlier released cultivars (Carlsson, 2009).

1.2.2 Breeding in Europe

In the beginning of the 1990s crambe breeding in Europe was resumed at Plant Research International (PRI), Wageningen University & Research Centre the Netherlands (Mastebroek et al., 1994). About 20 accessions of old European landraces and American cultivars from the 70s and 80s were evaluated for agronomical characteristics. This study showed that the breeding effort in the USA had resulted in a 15% oil yield increase per hectare. The breeding targets of PRI aimed at improving seed and oil yield, enhancing the erucic acid content in the oil, improving the disease resistance and decreasing the glucosinolate content in the seeds. The materials selected for further breeding at PRI were one late flowering old European landrace and two early flowering newer American lines. The three crambe-lines were intercrossed and, from F3 generation onwards, selection was performed for agronomical characteristics. From that research, several cultivars have been released during the 1990s. Independently, there was another genotype (Mario) released in 1995 by the Institute Sperimentale per le Colture Industriali in Bologna, Italy (Fontana et al., 1998).

1.3 Potential use of crambe as oil seed crop

The main argument for developing crambe into a cultivated oil seed crop is the demand for high erucic acid-containing plant oil (Vargas-Lopez et al., 1999). Erucic acid that constitutes up to 60% of crambe oil is an expensive chemical compound compared with other fatty acids, due to its

various utilities in the chemical industry. It belongs to the very long chain fatty acid (VLCFAs; very long chain fatty acids are fatty acids with aliphatic tails longer than 22 carbons) class. Although erucic acid is a fatty acid compound, which is not considered to be suitable for the human diet, there is certainly a global demand for specific and significant quantities as an important industrial feedstock. At present, the high erucic acid plant oil used in industry is mainly produced by HEAR. However, HEAR cultivation poses some problems, because of the risk of cross-contamination of seeds during processing and of genetic crossing with food quality rapeseed (canola). The canola used primarily in foods belongs to the kind of low erucic acid rapeseed (LEAR). The occurrences of crossing or cross-contamination between HEAR and LEAR will have a severe negative impact on the commercial applications and value of both crops. Currently, the upper limit of erucic acid in edible oil has been set to 5% in most countries. For LEAR, the increase in erucic acid content resulting from crosses with HEAR can badly influence its edible oil quality. On the other hand, for HEAR, the decrease of erucic acid content will also make its oil less valuable. An additional problem will be caused by the changes in omega 3 (linoleic acid) and omega 6 (alpha linoleic acid) unsaturated fatty acids which make up to 37% in LEAR oil but only a mere 20% in HEAR (Renner et al., 1979). Because these fatty acids belong to the class of polyunsaturated fatty acids (PUFAs, having two or more double bonds in their carbon chain), they are believed to be valuable components with health promoting effects in the human diet (Roche, 1999). On the other hand for erucamide production, a higher level of PUFAs just means more costs for their elimination in the downstream processing phase.

If crambe would be used to produce high erucic acid plant oil, there will be no such consequences, because it is very difficult for crambe to cross-pollinate with *Brassica* species including oilseed rape (Youping and Peng, 1998). Along with this fact, crambe has several other advantages over HEAR for producing erucic acid: 1) It already has a higher amount of erucic acid in its seed oil than HEAR; 2) it is useful in crop rotations for alleviating weed, pest and disease build-up in areas where rapeseed was planted; 3) crambe is a moderate input crop, requiring fewer pesticides. Pests are easily dealt with by tillage and/or pesticides; 4) the oil profile is of interest to the chemical industry as it contains fewer PUFAs than HEAR; 5) It grows well on all soil types from heavy clays to light sands, and is more tolerant to late-season drought (White and Higgins, 1966; Stymne and Dyer, 2007; Falasca et al., 2010). Although crambe apparently has so many superior characteristics, there is still a major disadvantage that obstructs large-scale, global cultivation of crambe instead of HEAR. This is the fact that crambe is not yet competitive with HEAR with respect to its lower seed yield and less value for the seed cake. Therefore, to make crambe competitive with HEAR, its economic value needs to be improved (Stymne and Dyer, 2007).

1.3.1 Use of crambe instead of rapeseed for current erucic acid production

One strategy for the improvement of crambe's economic value is to increase the erucic acid content and decrease the amounts of PUFA further in crambe seed oil. This will mean more erucic acid yield per hectare, and less input required for the following downstream chemical modifications. With increasing erucic acid content in crambe to more than 66%, e.g. by genetic engineering, this crop can be an economically attractive alternative to HEAR. As a result of the research performed on the lipid biosynthesis pathway in plant seeds, several important genes have been identified and proposed critical to control the level and composition of erucic acid and PUFA, accumulating in seeds of *Brassicaceae* species.

1.3.2 Genes positively modulating erucic acid accumulation

Erucic acid is synthesized in the cytosol by extension of oleic acids. After the C18:1 CoA is synthesized in the plastid and released into the cytosolic pool, the fatty acid elongase complex catalyzes its elongation reaction to produce C20:1 CoA and then C22:1 CoA. Every cycle of fatty acid elongation attaches two carbons to the acyl moiety (Ohlrogge and Browse, 1995). Within each cycle of reaction there are four steps, firstly condensation of malonyl-CoA with an acyl primer, followed by a reduction, then a dehydration, and finally a second reduction. The four enzymes catalyzing this biosynthesis are known as the fatty acid elongase complex (Whitfield et al., 1993). The 3-ketoacyl-CoA synthase (KCS) (EC 2.3.1.199) catalyzing the condensation reaction plays a key role in determining the chain length of fatty acid products found in seed oils (Katavic et al., 2000). It has been reported that crambe's own *KCS1* is most likely responsible for its high seed-oil erucic acid content compared to the other members of the *Brassicaceae* family. This was also supported by the introduction and expression of crambe *KCS1* into *Arabidopsis* by genetic modification, which resulted in an elevation of the erucic acid content (Mietkiewska et al., 2007). Therefore, a stronger expression of its own *KCS1* (also known as fatty acid elongase *FAE*, *CaFAE;* in this thesis, KCS will always be referred to as FAE) might be helpful for increasing the erucic acid content in crambe seed. However, this approach was not followed in the research for this thesis.

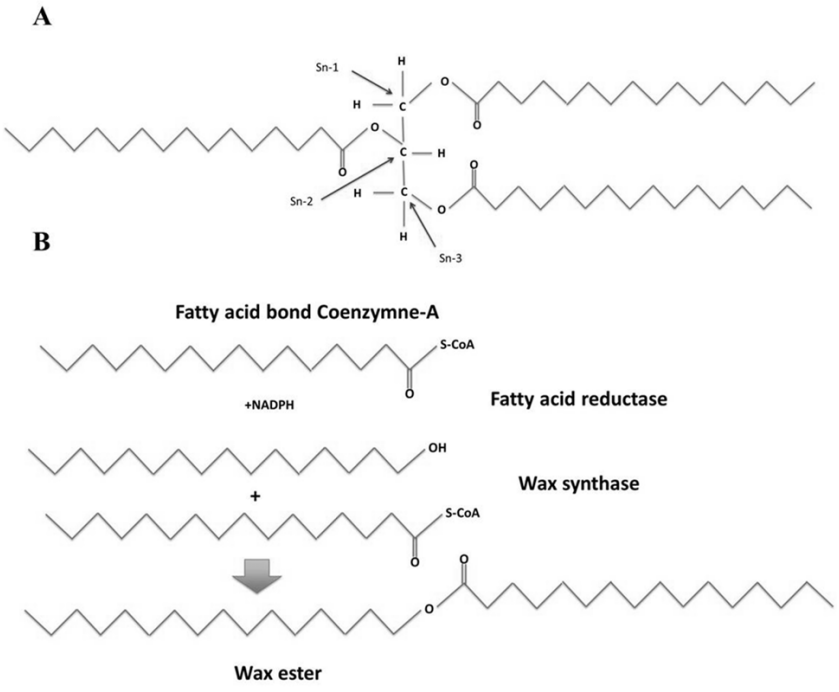

Figure 1.2 Difference between triacylglycerol and WE synthesis: Normally, oil seed plants accumulate TAG as main storage of lipids for use in seed germination. TAG is synthesized by the Kennedy pathway, in which the glycerol backbone is acylated at the positions sn-1, sn-2 and sn-3 (A). In jojoba seed, WE make up > 90 % of the seed oil (B). A fatty acid reductase or fatty acyl reductase (FAR) converts acyl-moieties from fatty acyl-CoAs to fatty alcohols: fatty alcohol acyltransferase (wax synthase, WS) catalyzes the esterification of the fatty alcohols with fatty acyl-CoAs.

Another bottleneck for raising the erucic acid content in the seed oil of crambe or even in any other *Brassicaceae* species is the endogenous lysophosphatidate acyltransferase 2 (LPAT2) (EC 2.3.51). This enzyme catalyzes acyl-CoA attachment to the sn-2 position (Figure 1.2 A) of the glycerol backbone for TAG, by an esterification bond. *In vitro* experiments indicated that the LPAT2 from *Brassicaceae* species, all have extremely low affinities to erucoyl-CoA (Kuo and Gardner, 2002), while the enzymes (glycerol phosphate acyl-transferase and diacylglycerol acyl-transferase) (Kennedy, 1961) catalyzing the sn-1 and sn-3 positions are without such kind of limitation (Kuo and Gardner, 2002). It means that the highest percentage of erucic acid within seed oil will be around 66%. Interestingly, Cao et al. (1990) found in the seed of *Limnanthes douglasii* a LPAT2 (LdLPAT2) that has a high affinity to erucoyl-CoA (Cao et al., 1990). The insertion of a gene encoding a *L. douglasii* LPAT2, capable of acylating erucic acid at the sn-2 position, led to a redistribution of erucic acid esterified at sn-2 in the oil of the transgenic seeds, and a slight increase of the total amount of erucic acid even in combination with an extra copy of *FAE1* from rapeseed (Lassner et al., 1995; Han et al., 2001; Jadhav et al., 2005). To produce a GM crambe with high erucic acid content (more than 66%) in its seed oil, the *LdLPAT2* is likely to be an essential factor that should be incorporated.

1.3.3 Genes negatively modulating erucic acid accumulation

The fact that if the erucic acid content can be as high as 66% means a sufficient supply of erucoyl-CoA, and hardly any other fatty acid moieties than erucoyl are built in on the sn-1 and sn-3 positions in crambe. When *LPAT2* from *L. douglasii* is introduced, theoretically it becomes possible to be able to achieve 100% C22:1 on the sn-2 position, and then plant oil with 100% erucic acid could be obtained. However, in practice, *L. douglasii LPAT2* transformation of *Brassica napus L.* has so far never led to an erucic acid level higher than 80% of the seed oil (Brough et al., 1996; Wilmer et al., 2000; Han et al., 2001; Nath et al., 2009). The reason why this is not 100% might be that the endogenous CaLPAT acts as a competitor to the introduced exogenous LdLPAT. It is proposed to test this hypothesis by using RNA interference to down-regulate specifically the endogenous *LPAT2*; if competition for the sn-2 position plays a role, the down regulation of the crambe endogenous *LPAT2* (*CaLPAT2*) should lead to higher erucic-acid content at the sn-2 position because of the action of *LdLPAT2* and to a higher overall percentage of erucic acid in the seed oil. An endogenous *LPAT2* cDNA of crambe has been cloned and sequenced and to down regulate this gene an RNA-interfering DNA fragment (*CaLPAT2-RNAi*) will be designed according to this sequence. A suitable conserved region has been identified consisting of the NHXXXD box, FP/VEGTR box and the sequence between them (Murphy, 2009).

In addition to the endogenous LPAT, endogenous Fatty acid desaturase 2 (FAD2) (EC 1.14.19.1) has also been found to play a limiting role in erucic acid biosynthesis. Fatty acid desaturase 2 is an enzyme that catalyzes oleic acid (C18:1) conversion into linoleic acid (C18:2) (Okuley et al., 1994; Dyer and Mullen, 2001; Yang et al., 2012). After that, the produced linoleic acid will enter the PUFA pool followed by several steps of modification (further desaturation and/or elongation), which means that once the C18:1 is transformed into C18:2, those carbon chains will no longer be available for C22:1 biosynthesis. This competition between FAD2 and erucic acid biosynthesis for C18:1 oleic acid as substrate has been proven to occur indeed by the transgene *Brassica carinata* with native *FAD2* silenced, which allowed it accumulated more erucic acid in its seeds than wild type (Jadhav et al., 2005). The erucic acid content also further increased (to 70 %) by crossing the mutant line defective in *FAD2* with GM rapeseed lines carrying the *L. douglasii LPAT* and *FAE* (Nath et al., 2009). So, another alternative for engineering increased erucic acid

accumulation in crambe seed oil would be by inhibiting the desaturation of oleic acid by knocking down *FAD2* (*CaFAD2-RNAi*) when the *L. douglasii LPAT* or/and a strong expression of native *KCS* are being introduced. Simultaneously with such increases in erucic acid, a decrease in the PUFA content will be expected.

1.3.4 Producing *Simmondsia chinensis* (jojoba) like wax esters in crambe

Another option for increasing the economic value of crambe is to have some other precious chemical compounds produced in its seed. Jojoba-like wax esters (WE; Figure 1.2B) are good candidates. Such long-chained wax esters have important applications in cosmetics and lubrication with high added value (Stymne and Dyer, 2006). An amazing characteristic jojoba, a desert plant from North America, is that it has WE as the main storage lipid in its seeds (Gentry, 1958; Miwa, 1971), instead of having TAG (Figure 1.2A) that accumulates in seeds of most other plants. Compared to conventional vegetable oils (TAGs), jojoba oil has many superior properties and can be extensively used in lubrication and transmission fluids. Jojoba WEs are ester molecules between fatty alcohols and fatty acids with long carbon chains both (C20:1, C22:1 and C24:1). The key steps of WE assembly in jojoba seeds are: first oleic acid is converted into eicosanoic acid, erucic acid or even nervonic acid (24:1) CoA by jojoba fatty acid elongase (ScFAE) (Lassner et al., 1996); and then fatty acid reductase (ScFAR) catalyzes those fatty acids to become fatty alcohols; finally, fatty acids and fatty alcohols are joined together by an esterification bond (Figure 1.2B), catalyzed by jojoba wax synthase (ScWS). The genes encoding the enzymes for each step have been cloned from the mRNAs isolated from developing jojoba seed (Lassner et al., 1999; Metz et al., 2000).

Presently, the amount of jojoba WEs that is used worldwide is very small due to the high price and low production. The price of the jojoba oil is about 5000 €/ ton. The global production of jojoba oil is about 3500 tons per year. The biggest markets for the use of jojoba oil are cosmetics (2000 tons per year) and the pharmaceutical sector (jojoba oil is said to heal acne, pimples and warts.). The jojoba oil consumption for lubrication is only around 100 tons annually. Although WE compounds comparable to jojoba oil can also be produced enzymatically from alcohols and fatty acids (bio-based or mineral-based) by artificial chemical engineering, this production system is rather expensive. To find another, cheaper alternative for production is very lucrative and necessary, if people want to extend the range of its uses, especially to the global lubricant market (polyalphaolefins 0.5 million tons and organic esters 0.05 million tons annually). One way of achieving this could be to transform *ScFAE*, *ScFAR* and *ScWS* into another plant species and use it as bio-platform to produce large quantities of jojoba WE (Lassner et al., 1999). A preliminary study has shown the encouraging result that insertion of *ScFAR* and *ScWS* from jojoba and *FAE1* from *Lunaria annua* in *Arabidopsis* resulted in transgenic plants producing seed oil with up to 70% of WE instead of the common TAGs (Lardizabal et al., 2000). An alternative approach that would bypass the need to use an elongase gene like *FAE1* is to choose plants that naturally contain high amounts of erucic acid (C22:1). Crambe, which has 60% of erucic acid in its seed oil, thus has an ideal fatty acid composition for production of jojoba type WE and becoming the desired bio-production platform (Mietkiewska et al., 2007).

1.4 European Union 7th Frame Work Project ICON and the scope of this thesis

Crambe is one of the target crops within EU 7th Frame Work Project 'Industrial Crops producing added value Oil for Novel chemicals' (ICON, website: http://icon.slu.se/ICON/). ICON was a 4-year international cooperative research project in which multiple partners from different countries were involved. The main goal for the ICON researchers was to find possibilities to replace non-renewable fossil oil as industrial chemistry feedstock with renewable plant oils. The main targets for the new crop that ICON proposed, i.e. crambe, were to increase the erucic acid content and produce jojoba-type WE in its seeds by genetic modification. All the work described in this thesis belonged to three work-packages (WP) of the ICON project.

Chapter 2: *Crambe abyssinica in vitro* regeneration and transformation

The work described in this chapter was part of the ICON WP1.1 'Increase efficiency of Crambe transformation method and determine optimal selectable marker gene'. A reliable protocol for transformation of crambe is crucial for further genetic engineering. By testing different tissues of crambe seedlings with various combinations of hormones and *Agrobacterium* inoculating procedures, we have developed a protocol of *Agrobacterium*-mediated transformation using cotyledonary nodes as explants, based on a protocol developed by Krens and coworkers for sugar beet (Krens et al. 1996). In addition to this, an efficient amplification method for *in vitro* crambe material was also set up. Both protocols were applied to the work described in the following chapters.

Chapter 3: Test the Marker-free system in the transformation of *Crambe abyssinica*

The work described in this chapter was part of the ICON WP1.2 'Test clean vector technology of PRI in Crambe'. To explore the possibilities to make GM crambe more consumer-friendly, the marker-free technology of PRI, WUR (Schaart et al., 2004) was tested in crambe transformation. In this part of the research, we first made GM plants using a pMF1-based vector (marker-free system). Then, two independent lines were selected from them, and after multiplication by *in vitro* propagation they were treated with DEX to trigger marker elimination by recombination. After applying 5-FC as the selection agent, marker-free plant material was obtained.

Chapter 4: Improve the erucic acid content of *Crambe abyssinica* seed-oil with genetic modification

Table 1.3 Gene combinations for improvement of the erucic acid content in crambe seeds

Goals	Gene combinations
Increase erucic acid content in seed oil	LdLPAT + BnFAE1
Increase erucic acid and decrease PUFA content	LdLPAT + BnFAE1 + CaFAD2-RNAi
Identify the proper domain in the mRNA of CaLPAT2 as target for RNAi	CaLPAT2-RNAi
Increase erucic acid and decrease PUFA content	LdLPAT + BnFAE1 + CaFAD2-RNAi+CaLPAT2-RNAi

The work described in this chapter was partially of the ICON WP2.2 'Production of ultra-high amounts of erucic acid in Crambe and *B. carinata*'. In this chapter, by using the protocol developed in Chapter 2, we introduced *L. douglasii LPAT*, *and B. napus FAE* and crambe *FAD2-RNAi* genes (Table 1.3) all driven by a napin promoter (Rask et al., 1998) into crambe. The T1 offspring seeds were analyzed by gas chromatography for their fatty acid composition. In this chapter we also report on the work of selecting specific sequences of crambe *LPAT2* for RNAi targeted silencing. The target sequence of the RNAi approach was from a conserved region of

Brassicaceae LPAT2 genes. It contained the conserved region consisting of the NHXXXD box, FP/VEGTR box and sequences between them (Murphy, 2009). The RNAi vector (Table 1.3) was used for transformation of crambe and *Arabidopsis*. The efficiency of RNAi was indicated by the knockdown of the endogenous *LPAT2* (*CaLPAT2*) in transformed crambe and *Arabidopsis*. Finally, this *CaLPAT2-RNAi* was assembled together with *LdLPAT, BnFAE1* and *CaFAD2-RNAi* into one construct for transforming crambe.

Chapter 5: Production of Wax ester in *Crambe abyssinica*

The work described in this chapter were for ICON WP4.2 'Production of long-chain wax esters in Crambe and *B. carinata*; Expression of ScWS+ScFAR in Crambe', and WP5.4 'Expression of [Jojoba WS]+[Jojoba FAR]+[FAD2-RNAi] in Crambe'. The genes needed to enable WE production are the jojoba *WS* and *FAR* either with or without the jojoba *FAE* (Table 1.4). Two constructs with these genes were available from the ICON project, provided by Dr. Edgar Cahoon (University of Nebraska-Lincoln, USA) with kanamycin as selectable marker (in one case with DsRed as additional visual marker for selection) and with a glycinin promoter from soybean (Iida et al., 1995) to drive the functional genes. After the establishment of GM plants carrying either *ScWS+ScFAR* or *ScWs+ScFAR+ScFAE*, they were maintained in the greenhouse for further seed ripening. The offspring seeds from T1 to T3 were analyzed and selected for increased WE content. The influence of WE content on seed germination and seedling vigor and growth was studied. In addition to the introduction of the jojoba WE genes (*FAR* and *WS*) and the introduction of the jojoba *FAE* to increase the conversion of C18:1 to C22:1, it was tested whether suppression of the native *FAD2* using RNAi can increase the substrate, C18:1, availability for conversion into C22:1 for WE yield in developing seeds.

Table 1.4 Producing jojoba-type WE in crambe

Goals	Gene combinations
WE accumulation in seeds of crambe	ScWS + ScFAR
Production of very long chain WE of the jojoba type to at least 20% in the seed oil of crambe	ScWS + ScFAR + ScFAE
Suppression of the native FAD2 to increase the supply of C18:1 for C22:1 conversion and uptake in WE	ScWS + ScFAR+ CaFAD2-RNAi

In summary, we describe a series of studies on transgene breeding of crambe, from the establishment of the protocol to the actual transformations with functional genes. These experiments demonstrated the potential value of GM crambe as oil seed platform crop and opened up the way for continuation of this work in the future.

Chapter 2: *Crambe abyssinica in vitro* regeneration and transformation

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Abstract:

In this chapter, we describe the development of a series of protocols for all steps that are required in the process of generating genetically modified plants in *Crambe abyssinica* (crambe). Explants of cotyledons, cotyledonary nodes and hypocotyls from 7-day-old crambe seedlings were tested in different hormone-combinations for shoot regeneration. The cotyledonary nodes on basic medium with 0.5 μM NAA and 2.2 μM BAP gave the highest percentages of shoot regeneration. For maintenance and propagation by tissue culture, explants of stems, petioles, leaves and axillary buds of *in vitro* plantlets were tested for regeneration using the optimal medium described above. The result was that the axillary buds had the highest efficiency of shoot proliferation. Cotyledonary nodes and/or axillary buds were used to test the proper concentration of antibiotics (kanamycin, hygromycin and bialaphos) to be able to select for transformation events, and 10 to 25 $mg \cdot L^{-1}$ for Kanamycin, 10 $mg \cdot L^{-1}$ for hygromycin and 2 $mg \cdot L^{-1}$ for bialaphos were identified as the appropriate concentration (range) for selection. The cotyledonary nodes and cotyledons from 7-day-old seedlings were used as explants for *Agrobacterium*-mediated transformation with two kinds of kanamycin selection strategies (shifting or consistent). It showed that, using the shifting selection method (two weeks on 10$mg \cdot L^{-1}$ kanamycin followed by four weeks on 25 $mg \cdot L^{-1}$, and then back to 10 $mg \cdot L^{-1}$ for at least four months) the cotyledonary node inoculations gave 10% transformation frequency, and the cotyledons 1%, in comparison with the consistent method that had a lower frequency (4% of cotyledonary nodes and 0% of cotyledons). In later experiments, *in vitro* plant axillary buds were also used as explants for the *Agrobacterium*-mediated transformation using kanamycin selection, and a rather low frequency of transformation was found ranging from 0.6 to 2%. In total, six different vectors and two kinds of *Agrobacterium* strains were tested in crambe transformation using the cotyledonary node explant type in combination with kanamycin selection. Here, the transformation efficiency varied from 1% to 10% with an average frequency of 4.4%. The results of T0 plant T-DNA insertion copy-number determination by Southern blotting showed that, there was a 50/50% chance of having multi-insertion lines and single-insertion lines.

Key words: *Crambe abyssinica*, regeneration, *Agrobacterium* mediated transformation, cotelydonary node and axillary bud.

2.1 Introduction

The industrial oilseed crop *Crambe abyssinica* (crambe) is a non-food oilseed crop from the *Brassicaceae* family, which includes crops such as rapeseed (canola and industrial rapeseed oil) and mustard (White and Higgins, 1966). Because it is a specific non-food crop, its production and processing chain has no overlap with the food production chain at all. 33% to 39% of the whole crambe seed including the pod consists of oil of which 55% to 63% is erucic acid (C22:1) (White, 1975; Rudloff and Wang, 2011). Erucic acid and its derivative erucamide (Ramírez et al., 2001) are widely used in industry and determine largely the high value of the crambe oil. Its erucic content is higher than that in the oil of most other *Brassicaceae* species, including high erucic acid rapeseed (HEAR). Currently, the main production of erucic acid is still from HEAR, which might present, however, some potential risks. These potential risks reside in intermixing of industrial HEAR with low erucic acid rapeseed (LEAR) which is used for edible oil. Due to their intrinsic different utilities, cross-contamination between them must be strictly avoided. Because it will not only decrease both their economical values (qualities), but it might also potentially threaten food security because the amount of erucic acid allowed in food oil has to be lower than 5% (w/w). To prevent such problems, crambe has been considered to replace HEARs, because it can hardly cross with any current *Brassica* oilseed crop or vegetable crop (Youping and Peng, 1998). However, an obstacle for this replacement is that crambe cultivation and production is economically not comparable to HEAR. Addressing this disadvantage of crambe, the EU funded project ICON planned to overcome this by raising its erucic content through genetic modification (GM). Higher erucic contents can reduce the total production costs including downstream processing of erucic acid, and then accordingly increase profits. Furthermore, another target of ICON is to turn crambe into a platform crop for different kinds of oils as needed by industry, with the facility of transgene technique (Stymne and Dyer, 2007).

The establishment of *Agrobacterium tumefaciens* mediated GM (Fraley et al., 1983) in plants has changed plant science studies remarkably. It also brought new strategies and methods to make molecular breeding based on GM possible within modern crop breeding research. After the first GM crop, the Flavr Savr™ tomato, which carried an artificial antisense gene copy targeted against polygalacturonase in order to prevent premature fruit softening, was put on the market in the USA, 1994 (Kramer and Redenbaugh, 1994), other GM crops were commercialized around the world in the past 20 years. In 2011 the area of GM crop cultivation was around 160 million hectares (Nábrádi and Popp, 2011). The U.S.A., Brazil and Argentina are the top-3 countries that have the biggest area of GM crops planted. GM soybean, maize and cotton ranked as first to third for their cultivation scale globally. However, in spite of this success, there are still many regions in the world, e.g. the European Union, where the policies against GM food crops are very strict. There has been a general concern of GM crops threatening people's health and the ecosystem, which exists globally and cannot be ignored. So, worldwide acceptance of food produced from GM crops is far from being a reality. On the other hand, GM crops not targeted for human consumption but e.g. for industrial feedstock production, like crambe, might be more easily accepted by consumers.

To achieve the goals of ICON mentioned above, it is important to have efficient transformation and *in vitro* regeneration/propagation protocols available for this crop. Here, we describe the development of a series of protocols for crambe for all steps that are required in the process to obtain GM crambe. It includes *in vitro* tissue regeneration, micro-propagation, selection regimes and *Agrobacterium*-mediated transformation.

2.2 Materials and methods

2.2.1 Plant materials, seed sterilization & germination

For sterilization, a gram of seeds of crambe cv. 'Galactica' were put into a 50 ml blue cap tube, firstly with 30 ml 70% (v/v) ethanol and shaken for 30 seconds for surface sterilization.

Subsequently, the ethanol was removed, and 30 ml 3% (w/v) NaClO with 300 µl Tween20 was added to the tube. With the cap closed the tube with seeds was put into a water bath at 42.5°C for 20 minutes. After that, the seeds were rinsed with sufficient sterile demi water four times. Finally, the sterilized seeds were placed onto germination medium consisting of salts and vitamins (MS) (Murashige and Skoog, 1962), 2% (w/v) sucrose and 0.8% (w/v) phytoblend at pH 5.8 in a plastic high container (50 seeds/container). The seeds were incubated at 7 °C overnight in the dark, followed by placing in the dark at 24°C for three days, and further culture at 24°C in 16h Light (with a light intensity of 33 $\mu mol \cdot m^{2} \cdot s^{-1}$) 8h Dark period (16hL/8hD) for another three days (In total, seven days of germination after seed sterilization).

2.2.2 Optimization of crambe regeneration medium

This experiment focused on verification of the appropriate concentrations for regeneration of auxin and cytokinin, and comparing two gelling agents (microagar and phytoblend) for three different explants types, cotyledons, cotyledonary nodes and hypocotyls (Figure 2.1). These explants were isolated from 7-day-old seedlings of wild type (WT) crambe, and inoculated into two series (with gelling agents of microagar and phytoblend) regeneration media with various combinations of auxin NAA of 0, 0.5, 2.5 and 5 µM and cytokinin BAP of 0, 0.44, 2.2, 4.4 and 22 µM (Supplementary data, Media components and observed results of regeneration experiments). The basic medium composition was full MS, 2 $mg \cdot L^{-1}$ $AgNO_3$ and 20 $g \cdot L^{-1}$ sucrose, pH 5.8. Medium was refreshed after four weeks and final regeneration was scored after seven weeks of culture at 24 °C with 16hL/8hD. Each combination was tested with at least 40 to 60 explants (20 explants/petri-dish) of each type. The effect of gelling agents and hormone concentrations on direct shoot regeneration and indirect shoot regeneration from the explants were evaluated by two-way ANOVA.

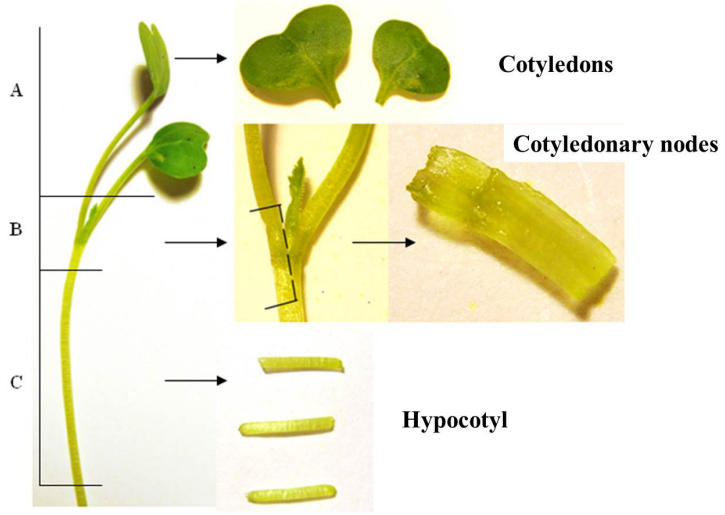

Figure 2.1 Cotyledon, cotyledonary node and hypocotyl explants from a 7-day-old crambe seedling: The A, B and C parts of the seedling are cotyledons, cotyledonary nodes and hypocotyls respectively. When cutting the cotyledonary node explants, the apical bud (indicated by the asterisk) should be removed thoroughly from the explant.

2.2.3 Regeneration capacity of *in vitro* plants

Rooted *in vitro* seedling plants of eight-weeks-old were taken for testing the regeneration potential of explant types, such as leaves, petioles, stems and axillary buds. Here, only one medium was used, i.e. the optimal regeneration medium as determined in the previous section, which was basic medium with 0.5 µM NAA and 2.2 BAP. The leaves were cut into square pieces

of 1 cm^2 and both petioles and stems were cut into small pieces with a length of 2 mm. Axillary buds were isolated by cutting stem parts carrying a petiole and leaf, removing the leaf and cutting the petiole at the part where it is connected to the stem; in this way, the axillary bud is at the end of the cut petiole. This part was used as explant and put on the regeneration medium.

2.2.4 Testing selectable agents

For this as a standard medium, full MS (salts and vitamins) with 2 $mg \cdot L^{-1}$ $AgNO_3$, 0.5µM NAA, 2.2 µM BAP, 20 $g \cdot L^{-1}$ sucrose, and 8 $g \cdot L^{-1}$ Phytoblend at pH 5.8 was used (this formula was from the optimization of regeneration medium, see the Results section 2.3.1).

For testing kanamycin (Pestka, 1974), hypocotyls, cotyledons and cotyledonary nodes of 7-day-old WT seedlings were used as explants. All explants were cultured with different kanamycin concentrations (0, 10, 25, 50 $mg \cdot L^{-1}$). Hygromycin (Blochlinger and Diggelmann, 1984) was tested with cotyledonary node explants only in a concentration range of 5, 10, 20 and 40 $mg \cdot L^{-1}$. There were 80 explants per treatment. Survival and regeneration response were recorded after two weeks.

Two kinds of herbicides, bialaphos (Murakami et al., 1986) and phosphinothricin (PPT) (Avila et al., 1998), were used to test their potential as selectable agent at a concentration range of 0, 2, 5 and 10 $mg \cdot L^{-1}$. Scoring was after five weeks of culture (without transferring to fresh medium). To fine-tune the optimal bialaphos concentration, a second experiment was done with the concentrations 0, 1, 1.5 and 2 $mg \cdot L^{-1}$. There were 80 explants (20 explants/petri-dish) for each concentration of treatment.

2.2.5 *Agrobacterium*-mediated transformation via seedling parts based on kanamycin selection

Vector and *Agrobacterium* strain: There were two vectors used for testing the crambe transformation. One is pMF1-gfp-gus, in which the *GFP* gene and *GUS* gene with the apple Rubisco-promotor (Schaart et al., 2011), fusion gene of *Neomycin phosphotransferase II* (*NPTII*) (Beck et al., 1982) and *CodA* (Schaart et al., 2004) driven by the 35S promoter. The vector was transformed into *Agrobacterium* strain AGL0 (Lazo et al., 1991). The other vector is pBinGlyRed ScFAR+ScWS (AscI) with *NPTII* and *DsRed* (Baird et al., 2000) as markers (Chapter 5, Materials and Methods section), which were driven by 35S promoter and Cassava vein mosaic virus (CMV) promoter (Verdaguer et al., 1998) respectively. The second was in *Agrobacterium* strain AGL1 (Lazo et al., 1991).

Inoculation with *Agrobacterium* and co-cultivation: An aliquot of a -80°C *Agrobacterium* stock was taken and cultured overnight in 10 ml liquid culture medium (Luria-Bertani <LB> medium + 50 $mg \cdot L^{-1}$ kanamycin + 50 $mg \cdot L^{-1}$ rifampicin) at 28 °C with constant shaking. The agrobacteria were collected by centrifugation at 3500-4000g for 10 min, subsequently the pellet was re-suspended in liquid MS medium with 2% sucrose and 100 µM acetosyringone (AS) till an optical density (600 nm) of 0.4. The cotelydonary nodes were cut from 7-day-old seedlings in liquid MS20 plus 100 µM AS. After cutting, the cotyledonary nodes were placed in a tea strainer, which was placed in the *Agrobacterium* suspension. After inoculation for 30 minutes, the cotyledonary nodes are blotted dry and placed with the curved side (outer side) on co-cultivation medium, MS20 + 100 µM AS + 0.5 µM NAA and 2.2 µM BAP, 2 $mg \cdot L^{-1}$ $AgNO_3$ and 8 $g \cdot L^{-1}$ Phytoblend, pH 5.8. The cotyledonary nodes were cut in a Petri dish with liquid MS20 containing 100 µM AS. After cutting, the cotyledon explants were also placed in a tea strainer in the *Agrobacterium* suspension. After inoculation the explants were blotted dry and placed with the cut side on co-cultivation medium. The Petri dishes were placed for 2 days at 24°C and 16hL/8hD under shaded conditions (a cheese cotton cloth on top of the Petri dishes).

Selection: After co-cultivation the explants were transferred to selection medium containing cefotaxime (200 $mg \cdot L^{-1}$) and timentin (150 $mg \cdot L^{-1}$) to kill the *Agrobacterium* and kanamycin for

the selection of the transgenic cells/shoots (20 explants/dish). Petri dishes were closed with leucopor tape and placed at 24°C and 16hL/8hD. The explants were transferred every 2 weeks to fresh selection medium. There were 2 kinds of hormone combinations used in the selection media: Formula A was 0.5 μM NAA + 2.2 μM BAP; formula B was 2.5 μM NAA + 22 μM BAP. Simultaneously, 2 selection regimes were used. Method 1 (shifting) was: selection on 10 $mg \cdot L^{-1}$ kanamycin for the first two weeks, then followed by four weeks selection on 25 $mg \cdot L^{-1}$ kanamycin, followed by further selection for at least four weeks on 10 $mg \cdot L^{-1}$ kanamycin again. In method 2 (consistent) the antibiotic concentration was high at 25 $mg \cdot L^{-1}$ continuously, and the selection period was minimally ten weeks. Table 2.1 provides an overview of the different conditions used and the explant numbers tested.

Table 2.1 Experimental design for setting up *Agrobacterium*-mediated transformation of seedling explants

Explant	Medium	Selection (kanamycin, $mg \cdot L^{-1}$)	No. of explants
Cotyledonary node	A	10→25	111
		25	116
	B	10→25	114
		25	110
Cotyledon	A	10→25	106
		25	104
	B	10→25	110
		25	98

Note: In the form, 10→25 $mg \cdot L^{-1}$ Kanamycin means the shifting selection; and 25 $mg \cdot L^{-1}$ Kanamycin means the consistent selection.

Rooting and transfer to soil: After selection, green, regenerated shoots were isolated and put onto rooting medium. The rooting medium consists of MS20 with 0.5 μM NAA and 8 $g \cdot L^{-1}$ Phytoblend. It contained cefotaxim and timentin but no kanamycin. After the *in vitro* shoots had developed adventitious roots, they were moved to the soil for further development. For this, the plants were taken carefully from the tissue culture containers and freed from medium without damaging the roots. The leaves at the bottom and middle part of the plant were removed, leaving the top three or four leaves only. Transferred shoots were covered with a beaker at first to maintain high humidity. The beaker was gradually lifted to allow acclimatization for two to three days and then replaced with a transparent and permeable cover for protecting the plant from insects till they had grown 10 cm high.

2.2.6 *Agrobacterium*-mediated transformation via axillary buds

First, *in vitro* plants were prepared by transferring regenerated shoots to rooting medium. After they grew bigger with well-developed roots and more than two visible axillary buds, they were considered ready for preparing axillary bud explants. The stem parts with an axillary bud were cut and separated. It was further divided up by transversely cutting along the petiole. The petiole side is the axillary bud explant needed for inoculation. These explants were collected and put in a tea strainer, which was submerged in the *Agrobacterium* suspension for a maximum of 30 minutes. After inoculation, the explants were blotted dry before placing them with the cut side on co-cultivation medium (20 explants per dish). The Petri dish was sealed with leucopor. Three selection schemes were used for axillary bud transformation: 1) 10 $mg \cdot L^{-1}$ consistent; 2) shifting I, i.e. 10 $mg \cdot L^{-1}$ for two weeks, 25 $mg \cdot L^{-1}$ for four weeks, and then back to 10 $mg \cdot L^{-1}$; 3) shifting II, i.e. 10 $mg \cdot L^{-1}$ for two weeks, 20 $mg \cdot L^{-1}$ for four weeks, and then back to 10 $g \cdot L^{-1}$. The vectors pBinGlyRed *ScFAR+ScWS* (ASCI) (Chapter 5, Materials and Methods 6.2.1) in *Agrobacterium* strain AGL1, and pMF1-gfp-gus in AGL0 were used to test for transformation.

2.2.7 Transformation frequency using different vectors

In further experiments to transform functional, economically important genes into crambe, six different vectors (Table 2.9) were used. These vectors were present in either *Agrobacterium* strain AGL1 or AGL0. The standard transformation protocol as described earlier was used for these vectors. The selection scheme was the one with a shift in kanamycin concentration, i.e. first two weeks on 10 $mg \cdot L^{-1}$ kanamycin followed by four weeks on 25 $mg \cdot L^{-1}$ cultivation; for the rest of the selection period, the kanamycin selection pressure was kept at 10 $mg \cdot L^{-1}$. Green shoots from regeneration clusters on the explants were cut off after four weeks on selection and placed into regeneration medium including the selective agent directly. These shoots were kept on selection medium at least ten weeks. If at that time they were still not bleached, they were transferred into rooting medium for rooting. After establishment of a rooting system, they were prepared for transfer into soil and to a greenhouse. At this stage material was harvested for DNA isolation for PCR and Southern blotting. Plants in the greenhouse (T0's) were allowed to self and set seed (T1 seed). All these vectors contained the *NPTII* as selectable marker, and in the vector pBinGlyRed ScFAR+ScWS (ASCI) there is an active reporter integrated, the fluorescent protein gene DsRed driven by cassava mosaic virus promoter; in pMF1-Gus-Rubscgfp, there are a *GUS* gene and a *GFP* gene driven by the apple Rubisco promoter.

2.2.8 Verification of transgenic nature of plants

GUS-staining: Histochemical GUS staining of leaves was carried out as described by Jefferson (Jefferson, 1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D- glucuronide (X-gluc) in 50 mM sodium phosphate buffer, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM potassium ferricyanide, 5% (w/v) polyvinylpyrrolidone-40 and 0.1% (v/v) Triton X-100. Chlorophyll was removed by washing with 70% (v/v) ethanol.

PCR: Genomic DNA was isolated from young leaves of candidate T0 plants with the method described by Aldrich and Cullis (Aldrich and Cullis, 1993) but with 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. *NPTII* primers were used to demonstrate the presence of the selectable marker gene. *VirG* primers were used for amplifying the *virG* gene (Stachel and Zambryski, 1986) from the virulence plasmid to test for the residual contamination by agrobacteria in the plant material. If the result of *virG* was positive, the material cannot be moved to the greenhouse into soil and for seed ripening.

The primer sequences were: for *NPTII*, forward 5'- TGGGCACAACAGACAATCGGCTGC-3' and reverse 5'-TGCGAATCGGGAGCGGCGATACCG-3', while for *virG,* forward 5'-GCCGGGGCGAGACCATAGG-3' and reverse 5'-CGCACGCGCAAGGCAACC-3'.

Southern blotting: Genomic DNA was isolated as described above. The design of the probe and the choice of the appropriate restriction enzyme were based on the sequence of specific vectors. The labelling system was the DIG-High Prime DNA Labelling and Detection Starter Kit I, Roche. For copy number determination (Höltke et al., 1995), a total of 40 µg of DNA was digested overnight with restriction enzyme, either *Eco*RI or *Hin*dIII, and fractionated on a 0.8% (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, UK) according to the manufacturer's recommendations. The membrane was hybridized at 65°C overnight with 20 ng of the labelled probe, either targeting to *NPTII* or 35S promoter, and washed for 2 times 30 minutes with 0.1 × saline-sodium citrate (SSC) buffer, 0.1% (w/v) SDS at 65°C.

2.2.9 Fluorescence observation

DsRed fluorescence from the plant material transformed with the construct pBinGlyRed ScFAR+ScWS (AscI) was checked using UV fluorescence microscopy (Zeiss, SteREO

Discovery.V8 equipped with PentaFluar S fluorescence equipment and PROIR, Lumen 200 illumination system).

2.3 Results

2.3.1 Shoot regeneration from seedling parts

Table 2.2 Cotyledon regeneration result from media with Microagar and Phytoblend

Microagar	NAA 0			NAA 0.5			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 0	100%	0%	0%						
BAP 0.44				100%	0%	0%	100%	0%	0%
BAP 2.2				100%	0%	0%	95%	0%	0%
BAP 4.4				100%	0%	0%	100%	0%	8%
BAP 22				100%	0%	0%	100%	0%	0%
Phytoblend	NAA 0.5			NAA 2.5			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 2.2	55%	3%	50%	98%	0%	48%	100%	0%	53%
BAP 4.4	95%	0%	35%	100%	0%	45%	100%	0%	58%
BAP 22	30%	0%	35%	80%	0%	13%	48%	0%	20%

Note: the unit of hormone concentration is μM; CI, callus induction; DSR, direct shoot regeneration; ISR, indirect shoot regeneration; shoots regenerated from callus culture.

Table 2.3 Cotyledonary node regeneration result from media with Microagar and Phytoblend

Microagar	NAA 0			NAA 0.0			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 0	0%	0%	0%						
BAP 0.44				25%	90%	0%	78%	53%	10%
BAP 2.2				48%	98%	0%	85%	23%	5%
BAP 4.4				30%	85%	3%	57%	73%	7%
BAP 22				7%	90%	0%	83%	33%	5%
Phytoblend	NAA 0.5			NAA 2.5			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 2.2	35%	100%	15%	50%	100%	3%	45%	100%	3%
BAP 4.4	35%	98%	8%	53%	95%	0%	58%	100%	0%
BAP 22	15%	100%	3%	35%	100%	0%	8%	50%	0%

Note: see explanation in Table 2.1

Table 2.2 shows frequencies of callus formation, direct shoot regeneration (DSR) and indirect shoot regeneration (ISR) of cotyledon explants with different hormone combinations and gelling agents after four weeks of culture. Although cotyledons formed callus on all hormone combinations in the media with Microagar, no shoot regeneration occurred after seven weeks of culture with one exception, while the explants on Phytoblend-solidified media did show shoot regeneration. The shoot regeneration of cotyledons mostly took place indirectly from the callus. DSR happened only on medium with 0.5 μM NAA and 2.2 μM BAP at a low percentage. The percentage of indirect shoot regeneration from callus decreased with increasing BAP

concentrations. The results of two-way ANOVA analysis showed that 1) the explants on Phytoblend medium gave significantly ($p<0.01$) more ISR than those on Microagar medium; among the media with Phytoblend, the BAP concentration significantly influenced the ISR ($p<0.05$), but the NAA did not; 3) neither gelling agent nor hormones had an effect on the DSR.

In comparison with cotyledons, the cotyledonary node explants (Table 2.3) gave less callus and ISR but more DSR, varying from 23% to 90% on medium with Microagar. With Phytoblend as gelling agent, the explants gave extreme high levels of DSR among all treatments and the efficiencies ranged from 50% to 100%. The combination of 0.5 μM NAA with Phytoblend showed more ISR than the other combinations. The Phytoblend medium with 0.5 μM NAA and 2.2 μM BAP had explants with 100% DSR and 15% ISR, which was the best. In the Phytoblend mediums, the frequency of ISR went down when BAP and NAA concentration were increased. According to the two-way ANOVA analysis, the conclusions are 1) in the Microagar media, the NAA concentration influenced the DSR and ISR significantly, negative for DSR but positive for ISR; 2) in the Phytoblend medium, neither NAA nor BAP influenced the direct shoot-regeneration; 3) within Phytoblend media, the NAA concentration significantly affected the ISR ($p<0.05$).

Hypocotyl explants (Table 2.4) also showed a lot of callus formation on media with Microagar plus 0.5 μM NAA, varying from 93.3% to 100%, fewer calli were formed on media with 5 μM NAA. No DSR or RSR was found on the media with Microagar with one exception (5 μM NAA + 2.2 μM BAP). Using Phytoblend, less callus induction was observed on 0.5 μM NAA than on 2.5 and 5 μM NAA. On the hypocotyl explants, only one direct shoot was formed on medium with 0.1 $mg \cdot L^{-1}$ NAA and 5 $mg \cdot L^{-1}$ BAP. On medium with 0.5 μM NAA and 2.2 μM BAP, 3% indirect shoots were formed. According to the result of two-way ANOVA analysis, no effector (gelling agent or hormone) was significant for DSR or ISR.

Table 2.4 Hypocotyl regeneration result from media with Microagar and Phytoblend

Microagar	NAA 0			NAA 0.5			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 0	0%	0%	0%						
BAP 0.44				100%	2%	0%	58%	0%	0%
BAP 2.2				100%	0%	0%	87%	0%	2%
BAP 4.4				93%	0%	2%	72%	0%	0%
BAP 22				100%	0%	0%	28%	0%	0%
Phytoblend	NAA 0.5			NAA 2.5			NAA 5		
	CI	DSR	ISR	CI	DSR	ISR	CI	DSR	ISR
BAP 2.2	88%	0%	0%	100%	0%	3%	100%	0%	0%
BAP 4.4	95%	0%	3%	100%	0%	3%	100%	0%	0%
BAP 22	88%	0%	0%	100%	3%	0%	100%	0%	0%

Note: see explanation in Table 2.1

In summary, according to the results above, the optimal medium for crambe shoot-regeneration induction is full MS, with 20 $g \cdot L^{-1}$ sucrose and 2 $mg \cdot L^{-1}$ $AgNO_3$, using 8 $g \cdot L^{-1}$ Phytoblend as gelling agent, with hormone-combination of 0.5 μM NAA and 2.2 μM BAP. Cotyledons had the highest ISR ratio, and cotyledon nodes had the highest DSR ratio and moderate ISR ratio, while explants from hypocotyls had very limited DSR and ISR regeneration.

2.3.2 Regeneration capacity of *in vitro* plant parts

The results of testing the regeneration capacity of explants derived from established *in vitro* plants are shown in Figure 2.2 in which the direct and indirect shoot regeneration numbers were counted together. *In vitro* leaf parts gave no shoot regeneration at all on the pre-selected regeneration medium, but merely some calli. The petioles had a very limited regeneration capacity of 2.5%. Stem explants showed a moderate frequency of shoot regeneration at 30%, which was all indirectly from callus; only the explants of axillary buds reached a high level of shoot regeneration. After two weeks, 70% of the cultured explants showed shoot regeneration, which was mostly direct. After five weeks, a regeneration percentage of 95% was observed; regeneration was both direct and indirect. In this whole procedure, axillary nodes always obtained significant highest shoot-regeneration ratio (test by one-way ANOVA $p<0.01$).

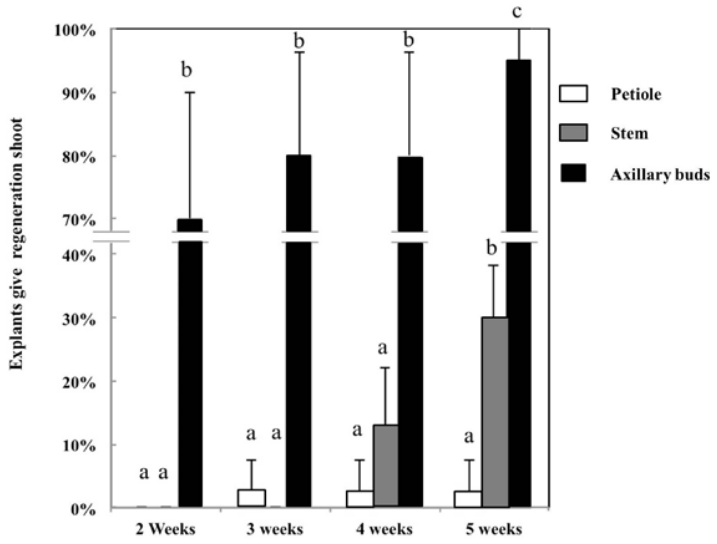

Figure 2.2 Regeneration capacity of explant types from *in vitro* plants: After being subjected to regeneration medium for two weeks, the axillary buds began to give shoot regeneration (direct and indirect), but the other kinds of explants just started their shoot regeneration after three weeks. After five weeks, the stem explants also have significantly higher shoot-regeneration frequency than the petiole slices, but lower than the axillary buds. Regeneration was scored at different time intervals for four different explant types. The letters above the error bars showed the result of one-way ANOVA analysis.

2.3.3 Selectable agent testing

The results of testing kanamycin treatments on WT seedling explants cv. 'Galactica' are shown in Table 2.5. The controls, growing on medium without kanamycin for four weeks, showed vigorous growth and callus and shoot development for all three explant types. On medium with 10 $mg \cdot L^{-1}$ kanamycin, the number of surviving explants (stay green and with green regenerated shoots) decreased, as well as the callus formation and shoot regeneration, but still some explants with green regeneration-shoot clusters were left. Kanamycin at the concentration of 25 and 50 $mg \cdot L^{-1}$ completely inhibited shoot regeneration from all types of explants.

Hygromycin was also tested for the appropriate selection conditions on cotyledonary node explants from 7-day-old seedlings of WT cv. 'Galactica' and the results are shown in Table 2.6. Hygromycin concentrations of 20 $mg \cdot L^{-1}$ and higher could eliminate 100% of the WT explants,

while there was still 20% survival on 10 mg·L^{-1}. 5 mg·L^{-1} in the medium is not enough to inhibit growth of WT explants with a survival rate of 100%.

Table 2.5 Effect of different kanamycin concentrations on the explants of hypocotyl, cotyledonary node and cotyledon

	Percentage of explants with green regenerating shoot			
	0 mg·L^{-1}	10 mg·L^{-1}	25 mg·L^{-1}	50 mg·$^{-1}$L
Hypocotyl	100%	35%	0%	0%
Cotyledonary node	100%	17%	0%	0%
Cotyledon	100%	19%	0%	0%

Note: the explants are from 7-day old in vitro seedlings, as in the research on shoot regeneration above.

Table 2.6 Effect of different hygromycin concentrations on regeneration of cotyledonary node explants of crambe

Hygromycin concentration (mg·L^{-1})	0	5	10	20	40
Rate of surviving explants with green shoots	100%	100%	20%	0%	0%

Note: only cotyledonary node explants from 7-day-old in vitro seedlings were used.

Finally, herbicide selection was tested in the same system and the results of PPT and bialaphos are shown in Table 2.7. These two kinds of herbicides were effective in eliminating growth of WT explants after five weeks at concentrations of 5 and 2 mg·L^{-1} respectively. The 21.3% of the explants with 2mg·L^{-1} PPT selection had regeneration of tiny shoots or primordial. At a concentration of 2 mg·L^{-1} bialaphos, there were only a few explants showed callus growth, but without any shoots regeneration. The rest explant with higher concentration of bialaphos had even no callus regeneration.

Table 2.7 Effect of different PPT and bialaphos concentrations on regeneration of cotyledonary node explants of crambe

Herbicide concentration (mg·L^{-1})	Explants with green shoot-regeneration (Direct and indirect)
0	100%
2 PPT	21%
5 PPT	0%
10 PPT	0%
2 bialaphos	0%
5 bialaphos	0%
10 bialaphos	0%

Note: only cotyledonary node explants from 7-days-old in vitro seedlings were used.

2.3.4 Transformation

As displayed in Table 2.8, cotyledons and cotyledonary node explants, with two methods of selection (shifting: 10→25 mg·L^{-1} kanamycin; consistent: 25 mg·L^{-1} kanamycin) and two hormone combinations (A: 0.5 μM NAA + 2.2 μM BAP; B: 2.5 μM NAA + 2.2 μM BAP) were used for transformation, and the explants were tested for GUS activity after eight weeks of

selection after *Agrobacterium* inoculation. GUS-positive shoots were detected on cotyledonary nodes on both media with shifting selection; but only the explants on medium A with shifting selection showed GUS staining and were PCR positive (Figure 2.3). For cotyledons, GUS-positive shoots were only obtained by explants on media A, and shifting selection showed a higher frequency than the consistent. Within these combinations, the highest percentage of GUS positive shoots was 10% for cotyledonary nodes from medium A with shifting selection. In summary, for all kinds of media and explant types, there were more GUS-positive shoots observed with the shifting selection method; the explants on medium A gave higher rates of transformation for both cotyledonary nodes and cotyledons. The cotyledonary nodes gave more positive shoots then the cotyledons, but not all plants that survived kanamycin selection for eight weeks proved to be GUS positive.

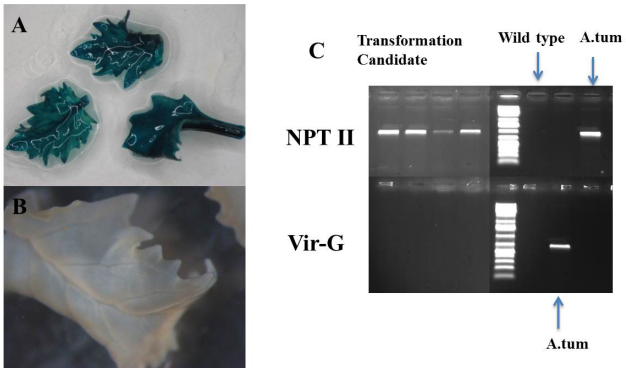

Figure 2.3 GUS staining and PCR test of putative transgenic shoots: A, GUS staining of leaf samples from a transformation candidate obtained from cotyledonary nodes with a shifting selection scheme; B, GUS staining of a leaf sample from the WT control; C, PCR using either primers for *NPTII* (upper panel) or *virG* (lower panel). DNA samples from four randomly chosen transformation candidates obtained from cotyledonary nodes on medium A were tested. DNA from WT crambe was used as negative control and *Agrobacterium* as positive control.

Table 2.8 Effect of different explant types and selection methods on transformation frequencies

Explant type	Medium	Selection	No. of Explants	No. of explants with regenerating shoots	No. of explants with GUS+ regenerating shoots	Transformation Frequency
Cotyledonary node	A	10→25	111	17	11	10%
		25	116	1	1	1%
	B	10→25	114	16	4	4%
		25	110	0	0	0%
Cotyledon	A	10→25	106	4	4	4%
		25	104	1	1	1%
	B	10→25	110	0	0	0%
		25	98	0	0	0%

Note: transformation frequencies are calculated as the percentage of GUS+ shoots per number of explants.

2.3.5 Transformation of axillary bud explants

Axillary buds of *in vitro* grown plants could potentially also be used as explant for inoculation (Table 2.9). To test their suitability some preliminary transformation experiments were done in

which different vectors were used as well as different selection schemes. The numbers of surviving, green shoots were scored after 20 to 30 weeks of selection, which were subsequently screened for GUS activity or DsRed fluorescence. The results are summarized in Table 2.9. Transformation events were obtained, however at lower frequencies when compared to cotyledonary node explants. The highest frequency of transformed shoots (2.0%) was obtained when the kanamycin shifting selection method with 10 mg·L^{-1} two weeks, followed by 20 mg·L^{-1} for four weeks, and then back to 10 mg·L^{-1} was used. The experiment with a slightly higher kanamycin concentration in the second period (10→25 mg·L^{-1}) yielded a very low transformation rate of 0.5%. Consistent selection with 10 mg·L^{-1} required a longer selection period of 30 weeks. A regenerated transgenic shoot from the transformation with pBinGlyRed (ASCI) is shown in Figure 2.4. All putative transgenic shoots were confirmed by PCR for the presence of *NPTII* gene.

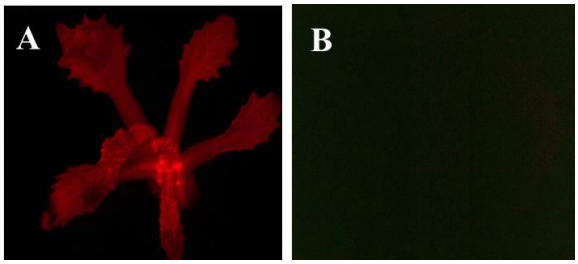

Figure 2.4 DsRed fluorescence in a transformation candidate after transformation by pBinGlyred (ASCI) (A) and a WT control (B): The fluorescent shoot in chart A was obtained from the transformation of pBinGlyred (ASCI) using the axillary bud explant from *in vitro* plant for inoculation.

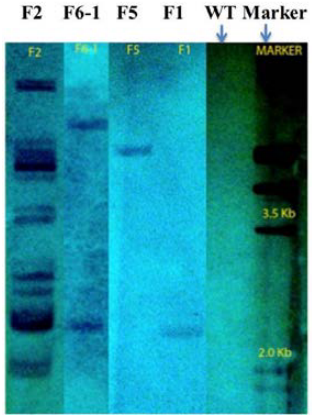

Figure 2.5 Southern blotting: DNA samples from four transgenic candidates (coded as F2, F6-1, F5 and F1 from left to right) of a pBinGlyRed transformation are presented. The restriction enzyme was *Dra*I and the probe was generated using the same *NPTII* primers as used for PCR. Next to the molecular weight marker lane control DNA from WT crambe is loaded. The lower size limit of fragments to be visualized is 2.3 kb.

Table 2.9 Transformation frequencies using axillary buds from *in vitro* grown crambe plants

Experiment ID	Kanamycin treatment	Vector	Term	Frequency
Tr11-05	10 mg·L^{-1}, Consistent	pBinGlyRed (ASCI)	30 weeks	1.6%
Tr12-01	10 → 25 mg·L^{-1}	pMF1-gus-Rubscgfp	20weeks	0.5%
	10 → 20 mg·L^{-1}		20 weeks	2.0%

Note: the success of transformation was verified by PCR, GUS staining or fluorescence.

2.3.6 Differences in frequencies of cotyledonary node explant based transformation with different binary vectors

In total, twelve different transformation experiments are listed in Table 2.10. The binary vectors used all contained the *NPTII* gene as selectable marker but differed in backbone (origin) and reporter genes or genes-of-interest involved in the regulation of seed oil composition. The vectors were present in two comparable *Agrobacterium* strains, either AGL0 or AGL1. AGL0 is an EHA101 with the T-region deleted, which also deletes the *aph* gene; for AGL1, genotype is AGL0 (C58 pTiBo542) recA::bla, T-region deleted Mop(+) Cb (R) (Lazo et al., 1991). All of the transformation events were confirmed by PCR, and representative samples were proven for their transgenic nature by Southern blotting (Figure 2.5). The average frequency was 4.4%, after an average selection period of 19 weeks. The pHan2 in AGL0, and pBinGlyRed ScFAR+ScWS+ScFAE (*Kpn*I) in AGL1 showed relatively low frequencies, i.e. lower than 2%. The variation from one experiment to the other is also evident for pMF1-gus-gfp and pBinGlyRed ScFAR+ScWS (*Asc*I). The T-DNA insertion copy-number of T0 GM crambe was partially determined by Southern blotting (Supplementary Table 2).

Table 2.10 Using different vectors in two *Agrobacterium* strains

Experiment Code	Vector	Vector Size/bp	*A.tum*	Period /week	Percentage of transformation
Tr08-01	pMF1-gus–gfp	21,071	AGL0	20	9.9%
Tr08-02	pMF1-gus–gfp	21,071	AGL0	19	4.4%
Tr12-01	pMF1-gus–gfp	21,071	AGL0	20	4.0%
T11-03	pMF1-gus–gfp	21,071	AGL0	19	7.5%
T10-01	Phan2	16,783	AGL0	24	1.5%
T09-10	pBinGlyRed ScFAR+ScWS (*Asc* I)	14,937	AGL1	19	4.0%
T10-02	pBinGlyRed ScFAR+ScWS (*Asc* I)	14,937	AGL1	16	3.8%
T10-03	pBinGlyRed ScFAR+ScWS (*Asc* I)	14,937	AGL1	18	5.8%
T09-10	pBinGlyRed ScFAR+ScWS+ScFAE (*Kpn* I)	17,662	AGL1	19	1.0%
T10-04	pBinGlyRed ScFAR+ScWS+ScFAE (*Kpn* I)	17,662	AGL1	18	2.0%
T12-02	pHellsgate LPAT2-RNAi	15,392	AGL1	20	2.5%
Tr12-05	pWatergate-3G	23,746	AGL1	20	2.3%

The average term of the Cotyledonary node explant transformation protocol is as long as 20 weeks.

Note: in pMF1-gus-gfp and pBinGlyRed ScFAR+ScWS (*Asc*I) there are extra selectable markers; Rubisco::GUS in pMF1-gus-GFP and Rubisco::GFP (Chapter 3, Material and Method); CMV::DsRed in pBinGlyRed ScFAR+ScWS (*Asc*I) (Chapter 5, Materials and Methods).

2.4 Discussion

In this chapter a series of protocols for crambe *in vitro* regeneration, selection and transformation are described. The research presented here showed that the most efficient regeneration was obtained from cotyledonary nodes isolated from seedlings and axillary buds taken from *in vitro* grown plantlets as explant types. A combination of the plant growth regulators NAA (0.2 µM) and BAP (2.2 µM) in MS, 20 $g \cdot L^{-1}$ sucrose and 2 $mg \cdot L^{-1}$ $AgNO_3$, pH 5.8 (referred to here as MS20) was shown to be the most optimal. Furthermore the gelling agent used was found to play an important role in regeneration efficiency; with Phytoblend being better than Microagar. An alternative could be Gelrite, which was used by Li (Li et al., 2010) and Chhikara (Chhikara et al., 2012) for crambe. In their protocols, hypocotyl explants from seedlings were successfully used

for shoot regeneration. In our hands, no efficient regeneration was found from hypocotyls. In this study, the regeneration frequency with cotyledonary nodes and with axillary buds was higher than with hypocotyls, however, the mode of regeneration might be different. Cotyledonary nodes and axillary buds contain meristematic tissue. They consist of undifferentiated cells, intended to grow into a shoot. This explains why they showed more direct shoot regeneration than other explant types both from seedlings as well as from *in vitro* plants (leaf, petiole, cotyledon, stem and hypocotyl). Meristem outgrowth, especially regeneration from axillary buds is a useful tool for *in vitro* plantlet multiplication or propagation (Walkey, 1972). *In vitro* propagation can be extremely useful in multiplying any putative transgenic crambe events, generating sufficient clonal material for either DNA isolation or rooting and transfer of T0 lines into the greenhouse. Cloned individuals can be subjected to further antibiotic selection, thereby decreasing the chance of getting escapes or chimeras. The latter is important because using explant types containing meristematic tissue showing little callus-formation and indirect regeneration are considered prone to producing chimeric plants. In our hands, the clonally propagated putative transgenic lines were subjected to continued selection on 10 $mg \cdot L^{-1}$ kanamycin for approximately three weeks, and when the entire clone kept green (entire regenerating cluster), it meant that the candidate was transgenic; if some bleached, it meant that the original shoot was either an escape or a chimera, for which longer selection was needed. However, prolonging the selection period does not guarantee elimination of all chimeric tissue. To exclude totally any chance of working with chimeric plants, going through a seed phase is considered to be essential in order to get stable, homogeneous transgenic plant lines.

For efficient plant transformation protocols, the choice of the proper selection agent and the optimal concentration to use is crucial. In the present study, we tested killing curves for kanamycin, hygromycin and the related herbicides phosphinothricin (PPT) and bialaphos on cotyledonary node explants and determined the concentrations at which regeneration would be totally abolished, preferably without complete killing of the original explant. In addition to determining the optimal concentration also different schemes were tested, varying from a specific concentration continuously throughout the entire selection to shifting from one starting concentration, to another second phase concentration and back to the original concentration again. The results indicated that the shifting treatments gave better results for transformation efficiency than the consistent one in case of kanamycin selection. The shift in concentrations originated from the notion, that although in the tolerance test on WT material, 10 $mg \cdot L^{-1}$ kanamycin still allowed escapes up to a level of more than 15% after four weeks, the consistently high selective pressure of 25 $mg \cdot L^{-1}$ kanamycin might be too harsh for the selection as only a limited number of transformants could be obtained in this way, both with cotyledon as well as with cotyledonary node based transformation. Therefore, the strategy of changing the concentration during the selection period herewith providing a window of stringent selection temporarily was developed and indeed higher transformation efficiencies were obtained. It is still unknown how after transformation the untransformed cells mixed with the transformed ones influence each other in their response towards the selective antibiotics. We expect that in an explant the majority of untransformed cells surround a minority of transformed cells. Dying untransformed cells might negatively influence the viability of transformed cells hampering regeneration or untransformed cells might benefit from the detoxifying capabilities of transformed cells and survive selection giving rise to escapes or perhaps chimeras depending on the regeneration process. GUS staining and measuring DsRed fluorescence shortly after co-cultivation demonstrated that there were only a few areas on the cotyledonary node explants that showed evidence of gene transfer (Figure 2.6). In the following selection period, those regions could show further growth and development

indicating stable integration of the transferred DNA, but most of them disappeared which was an indication for only transient expression. Our research showed that a moderate selection scheme was favorable for CNE–based transformation. On the other hand, in the kanamycin test, the escape rate for hypocotyl explants at a concentration of 10 $mg \cdot L^{-1}$ was much higher (37%) than for the other explant types tested (19% for cotyledons and 17% for cotyledonary nodes). This could suggest that the tolerance level of hypocotyls to kanamycin was higher than that of other explants. According to Li's protocol of crambe transformation (Li et al., 2010) which was based on hypocotyl explants, 25 $mg \cdot L^{-1}$ kanamycin was used for selection, while it gave 1% to 3% transformation frequency. Hence, the consistent selection at 25 $mg \cdot L^{-1}$ could be suited for selection in hypocotyls, but not for selection in cotyledons and cotyledonary nodes. In addition to kanamycin, we also tested hygromycin and the herbicides PPT and bialaphos. Sudesh *et al,* 2010 have reported a protocol of crambe transformation based on hygromycin (Chhikara et al., 2012). The concentration they used is 20 $mg \cdot L^{-1}$, which is comparable to the optimal concentration we found in our killing curve result with WT explants. As far as the herbicides are concerned, the results suggested that bialaphos performed better than PPT, with 5 $mg \cdot L^{-1}$ being sufficient for selection. We also performed some transformation based on selection by hygromycin and or by bialaphos (data not shown). Generally, the systems did not work as well as kanamycin selection, although some transformants could be obtained.

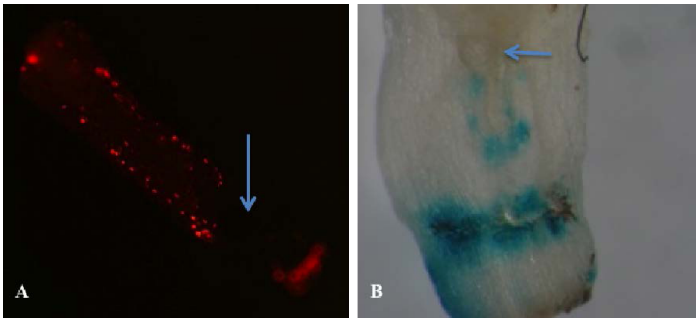

Figure 2.6 Cotyledonary node explants shortly after co-cultivation: A, DsRed fluorescence in a cotyledonary node explant after co-cultivation with *pBinGlyRed ScFAR+ScWS* (AscI)/AGL1; B, GUS staining of a cotyledonary node explant after co-cultivation with pMF1-gus-gfp/AGL0; Arrows are showing the meristematic zone in the explants, note the absence of visible gene transfer in these zones as judged by the absence of color.

In the transformations where we did use kanamycin selection, the transformation frequencies using cotyledon explants or *in vitro* axillary bud explants ranged from 0.5 to 4%, while the cotyledonary-node method varied from 1 to 10% with an average of 4.4% with respect to the efficiency. Previously, similar protocols have also been used on transformation of sugar beet (Krens et al., 1996) and caraway (Krens et al., 1997). In caraway, the cotyledonary-node transformation method yielded confirmed transformants through a seemingly direct shoot regeneration process. On the other hand for sugar beet, no positive transformed shoot was obtained from the first series of direct regenerants, but only from later series with or without visible signs of dedifferentiation. In this research, similar to sugar beet, we never found any GUS positive transformants using reporter constructs such as pMF1-GFP-RubGUS or other vectors among the direct regenerating shoots from cotyledonary nodes. All the GUS positive and DsRed fluorescing shoots were obtained later after the first, directly regenerating shoots had been removed. During the process of cotyledonary node and axillary bud regeneration and transformation, we noticed that from these meristematic explants, all the callus and indirect regenerating shoots were formed in the region around the original meristematic zone, where direct

regenerating shoots emerged. This might suggest that the actively growing meristem cells can influence the adjacent cells inducing them to differentiate into developing shoots. Meanwhile, the meristem cells themselves are only rarely transformed during inoculation (Figure 2.6). Therefore, we believe that the transformed shoots were mostly derived through indirect regeneration, thereby reducing the chance of finding chimeras composed of transgenic and non-transgenic cells. Crucial in this phenomenon is whether the regeneration comes from a single cell or from a cluster of multiple cells. The latter cannot be excluded, especially not in case of the axillary bud explants. Although cotyledons gave more indirect shoot regeneration from primary formed callus, there were just too few transgenic events leading to the generation of transgenic shoots. Accordingly, the cotyledonary-node method was chosen as the main protocol for the transformation of crambe. Meristematic tissues have also been used for sunflower (Schrammeijer et al., 1990), cotton (Chlan et al., 1995) and soybean (Christou et al., 1990) transformation.

This new protocol (using crambe cotyledonary nodes as explant, $8g \cdot L^{-1}$ phytoblend gelling agent with full MS, 0.5 µM NAA and 2.2 µM BAP as basic regeneration medium, shifting selection method) was applied for transformation with several different vectors. No correlation could be found between vector traits such as size or origin and the transformation frequency, nor was any difference observed between the two *Agrobacterium* strains used (AGL0 and AGL1). *GUS* driven by the apple Rubisco promoter and *DsRed* driven by CMV promoter (Verdaguer et al., 1998), proved to be both good visible markers for monitoring transformation. However, GFP activity could not be scored satisfactory in crambe using this transformation system because of too high levels of background fluorescence. Finally, with all vectors used we found T-DNA insert copy numbers ranging from one single copy to more than six multiple copies. The chance of acquiring multiple T-DNA insertion lines (51%) was similar to getting single copy-insertion lines (49%).

Although axillary bud transformation showed low efficiencies compared to the cotyledonary nodes, this method might serve as a more convenient alternative method for retransformation using different selection agents or after generating marker-free plants (Chapter 3). However, further improvements are still needed to make it into a stable and reliable method. If the transformation frequency can be stabilized around 2%, it will be good enough to become a proficient tool for the retransformation of crambe.

Supplementary Table

Supplementary table 1: Media components and observed result of regeneration experiments

Microagar			No. of explants evaluated	Callus induction		Direct shoot regeneration		Regeneration from callus cultures	
Hormones (µM)		Explant type		No.	%	No.	%	No.	%
NAA 0.5	BAP 0.44	Cotyledons	40	40	100	0	0	0	0
		petcot	40	10	25	36	90	0	0
		hypocotyl	60	60	100	1	2	0	0
	BAP 2.2	Cotyledons	35	35	100	0	0	0	0
		petcot	40	19	48	39	98	0	0
		hypocotyl	40	40	100	0	0	0	0
	BAP 4.4	Cotyledons	40	40	100	0	0	0	0
		petcot	40	12	30	34	85	1	3
		hypocotyl	60	56	93	0	0	1	2
	BAP 22	Cotyledons	40	40	100	0	0	0	0
		petcot	30	2	7	27	90	0	0
		hypocotyl	40	40	100	0	0	0	0
NAA 5	BAP 0.44	Cotyledons	40	40	100	0	0	0	0
		petcot	40	31	78	21	53	4	10
		hypocotyl	60	35	58	0	0	0	0
	BAP 2.2	Cotyledons	40	38	95	0	0	0	0
		petcot	40	34	85	9	23	2	5
		hypocotyl	60	52	87	0	0	1	2
	BAP 4.4	Cotyledons	40	40	100	0	0	3	8
		petcot	30	17	57	22	73	2	7
		hypocotyl	60	43	72	0	0	0	0
	BAP 22	Cotyledons	40	40	100	0	0	0	0
		petcot	40	33	83	13	33	2	5
		hypocotyl	60	17	28	0	0	0	0
NAA 0	BAP 0	Cotyledons	40	0	0.0	0	0	0	0
		petcot	40	0	0.0	0	0	0	0
		hypocotyl	60	0	0.0	0	0	0	0

Phytoblend			No. Of explants evaluated	Callus induction		Direct shoot regeneration		Regeneration from callus cultures	
Hormones (µM)		Explant type		No.	%	No.	%	No.	%
NAA 0.5	BAP 2.2	Cotyledons	40	22	55	1	3	20	50
		petcot	40	14	35	40	100	6	15
		Hypocotyl	40	35	88	0	0	0	0
	BAP 4.4	Cotyledons	40	38	95	0	0	14	35
		petcot	40	14	35	39	98	3	8
		Hypocotyl	40	38	95	0	0	1	3
	BAP 22	Cotyledons	40	12	30	0	0	2	5
		petcot	40	6	15	40	100	1	3
		Hypocotyl	40	35	88	0	0	0	0
NAA 2.5	BAP 2.2	Cotyledons	40	39	98	0	0	19	48
		petcot	40	20	50	40	100	1	3
		Hypocotyl	40	40	100	0	0	1	3
	BAP 4.4	Cotyledons	40	40	100	0	0	18	45
		petcot	40	21	53	38	95	0	0
		hypocotyl	40	40	100	0	0	1	3
	BAP 22	Cotyledons	40	32	80	0	0	5	13
		petcot	40	14	35	40	100	0	0
		hypocotyl	40	40	100	1	3	0	0
NAA 5	BAP 2.2	Cotyledons	40	40	100	0	0	21	53
		petcot	40	18	45	40	100	1	3
		hypocotyl	40	40	100	0	0	0	0
	BAP 4.4	Cotyledons	40	40	100	0	0	23	8
		petcot	40	23	58	40	100	0	0
		hypocotyl	40	40	100	0	0	0	0
	BAP 22	Cotyledons	40	19	48	0	0	8	20
		petcot	40	3	8	20	50	0	0
		hypocotyl	40	40	100	0	0	0	0.

Supplementary table 2: **T-DNA insertion copy-number of the T0 Crambe**

	Copy number of T0 Plant						
Vector	Number of T0 Plant with Certain T-DNA insertion Copy-number	1	2	3	4	5	≥6
pMF1-gus -gfp		2	2	2	0	0	1
Phan2		0	1	1	0	0	1
pBinGlyRed ScFAR+ScWS (*Asc* I)		14	1	2	2	1	1
pBinGlyRed ScFAR+ScWS+ScFAE (*Kpn* I)		2	0	1	1	1	0

Chapter 3: The generation of marker-free, genetically modified plants in *Crambe abyssinica*

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Abstract

The vector pMF1-RbcSGFP-RbcSGUS (pJS-M14) used in the transformation described in chapter 2 belongs to the marker-free genetic modification system developed by Wageningen UR Plant Breeding. The T-DNA region of the vector has a *Recombinase R-LBD* gene fusion and a *codA* and *nptII* gene fusion between two recombination sites. After transformation, the application of dexamethasone (DEX) can activate the recombinase, which will remove the two gene fusions mentioned above. Marker-free plants, generated by recombination, can be obtained through negative selection on 5-fluorocytocine (5-FC), because the *codA* part encodes an enzyme that can convert 5-FC into the toxic compound 5-fluorouracil (5-FU). In the research described in this chapter, firstly, we studied the effect of different DEX concentrations on regeneration and found that up to 25 μM the regeneration from axillary buds was not affected negatively. Similarly, the effect of different 5-FC concentrations on regeneration was assayed and concentrations up to 200 $mg \cdot L^{-1}$ were proven to be without any negative effect. To confirm toxicity of 5-FU for *Crambe abyssinica* (crambe) plant material we subjected axillary bud explants to increasing concentrations of it and we found that 50 $mg \cdot L^{-1}$ 5-FU is enough to induce total cell death in wild type explants, hence a concentration of 200 $mg \cdot L^{-1}$ 5-FC was chosen as selection in further experiments. Among the T0 lines obtained using pJS-M14, one with a single T-DNA insertion and another with a double insertion were chosen for producing marker-free plants by DEX-induced recombination and 5-FC selection. A series of DEX concentrations (0, 5, 15, 25 μM) was tested in order to identify the optimal concentration in generating marker-free plants. In contrast to earlier experiments with other crops, DEX treatment of crambe was administered through the rooting medium instead of by submergence. DEX treatment was continued into the subsequent axillary bud regeneration phase followed by negative selection on 5-FC containing regeneration medium. Marker-free (MF) plants were indeed obtained. The application of DEX was essential for production of marker-free plants but no significant difference was found among the different concentrations used. Although the 5-FC selection clearly reduced the number of non-MF regenerants, still numerous non-recombined plants survived and also chimerism was observed, both for transgenic and wild type cells in the original transgenic lines as well as for marker-free and non marker-free cells after DEX and 5-FC selection. Ultimately, by going through a seed phase the fully homogeneous marker-free nature of plants was guaranteed.
In summary, the results showed a successful application of the pMF1-based vector system to make marker-free transgenic crambe.

Key words: *Crambe abyssinica*, marker-free, recombination induction, dual selection, chimerism, seed.

3.1 Introduction

There is a lot of controversy about genetic modification (GM) of crops, while the research on GM crops is still going on. From scientific literature it is clear that GM crops can be beneficial for people, planet and profit with sustainable improvements of quantity or quality of plant products (Franke et al., 2011). However for the continuation of the GM research and the application of its products in future, wide general social approval is still essential, something which cannot be achieved soon. The main problem is the doubt that still exists with many people, non-governmental organizations and governments around the world on the safety of GM crops. A common argument is that the food produced from GM organisms might be potentially harmful to human health, because proteins, which are encoded by the inserted genes, could prove to be toxic or allergenic to humans. This can be tested before a new GM crop is taken into production (Halford and Shewry, 2000). So with a proper test system, this risk can be decreased. However, because food is directly consumed entering straight the human metabolism, there is always a chance that people will remain sceptic about GM foods. In comparison to the food crops, GM non-food crops might have better possibilities for acceptance by the general public. Another vital point in the discussion on GM crops is concerned with the marker genes used for selecting transformation events. At present, those markers are mainly genes coding for antibiotic or herbicide resistance. There is concern about a hypothetical possibility that when GM crops with marker genes are grown in fields, there will be a chance of horizontal gene flow of the antibiotic resistance genes into the genomes of the microorganisms living in the soil leading to the development of antibiotic resistant pathogens (Allen et al., 2010). Similarly for herbicide resistance some people fear that by crosspollination between a GM crop and wild (weedy) relatives a kind of super weed will be created (Dale et al., 2002).

Figure 3.1 The T-DNA part of vector pJS-M14: RB is right border; LB is left border; there are two recombination-sites (RS), and in between them there are 3 genes (combinations), i.e. *Recombinase R-LBD*, *codA-nptII* and *gfp*. Outside the RS sites there is the marker gene *gusintron,* acting as gene-of-interest. The *gusintron* and *gfp* were both driven by apple 1.6kb Rubisco promoter and apple Rubisco terminator. After recombination, the genes between the RS sites will be removed, while the *gusintron* gene will remain. The unique restriction site *Eco*RI is used for digestion prior to Southern blotting; the *gfp* gene is the target.

To avoid the above-mentioned risks, it is better to produce transgenic crops without antibiotic and/or herbicide resistance genes or any other sequences that are not desired in the final product. Some novel selection strategies making use of other selective agents than herbicides and antibiotics have been developed, for example the positive selection method using the *Streptomyces rubiginosus xyl*A gene in the T-DNA (Haldrup et al., 1998). These new marker genes are regarded as less risky, but because they are mostly from microbiological origin, they probably still run the risk of being disliked by the public. Therefore, other strategies for transformation have been developed, such as the marker-free system. Till now, several systems have become available to obtain marker-free GM crops (Sugita et al., 2000; Daniell et al., 2001; Hare and Chua, 2002; de Vetten et al., 2003; Puchta, 2003; Schaart et al., 2004; Vanblaere et al., 2011). Wageningen UR Plant Breeding developed some of these. One of them based on marker excision consists of a *Recombinase* gene from *Zygosaccharomyces rouxii* that is fused to the ligand-binding domain (LBD) of the rat glucocorticoid receptor. This gene fusion is under the control of a 35S promoter (Odell et al., 1985) that results in a continuous and ubiquitous expression of the combined gene in the transformed plant. Because cytosolic factors will bind to the LBD, the R recombinase-LBD protein cannot enter the nucleus. When transformed plant cells are exposed to the chemical dexamethasone (DEX), this will initiate competition for the LBD binding sites. With DEX bound to the LBD, the R recombinase-LBD protein is able to enter the nucleus where it induces recombination of DNA that lies between the recombination sites (RS).

Gene sequences between these recombination sites, so including the marker gene, can be removed in this way. The PRI system uses an (*neomycin phosphotransferase II*) *nptII* gene (Beck et al., 1982) still as the selectable marker, but it is fused to a cytosine deaminase gene (c*od*A) (Mullen et al., 1992) of *E. coli*, which allows negative selection against transformed cells without recombination (Schaart et al., 2004). This is done by placing transformants on a medium with non-toxic 5-fluorocytosine (5-FC). The 5-FC will be converted into the toxic compound 5-fluorouracil (5-FU) by action of the *cod*A protein part enabling selection of successfully recombined cells but eliminating those without recombination. The CaMV 35S promoter drives the combined c*odA-nptII* gene for the expression in all tissues. Both the R-recombinase-LBD gene and the *cod*A-*nptII* gene are placed between the recombination sites so that they can be removed after recombination and subsequent selection. This entire system is present in a binary vector called pMF1 (Figure 3.1). In addition to the marker removal system between the recombination sites this vector also contains a multiple cloning site (MCS) that can be used for insertion of genes of interest.

Here we report applying the pMF marker-free system of Wageningen UR Plant Breeding to *Crambe abyssinica* (crambe) genetic modification. Crambe is a non-food oil seed crop (White and Higgins, 1966; Falasca et al., 2010; Rudloff and Wang, 2011). Its seed oil has wide utilities in chemical industry because of the high erucic content (Bruun and Matchett, 1963; Lazzeri et al., 1994; Lalas et al., 2012). Furthermore, it is also a potential oil crop as a platform for various kinds of feedstock oil for industry based on genetic modification (Stymne and Dyer, 2006; Carlsson, 2009). Hence, producing marker-free crambe is considered to be very interesting with respect to increasing consumer acceptance and to allowing retransformation for further improvements if required. In present research, a model construct of the pMF marker-free system carrying two reporter genes to monitor individual steps in the process in transformation of the non-food oil seed crop crambe, was used. After the treatment with DEX and selection on 5-FC, we found marker-free GM plants after induction of recombination. This demonstrated the potential of using this vector system to produce, in the future, a marker-free GM crambe crop with altered traits, e.g. in oil composition that might be more acceptable to the general public.

3.2 Materials and methods

3.2.1 Plant materials, vector and transformation

Seeds of crambe cv. 'Galactica' (seeds harvested in July 2009 from a seed production field in Wageningen, The Netherlands) were sterilized and germinated *in vitro* to obtain cotyledonary nodes as starting material for transformation and regeneration (see Chapter 2, 2.2.3). The *in vitro* material was cultivated on solid MS20 medium (full MS, 20 $g \cdot L^{-1}$ sucrose + 8 g/l Phytoblend; pH 5.8) in growth chambers with a photoperiod of 16 h with a light intensity of 33 $\mu mol \cdot m^2 \cdot s^{-1}$ and a temperature of 24°C. In total, 300 cotyledonary node explants were inoculated with *Agrobacterium*. The transformation was carried out as described in Chapter 2.

Binary vector pMF1-RbcSGFP-RbcSGUS (pJS-M14) was used in this study. Figure 3.1 shows the T-DNA part of that vector. There are two recombination sites in the T-DNA, one of them just beside the right border. Between the two recombination-sites, there are three genes, the *R recombinase-LBD* gene fusion, the c*odA-nptII* and *gfp*. The first two are driven by the CAMV 35S promoter and contain a NOS terminator, the *gfp* gene (Chalfie, 1995) is driven by the promoter and terminator of the apple Rubisco Small subunit gene (Schaart et al., 2011). Outside the recombination region, there is the *gus* gene, which is also driven by the apple Rubisco promoter and terminator and acts as gene-of-interest in this case. *Agrobacterium tumefaciens* strain AGL0 (Lazo et al., 1991) was used for transfer of the vector.

3.2.2 Determination of the effect of dexamethasone on regeneration

To test the influence of the DEX treatment on the regeneration of *C. abyssinica* explants, axillary bud explants were cut from rooted, *in vitro* grown plants and subjected to regeneration media

containing various concentrations of DEX (5, 10, 15 and 25 μM). After 4 weeks, the general condition of these explants and their regeneration responses were scored.

3.2.3 Determination of the effect of 5-FC and 5-FU on regeneration

Similarly as in the previous paragraph, axillary bud explants were taken and subjected to regeneration media, this time containing 5-FC in concentrations of 0, 10, 50, 100 and 500 $mg \cdot L^{-1}$ or 5-FU in the concentrations of 0, 5, 10, 50, 100 $mg \cdot L^{-1}$. After 4 weeks, the general condition and regeneration responses of these explants were scored.

3.2.4 Transformation of crambe with the model construct pJS-M14

Cotyledonary node explants were infected with a suspension of *Agrobacterium* in liquid MS20 (OD_{600} 0.4~0.5). After two days co-cultivation, the explants were put on selection medium (MS20 + 0.5 μM 1-naphthaleneacetic acid (NAA) and 2.2 μM 6-benzylaminopurine (BAP) + 8 $g \cdot L^{-1}$ Phytoblend + Kanamycin (Km) for regeneration and selection; in the first 2 weeks, the Km concentration is 10 $mg \cdot L^{-1}$, then the explants are transferred onto 25 $mg \cdot L^{-1}$ for further selection for approximately 1 month. Subsequently, the non-bleached shoots are isolated and placed on selection medium of 10 $mg \cdot L^{-1}$ Km for 4 months. After that the non-bleached shoots were placed on rooting medium (MS20 solid + 0.5 μM NAA + 8 $g \cdot L^{-1}$ Phytoblend). During the following 4 weeks, the shoots grow bigger and form roots. When the plants reached this phase, their axillary buds were isolated and torn apart, and put on regeneration medium. New callus and regenerating shoots were formed in 2 to 4 weeks, and each original primary transformed shoot can be amplified into dozens this way. Amplified shoots were put on selection medium with 10 $mg \cdot L^{-1}$ Km again. In two weeks' time, when all shoots derived from one original transformant remained alive and green on selection medium without any bleaching, the original T0 transformant could be considered as a confirmed transformation event.

3.2.5 Verification of transformation

GUS-staining: Histochemical GUS staining of leaves was carried out as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM potassium ferricyanide, 5 % (w/v) polyvinylpyrrolidone-40 and 0.1 % (v/v) Triton X-100.

PCR: Genomic DNA was isolated from young leaves of candidate T0 plants with the method described by Aldrich and Cullis (1993) but with 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. The *nptII* primers were used to confirm the transgenic nature of candidate material (Aldrich and Cullis, 1993). The *virG* primers are used for amplifying the *virG* gene (Stachel and Zambryski, 1986) from the virulence vector to test for the presence of remaining agrobacteria in the plant material. In case of positive results with the *virG* primers, the plant cannot be counted as GM candidate nor can it be moved into soil for seed ripening. Later *nptII* primers and *gfp* primers were used to identify the marker-free nature of plant material after DEX treatment. The primer sequences were: for *nptII*, forward 5'-TGGGCACAACAGACAATCGGCTGC-3' and reverse 5'-TGCGAATCGGGAGCGGCGATACCG-3', for *virG,* forward 5'-GCCGGGGCGAGACCATAGG-3' and reverse 5'-CGCACGCGCAAGGCAACC-3', and for *gfp* forward 5'- CGACAAGCAGAAGAACGGCATC- 3' and revers 5'-CGGCGGTCACGAACTCCAG- 3'. The reaction was performed in a volume of 15 μl with 0.4 μl of Dreamtaq™ DNA polymerase (Fermantase), 0.5 μl of each primer, 1.5 μl 10 x Buffer and 70 ng DNA. Cycling conditions were 94 °C for 1 min, followed by 35 cycles of 94 °C (30 sec), 59 °C for *nptII* and *virG* or 58 °C for gfp (20 sec), and 72 °C (30 sec) with a final extension at 72 °C for 5 min. for both genes. The conditions of gel electrophoresis were as: 1% (w/v) TBE agarose gel, 110 V for 30 min. The expected fragment size is about 650 bp for *nptII*, 550 bp for *virG* and 218 bp for gfp. Staining was with GelRed.

Southern blotting: Isolation of genomic DNA was as described in the PCR section. Because a lot of DNA is required for loading in case of crambe, we used 1 gram of fresh young leaves from *in vitro* amplified shoots or greenhouse grown plants. Probe design and restriction enzyme selection were based on the sequence of *GFP*. The location of the probe (566 bp) is given in Figure 3.1 as are the restriction sites of *Eco*RI. For labelling the DIG-High Prime DNA Labelling and Detection Starter Kit I, Roche was used. For copy number determination, a total of 40 µg of DNA was digested overnight with *Eco*RI and fractionated on a 0.8% (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, UK) according to the manufacturer's recommendations. The membrane was hybridized at 65°C overnight with 20 ng of the labelled probe and washed two times for 30 minutes with 0.1 × saline-sodium citrate (SSC) buffer, 0.1% (w/v) SDS at 65 ºC. The primers of amplifying the probe are: forward 5'-ACAAGTTCAGCGTGTCCG-3', and reverse 5'-CTCGTTGGGGTCTTTGCT-3'.

3.2.6 qRT-PCR

Two pairs of primers, specific for the *nptII* gene and *codA* gene respectively, were developed based on their sequences for qRT-PCR. The primer pairs were for *nptII* forward 5'-AGGAAGCGGTCAGCCCAT-3' and reverse 5'-GCGTTGGCTACCCGTGATAT-3', and for *codA* forward 5'- TAACGCTTTACAAACAATTATTAACGCCCG -3' and reverse 5' ATTTTTCCGTCCTGCAGATGAATCT -3' giving fragments of respectively 58 and 81 bp. The gene β-actin 2 (GI: 20465834) was used as a reference gene (forward 5'-GATGGAGACCTCGAAAACCA-3', reverse 5'-AAAAGGACTTCTGGGCACCT-3'). For RNA isolation, total RNA was extracted from 0.5 g leaf material of *in vitro* plant with RNeasy Plant Mini Kits (Qiagen, Germany) according to the manufacturer's instructions. The isolated RNA was treated with RNase-free TURBO DNase (Ambion, USA) to remove residual genomic DNA. First-strand cDNA was synthesized in 20 µl from 1 µg of total RNA with iScript™ cDNA Synthesis Kit (Bio-rad, USA). The cDNA samples were 20 × diluted and used as templates for real-time PCR. The PCR reaction contains 2 µl template, 5 µl SYBR Green Super Mix (Bio-rad, USA), and 1 µl of each of the forward and reverse primers (3 µM) in a total reaction volume of 10 µl. Cycling conditions were 1 cycle at 95°C for 3 min followed by 30 cycles at 95°C for 10 s, 60°C for 1 min, then a final melting step ramp from 65°C to 95°C with 0.5 °C increments per cycle to monitor specificity. PCR reactions were performed in triplicate. The expression of each replicate was normalized by the reference gene, β-actin 2, which has shown to be stably expressed in both crambe seedlings under no and arsenate stress as well as in various *Brassica napus* cultivars (Hu et al., 2009; Paulose et al., 2010; Cheng et al., 2013). The relative expression level of each replicate was calculated according to the comparative CT method (User bulletin no. 2, ABI PRISM 7700 Sequence Detection System, December 1997; Perkin-Elmer, Applied Biosystems). The mean of three biological replicates represents the relative expression level of a line.

3.2.6 Application of DEX and subsequent selection steps

Table 3.1: The scheme of DEX treatments and 5-FC selection

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8
Time	6 weeks	2 weeks	2 Weeks	3 weeks	3 weeks	3 weeks	3 weeks	6 weeks
Medium	Rooting	Regeneration	Regeneration	Regeneration	Regeneration	Regeneration	Regeneration	Rooting
Agent	DEX	DEX	DEX+5FC	5FC/Km	5FC	5FC	5FC	None
Explants	Shoot	Axillary buds		Shoots	Shoots	Shoots	Shoots	Shoots
Selection Subcycle				Subcycle 1	Subcycle 2	Subcycle 3	Subcycle 4	
Analysis						PCR+GUS	PCR+GUS	

Note: the regenerating shoots from the axillary buds were divided in Step 4, and some of them were subjected to 5-FC selection; and the rest to Km selection. In the other subcycles, only 5-FC was used for selection. At the end of subcycle 3 and 4, all of the surviving regenerating shoots were checked by GUS-staining, some of them were also checked by PCR.

The general setup of this experiment with its individual treatments is displayed in Table 3.1.

DEX treatment to induce recombination (Step 1, 2 and 3)

Two independent T0 lines were chosen, of which one had a single T-DNA insertion (line 1), the other had a double insertion (line 2). The two T0 lines were vegetatively multiplied according to the *in vitro* axillary bud regeneration method described in Chapter 2, to have enough regenerating shoots for the DEX treatment. The DEX treatment was applied through incorporation in the rooting medium on which multiplied shoots were placed. The DEX concentrations tested were 0, 5, 15 and 25 μM. Table 3.2 shows the number of rooting shoots from each line for each DEX concentration (each shoot was marked with a unique number); the shoots were subjected to DEX through the rooting medium for six weeks; after that, axillary buds from these rooted shoots were cut and put onto regeneration medium with the same DEX-concentration as the rooting medium they were from, for two weeks. After these two weeks of DEX treatment of axillary bud explants, the regeneration medium was renewed, and the new medium was supplemented with 200 $mg \cdot L^{-1}$ 5-FC as selectable agent together with DEX for another two weeks (each axillary bud had a unique code to mark its origin).

Table 3.2 The DEX treatments on the chosen T0 lines

T0 line	DEX Con. (μM)	Rooting Plants	Axillary buds
1	0	16	62
	5	18	60
	15	19	62
	25	13	28
Total		66	212
2	0	8	23
	5	9	19
	15	16	40
	25	13	26
Total		46	108

Selection for marker-free shoots (Step 4 to step 8)

After Step 3, regenerating shoots from the axillary-bud explants were isolated and taken for further selection. The regeneration medium was used as basic medium in Step 4. The regenerating shoots were divided into two groups evenly, one was for 5-FC (200 $mg \cdot L^{-1}$) selection (subcycle 1), and the other for Km (10 $mg \cdot L^{-1}$) selection in order to get an idea about the efficiency of the DEX treatment in generating marker-free shoots capable of survival on 5-FC medium but bleaching or dying on Km medium. After 3 weeks the condition of the shoots was monitored. During this Step 4 as expected, each surviving shoot had developed into a regeneration cluster. At the end of subcycle 1, the regeneration clusters on 5-FC selection were split into individual shoots and the healthy ones were kept for the next 5-FC selection subcycle. The clusters from Km selection, however, were discarded after their condition was recorded. In subcycles 2 to 4, only 5-FC selection was applied at 200 $mg \cdot L^{-1}$. The term for every subcycle was three weeks. During every new subcycle, the isolated shoots grew into regeneration clusters again. At the end of each term, the condition of those clusters was recorded, and healthy shoots were taken for the next subcycle (every regenerating shoot in each subcycle received its own unique code for tracking its origin.)

At the end of subcycles 3 and 4, after the clusters were split up into individual shoots, tissue from every shoot was taken for GUS staining. In addition, some of these shoots were tested by PCR. The shoots for PCR at the end of subcycle 3 were randomly selected (all the clusters were covered), but the shoots for PCR at the end of subcycle 4 were picked only when they showed a

positive result in GUS staining. The primers used in the PCRs were for testing the presence or absence of the *npt*II gene. If the shoots were GUS positive and PCR negative, they were considered as potentially marker-free; in case the shoots showed positive for both tests, they were regarded as non marker-free; if negative for both, it was considered to be WT.

All surviving shoots at the end of subcycle 4 were transferred to rooting medium (Step 8). After the development of roots, the shoots were put into the soil and further cultured in the greenhouse to obtain seeds. The seeds were germinated in petri-dishes with 3 layers of filter paper wetted thoroughly and grown in 24ºC darkness for 5 days, which gave etiolated seedlings. And those seedlings of next generation were tested again for PCR, gfp fluorescence and GUS staining and analysis for identification of homogeneous marker-free individuals.

3.3 Results

3.3.1 Determination of the effect of dexamethasone on regeneration

Axillary buds from *in vitro* grown wild type (WT) plants were subjected to regeneration medium with various DEX concentrations (0, 5, 15, 25 μM). After 4 weeks, overall phenotype and regeneration frequency were scored. All explants treated with DEX, irrespective of the concentration used, showed no differences in visual appearance and showed similar regeneration frequencies as the ones without DEX treatment. In all cases, the percentage of explants giving regeneration was around 95%. So, there were no indications for a significant effect of the *in vitro* DEX treatment on the regeneration of shoots from axillary buds. The same DEX concentrations were used later in the DEX treatment given to the pJS-M14 transgenic plant material.

3.3.2 Determination of the proper concentration of 5-FC for selection

Regeneration of WT axillary bud explants on medium with 5-FC

Application of 5-FC in the regeneration medium at any of the concentrations (0, 10, 50, 100 and 500 $mg \cdot L^{-1}$) tested did not show any significant effect (positive or negative) on the regeneration from axillary bud explants in 4 weeks (data not shown).

Regeneration of WT axillary bud explants on medium with 5-FU

Table 3.3 The 5-FU treatment on WT crambe axillary bud explant regeneration

Treatment ($mg \cdot L^{-1}$)\ status	All Green	Half bleached	All bleached	Total
0	40	0	0	40
5	40	0	0	40
10	38	2	0	40
50	0	0	40	40
100	0	0	40	40

Note: In table 3.3 the status of regenerants from axillary bud explant on media with increasing 5-FU concentrations after 4 weeks is shown. With or without 5-FU, the explants kept regenerating, but at high concentrations of 5-FU the phenotype of the regenerating shoots was severely affected. 'All Green' means the regenerating shoots in a cluster were all green; 'Half bleached' means that in a cluster some of them were green, but the rest was bleached; 'All bleached' means that all regenerating shoots were bleached.

As an effect of the 5-FU, regenerating shoots from the axillary bud explants turned white (the bleaching started from the shoot tip and then to the bottom), while those on medium without 5-FU stayed green. The treatment of axillary bud explants with 5-FU in regeneration medium at concentrations of 50 $mg \cdot L^{-1}$ and 100 $mg \cdot L^{-1}$ showed complete bleaching in all subjected WT explants in 4 weeks (Table 3.3). The 5 $mg \cdot L^{-1}$ 5-FU treatment on axillary bud explants showed no visible effect, the regeneration of the explants and the color of the regeneration shoots were the same as that of the 0 $mg \cdot L^{-1}$ treatment. For the treatment with 10 $mg \cdot L^{-1}$ 5-FU, only two explants were found with some bleaching in regenerating shoots, which also indicated insufficient

selection. According to the results of 5-FC and 5-FU, in later selection for marker-free material, 200 mg·L^{-1} 5-FC was used in all experiments.

3.3.3 Transformation of crambe with the marker-free vector pJS-M14

From 400 inoculated explants, multiple green regenerating shoots were obtained after 20-weeks of Km selection. Sixteen independent transformation events were isolated, and GUS staining and PCR analysis proved their transgenic natures. The T-DNA insert copy-number of those events was verified by Southern blotting. One single T-DNA insertion line and one double T-DNA insertion line were chosen for triggering recombination by a DEX treatment (Figure 3.2). The qRT-PCR analysis on the expression levels of the *npt*II and *cod*A genes indicated that the introduced genes in the line with a double T-DNA insertion had a significantly stronger expression than those in the single T-DNA insertion line (Figure 3.3).

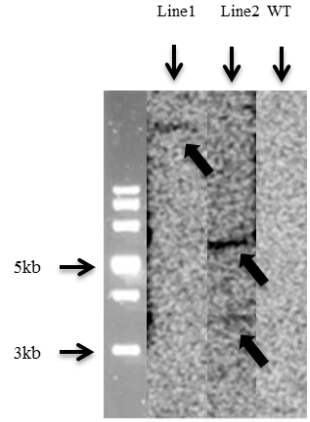

Figure 3.2 T-DNA insertion number verification by Southern blotting: The probe used was a fragment of the *gfp* gene and *Eco*RI was chosen for the restriction because this combination allowed copy number determination. Line 1 and line 2 represent two separate T0 events, with a single T-DNA insertion and double copy insertion, respectively. WT represent the wilt type, non-GM crambe cv. 'Galactica' control; the outer left lane shows a molecular weight marker. Hybridizing fragments should have a minimal size of 2.8 kb. The wide arrows in the chart marked the position of the bands.

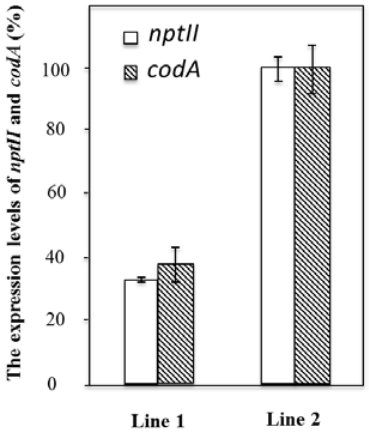

Figure 3.3 qRT-PCR analysis on *in vitro* vegetative material of line 1 and line 2: The chart provide the qRT-PCR data on the expression of the *nptII* gene and the *codA* gene in the *in vitro* leaf material of two selected original lines without any treatment. The average relative expression level of the highest performing line (Line 2 for both genes) was set at 100%. Statistical analysis doing a T-test (student t-test) showed that the difference in expression between both lines was significant ($p<0.01$) for both genes.

3.3.4 The effect of the DEX treatment on rooting of *in vitro* shoots

The different DEX treatments were administered to regenerating shoots through the *in vitro* rooting medium. Although in previous experiments, no effect of the DEX on regeneration from axillary buds was found, here, high concentrations of DEX did show a negative effect on the rooting of the inoculated shoots. As shown in Figure 3.4, DEX concentrations of 15 and 25 µM yielded lower rooting percentages. The negative correlation between rooting and DEX concentration was found to be significant by correlation analysis following Pearson (2-tailed).

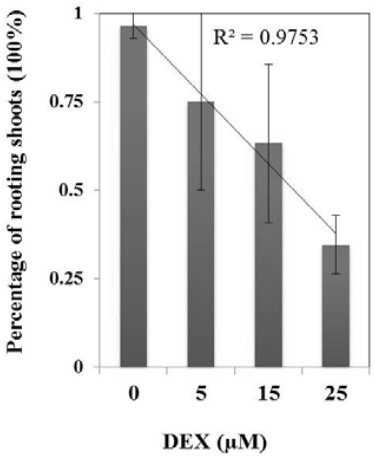

Figure 3.4 The effect of a 6-week DEX treatment on the rooting of shoots: The percentages of shoots giving roots on media with different DEX concentrations are given, together with the standard error of means as bars on the columns. The percentage of rooting shoots was significantly correlated (at 0.05 level) with the DEX concentration according to Pearson correlation analysis (2-tailed) in SPSS.

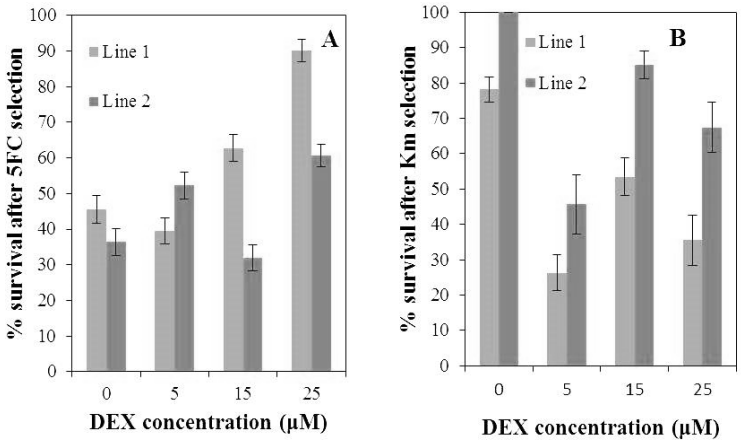

Figure 3.5 The effect on survival of regenerating shoot clusters of 5-FC and Km at subcycle 1: Chart A demonstrates the effect of 5-FC selection for both lines after different treatment with DEX; Chart-B gives the effect of Km selection. The bars on the column represent the standard error (SE).

3.3.5 The efficiency of marker-free plant generation as monitored by the treatments with 5-FC and Km at subcycle 1

As shown in Table 3.1, the regenerating shoots in subcycle 1 from the axillary bud explants were cut and subjected to cultivation on either 5-FC or Km for 3 weeks. During this selection, the

individual shoots kept regenerating and became regeneration clusters at the end of the term. The regeneration clusters consisted of green shoots, white shoots or a mixture. The survival rate is defined as the number of clusters that still had green shoots left. The survival rates after the 5-FC treatments for 3 weeks are presented in Figure 3.5A. According to a Chi-square test, the survival rates of DEX (5, 15, 25 μM) treated material of both lines were significantly higher than the ones without DEX. Comparing the two GM lines, their 5-FC survival rates were significantly different from each other, with the fraction of 5-FC survival being generally lower for line 2 than for line 1. Moreover, the Km treatments also showed differences related to the various DEX treatments. Figure 3.5B displays the rates of subjected explants giving no bleaching of shoots. Surprisingly, the explants of line 1 without DEX showed bleaching of regenerating shoots at 12.8%, while the materials without DEX from line 2 showed no bleached shoot at all, as expected. Chi-square tests also showed that on Km selection, the shoot-clusters on any treatment with DEX (5, 15, 25 μM) gave significantly lower percentages of survival shoots than the explants on 0 DEX; no significant differences were found between the various concentrations tested. The material of line 1 gave more serious bleaching than line 2.

3.3.6 Survival of regenerating shoot-clusters after each subcycle of 5-FC selection

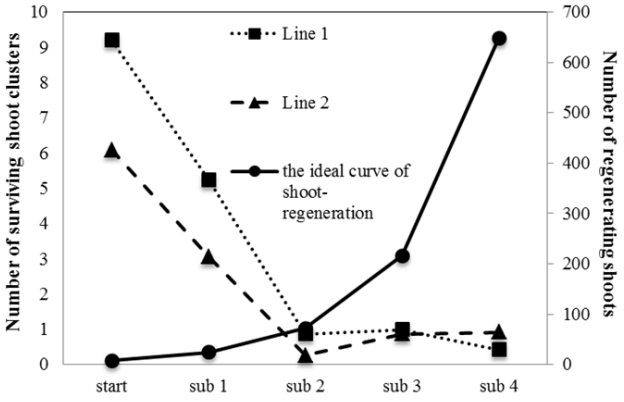

Figure 3.6 The efficiency of 5-FC selection: The efficiency of 5-FC selection is demonstrated by plotting survival at different subcycles, 1 to 4 against the theoretical multiplication rate that can be achieved without any selection. The dotted and dashed lines (with Y axis on the left) display the dynamics of shoot survival for respectively line 1 and line 2. For the X-axis, 'start' means the beginning of subcycle 1, and the rest means the ends of each subcycle. The start value was calculated as the total number of shoots starting 5-FC selection, from the axillary bud regeneration, divided by the number of initial rooting plants times three as the number of axillary buds isolated from them. The start number for line 1 is nine, and for line 2 is six. As start value for the ideal curve 8 was taken which is close to the average start-value of line1 and line 2. The full line (with Y axis on the right) shows the ideal shoot multiplication curve starting from eight shoot in the beginning, assuming that one shoot in regeneration medium for 3 weeks without selection will produce 3 shoot clusters on average. Then, after 4 rounds of shoot to shoot amplification, there will be 648 shoots in the end.

The regeneration medium was used as the basic medium for the selection in each subcycle. So, there was always regeneration in parallel with selection. According to the results of preliminary experiments, without selection, each shoot subjected to regeneration medium for 3 weeks would give rise to on average 3 newly regenerating shoots. Hence, the theoretical multiplication rate for a plant put on rooting medium, from which three axillary buds can be isolated which are put through four rounds of multiplication subsequently would lead to 243 shoots. The theoretical curve for this experiment based on the average number of axillary buds per rooting plant of both lines taken together is shown in Figure 3.6 and compared there with the actual number of regenerating shoots as obtained after treatment with DEX and selection on 5-FC visualizing the effect of selection in this stage. It is clear that in this experiment, because of the 5-FC selection, the number of surviving shoots was constantly decreasing step-wisely. In Figure 3.6, the dotted and dashed lines demonstrate the declining numbers of shoots on 5-FC for the two lines. The actual numbers of surviving shoot-clusters at the end of each subcycle are shown in the Table 3.4.

At the end of subcycle 4, there were 18 surviving shoots for line 1, and those were obtained from 8 regenerating axillary buds of step 2, and from 5 originally rooting plants in step 1; for line 2, there were 15 survivors, which were derived from 4 axillary buds of step 2, and from 4 rooting-plants of step 1(Table 3.1).

Table 3.4 The numbers of surviving shoot-clusters at the end of each subcycle

Line	# Number of plants put on rooting medium (step 1)	# Number of axillary buds isolated (step2)	# Number of axillary buds put on 5-FC (step 3)	# Number of axillary buds at the start of Sub 1	Sub 1	Sub 2	Sub 3	Sub 4*
1	66	212	212	201	346	57	32	18 (4;5)
2	46	108	108	101	141	12	26	15 (4;4)

Note: As shown in the table, from the step 3 to the start of Sub 1, a few axillary bud explants were discarded because that they gave no green regeneration shoots. Between brackets are the number of axillary buds at step 2 from which the surviving shoot cluster are derived and the number of original rooting plants at step 1,from which they are derived respectively.

3.3.7 GUS staining of surviving shoot-clusters after prolonged 5-FC selection

GUS staining was done on the surviving shoots at the end of subcycle 3 and subcycle 4. Among those survivors, GUS negative shoots (as white as WT material) were found in both lines, which implied that the starting material was chimeric, containing both transformed and untransformed cells in both cases. For line 1, 14 out of 18 total green shoot clusters showed no GUS staining (without any blue color) coming from three of the five original rooted shoots. For line 2, only one of the shoot clusters proved to be GUS negative derived from one original rooted plant, while the other 14 clusters stained positive. All in all, the overall percentage of GUS positive shoots from both lines, indicating putative transgenic, marker-free material was 54.5% of the remaining shoot cultures after prolonged selection on 5-FC. The GUS positive survivors of line1 originate from two Step-1 rooting plants of the 15 μM DEX treatment; those of line 2 were from three Step-1 rooting plants, one of the 0 μM DEX (spontaneous recombination) and two of the 15 μM DEX treatment.

3.3.8 Identification of true marker-free plant material

PCR analysis was performed on two separate leaves from each shoot culture at the end of subcycle 3 and at the end of subcycle 4 in parallel with the GUS staining. The results showed that the surviving shoot clusters consisted of marker-free, non-marker-free or WT material or a mixture of any of the three types. Marker-free shoots testified by PCR only presented a small fraction of the total number of plants. The PCR results at the end of subcycle 4 showed that from the GUS positive regenerating shoot clusters, 37.5% were marker-free and 62.5% were non-marker-free. All of those marker-free shoots were derived from line 2, and amounted up to 15 in total. Among them, one was from obtained from the 0 μM DEX treatment, the rest from 15 μM DEX treatment.

The putative marker-free shoots were evaluated again by taking another two leaves from the cluster for a new DNA isolation and PCR run, as well as for a GUS assay after subcycle 4. The GUS staining result was positive for all and proved the transgenic nature of this material to be consistent. However, the second PCR test showed that this time among the shoot clusters, previously found to be *nptII* negative for both leaves, only 30% could be reconfirmed as marker-free, which implied that among the surviving shoots, many of them were still chimeras of marker-free and non marker-free cells. All of the double confirmed marker-free shoots were from line 2, 15 μM DEX treatments. Figure 3.7 provides a flow-diagram demonstrating how a batch of marker-free shoots was finally acquired at the end of subcycle 4 starting from one rooting plant as

the result of the strategy used in the present research. Ultimately, five putative marker-free candidates (reconfirmed) were obtained from two original rooting plants.

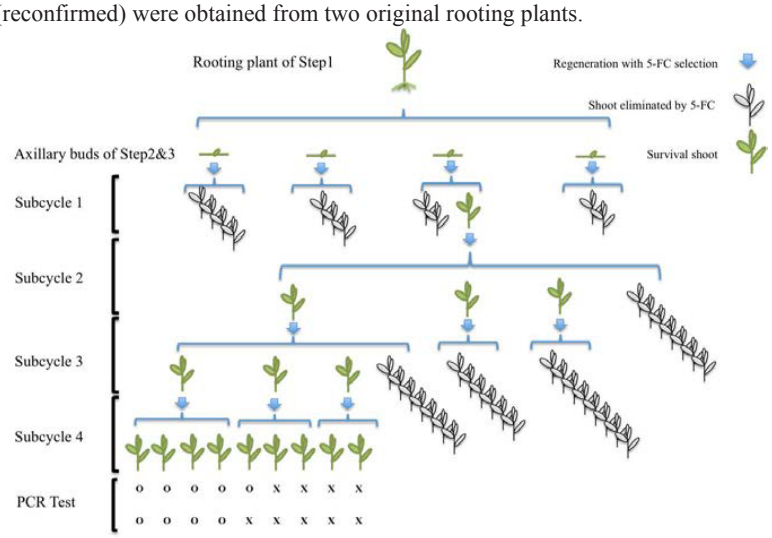

Figure 3.7 Flow chart exemplifying the isolation of marker-free shoots from one initial rooting plant. Individual marker-free shoots were obtained in several steps. From one shoot of a single rooting plant (Step1) of line 2 under 15 µM DEX treatment, four axillary buds were obtained. A blue arrow means a cycle of regeneration and selection with 5-FC. A pale plant symbol indicates plants eliminated by 5-FC. Each plant symbol indicates one tested plant. Gus-staining at the end of subcycles 3 and 4 were all positive for the green individuals. The 'PCR Test' shows the results of two PCR analyses on the surviving shoots. 'O' means marker-free, and 'X' means non marker-free. So, finally, from this specific starting plant, nine surviving shoots were obtained and within them, there were four double-confirmed marker-free individual shoots, one single-confirmed marker-free shoot and 4 non marker-free shoots. Seeds from two of these double-confirmed marker-free shoots were germinated to establish seedlings for further PCR, gfp fluorescence (Figure 3.8) and GUS staining analysis (Table 3.5).

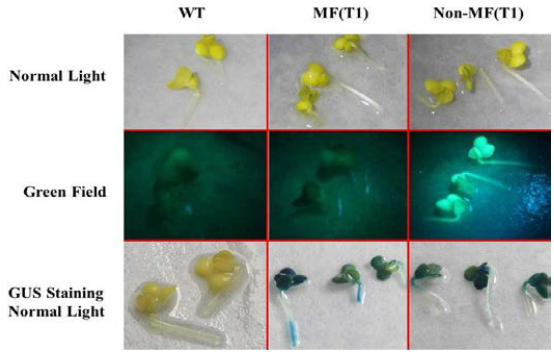

Figure 3.8 GFP and GUS staining analysis on T1 seedlings from a marker-free candidate T0 plant of Line 2. WT was the wild type control, Non-MF(T1) shows T1 seedlings from the T0 plant of Line 2 without DEX treatment and 5-FC selection. MF(T1) shows T1-seedlings obtained from the marker free candidate after 15 µM DEX treatment. In Green Field, fluorescence of gfp is visible in non-marker free plants and absent in marker free plants and non-GM wild type.

After PCR identification, all of the marker-free shoots were put onto rooting medium, and after they formed roots, they were transferred into soil and brought to the greenhouse to get rid of any remaining chimerism by going through a seed phase. As shown in Table 3.5, the marker-free nature was reconfirmed in the next seedling generation as proven by PCR (absence of *gfp* and *nptII)*, gfp fluorescence and GUS staining (as proof of presence of *gus)*. Two T1 seed families originating from two of the five putative marker-free shoots, earlier identified by PCR, were chosen for confirming their marker free nature, using T1 seeds from a line-2 plant without DEX treatment as control. For the GUS staining, the two marker-free T1 seed families (20 seedlings tested) acted similarly as seedlings from line 2 without DEX. However, in the PCR test for the

presence of *nptII* and *gfp*, the marker free families proved to be all negative, while the original line 2 seedlings tested positive in most seedlings. On the other hand, positive gfp fluorescence was observed on the seedlings originate from the plant without DEX treatment; while, the seedlings of the marker-free candidates were without fluorescence, but positive for GUS staining (Figure 3.8).

Table 3.5 GUS staining and PCR tests on T1 seedling obtained from the marker-free shoots identified earlier

Seedlings	GUS staining		PCR			
			gfp		*nptII*	
	Blue	White	Negative	Positive	Negative	Positive
T1MF1	19	1	20	0	20	0
T1MF2	18	2	20	0	20	0
Line 2 (Without treatment)	18	2	1*	19	1*	19

Note: Two T1 families (T1MF1 and T1MF2) from the marker-free shoot that was confirmed twice by PCR to be marker-free (showed in figure 3.8) were selected. T1 seedlings from the Line 2 shoot without DEX treatment and 5-FC selection were used as control. From each of the families, 20 seedlings were prepared for GUS staining and PCR testing respectively. The asterisks indicate that the results were on a same individual seedling.

3.4 Discussion

Here we showed that it is possible to produce marker-free transgenic crambe plants using the pMF1 marker-free system from PRI, WUR. The same system has been successfully used for making marker-free potatoes (Kondrák et al., 2006), strawberries (Schaart et al., 2004) and apple (Vanblaere et al., 2011) before. In those experiments generally leaf explants were taken and submerged overnight in liquid medium with DEX (10 to 50 μM). In the next step, the explants were put on regeneration medium with DEX at a concentration of 1 μM still being present and supplemented with 150 $mg \cdot L^{-1}$ 5-FC for selection for approximately four weeks. Marker-free regenerating shoots were obtained from these explants using this protocol. In preliminary tests, we have also performed the same protocol of submerging explants on crambe aiming to acquire marker-free plantlets, but failed. No marker-free shoots could be isolated (results not shown). Therefore, we tested application of the DEX treatment to intact crambe *in vitro* shoots at rooting phase, allowing uptake of DEX through the roots. This method was used earlier in *Arabidopsis* (Zuo et al., 2000). In our new protocol, the treatment with DEX is continued into the next phase where axillary bud explants are isolated from the rooted shoots and put on regeneration medium. The total exposure period of the plant material to DEX is 10 weeks with 5-FC selection initiated in the last two weeks in addition to the DEX treatment. Comparing the present method with the one applied to strawberry, the DEX concentration is higher and the 5-FC selection was longer and more stringent. The most significant difference was the subcycle selection strategy aiming to enrich for marker-free cells and shoots, and to get rid of the non marker-free cells. In the end, albeit at low frequency, the protocol based on DEX uptake by roots was successful in acquiring marker-free plants in crambe.

The recovery at the end of subcycle 4 of such a high percentage of WT non-transformed shoot clusters demonstrated that the transformation protocol developed in our lab for crambe using cotyledonary node explants (CNE) with meristematic tissue may give rise to chimeras. However, such chimerism was never observed in earlier or later transformation experiments when looking e.g. at segregation ratios in T1 or T2 progenies of transgenic lines. A possible reason can be that here a less stringent (shorter) Km selection was applied and the continued 5-FC selection without of course Km allowed the scarce WT cells to proliferate and become prominent. Still, it was obvious that in the experiments described here chimerism did occur in the original shoots put on rooting medium and subjected to DEX; line 1 showing a higher percentage of WT cells (2.4% of the original number of axillary buds contained WT cells) than line 2 (0.9%). The two originally selected lines were analyzed at the start by GUS staining and molecular analysis by PCR and Southern hybridization and proven to be transgenic. Nevertheless, these analyzes apparently

cannot exclude the possibility of some WT cells to be still present. There was no evidence indicating that any axillary-bud explant from the rooting shoots in the beginning was totally non-transgenic (WT). Using the CNE protocol of transformation in crambe, a stringent and prolonged selection period on Km seemed to be required in order to avoid the occurrence of WTs. Final assurance for obtaining fully transformed plants is to go through a seed phase and working with T1 or T2 generations. GUS staining and PCR assays on the T1 seedlings of the marker-free shoots confirmed that those original shoots still contained some non-GM cells; the T1 seedlings themselves should now be homogeneously GM and marker-free (90 to 95%) or WT (5 to 10%).

Using a multicellular organ or tissue to treat with DEX also allows for the generation of random mosaic chimeras existing of marker-free and non-marker-free cells. If not followed by a regeneration protocol based on the outgrowth of shoots from one cell (adventitious shoot formation) the plant material will remain chimeric. This was what was found here, because in crambe the regeneration process is based on axillary bud explants carrying meristematic tissue and shoots will originate from multiple cells. A stringent selection scheme is required to eliminate non-MF cells and although it was clear that in our experiments 5-FC selection really helped in reducing regeneration of non-MF cells, it did not totally prevent it. For example, at the end of subcycle 4, the surviving shoot clusters were very likely still chimeras mostly, as clear from the two separate PCR tests performed on DNA samples taken from different individual leaves. The results clearly showed that both the DEX treatment and the 5-FC selection did work but not to a full 100%. In our experiments we could not find an optimal DEX concentration when checking survival on Km and 5-FC at step 4. For line 1 the survival on 5-FC suggested that 25 µM was best but this was not verified by survival or rather bleaching on Km. For line 2, 15 µM gave the lowest level of survival on 5-FC and the highest survival on Km, suggesting poor performance for generating marker-free plants and in the end most MF shoots were obtained from this particular treatment. The effect of Km was expected to be opposite to the effect of 5-FC. Such a straight negative correlation was not found. Statistical analysis taking all DEX treatments together and comparing to non-treated controls showed that there was a significant difference between with or without DEX treatment. As mentioned earlier, most marker-free candidates were isolated from shoots and explants treated with 15 µM DEX, so for further research this concentration can be recommended. The one marker-free line obtained from the control treatment without any DEX can be explained by spontaneous recombination; a phenomenon that was observed earlier in lily (Krens pers. commun.) and could come from the Recombinase–LBD fusion protein being able to pass the nuclear membrane without DEX attached to it nor any other proteins.

No marker-free shoots were found for line 1, the line with the one copy T-DNA insert where recombination should result in a simple one copy remaining T-DNA with the *gus* gene as single gene-of-interest left between the T-DNA borders. A possible explanation for this could be the generally low efficiencies of the DEX treatment and the subsequent 5-FC selection in this system and the presence of a relatively large portion of non-transgenic cells competing with the marker-free cells for growth and regeneration on 5-FC medium. The position effect of the T-DNA insertion (Matzke and Matzke, 1998) might play a role in determining accessibility of the Rs sites in the T-DNA to the Recombinase. Moreover, we demonstrated that the expression of the introduced genes (Figure 3.3) was higher in line 2 carrying two copies of the T-DNA than it was in line 1 (one copy insert). The higher expression in line 2 could have resulted in a higher excision rate and could explain the more efficient recovery of marker-free plants in that line. Therefore, the results suggested that for making marker-free plants with the pMF1 system, the nature of the original transgenic material is vital for the final success and multiple independent transgenic lines should always be taken. A passage through a seed-phase should ensure the homogeneous marker-free nature of the subsequent generations after DEX treatment and 5-FC selection.

In conclusion, using a different way of applying DEX we managed to generate marker-free, transgenic crambe plants. Frequencies were low and the DEX and 5-FC treatments were not fully effective. One should be aware of the possible occurrence of chimeras and always go through a seed-phase and check carefully. Multiple independent transgenic lines should be taken as starting

material. Spontaneous recombination can occur but that does not present any problem, on the contrary. The MF crambe genetically modified lines might be more acceptable to the general public for cultivation and generating plant-derived special oils or wax esters.

Chapter 4: Increasing erucic content of *Crambe abyssinica* seed-oil by genetic modification

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Abstract

Crambe abyssinica has a high erucic acid (C22:1) content of about 60% in its seed oil, but industry would benefit highly from ultra-high erucic acid oils. Increasing erucic acid beyond 66 % in crambe seed oil is impossible by classical breeding as the crambe enzyme lysophosphatidic acyltransferase 2 (LPAT2) building fatty acids into the middle (sn-2) position of triglycerides does not use longer chain (> C18) fatty acids as substrate in the developing seed. An RNA interference (RNAi) construct was developed targeting a conserved sequence of LPAT2 of crambe as well as of other *Brassicaceae* species, including *Arabidopsis*, under control of the 35S promoter. Transformed *Arabidopsis thaliana* harboring this construct had an efficiently suppressed *LPAT2* expression in leaves and the expected phenotype (shorter pods and a high proportion of seed abortion), in comparison with the wild type. The transformed *C. abyssinica* containing the same RNAi construct showed a highly reduced *LPAT2* expression both in leaves and developing seeds as well, but no seed abortion or difference in seed size. Oil composition analysis of the transgenic seed oil showed that it contained a higher erucic acid content (max 66.3%) and corresponding lower polyunsaturated fatty acid (PUFA) content (min 10.6%), which both were significantly different. To increase crambe seed oil erucic content further, these LPAT2-RNAi transformants were retransformed with three different genes in one vector under control of the seed specific Napin promoter (termed pWatergate-4G (*LdLPAT+BnFAE1+CaFAD2-RNAi+CaLPAT-RNAi*)): 1) *Limnanthes douglasii* LPAT (*LdLPAT*) that can incorporate erucic acid on sn-2, 2) *Brassica napus* fatty acid elongase (*BnFAE1),* in charge of prolonging oleic acid into erucic acid, and 3) an RNAi construct targeted at native *fatty acid desaturase 2* (*FAD2-RNAi*) that is in charge of desaturating oleic acid into linoleic acid and linolenic acid. Simultaneously, vector pHan2 (*LdLPAT+BnFAE1*) and vector pWatergate-3G (*LdLPAT+BnFAE1+CaFAD2-RNAi*) were also transformed into crambe respectively. Oil composition assays on individual T1 transgene seeds showed that all of the transformations could elevate the erucic acid level in the seed oil. The highest erucic content measured was up to 79%, while 43% tri-erucin content in seed oil was found. The GM seeds of pWatergate-4G and pWatergate-3G transformants (which contained *FAD2-RNAi)* accumulated less PUFA in the seed oil composition, than seeds of wild type, pHan2 and pHellsgate CaLAPT2-RNAi transformants. On the other hand, super low erucic acid content in some seeds of pHan2, pWatergate-3G and pWatergate-4G were also frequently detected during the assay. The lowest erucic acid level of individual seeds observed was less than 1% when there was 85% oleic acid, which indicated that the elongation from C18:1 to C22:1 was almost blocked. This phenotype implied that the exogenous *BnFAE1* introduced by the transgene might cause co-suppression against the endogenous one.

All these results taken together indicate that due to the modulation of the transgenes, more C18:1 could be channeled to C22:1 and less C18:1 was converted to poly-unsaturated fatty acids (PUFA, C18:2+C18:3 mainly); but the unexpected CaFAE co-suppression by overexpression of BnFAE1 could reverse the phenotype into the opposite way completely. Therefore, in summary, the present study suggests that the best way to obtain increased erucic acid content would be by combining LdLPAT+CaLPAT2-RNAi+CaFAD2-RNAi as the addition of BnFAE1 overexpression can lead to co-suppression of KCS thus negating the desired effect.

Key Words: *Crambe abyssinica, Brassicaceae,* lysophosphatidic acid acyltransferase; RNA interference, *Limnanthes douglasii*; β-ketoacyl-coenzyme A synthase; fatty acid elongase; fatty acid desaturase 2.

4.1 Introduction

Erucic acid (omega-9 C22:1 fatty acid) and its derivatives like erucamide, behenic acid or alcohol have wide industrial uses as for example surfactant, lubricant and pour point depressant (Vargas-Lopez et al., 1999; Rudloff and Wang, 2011). Erucic acid only occurs in the seed oil of the plant families *Brassicaceae* and *Tropaeolaceae* and is produced from C18:1 CoA by ketoacyl-CoA synthase (KCS or FAE) of the cytosolic fatty acid elongase complex in the developing seed embryo of *Brassicaceae* plants. Subsequently, erucic CoA is integrated into the glycerol backbone by the acyltransferase of Kennedy pathway which takes place in endoplasmic reticulum (Kennedy, 1961; Murphy and Vance, 1999). In the first two steps, glycerol-3-phosphate is acylated by glycerol-3-phosphate acyltransferase (GPAT) into lysophosphatidic acid (Murata and Tasaka, 1997; Zheng and Zou, 2001) and it is then acylated further by lysophosphatidate acyltransferase (LPAT) into phosphatidic acid (Khaik-Cheang and Huang, 1989; Kim et al., 2005; Soupene et al., 2007) this is followed by dephosphorylation of phosphatidic acid catalyzed by phosphatidate phosphatase (PAP) to release diacylglycerol (DAG) (Kocsis et al., 1996) The final acylation of DAG catalyzed by diacylglycerol acyltransferase (DGAT) (Cases et al., 1998; Routaboula et al., 1999) generates triacylglycerol (TAG) that is transferred to oil body for storage (Anthony H, 1996; Murphy and Vance, 1999). From C18:1 CoA to polyunsaturated fatty acids (PUFA, as linoleic acid, linolenic acid and so on) catalyzed by fatty acid desaturase (FAD) is a well-known substrate-competition to erucic acid biosynthesis (Okuley et al., 1994; Jadhav et al., 2005; Cheng et al., 2013).

Vegetable oil containing erucic acid higher than 55% is considered as high erucic acid vegetable oil which is a valuable feedstock for the chemical industry. Nowadays, most of high erucic acid vegetable oil supplied for chemical industry comes from high erucic acid rapeseed (HEAR). The cultivation of HEAR however, is problematic because of the risk of contaminating food quality rapeseed (Canola) by either inadvertent mixing or cross-pollination. Therefore, HEAR cultivars are cultivated in Europe (about 40,000 hectares in 2006/2007) and in the USA and Canada as an identity preserved crop, because oil from HEAR (high erucic acid rapeseed oil, HERO) should not enter the food chain. The upper limit of erucic acid in food oil rapeseed has been set to 5% in the EU (Council Directive 76/621/EEC), although the risk of erucic acid consumption was only inferred from effects on rats and in humans no epidemiological evidence is available that erucic acid consumption leads to an increase in cardiovascular diseases (Schierholt et al., 2000; Hu et al., 2006). For infant formulas, EC Directive 2006/141/EC (2006) states a limit of 1 % erucic acid of total fatty acids. Because of the risk, another member of the *Brassicaceae* family, *Crambe abyssinica* (crambe) is considered as a candidate to substitute HEAR for high erucic acid vegetable oil production (Bruun and Matchett, 1963; Rudloff and Wang, 2011). The main advantages of crambe concerning the risk of erucic acid vegetable oil entering the food chain are: 1) identity preservation is easy as it is morphologically very distinct from rapeseed, both as a crop and as seed, 2) it does not outcross with rapeseed as HEAR can with Canola. Technically, crambe also has advantages over HEAR as it has a higher erucic content (59-65%) in its seed oil than HEAR (erucic content is 50-55%); and the oil contains relatively less polyunsaturated fatty acids (PUFAs) than HEAR oil. Furthermore, it grows well on all soil types from heavy clays to light sands, and is more tolerant to late-season drought than HEAR (Falasca et al., 2010). Crambe is also useful in crop rotations for alleviating weed, pest and disease build-up in areas where rapeseed was planted (Stymne and Dyer, 2007). Unlike HEAR, crambe is a spring crop and can already be economically attractive to farmers in spring crop areas. Crambe could become even more attractive to farmers and end users, if the erucic content in the oil of crambe seed could be increased.

The content of erucic acid in the seed oil of crambe can be up to 65 % depending on growth conditions and cultivars and has almost reached its maximum. Enzymatic analysis on *Brassicaceae* developing seeds show that the LPAT2 enzyme in charge of catalyzing the incorporating of fatty acids into triglycerides on the sn-2 position cannot use erucic acid as substrate (Kuo and Gardner, 2002). It means erucic acid can only be incorporated on the sn-1 and

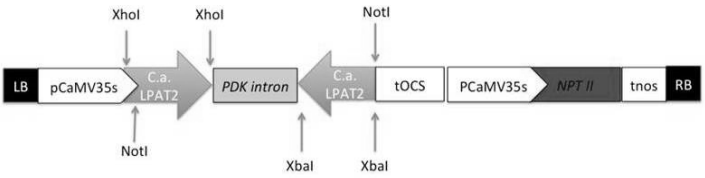
sn-3 positions of glycerol and not on the sn-2 position of triglycerides, which limits the potential erucic acid content in *Brassicaceae* crop seed oil to 66.7 % (2/3). Because of this sake, genetic modification strategies have been developed to enable incorporating erucic acid on the sn-2 position by introducing a *Limnanthes douglasii* LPAT (LdLPAT) that takes erucic acid as substrate for lysophosphatidate sn-2 acylation (Cao et al., 1990; Laurent and Huang, 1992; Lassner et al., 1995). Previous research on *Brassica napus* (Han et al., 2001) and crambe (Li et al., 2012) shows that by introducing *LdLPAT* gene, overexpressing *KCS* gene and suppressing *fatty acid desaturase* expression, the erucic acid level of the transgene seed oil could be elevated moderately.

In the present study, endogenous *LPAT* (*CaLPAT*2) suppression was combined into the new approach to increase crambe seed oil erucic content by genetic modification. Because the endogenous LPAT2 of crambe (CaLPAT2), that only incorporates C18-fatty acids in the sn-2 position, can act as competitor with the introduced LdLPAT for substrate and spatial position. Firstly, an RNA interference (RNAi) construct (Dyer and Mullen, 2001) was developed targeting at a conserved sequence of LPAT2 of crambe as well as that of other *Brassicaceae* species, under control of the 35S promoter. Later, to increase crambe seed oil erucic content further, this LPAT2-RNAi was combined with 1) *Limnanthes douglasii* LPAT (*LdLPAT*) that can incorporate erucic acid on sn-2, 2) *Brassica napus* fatty acid elongase BnFAE1, in charge of prolonging oleic acid into erucic acid (Mietkiewska et al., 2007), and 3) the RNAi sequence targeted at native *fatty acid desaturase 2* (*FAD2-RNAi*) that is in charge of desaturating oleic acid into linoleic acid and linolenic acid (Cheng et al., 2013), into one vector (pWateragte-4G) under control of a seed specific promoter. Finally, crambe was transformed with this vector (*LdLPAT+BnFAE1+CaFAD2-RNAi+CaLPAT-RNAi*). Simultaneously, vector pHan2 (*LdLPAT+BnFAE1*) and vector pWatergate-3G (*LdLPAT+BnFAE1+CaFAD2-RNAi*) were also transformed into crambe respectively.

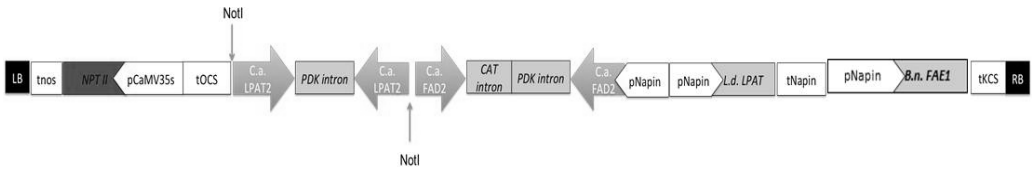
4.2 Materials and Methods

4.2.1 Vectors used in present approach

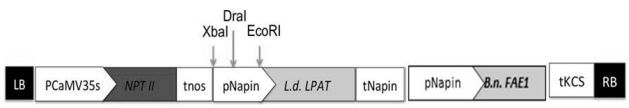
A. pHellsgate CaLPAT2-RNAi



B. pWatergate-4G



C. pHan2



D. pWatergate-3G

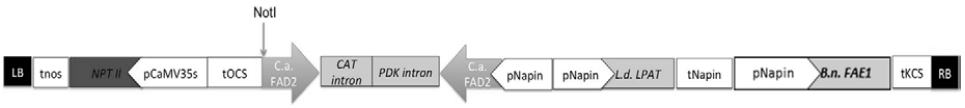


Figure 4.1 the functional genes contained by the 4 vectors used in the present research: There are 4 vectors were used in present study. And all of them have *NPTII* as the selectable marker for plant transformation. In pHellsgate *CaLPAT2-RNAi* (**A**) the *CaLPAT2 RNAi* was controled by pCaMV35s promoter. In the rest vectors genes adjusting plant seed oil compositon in pWatergate-4G (**B**), pHan2 (**C**) and pWatergate-3G (**D**) were ruled by *Brassica napus* Napin promoter (pNapin). (abbreviations: LB - left border; RB – right border; tnos- NOS terminator; tOCS - OCS terminator; tKCS – KCS terminator (KCS=FAE)

As shown in Figure 4.1, four binary vectors were used in this study. The vector pHellsgate CaLPAT-RNAi was composed of the RNA interference gene against *CaLPAT2* (*CaLPAT2-RNAi*), which was controlled, by the 35S promoter and terminator. The pHan2 and pWatergate-3G vectors were acquired from the Unit of Botany, Aachen University of Technology. The pHan2 vector is carrying two genes which are *LdLPAT* and *BnFAE1*; and pWatergate-3G contains *LdLPAT*, *BnFAE1* and *CaFAD2-RNAi*. The pWatergate-4G construct contains *LdLPAT*, *BnFAE1*, *CaFAD2-RNAi* and *CaLPAT2-RNAi* was acquired after the molecular modification of pWatergate-3G. Functional genes in pHan2, pWatergate-3G and pWatergate-4G were controlled by the Napin promoter. All of those vectors contained the *NPTII* gene as selectable marker. For inoculating plants, pHellsgate CaLPAT-RNAi, pWatergate-4G and pWatergate-3G were transformed into *Agrobacterium* strain AGL1, while pHan2 was introduced in AGL0 (Lazo et al., 1991).

Construction of pHellsgate CaLPAT-RNAi

The target coding region of CaLPAT2 was cloned from cDNA of developing seeds (20 days after pollination) of *C. abyssinica* cv. Galactica by PCR using the specific primers (Forward primer: CACC GGGTAAAGAACATGCTCTTG, Reverse primer: AGTTTAGCCTCAGTAAATCG, designed according to the cDNA sequence of crambe LPAT2 acquired from NCBI, GI: 124378834) (Figure 4.2). The amplicon was verified by sequencing which proved that it was the wanted. And then it was ligated into donor vector pENTR/D-TOPO using the Gateway® BP ClonaseTM II enzyme mix (Invitrogen) to construct entry vector pENTR/D-TOPO::CaLPAT2. The target fragment in the entry vector was subsequently exchanged into Gateway destination vector pHellsgate8 (Dyer and Mullen, 2001) by using the Gateway LR® ClonaseTM II enzyme mix (Invitrogen). Both constructs were transformed into *E. coli* (strain: TOP10, Invitrogen, Cat. no. 4040-50) for amplification. The transformants of pENTR/D-TOPO::CaLPAT2 was checked by colony PCR analysis using primers M13 forward and reverse (GTAAACGACGGCCAG and CAGGAAACAGCTATGAC). The transformants of pHellsgate CaLPAT2-RNAi were checked by restrictions of *Xba*I and *Xho*I respectively. Finally, the accurate pHellsgate-CaLPAT2-RNAi construct was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation.

Modifying pWatergate-3G into pWatergate-4G

As shown in Figure 4.1, in the DNA molecule of pWateragte-3G there is only one restriction site of *Not* restriction enzyme in the connection region of CaFAD2-RNAi palindromic sequence to the Napin promoter; and in the DNA molecule of pHellsgate CaLPAT-RNAi, there are two *Not*I restriction sites between which the palindromic sequence of *CaLPAT-RNAi* locates.

The pHellsgate CaLPAT-RNAi vector DNA was digested by *Not*I. And then the digested DNA fragments were separated by electrophoresis with 0.8% agarose gel, by which the desired palindromic fragment (364 bp) was separated. After reclaiming the fragment from gel, it was ligated with the linear pWatergate-3G molecule cut by *Not*I. From the modification, the pWatergate-4G vector was constructed. In this new vector, there are two parlindrom structures (CaFAD2-RNAi and CaLPAT-RNAi) side-by-side, sharing the same promoter (Napin) and terminator (tOCS). The ligation product was transformed into *E. coli* competent cells (strain: TOP10 Chemical, Invitrogen) by electroporation. Candidate colonies' plasmid DNA was isolated and digested again by *Not*I. Those giving a unique band of the original size (364 bp) were chosen for transforming *Agrobacterium* strain AGL1.

4.2.1 Plant transformation

Crambe abyssinica seeds (cv. Galactica, harvest July 2009 from a seed production field in Wageningen, The Netherlands) were germinated to obtain cotelydonary node explants as starting material for transformation and regeneration. The *in vitro* material was cultivated in growth chambers with a photoperiod of 16 h with a light intensity of 33 $\mu mol \cdot m^{2} \cdot s^{-1}$ and a temperature of 24°C. The transformation and selection for GM events of *C. abyssinica* in this chapter were performed as described in chapter 3.

Arabidopsis thaliana plants (Accession Columbia) were grown in the greenhouse or in the climate chamber with the following settings: 22/19°C with 70% relative humidity and 16/8 hour day/night periods, respectively. If the light intensity dropped below 150 $W \cdot m^{-2}$, it was compensated with an extra 100 $W \cdot m^{-2}$ supplemental light. *A. tumefaciens* with target pHellsgate CaLPAT2-RNAi construct were used to transform *Arabidopsis* plants with the method of floral dip (Clough and Bent, 1998). Kanamycin selection of positive transformants was performed (Harrison et al., 2006).

4.2.3 Identifying transformants

Southern blotting

From pHan2 T0 candidate transformants genomic DNA was isolated from the leaf material of *in vitro* plants with the method described by Aldrich and Cullis (1993) but with 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer (Aldrich and Cullis, 1993). For each transformant, the DNA isolation was performed 3 times independently for each candidate. For each time of DNA isolation, 1 g fresh leaf material was used. There were 3 pieces of DNA samples (40 µg per piece) from each candidate transformant digested by EcoRI, DraI and *Xba*I respectively. The T-DNA region of pHan2 is shown in Figure 4.1. The *NPTII* gene is located near the left border. The nearest restriction sites of *Eco*RI, *Dra*I and *Xba*I to the left border are marked in Figure 4.1 as well. For copy number determination, a probe (516 bp) was designed based on the sequence of *NPTII* gene and was labelled with $^{[32P]}$ ATP. The digested DNA samples were fractionated on a 0.8% (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, UK) according to the manufacturer's recommendations. The membrane was hybridized at 65°C overnight with 20 ng of the *nptII* probe and washed for 2 times 30 minutes with 0.1 × saline-sodium citrate (SSC) buffer, 0.1% (w/v) SDS at 65°C. The DNA gel blots were exposed to a phosphorimager screen and subsequently scanned into a Bioimager device (Fujix BAS2000).

The primers of probe amplification were forward TCCAAGATGTAGCATCAAGAATCC, and reverse TGGTTTCGATCCACTTTCTTAC.

PCR: Genomic DNA was isolated from young leaves of each candidate T0 plant with the same method as used for the Southern blotting. The NPTII primers were used to identify the transgenic nature of candidate materials. The *virG* primers are used for amplifying the *virG* gene from the vector backbone to test the presence of it in the material. If the result of *virG* is positive then, the material cannot be counted as GM candidate or moved into soil for seed ripening. They should stay in the medium with cefotaxime and timentin until such time that no positive PCR result of *virG* is showing up anymore.

The primer sequences were: for *NPTII*, forward 5'- TGGGCACAACAGACAATCGGCTGC-3' and reverse 5'-TGCGAATCGGGAGCGGCGATACCG-3', while for *virG* forward 5'-GCCGGGGCGAGACCATAGG-3' and reverse 5'-CGCACGCGCAAGGCAACC-3'.

qRT-PCR: Two pairs of primers specific for the crambe *LPAT2* genes were developed based on the cDNA sequences in *C. abyssinica* (GI: 124378834), and two pairs of primers of *Arabidopsis LPAT2* gene were developed on the cDNA sequences of AtLPAT2 (GI:145339616). The gene β-

actin 2 (GI: 20465834) was used as a reference gene. All primers used in real time PCR are listed in Table 4.1. For the crambe developing seed RNA isolation, total RNA was extracted from bulked seeds of T0 plants (10 seeds per plant, 20 days after flowering/ 20 DAF) with RNeasy Plant Mini Kits (Qiagen, Germany) according to the manufacturer's instructions. For the leaf RNA isolation of crambe and *Arabidopsis*, total RNA was extracted form 0.5g fresh leaf material from the greenhouse plant with the same kit. The isolated RNA was treated with RNase-free TURBO DNase (Ambion, USA) to remove residual genomic DNA. First-strand cDNA was synthesized in 20 µl from 1 µg of total RNA with iScript™ cDNA Synthesis Kit (Bio-rad, USA). The cDNA samples were 20 × diluted and used as templates for real-time PCR. The PCR reaction contains 2 µl templates, 5µl SYBR Green Super Mix (Bio-rad, USA), and 1 µl of each of the forward and reverse primers (3 µM) in total 10 µl reaction. Cycling conditions were 1 cycle at 95°C for 3 min followed by 30 cycles at 95°C for 10 s, 60°C for 1 min, then a final melt step from 65°C to 95°C ramp with 0.5°C increments per cycle to monitor specificity. PCR reactions were performed in triplicate. The expression of each replicate was normalized by the reference gene, β-actin 2, which has shown to be stably expressed in crambe seedlings under arsenate stress and various *Brassica napus* cultivars (Hu et al., 2009; Paulose et al., 2010). The relative expression level of each replicate was calculated according to the comparative CT method (User bulletin no. 2, ABI PRISM 7700 Sequence Detection System, December 1997; Perkin-Elmer, Applied Biosystems). The mean of three replicates represents the relative expression level of a line.

Table 4.1 The primers of Q-PCR for testing *LPAT2* expression level in *Arabidopsis* leaf, crambe leaf and crambe developing seed

Target Gene	Primers name	Sequence
Arabidopsis LPAT2 (GI:145339616)	AtLPAT2_F1	GGT GGT TGC AGA AAC CTT GT
	AtLPAT2_R1	AGC ATG TTC TTT GCC CAT TC
	AtLPAT2_F2	GGT GGT TGC AGA AAC CTT GT
	AtLPAT2_R2	GAG CAT GTT CTT TGC CCA TT
Arabidopsis β-actin 2 (GI: 20465834)	AtActin2_F	GATGGAGACCTCGAAAACCA
	AtActin2_R	AAAAGGACTTCTGGGCACCT
Crambe *LPAT2* (GI: 124378834)	CaLPAT2_F1	CGC ATT GGC TGT AAT GAA GA
	CaLPAT2_F2	CCA GTC ATA GGC TGG TCC AT
	CaLPAT2_R1	TTC ATC CTT TGC CCA ATT TC
	CaLPAT2_R2	CCA CAA AAA GGG CTA ACC AG
Crambe *FAD2* (Li et al., 2012)	CaFAD2-F1	CCGTGAACGTCTCCAGATAT
	CaFAD2-R1	CGTTGACTATCAGAAGCCGA

4.2.4 Gas chromatography analysis

Gas chromatography (GC) was used to analyze the oil composition of crambe seed. Individual seeds were crushed at room temperature in a 1.5 ml Eppendorf tube with 600 µl of hexane to extract the seed oil. After centrifuging at 18,000 g for 5 minutes, 200 µl of the hexane supernatant was taken out and saved into a GC vial for later triacylglycerol analysis; the remaining 400 µl hexane supernatant was transferred to a new Eppendorf tube and 40µl methanol (with 5M KOH) was added and the closed tube was incubated at 60 ºC with shaking to hydrolyze the TAG to free fatty acids, and to methylate the fatty acids to fatty acid methyl esters (FAME). The FAME composition was determined using GC, (column DB-23, Agilent). A 1 µl sample was injected into the GC with a flame ionization detector (FID), using a split ratio of 1:20. A temperature gradient was used starting with 10 minutes at 180°C, a temperature increase to 240°C for 8 minutes and 7 min at 240 °C. Identification of FAME was based on retention time of standards and checked by separate GC-MS analysis with the same conditions. The relative amounts of different FAME were

determined as the relative peak area of the components in the total peak area of FAME. Table 4.2 shows how much seeds were put into the FAME assay.

Table 4.2 The amount of seeds used for FAME analysis

Generation	Vector	Seed Family No.	Seed No.
	Wild type	3	60
T1	pHellsagate CaLPAT2-RNAi	2	60
T1	pHan2	3	100
T1	pWatergate-3G	5	120
T1	pWatergate-4G	4	120

Note: Seed Family No. means how many seed families were chosen for the assay. The Seed No. means how many seeds from those families were analyzed.

The trierucin content was analyzed by GC with high temperature column (RTX-65, 15m x 0.25mm x 0.1µm, Cat. 17005). H_2-gas at 160kPa was the carrier with velocity of 138.46 cm·sec^{-1}. The oven temperature program was like 340°C for 5min and then average increase to 360°C for 10 min, and then held for 1 min. A 1µl sample was injected into the GC with FID with a split ratio of 1:10. Identification of trierucin was based on retention time of standards and checked by separate GC-MS analysis using the same column and temperature profile. The relative amount of trierucin was determined as the relative peak area of the components in the total peak area of revealed.

4.3 Results

4.3.1 Cloning the conserved sequence of CaLPAT2 to construct the RNAi vector

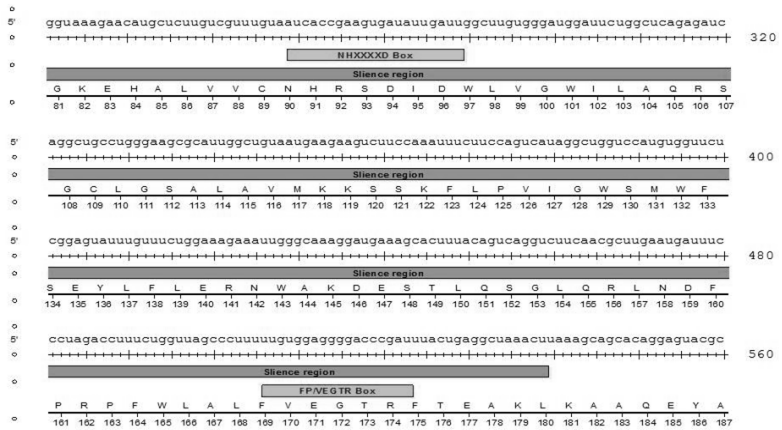


Figure 4.2 the target sequence picked up for *C. abyssinica* endogenous LPAT2 RNAi: A part of mRNA of *C. abyssinica LPAT2* and the corresponding peptide were displayed in the chart. The long bar marked the whole sequence (360bp) incorporated into the RNAi vector later; and two short bars showed two conserved regions the NHXXXD box and FP/VEGTR box.

The conserved region encoding the NHXXXXD box, FP/VEGTR box and the connecting region in between of *CaLPAT2* mRNA was chosen as the target of RNAi (Figure. 4.2) (Murphy, 2009). The sequence was cloned from the cDNA of 20 DAF developing crambe seed by PCR. It was integrated into clone/entry vector pENTR/D-TOPO, of which the success was verified by

sequencing. Finally the target fragment in pENTR/D-TOPO was recombined into the empty pHellsgate 8 vector, to build the pHellsgate CaLPAT-RNAi. The wanted RNAi vectors were identified by restriction of *Xba*I and *Xho*I respectively. As shown in Supplementary Figure 4.1, both of the digestions gave the bands of expected sizes, which was a sign of successfully constructing pHellsgate CaLPAT2-RNAi.

4.3.2 Transformation of *Arabidopsis* and crambe with pHellsgate CaLPAT-RNAi

Arabidopsis (accession: Columbia) and crambe were transformed with construct pHellsgate CaLPAT2-RNAi using *Agrobacterium*. There were 5 independent T0 crambe lines and 20 T1 *Arabidopsis* lines acquired finally after Kanamycin selection, and confirmed by PCR.

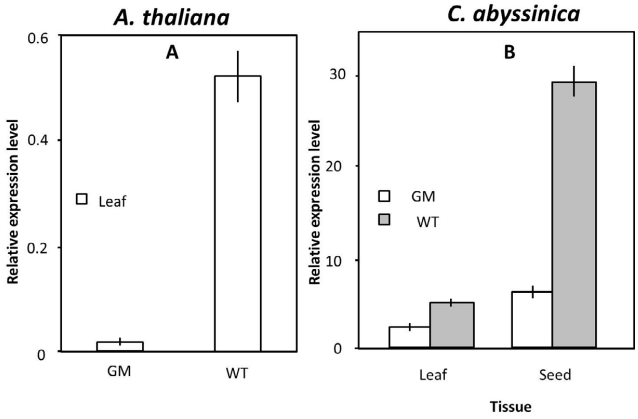


Figure 4.3 qRT-PCR tests on pHellsgate-CaLPAT2-RNAi GM plant materials of *Arabidopsis* and crambe: Endogenous LPAT2 expression levels of leaf material from T1 *Arabidopsis* and T0 crambe plants and of developing T1 crambe seeds were analyzed by qRT-PCR. The GM lines that showed the largest decreases are shown in the charts. The column is the average of repeats and the bar is the standard deviation. In comparison with the wild-type, the *LPAT2* expression in transgenic *Arabidopsis* and crambe was suppressed significantly in different tissues (according to the student T-test).

Figure 4.4 The phenotype of LPAT2 knockdown in *Arabidopsis* by RNAi: The T1 plant of *Arabidopsis* transformed with pHellsgate-CaLPAT2-RNAi showed shorter siliques (panel A) than those of the wild type (panel B). Inside developing siliques, some developing seeds were aborted (panel C, marked by arrows).

The expression of endogenous *LPAT2* in the transgenic plants was down regulated because of the RNAi, which was confirmed by the qRT-PCR. Among the 20 T1 lines of *Arabidopsis*, the most efficient RNAi effect is shown in Figure 4.3 (panel A). The LPAT2 expression level in the GM plant leaf tissue was about 20-fold lower than the wild type (significant, $p<0.01$, by student T-

test). The phenotypes of shorter silique and seed abortion were observed in parallel with the low expression level of *LPAT2* in the T1 *Arabidopsis* plants (Figure. 4.4).

Comparing with the WT, the T0 crambe with the lowest LPAT2 expression was around 50% decreased in leaf tissue, (significant, $p<0.01$, by student T-test); and 80% down regulated in developing seeds (Figure 4.3 panel B). Crambe has only one seed per pod, so abortion of a seed results in empty pods. No difference in seed abortion or seed size between WT plant and the CaLPAT-RNAi transgenic plant was found in this experiment.

4.3.3 Identification of transgenic events of pHan2, pWatergate-3G and pWatergate-4G

The vectors pHan2, pWatergate-3G and pWatergate-4G were transformed into *C. abyssinica* respectively. After selection by Kanamycin and verification by PCR using primers of *NPTII* and *VirG*, there were three independent T0 lines of pHan2, eight of pWatergate-3G and four of pWatergate-4G obtained. The T-DNA insertion number of pHan2 T0 lines was verified by Southern blotting (Figure 4.5). Among the pHan2 independent transformants, one was with two T- DNA inserts, and one with three (or four) T-DNA insertions; and the other one had more than 6 T-DNA inserts.

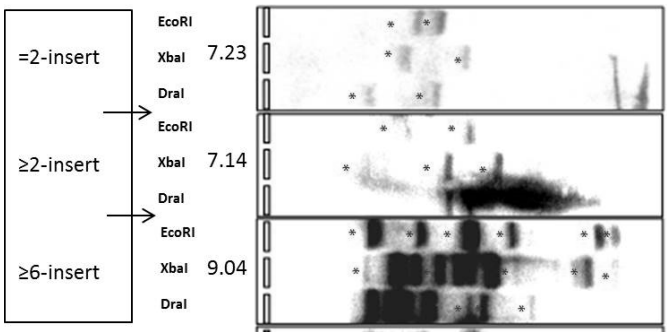


Figure 4.5 Southern blotting analysis on T0 crambe plant of pHan2 transformation: The probe is designed targeting the *NPTII* gene, and three restriction enzymes (*Dra*I, *Xba*I and *Eco*RI) were used for DNA digestion. The Southern blotting demonstrated that one line (ID: 9.04) carried more than 6 T-DNA insertions; line 7.23 contained 2 T-DNA insertions and the T-DNA insertion number of 7.14 was 2 or 3. (Distinguishable bands on the blot are marked with an asterisk; the arrows indicate the direction of the electrophoresis.).

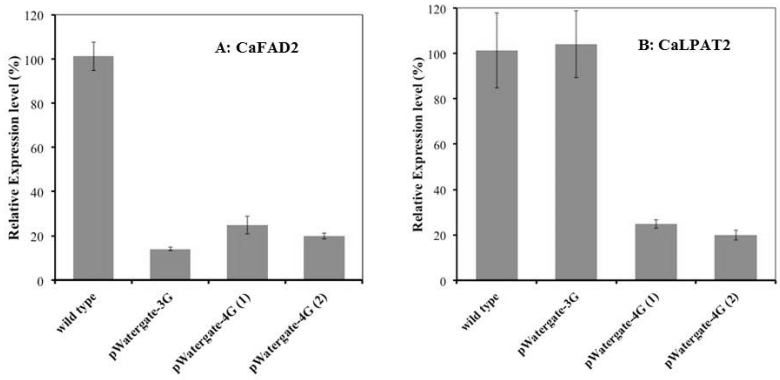


Figure 4.6 qRT-PCR analysis on the developing GM seeds of pWatergate-3G and pWatergate-4G transformations: T1 developing seeds of 20 days after pollination were used for this assay. Two T0 pWatergate-4G plants and one T0 pWatergate-3G plant were selected. The wild type seeds were acquired from control plants after a tissue culture phase as were the transformed ones. Panel A shows the expression level of CaFAD2, panel B shows the expression level of CaLPAT2.

The expression of *CaFAD2* and *CaLPAT2* were assayed by qRT-PCR in developing seeds (20 DAP) of WT, a T0 line of pWatergate-3G and two T0 lines of pWatergate-4G. In comparison to the WT, the expression of *CaFAD2* in the transgenic developing seeds of pWatergate-3G and pWatergate-4G was about 4 to 5 folds down regulated (Figure 4.6 A). The expression of *CaLPAT2* was only around 4-fold decreased in the developing seeds from pWatergate-4G (Figure 4.6 B).

4.3.4 Oil composition of T1 transgene seeds

The individual T1 seed oil composition of pHellsgate CaLPAT2-RNAi, pHan2, pWatergate-3G and pWatergate-4G were analyzed by GC. The average content and variation range of the fatty acids are shown in Table 4.3.

Table 4.3 Fatty acids content variation in the seeds of T1 generation transgene and wild type control

Plasmid	Fatty acid (%)	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0
WT	Mean	1.8	0.6	13.1	8.8	5.8	2.7
	Range	1.3-2.5	0-1.4	9.3-21.7	6.7-11.2	4.2-7.6	0.7-13.3
pHellsgate CaLPAT2-RNAi	Mean	1.8	0.5	14.0	6.8	4.6	0.8
	Range	1.3-2.5	0.3-0.6	12.2-15.2	5.9-7.9	4.2-5.2	0.6-1.0
pHan2	Mean	1.9	0.9	20.6	7.9	3.6	3.2
	Range	1.2-5	0-4.2	11.2-74.3	5.5-11.6	1.4-6.9	1.2-13.1
pWatergate-3G	Mean	2.4	1.5	50.1	3.4	3.6	5.9
	Range	1-3.8	0.2-4	6.2-85.7	1-8.1	1.6-5.9	1.3-14.5
pWatergate-4G	Mean	1.5	0.8	17.4	5.3	2.5	0.8
	Range	0.9-3.1	0.0-3.1	10-32.7	2.3-12.5	0-5.6	0-2.6

Plasmid	Fatty acid (%)	C20:1	C22:0	C22:1	C22:2	C24:0	C24:1
WT	Mean	0.7	2.2	61.0	0.8	0.8	1.7
	Range	0-1.3	0.9-2.9	44.6-64.6	0.3-1.4	0.4-1.3	0.9-2.5
pHellsgate CaLPAT2-RNAi	Mean	2	2.4	64.5	0.5	0.8	1.5
	Range	1.3-2.8	2.0-3.2	63.1-66.3	0.7-0.9	0.7-0.9	1.2-1.7
pHan2	Mean	0.9	2.3	55.7	0.6	0.9	1.6
	Range	0-3.8	0.4-4.3	2-66.5	0-1.5	0.3-2.3	0.3-2.9
pWatergate-3G	Mean	0.8	1.2	29.5	0.3	0.4	0.8
	Range	0-2.1	0.3-2.9	1.0-79.2	0-1.2	0.1-1	0.2-3.5
pWatergate-4G	Mean	2.1	2.7	63.3	0.3	1.2	2
	Range	0-11.1	0-6.6	42.0-71.6	0-0.9	0-3.9	1.5-3.3

Note: The value in the table is in percentage. It means the relative content of certain fatty acid compound to the total seed oil.

Variation of polyunsaturated fatty acid (PUFA) content of the T1 seeds' oil

According to the crambe seed oil fatty-acid composition, its PUFA consists of C18:2, C18:3 and C22:2 mainly. In WT seed oil, the PUFA content was 15.4% on average and varied from 12.0% to 19.2%. In comparison with the WT, the PUFA content of the T1 GM crambe seeds' oil was decreased significantly in all categories of vector-transformation (One-way ANOVA following

Tukey analysis). As shown in Figure 4.7, the WT seed oil had the highest PUFA content. The pHan2 and pHellsgate CaLPAT2-RNAi transgenes were in the middle class, which had a decreased PUFA content of 12.1% on average and ranging from 7.6% to 18.6%. The T1 seeds oil of pWatergate-3G and pWatergate-4G had the lowest PUFA content, which was 7.8% (ranging from 2.3% to 15.2%).

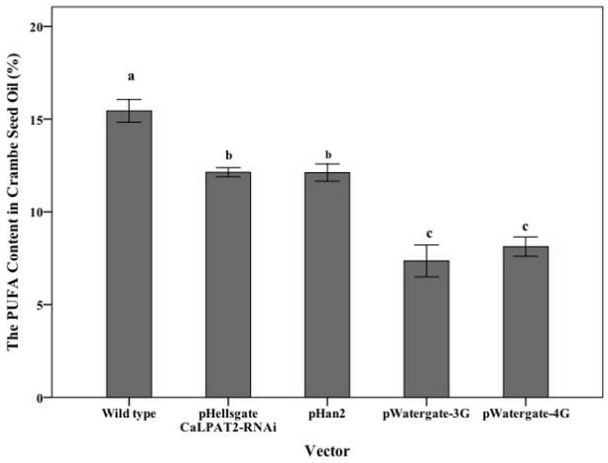


Figure 4.7 PUFA content of T1 seeds: The PUFA content is the sum of C18:2, C18:3 and C22:2 fatty acids. In the panel, the column presents the mean and the bars the standard deviation; different letters on the top of bars show significant differences based on an ANOVA analysis (Method: Tukey). All of the transgenic seeds had lower PUFA accumulated than the wild type.

The variation of erucic acid and other fatty acids in the T1 seeds' oil of transformants

pHellsgate CaLPAT2-RNAi

According to the result of qRT-PCR, seeds from the pHellsgate CaLPAT2-RNAi T0 plant with the most significant *CaLPAT2* suppression were selected for GC analysis. Comparing with wild type, the T1 seed oil had a generally stable and higher level of erucic content, which is 64.5% on average and ranged from 63.1% to 66.3%; the increase was significant, according to one-way ANOVA analysis following Tukey method.

pHan2

Comparing with wild type, among the three T0 lines of pHan2, two (7.23 and 7.14) with low copy number of T-DNA inserts (Figure 4.5) showed slight elevation of erucic content in their seed oil. Among the measurement on individual seeds, the top erucic content was 66.5%. The one (9.04) with the highest number of T-DNA inserts (Figure 4.5) indicated a reversed modulation to the GM seed fatty acid composition. About 70% of its seeds had their erucic content lower than 20.0%, and the lowest was 2.0%.

pWatergate-3G

Five T1 seed families of pWatergate-3G were analyzed. In comparison with the T1 seeds of pHan2 transformation, the Watergate-3G indicated an even wider distribution of seed oil erucic content variation from 79.2% to 1.1%. This abnormal low erucic content phenotype (lower than 44.6%) was generally found in four out of five selected seed families. The single seed with 79% erucic content of its oil was also from a family that had some seeds with very low erucic content phenotype. And this was the only T1 seed family that had seeds with an erucic acid content higher than 70.0%. On the other hand, the T1 family with the most seriously reversed phenotype of fatty acid composition showed its seed erucic content to range from 1.75% to 4.76%.

pWatergate-4G

There were four T1 seed families of pWatergate-4G assayed. Erucic content in single seed oil varied from 42.0% to 71.6%. Single seed with more 70% erucic acid in its oil were found in three families, the top value of single seed was 71.6%. In comparison with the lowest erucic content (44.6%) found in WT seed oil, the lowest of pWatergate-4G was 42.0%. It implied that erucic content suppression phenotype might happen in this transformation as well, but not as serious as pHan2 and pWatergate-3G.

In summary

As shown in Table 4.2, the erucic content in WT crambe seeds were found varying from 45% to 65%, while the mean was 61%, which is consistent to the results obtained in previous researches. Individual seeds with erucic content higher than 66.6% of the oil were found in the GM seeds of pWatergate-3G and pWatergate-4G. While, from pHellsgate CaLPAT2-RNAi and pHan2 transgenes, there were seeds with erucic content higher than 66% found. The highest erucic content of a single seed detected in the present research was 79.2%, which was of pWatergate-3G transformation. The highest erucic content of single seed in other categories of transgenes were found to be 66.3% for pHellsgate CaLPAT-RNAi, 66.5% for pHan2, and 71.6% for pWatergate-4G. The GM seed family with the highest erucic content on average (68.0%) was one of the pWatergate-4G transformants. On the other hand, the content of C24 fatty acids (tetracosanoic and nervonic) also increased slightly because of the overexpression of *BnFAE1*. The phenotype of super low erucic content was frequently found among the GM seeds of pHan2, pWatergate-3G and pWatergate-4G. The lowest erucic content of the pHan2 transformants was found to be as little as 2% with an increased C18:1 content of 74.3%. And the lowest of the pWatergate-3G was 1% with 85.7% C18:1 content. The large increase in C18:1 in those GM seeds in parallel with decreased C22:1 content seems due to co-suppression of *CaFAE*.

The fatty acid content variation because of genetic modification

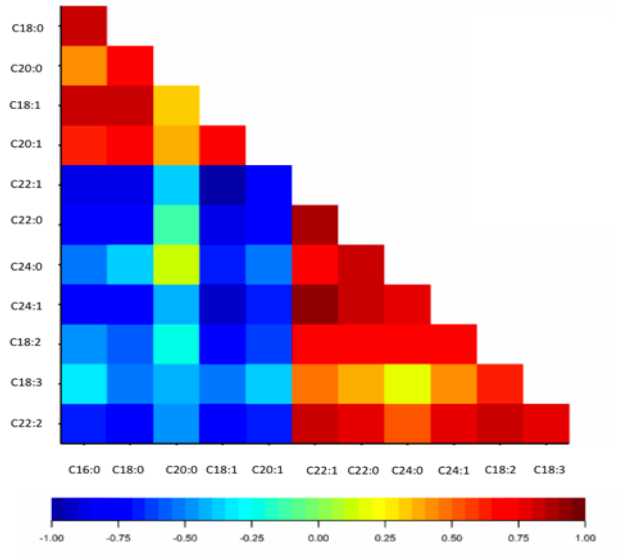


Figure 4.8 Correlation analysis between different fatty acid compounds in wild type and GM *C. abyssinica* seeds: The GC analysis data of all GM seeds and the wild type control were assembled to analyze the correlation effect between different fatty acids following the method of Pearson correlation. The number in each cell is the value of Pearson's R. From -1 to 0, then to 1, the color changed from blue to white, and then to red. Except the correlations of C20:0FA / C22:0 and C18:2 FA / C20:0, the other correlations (red is positive or blue is negative) were all significant ($p \leq 0.05$ or 0.01).

The correlations between different fatty acids (Figure 4.8) were assayed based on the total data acquired from the GC measurement mentioned above. The contents of C18:1 and C22:1 was highly variable, due to the effect of the introduced genes *BnFAE1*, *LdLPAT* and *CaFAD2-RNAi*. The correlation between C18:1 and C22:1 (negative) was the most pronounced (r=-0.98). C18:1 together with C16:0, C18:0, C20:0 and C20:1 form a group that showed positive correlation with each other (r = 0.23 to 0.80), and moderately to highly negative correlation with all other fatty acids, especially with the fatty acids with carbon chain longer than 20. This indicated that when C18:1 was not converted into longer fatty acid (≥C22), also the contents of short chain saturated and mono-unsaturated fatty acid increased correspondingly. Meanwhile, C18:1 negatively correlated with PUFA, which was likely due to the difference of PUFA content in between lines with and without *CaFAD2-RNAi*.

Trierucin content of the transgenic seeds

The analysis on the seeds' trierucin content was also partially conducted in parallel with the fatty acid content verification. No trierucin was detected in the wild type and pHellsgate CaLPAT2-RNAi T1 seeds. The highest trierucin content among the seeds of pHan2 transformation was 9.7%, while the corresponding seed erucic content was 61.1%. The highest trierucin content in the seed of pWatergate-3G transgene seeds was 42.6%, of which the corresponding erucic content was 79.2%. All the GM seeds with low erucic acid (≤20%) had no trierucin accumulation detectable by GC. Bivariate correlation analysis (Pearson, two-tailed) showed that, in the oil composition of one seed, the erucic content positively correlated with the trierucin content, which is significant at $p<0.01$.

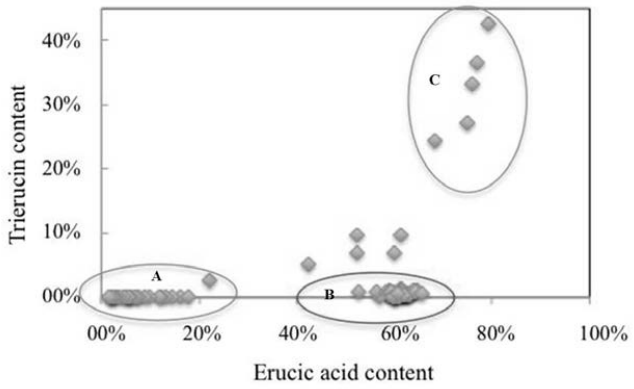


Figure 4.9 the trierucin content of pHan2 and pWatergate-3G transgene seeds: A fraction of seeds for fatty acid composition analysis was also analyzed for their trierucin content. The T1 seeds of pHan2 and pWatergate-3G transformation are shown in the chart. According to a Pearson correlation analysis for single seeds, the content of trierucin correlated significantly at $p< 0.01$ with the erucic acid content.

The erucic acid content in the GM seeds of pHan2 and pWatergate-3G, and their corresponding trierucin content are shown in Figure 4.9. These seeds could be grouped in 3 categories. Those in circle A were the seeds with *CaFAE* co-suppression like phenotype (from overexpression of *BnFAE1*); in circle B were the seeds similar to the WT (without promising phenotype of transgene); and those in circle C were the seeds with expected phenotype of erucic increase. The points out of any circles were those seeds without increased erucic content but with deviating trierucin content.

4.4 Discussion

In the current research, the GC analysis results of pHan2 and pWatergate-3G T1 seeds showed that the trierucin content significantly correlated with the erucic content (Figure 4.5). The highest

trierucin accumulation is 42.6%, and the erucic content is 79.2%, found in a T1 seed from a particular transformant of pWatergate-3G. If we consider there was an ideal seed, all its lipid compound was TAG; and among these TAG molecules, 42.6% were trierucin; all of the rest TAG had two erucyl moieties (1,3-dierucin). Accordingly the erucic content of this seed oil would be 80.7%, which is very close to 79.2%. It implied that in this single seed, the capacity for its oil containing erucic acid was almost saturated; the only way to efficiently improve the erucic acid content further is hence to make more other TAGs become trierucin, which is processed by LdLPAT. Many previous studies have expressed exogenous *LdLPAT* in oil seed plants like *Arabidopsis*, rapeseed and crambe, to replace the function of the endogenous LPAT, which gave moderate elevation of erucic acid in the seed oil. Regarding these results, the hypothesis could be that the endogenous LPAT could act as a competitor to the LdLPAT for subtracts (instance lysophosphatidic and fatty acyl.) and spatial position. But the possible endogenous competition to LdLPAT has not been proven yet.

There are five LPAT isoforms (LPAT1, 2, 3, 4 and 5) of *Arabidopsis* discovered (Kim et al., 2005). Besides LPAT1, the rest of them locate on the endoplasmic reticulum. Among the genes encoding LPAT isoforms, *LPAT2* always have much higher expression level in various tissues (callus, inflorescence, leaf, root and silique) than the others. The sequences which are highly homologous with the *Arabidopsis LPAT2* have also been cloned from the genome of other *Brassicaceae* species like *Brassica napus*, *Brassica rapa* and crambe. Present research indicated that the LPAT2 expression of *Arabidopsis* and crambe could be suppressed efficiently by the RNAi targeting on the encoding sequence of NHXXXXD box, FP/VEGTR box and the connecting region in between (Frentzen and Wolter, 1998). The pHellsgate CaLPAT2-RNAi transformed *Arabidopsis thaliana* had an efficiently suppressed *LPAT2* expression in leaves and the expected phenotype (shorter pods and a high proportion of seed abortion), as same as the T-DNA insert mutant. The transformed *C. abyssinica* also showed a significantly reduced *LPAT2* expression both in leaves and developing seeds as well, but no seed abortion or difference in seed size. Oil composition analysis of pHellsgate CaLPAT2-RNAi transgene crambe seed oil showed that it was with higher erucic acid (max 66.3%) and lower PUFA content (min 10.6%) than the wild type. It is clear that the CaLPAT-RNAi modulation let more carbon flux go to erucic acid, and less to PUFA. But, it is not clear why this happened. A hypothesis could be that in the developing seed, when the *LPAT2* was suppressed by RNAi, other functional LPATs were put in charge of TAG biosynthesis; and their substrate affinities were different from that of LPAT2, which lead to this oil composition variation.

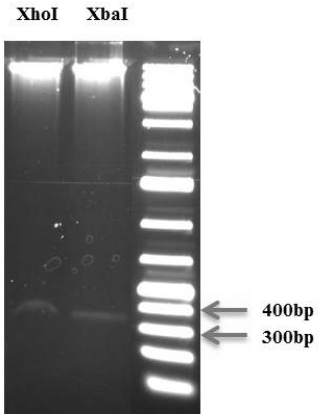
By inserting the palindrome sequence from the pHellsgate CaLPAT2-RNAi into the pWatergate-3G vector, pWatergate-4G vector was constructed. The qRT-PCR analysis on endogenous *CaFAD2* and *CaLPAT2* gene expression demonstrated that the double-RNAi was functional. The GC analysis also indicated that the T1 seed oil had enhanced erucic acid accumulation (highest value: 71.6%) and a suppressed PUFA level. We expected the pWatergate-4G transformant seed would give higher erucic content than the seeds of all the other transformants, because the CaLPAT-RNAi could diminish the endogenous competition to LdLPAT. However, the highest value (79.2%) in the present research was from the pWatergate-3G transformant. This may be due to the fact that the low number of T1 seed families (four in total) limited the variation of the GM phenotype. On the other hand, a report from Guan *et al. (*2013) showed that in the developing crambe embryos of pHan2 transgene, the erucic moiety was mainly locked in the phosphate choline (PC) fraction (Guan et al., 2013). Perhaps this might be a reason why the pWatergate-4G vector didn't work as well as expected. If that would be the case, the issue confronted will be how to channel the erucic moiety from PC to TAG.

According to the results of qRT-PCR analysis on developing seed and GC analysis on ripened seed, both the CaFAD2-RNAi of pWatergate-3G and pWatergate-4G functioned significantly. According to the correlation analysis on the content of different fatty acids, PUFA had a significant positive correlation with erucic acid (Figure 4.10). It is opposite to the generally acknowledged hypothesis that PUFAs biosynthesis is a competitor to C22:1 production for the

carbon flux. This paradox is mainly because of the interruption by co-suppression effect (Napoli et al., 1990; Ketting and Plasterk, 2000; Jadhav et al., 2005) of CaFAE, which generally happened in the transgene seeds of pHan2, pWatergate-3G and pWatergate-4G. If the oil composition data from the seeds with this co-suppression effect (erucic acid content lower than 44.6%) were excluded, the correlation analysis on the rest data showed that the PUFA content negatively correlated with the erucic acid content (data not shown).

The phenotype of sharply decreased erucic content has been generally found among T1 seeds of pHan2, pWatergate-3G and pWatergate-4G. The lowest content of erucic acid found was 1.0% corresponding to 85.7% oleic acid, which indicated the elongation from C18:1 to C22:1 was almost blocked by this effect. This phenotype was most likely due to the co-suppression induced by the transgene with *BnFAE1* overexpression. According to the T-DNA insert number and seed oil composition data, among three T1 seed families of pHan2, only the one with the sextuple T-DNA inserts had offspring seeds with supper low erucic content. It suggested that the high T-DNA insert number could be essential for inducing *CaFAE* co-suppression. Because those T-DNA insert loci will segregate during meiosis, the co-suppression genotype will thus disappear in part of its offspring. This hypothesis was firstly proven by the fact that in the pWatergate-3G T1-family that had 79% erucic content, there was a phenotype-variation of erucic content (less than 10% to more than 66%). Later it was proven again by the test on T3 seed families propagated from the T1 family of sextuple T-DNA, where some seeds showed an erucic content as high as 72%. Therefore, the present study suggests that the highest erucic acid content can be obtained when combining LdLPAT+CaLPAT2-RNAi+CaFAD2-RNAi without BnFAE1 overexpression.

Supplementary Figure



Supplementary Figure 4.1 the identification of the successful recombination for constructing pHellsgate-CaLPAT2-RNAi: A fragment from CaLPAT2 cloned in donor vector pENTR/D-TOPO was integrated into pHellsgate8 vector twice (in reverse orientation) by the kit (Gateway® LR Clonase™ II Enzyme Mix, Invitrogen). This Figure S4.1 demonstrates that the desired construct was made. Two *Xho*I restriction sites were present on the vector, and the CaLPAT2 fragment was placed in between; another fragment distinguishable after successful cloning can be identified by restriction with *Xba*I. *Xho*I and *Xba*I were used to digest the DNA of the construct made. The fragment sizes should be (and were) 390bp for *Xho*I digestion and 374bp for *Xba*I digestion. The electrophoresis verified that the proper vector had been constructed. There are two *Not*I restriction sites available located at both sides of the stem loop fragment for further cloning.

Chapter 5: Producing wax esters in *Crambe abyssinica*

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Abstract

The study presented here showed that it is possible to acquire jojoba wax ester (WE) from the seed of genetically modified *Crambe abyssinica*, in which jojoba genes encoding fatty acid elongase (ScFAE), fatty acid reductase (ScFAR) and wax ester synthase (ScWS) were introduced. By selecting the seeds with the highest WE content for propagation, the WE content of seeds could be further increased stepwise from generation to generation. This led to the highest WE content in the oil of single GM seeds of 252 mg/g (WE/seed). The transgenic seeds of triple construct transformants (ScFAE+ScFAR+ScWS) had more long fatty chain moieties with 24 carbon atoms incorporated into its lipid compound than the transgene seeds of the ScFAR+ScWS transformants. We further demonstrated that knocking down (RNAi) endogenous fatty acid desaturase 2 (FAD2) could enhance the WE accumulation in the transgenic seeds of ScFAR+ScWS transformants. However, this came at a cost, because the higher the WE content in the GM seed with FAD2-RNAi, the higher the risk was of germination problems or poor seedling development. During seed propagation of the transgenic lines, a reduction in germination vigor of seeds with high WE content was observed. Subsequent seed germination tests showed systematically that the more WE accumulated in a seed, the higher the chance that germination failed and/or that the resulting plants showed a severe delay in growth or even death at seedling stage.

Key Words: Transgenic crambe, Wax ester synthesis, jojoba genes, tailoring composition, seed vigor.

5.1 Introduction

Simmondsia chinensis (jojoba) is endemic to the Sonora and Mojave deserts of Arizona, California, and Mexico (Gentry, 1958). The most significant characteristic of this plant species is that in its seeds, the storage lipids are wax esters (WE) rather than triacylglycerol oil (TAG) which is usually stored by other oil-seed plants, such as rapeseed, sunflower, soybean and peanut (Gurr et al., 1974). Jojoba seed wax lipid is well-known for its outstanding quality and wide usages in body care and cosmetology (Van Boven et al., 1997). However, the low yields obtainable from jojoba plants make this plant wax oil a very expensive commodity. The price on the international market is around 5000 €·ton^{-1}. The global production of jojoba oil is about 3500 tons annually of which almost 3400 tons is used in cosmetics production and the pharmaceutical sector (Stymne and Dyer, 2006). Jojoba wax oil is an outstanding lubricating agent (Dyer et al., 2008). The amount of jojoba oil used for lubrication is limited to only around 100 tons per year mainly because of its high price. The narrow climate adaptation of the crop, low yields, and consequently, the high price are crucial factors that block jojoba wax from the international lubricant market. Although the same chemical compounds can be synthesized by chemical engineering, the prices of these chemicals are not significantly lower (Steinke et al., 2000; Stymne and Dyer, 2006). If jojoba wax yield could be substantially increased and at a lower cost, the lubricant market would potentially be a large consumer.

Biosynthesis of jojoba WE involves three essential enzymes. They are fatty acid elongase (FAE) (Lassner et al., 1996), fatty acid reductase (FAR) (Metz et al., 2000) and wax ester synthase (WS) (Lardizabal et al., 2000). The FAE is in charge of prolonging the fatty acid carbon chain from oleyl (C18:1)-CoA to gondoyl (C20:1)-CoA, C20:1-CoA to erucyl (C22:1) CoA and C22:1 CoA to nervonyl (C24:1)-CoA. FAR catalyzes the conversion of prolonged acyl-CoA into fatty alcohol. Finally, the wax ester synthase (WS) connects one fatty alcohol to one fatty acid with an ester bond to form a wax ester (Lassner et al., 1999). The affinity of FAR to C16:1 CoA and 18:1 CoA is very low, whereas, it is high to long acyl-CoAs like C22:1 and C24:1. By transferring the key genes of jojoba WE biosynthesis via genetic modification, it is possible to produce these valuable WE in other plants (Lardizabal et al., 2000; Metz et al., 2000). The European Union 7th Framework project 'Industrial Crops producing added value Oil for Novel chemicals (ICON)' proposed to produce jojoba WE by genetic modification (GM) in a potential oil-seed platform crop, such as *Crambe abyssinica* (crambe). Crambe was chosen because 1) crambe seed's characteristic of a high erucic acid content just fits well with the biosynthesis of jojoba-type WE (Lalas et al., 2012); 2) it is less restricted by climate conditions in cultivation (Falasca et al., 2010) and 3) it can be mechanically harvested. In the present research, the three essential jojoba genes *FAE*, *FAR* and *WS* were cloned into two constructs pBinGlyRed *ScFAR*+*ScWS* (FAR+WS) and pBinGlyRed *ScFAE* +*ScFAR*+*ScWS* (FAE+FAR+WS), under control of a seed specific promoter. The jojoba *FAE* is a fatty acid elongase capable of enhancing the C24:1-CoA biosynthesis. The transformation of FAE+FAR+WS is aiming to have GM crambe seed with wax ester containing more C24:1 moieties. Both constructs were transformed into crambe with the *Agrobacterium*-mediated protocol described in Chapter 3.

As outlined above, erucic acid is the main substrate for producing WE in the GM crambe seeds. In the metabolic network of developing seed oil, fatty acid desaturase 2 (FAD2) is a well-known competitor to erucic acid biosynthesis for the fatty acid flux. FAD2 desaturates C18:1 (oleic acid) into C18:2, a polyunsaturated fatty acid, enabling the further desaturation to C18:3 by FAD3. PUFAs (linoleic acid (C18:2) and linolenic acid (C18:3)), cannot be used for erucic acid

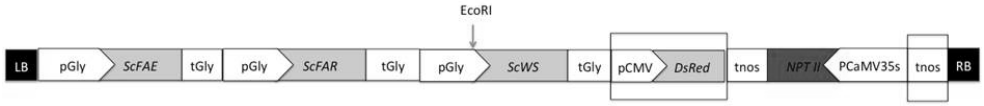
production by FAE and PUFA production therefore competes with the long chain fatty acid substrate production for WE. Hence, for tailoring the production of WE by crambe further, the interaction between the two different pathways of WE and PUFA and the resulting levels of WE and PUFA were studied, after crossing FAR+WS transformants with a *fatty acid desaturase* 2 (FAD2, the key enzyme of PUFA biosynthesis) knockdown plant.

5.2 Materials and Methods

5.2.1 Plant materials and transformation vectors

Crambe seeds *cv. Galactica* (Mastebroek et al., 1994) (harvested in July 2009 from a seed production field in Wageningen, The Netherlands), were germinated to obtain cotyledonary node explants as starting material for transformation and regeneration. The *in vitro* material was cultivated in growth chambers using the conditions as described in Chapter 2.

A: pBinGlyRed ScFAR+ScWS



B: pBinGlyRed ScFAE+ScFAR+ScWS

Figure 5.1 Two constructs used in present research: The construction of pBinGlyRed ScFAR+ScWS was indicated in chart A, and pBinGlyRed *ScFAE+ScFAR+ScWS* in B. In second vector, the function of DsRed gene (marked in two boxes) was broken by the insertion of *NPTII* gene. (abbreviations: LB - left border; RB – right border; pGly – glycinin promoter; pCMV – CMV promoter; tnos-NOS terminator; tGly - glycinin terminator; tCMV – CMV terminator)

Two binary vectors (shown in Figure 5.1) were used in this study, pBinGlyRed *ScFAR+ScWS*, and pBinGlyRed *ScFAE +ScFAR+ScWS*. In these constructs, each of the jojoba genes was driven by a seed specific soybean glycinin promoter (Chiera et al., 2007) individually. As a selectable marker, the *neomycin phosphotransferase II* (*NPTII*) gene (Beck et al., 1982) under the control of the NOS promoter was integrated. Vector pBinGlyRed *ScFAR+ScWS* also contained a functional *DsRed* gene (Baird et al., 2000) as a visual fluorescent marker driven by the CMV promoter (Odell et al., 1985). Although vector pBinGlyRed *ScFAE+ScFAR+ScWS* contained a CMV promoter, which controlled *DsRed* gene as well, it is inactive because of a gene insertion in its coding region. Hence, the DsRed fluorescence marker was not available for FAE+FAR+WS transformation. The binary vectors were transferred to the supervirulent *Agrobacterium* strain AGL1 (Lazo et al., 1991) for plant transformation.

Transformation and selection

One gram of crambe seeds with pods was put into a 50 ml blue cap tube (Greiner) with 20 ml 70% (v/v) ethanol, and rinsed for 30 seconds. After pouring off the ethanol, 30 ml 3% (w/v) NaClO with 300 µl Tween20 were added to the tube. Subsequently, the tubes (with closed cap) were placed in a shaking water bath at 42.5 °C for 20 minutes. In the end, the seeds were rinsed four times with sterilized milli-Q water, and put into a plastic, high-walled Petri dish (50/dish) filled with 50 ml 1x MS, 2% (w/v) sucrose (MS20), solidified with 0.8% (w/v) Phytoblend and

pH 5.8. After cold treatment at 7 °C overnight, the dishes with seeds were placed in the dark at 24 °C for three days, and then in 16h light/8h dark for another three days, at 24 °C.

Crambe explant inoculations were performed according to the cotyledonary node method as developed for sugar beet and caraway transformation with minor modifications (Krens et al., 1996; Krens et al., 1997) essentially as described in Chapter 3.

5.2.2 Identification of potential transformants

Screening for fluorescence

DsRed fluorescence from the plant materials transformed with the construct FAR+WS was checked using UV fluorescence microscopy (Zeiss, SteREO Discovery V8 equipped with PentaFluar S fluorescence equipment and PROIR, Lumen 200 illumination system).

Southern blotting

Genomic DNA (gDNA) was isolated from leaf materials of *in vitro* plants with the method described by Aldrich and Cullis (Aldrich and Cullis, 1993) but with 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. A probe (516 bp) was designed based on the CMV promoter driving the *DsRed* gene, and labelled with $^{[32P]}$ ATP. The primers for the probe preparation were F(orward) 5'-TCCAAGATGTAGCATCAAGAATCC-3', and R(everse) 5'-TGGTTTCGATCCACTTTCTTAC-3'. To determine the T-DNA insert number, a total of 40 µg of gDNA was digested overnight with *Eco*RI which digests the T-DNA only once (outside the probe sequence), fractionated on a 0.8% (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, UK) according to the manufacturer's recommendations. The membrane was hybridized at 65 °C overnight with 20 ng labelled probe and washed for two times 30 minutes with 0.1 × saline-sodium citrate (SSC) buffer, 0.1% (w/v) sodium dodecyl sulfate at 65 °C. The DNA gel blots were exposed to a phosphorimager screen and subsequently scanned into a Bioimager device (Fujix BAS2000).

5.2.3 Crossing

Transgenic crambe lines with *CaFAD2* knocked down by RNAi in developing seed (CaFAD2-RNAi) driven by the napin promoter (Stålberg et al., 1993) were made available by prof. Li Hua Zhu of the Swedish University of Agricultural Science (SLU), Alnarp, Sweden. Four lines representing independent transformation events (ID: Wg19-1, Wg19-3, Wg4-1 and Wg4-3) were used to cross with two FAR+WS transgenic lines (ID: Tr09-10, F5 A and Tr09-10, F5 B). For the crossing, all mature seeds, open flowers and buds that had already a white tip, were removed from the inflorescences on mother plants. Still closed flower buds were manually opened and anthers were removed. Styles and stigmas were exposed and used in pollinations. From the father plants, an open, mature flower with visible pollen shedding was taken. Anthers were tapped on the stigma to cover it with pollen grains. Subsequently, the artificially pollinated flowers on mother plants were marked. After 24 h, this process of pollination was repeated exposing the stigmas to pollen from fresh flowers taken from father plants for a second time.

The plants of FAR+WS are T1 plants from one T1 seed family obtained after selfing a single T-DNA insertion T0 plant. Meanwhile, the plants of CaFAD2-RNAi are from two T1 families, of which Wg19-1 and Wg19-3 are from a same T1 seed family, while Wg4-1 and Wg4-3 are from the other one. These two T1 seed families originate from two different multiple T-DNA insertion

T0 plants respectively. The T1 plants of FAR+WS were both proven to be heterozygous by the fluorescence segregation in their T2 seeds. The T-DNA insertion number in the CaFAD2-RNAi plants was unknown, and the plants for crossing were picked randomly. Each of the plants was used both as father and mother in reciprocal crosses. Ultimately, 34 F1 seeds were obtained. These F1 seeds were put into a Petri dish with three layers of filter paper wetted thoroughly with water, for germination at 24 ºC in the dark. After the cotyledons were found breaking the seed coats, they were checked by fluorescence microscopy for DsRed fluorescence. The seedlings with DsRed fluorescence were transferred to the soil in the greenhouse to obtain F2 seeds.

5.2.4 Analysis of fatty acid and fatty alcohol composition of the WE in GM crambe seeds

Gas chromatography

For single seed analysis of the fatty acid and fatty alcohol composition, the seed was crushed in a 1.5 ml Eppendorf tube with 300 µl hexane to extract the TAG and WE at 60 ºC for 30 min. After centrifuging at 13,000 g for five minutes, 250 µl of the hexane supernatant was taken and the hexane was evaporated under a flow of nitrogen. To the oil sample thus obtained 300 µl hexane containing 300 $mg \cdot L^{-1}$ of both C17:0 margaric acid and C22:0 behenyl alcohol as internal standards and 40 µl methanol (with 5 M KOH) were added. Than the mix was incubated at 60 ºC with shaking for five minutes to hydrolyze the TAG and WE to obtain the free alcohols (FA-OH) and free fatty acids, and to methylate the fatty acids to fatty acid methyl esters (FAME). For the analysis of 20 bulked seeds, all volumes were 20 times larger. The compositions of FA-OH and FAME were determined using gas chromatography (GC, column/DB-23, Agilent). One µl sample was injected into the GC with Flame Ionization Detector with a split ratio of 1:20. A temperature gradient was used starting with ten minutes at 180 ºC, a temperature increase to 240ºC for eight minutes and finally, seven minutes at 240 ºC. Identification of FA-OH and FAME was based on retention time of the standards checked by separate GC analysis using the same column and temperature profile. The relative contents of different FA-OH and FAME were determined as the relative peak area of the components in the total peak area of FA-OH and FAME taken together (= total fatty acyl compound), corrected for possible differences in recovery rate of FAME and FA-OH based on the internal standards. The WE content of the seed oil was calculated as two times that of the total fatty alcohol content. Therefore, the WE content presented here was the relative WE content of total fatty acyl compound.

Electrospray ionization coupled with tandem mass spectrometry (ESI-MS/MS)

The WE from GM crambe seeds was analyzed by electrospray ionization coupled with tandem mass spectrometry (ESI-MS/MS; at the laboratory of prof. Ivo Feussner, Georg-August University, Göttingen, Germany). In this research, three T 1 families, each a representative of the class with highest WE content among all of the transgenic plants, were chosen. Five seeds from each GM family were randomly selected. Each GM seed was assayed individually. The seed was weighted and then extracted for lipid compounds by hexane. The extraction was spotted on a TLC (plate silica gel 60 glass plates 0.25 mm × 20 cm × 20 cm, Merck) for separating WE from other lipid fractions. Then, the WE was scraped off the plate for further analysis by ESI-MS/MS in which individual species of WE (determined by the specific combination of a certain fatty acid and a certain fatty alcohol present) was determined and quantified (Heilmann et al., 2012).

5.2.5 Half-seed analysis and seedling growth evaluation

Seeds from the selected families were soaked in water for two hours. After that, they were cut with a fine knife under binoculars with ten times magnification. Under binoculars, the profile of the seed showed the big cotyledons at the outside and the radicle in the middle (Figure 5.2). The cutting must be on one side of the cotyledon but not in the middle. Any damage to the radicle must be avoided (Figure 5.2). After cutting, the chipped fragment of the cotyledon was immediately used for oil composition analysis with GC, as described above. The remaining part of the seed was sowed directly into a pot (diameter/14cm, height/14cm) with well-irrigated soil in the greenhouse (Unifarm, Wageningen University). There was a cover of gas-permeable mesh on the top of each pot to protect the emerging seedlings from insect infestation. After two weeks, the lengths of the seedlings were recorded.

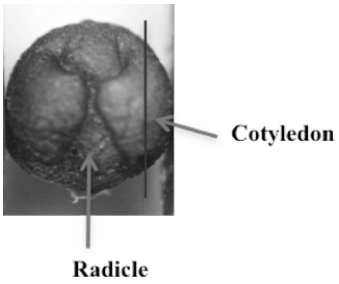


Figure 5.2 Crambe seed image under binoculars: The picture shows the cotyledons at the outer side of the seed, and between the cotyledons the radicle is visible. For chipping, one cotyledon is cut off along the verticle line as indicated. Any damage to the radicle should be avoided.

5.2.6 Statistical analysis

One-way ANOVAs and correlation analyzes in the present research were run with SPSS statistics 17.0.

5.3 Results

5.3.1 Identification of transformants

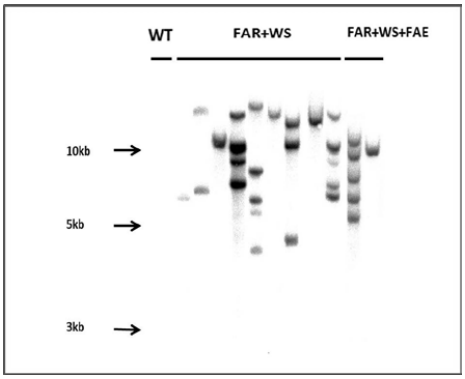


Figure 5.3 Southern blotting of DNA from T0 Plants: Insert numbers in transgenic crambe lines of either FAR+WS or FAE+FAR+WS were determined by Southern blotting. The DNA was digested with *Eco*RI and probed with a fragment derived from the sequence of the CMV promoter driving the *DsRed* gene.

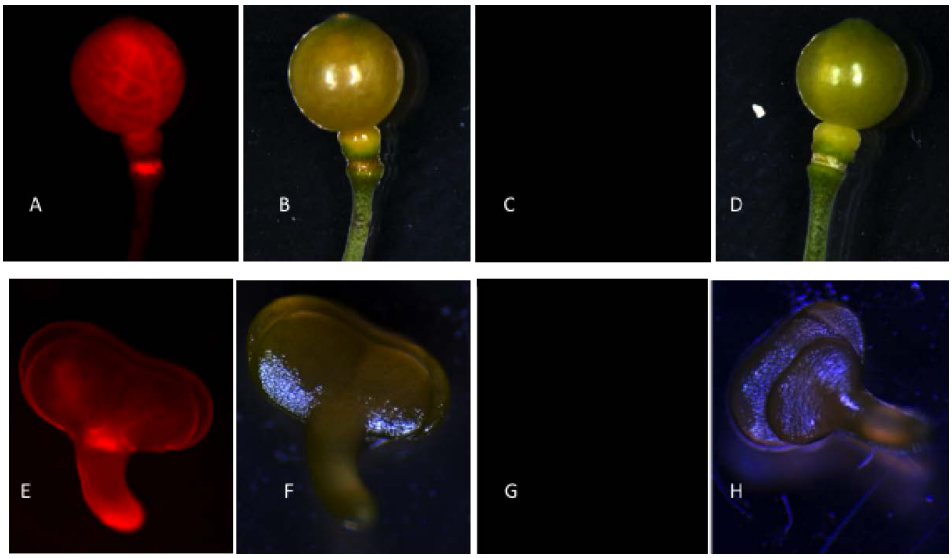


Figure 5.4 DsRed Fluorescence: Construct FAR+WS has a functional DsRed gene as a reporter gene, picture A and B show a T1 developing seed in UV and bright field microscopy, respectively, checking fluorescence of the pod; C and D show the WT; E and F shows developing T1 embryo; G and H show the developing embryo of WT.

In total, 22 independent transformation events for FAR+WS and five for FAE+FAR+WS were obtained and analyzed by Southern blotting. Among the transformants of FAR+WS, there were 13 with a single T-DNA insertion, two with two copies inserted, two with three, one with four, three with five and one with an insert number larger than six. Among the transformants of FAE+FAR+WS, copy number of the T-DNA insert varied from one to five. The Southern banding patterns of some of these T0 plants are shown in Figure 5.3. The transgenic character of T0 plants from FAR+WS and their offspring plants carrying T-DNA insertions were also confirmed by fluorescence of DsRed as shown in Figure 5.4.

5.3.2 Determination of WE content in the seed oil of different transgenic lines

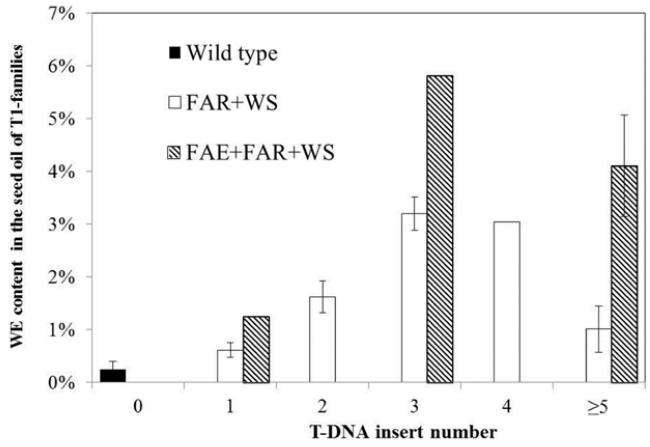


Figure 5.5 Relation between T-DNA insert number of FAR+WS and FAE+FAR+WS and WE content in GM crambe lines: The columns show the average WE content of the lines with similar insert number. The WE content data of individual lines were from bulks of 20 seeds. Bars indicate +/- standard error of the mean. The columns without error bar are based on only one line obtained for a certain T-DNA insert number. In total data on four WT families and 27 genetically modified events are presented.

The WE content of T1 families varied from 0% to 5.7%. The T1 seed family with the top WE content (5.7%) was from a FAE+FAR+WS transformant with a triple T-DNA insertion. The highest WE content found among the T1 families of FAR+WS was 3.7%, which was also from a transformant with a triple T-DNA insertion. When comparing lines with equal T-DNA insertion number, generally, T1 seeds of FAE+FAR+WS T0 plants had higher WE content than T1 seeds of FAR+WS T0 plants (Figure 5.5). Along with the increment of the T-DNA insertion number in T0 plant, the average WE content of T1 seeds increased and peaked at three inserts, but dropped when the insertion number increased further. The T1 seeds from a line with more than 12 T-DNA insertions, which was the highest number in this experiment, had no detectable WE accumulation.

5.3.2. Modulating the WE content by targeted selection in subsequent generations

From the FAR+WS transformants, we choose the T1 seed family (with triple T-DNA insertion) that had the highest average WE content. Only T1 seedlings that were verified by DsRed fluorescence were kept to propagate T2 seeds. Among the T2 seed families, two of them with highest WE content were chosen to produce T3 families. As shown in Figure 5.6, after the selection, the relative WE content for T3 seeds was significantly higher than the T1 and T2 and showed a sharp increase from T2 to T3 (Figure 5.6 A); the highest WE content record of individual families increased from 3.5% for T1 to 18.2% for T3 (Figure 5.6 B). Furthermore, the relative standard deviation of WE content of the acquired T3 families after selection was smaller than that of the T2-families. These results indicate a successful selection for higher WE content in the crambe lines of FAR+WS transformation (Figure 5.6).

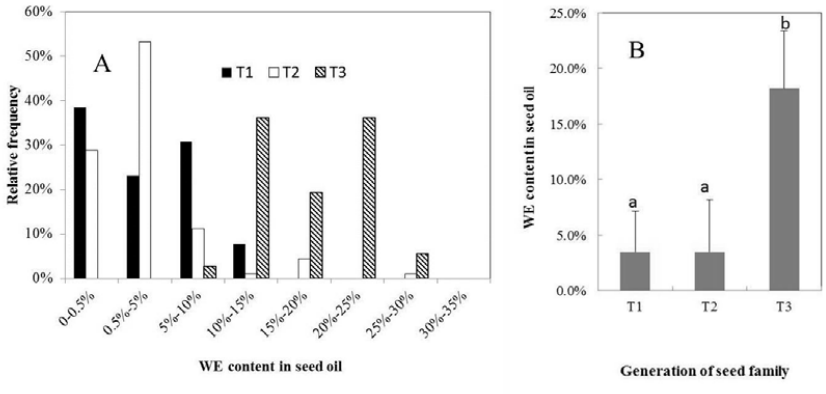


Figure 5.6 Effect of selection between and within different GM lines of crambe with the FAR+WS construct: A, T1 = Individual T1-seeds of a single T0-plant with a T-DNA insertion number of three; T2 = Individual T2-seeds from all T1-seeds that germinated (four T1-plants); T3 = individual T3-seeds of the best two T2 families; For T1 seed, the selection on their fluorescence nature was applied. The y-axis indicates the relative frequency of the appearance of the seed with its WE content in certain range marked in x-axis during the assay. The germinated seeds without fluorescence were discarded. From T2 to T3, the WE content is the criterion the two T2 lines with highest WE were chosen to generate the T3 families. B, After selection the T3 seed's relative WE content is significant higher than the T1 and T2, different letters on the top of error bars indicated a significant difference at the $p<0.01$ level according to a one-way ANOVA analysis.

The selection on progenies of FAE+FAR+WS transformants was performed on chipped seeds used for oil analysis and seedling establishment (Materials and Methods, 5.2.5). Sixty seeds from four T1 families were chipped. The half-seed WE content analysis showed that these T1 seeds had a wide distribution from less than 0.5% to higher than 45% (Figure 5.7). The half-seed sowing indicated that many of those with over 25% WE either failed to establish as seedlings, or the seedlings had very poor growth (Figure 5.6). Hence, the T1 plants from the seeds with WE content of 5% to 25% were selected for harvesting T2 generation, to select seed families giving better seedling growth as well as moderate WE content. After selection, the T2 seeds of the selection outcome showed a narrower distribution of WE content, which was from 0.5% to 25%, in comparison with the T1 (Figure 5.7).

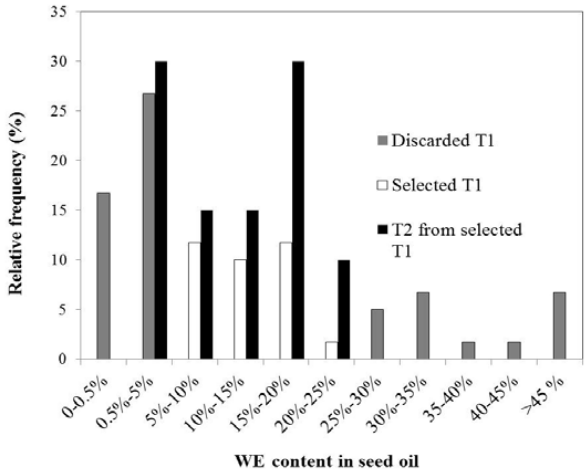


Figure 5.7 Effect of selection between and within different GM lines of crambe with the FAE+FAR+WS construct: In total there were 60 seeds from T1 families with one to five T-DNA insertions used for this research. The T1 seeds indicated WE content more than 25% were all from lines with five T-DNA insertions. The average of T1 seed WE content was 13.5%, but the T2's fell into 10.1% due to the loss of germination and selection.

5.3.3 Characterization of WE species in the GM crambe lines by ESI-MS/MS

Three GM crambe seed families with the highest WE content were selected for EMI-MS/MS analysis. Two of them were T3 families (W2T3B1 and W2T3B2) of FAR+WS. They are from the same T2 family, which originated from a T0 plant with triple T-DNA insertions. The other one was a T2 family of FAE+FAR+WS (ID: W3T2B1). It also originated from a T0 plant with triple T-DNA insertion. Five seeds from each family were analyzed individually.

Figure 5.8A shows the absolute WE content of every seed analyzed. Among them, the highest absolute WE content detected was 252.9 mg/g (WE/seed), which was 65% in terms of relative WE content. The different WE species (individual fatty acid and fatty alcohol constituents) in these GM seeds were also identified and quantified (related to the total WE molecules in the seed oil). There were 462 and 412 different WE molecules detected in the seed oil of FAR+WS and FAE+FAR+WS, respectively. In general, these detected WE molecules consisted of 28 kinds of fatty acid and 28 kinds of fatty alcohols.

Figure 5.8B shows the top-10 (in terms of concentration) WE molecules in these three seed families. In these GM seeds, the sum of those top-10 molecules added up to 50% of the total seed WE content. WE of 22:1/20:1 (fatty alcohol/fatty acid) had the highest concentration, and WE

22:1/22:1 was the second (Figure 5.8 B), in all of the analyzed seeds. Both the concentrations of 22:1/20:1 and 22:1/22:1 (reaching 32% of total WE) in the seed oil of FAR+WS, were significantly higher than those of FAE+FAR+WS (totaling up to 20% of the total WE) (Figure 5.8 B).

The WE species present in the seed oil of FAE+FAR+WS contained more C24:1 moieties than those of FAR+WS. The full profile of fatty acids and fatty alcohols bound in WE (Figure 5.8 C) demonstrates that the main alcohols incorporated into WE were C22:1 OH, C22:2 OH, C20:1 OH and C22:0 OH for FAR+WS transformants and C22:1 OH, C24:1 OH, C22:2 OH, C24:2 OH, C20:1 OH and C22:0 for FAE+FAR+WS. Among the fatty alcohols in GM crambe seeds of FAR+WS and FAE+FAR+WS, erucyl alcohol always had the highest concentration. Further, the C20:1 and C22:1 fatty acids were preferably integrated into WE, more than the other fatty acids.

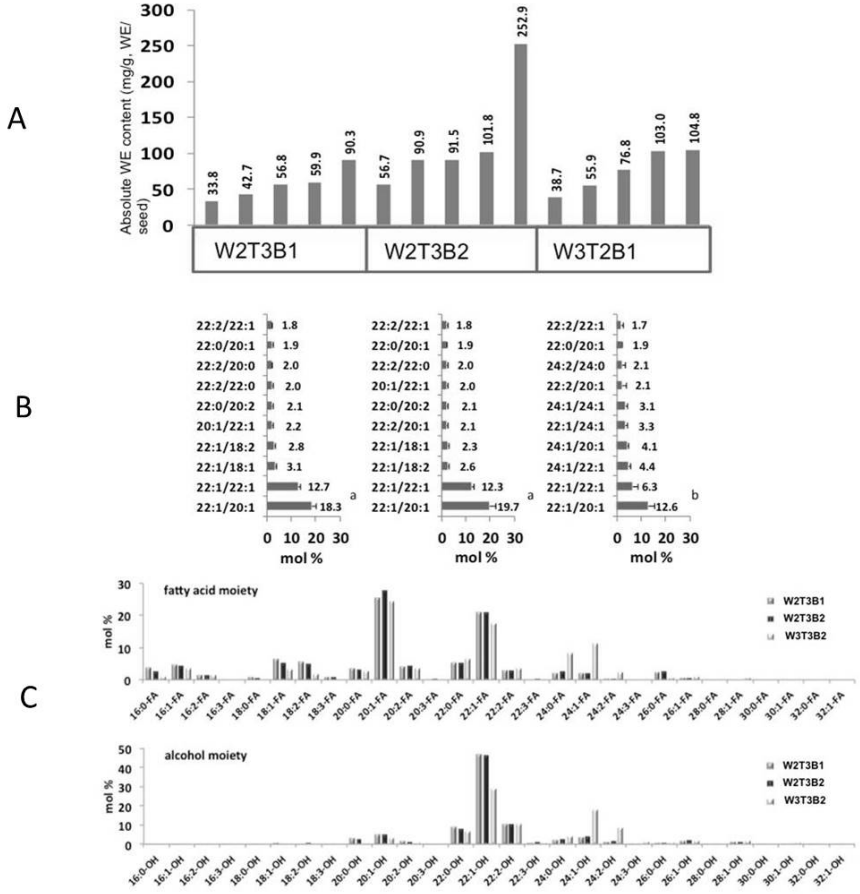


Figure 5.8 Analyzes of WE composition in GM crambe seeds by ESI-MS/MS: Individual seeds from 3 families (W2T3B1; W2T3B2 and W3T2B1 respectively) with the highest WE content after selection were taken. A is the absolute seed WE content of the families out of the selection. W2T3B1 and W2T3B3 are T3 families of FAR+WS; and W3T2B1 is T2 family of FAE+FAR+WS. Five seeds were randomly selected from each family. B shows different WE species' relative content in the total WE of the GM crambe seeds demonstrated by chart A; the columns show the WE species of top-10 concentration; the left part is fatty alcohol moiety and right fatty acid; The letters between 22:1/20:1 and 22:1/22:1 means that follow the one-way ANOVA test, the concentrations of these two WEs in FAE+FAR+WS are significantly different from the other two FAR+WS families, respectively with $p<0.01$. C displays the fatty alcohols and fatty acids built into the WEs, and their concentrations contained by the seeds of those three families.

5.3.4 Tailoring WE content after crossing FAR+WS GM plants with FAD2-RNAi GM plants

To test the effect of the gene combination of *ScFAR, ScWS* and *CaFAD2-RNAi* on WE content and composition, FAR+WS GM plants were crossed with FAD2-RNAi GM plants. In total, 33 F1 seeds were obtained from these crossings. After germination and seedling establishment, 24 seedlings showing DsRed fluorescence were kept for F2 seed harvesting; the remaining nine seedlings did not show DsRed fluorescence and were discarded. The number of F1 fluorescing plants from each cross is shown in Table 5.1. The two parental plants of FAR+WS were T1 generation, and from a same single insert T0 plant. The segregation of DsRed fluorescence phenotype in their progenies indicated that both of them were heterozygous. On the other hand, the CaFAD2-RNAi parents (Wg4-1; Wg4-2; Wg19-1; Wg19-3) contained multiple T-DNA insertions. It is not known whether they are present in one locus or in multiple loci.

Table 5.1 Crossing combinations and numbers of F1 plants used for further research

Crossing ♂ x ♀	No. of kept F1 Plants
Tr09-10,F5A×Wg19-3	10
Tr09-10,F5B×Wg19-1	1
Tr09-10,F5A×Wg4-1	2
Tr09-10,F5A×Wg4-1	2
Wg4-2×Tr09-10,F5B	2
Tr09-10,F5A×Wg19-1	6
Tr09-10,F5A×Wg4-2	1

Two F2 families from the crossing between Wg4-2×Tr09-10, F5B were chosen for GC analysis. Twenty seeds of each line were analyzed individually, with seeds from their parents and the wild type (untransformed cv. 'Galactica', WT) as controls, as shown in Figure 5.9. The average WE content of F2 family seeds was 4%. In the FAR+WS parental family, the average T2 seed WE content was 2%. The highest WE content of individual F2 seeds was found to be as high as 12%, while the highest WE content obtained from individual T2 seeds of the FAR+WS parent was 6%. Clearly, WE levels can be increased by knocking down FAD2 activity. The effect of CaFAD2-RNAi on GM crambe seed with or without WE was evaluated according to the decrement of its PUFA content. The average content of PUFA in WT seed (the control) was 13.6%; and 11.7% in T2 seeds (Figure 5.9) from the FAR+WS parent. The PUFA content of F2 seeds was 4.6% on average. This was similar to the 5.3% PUFA content in the T2 seeds from the CaFAD2-RNAi parent.

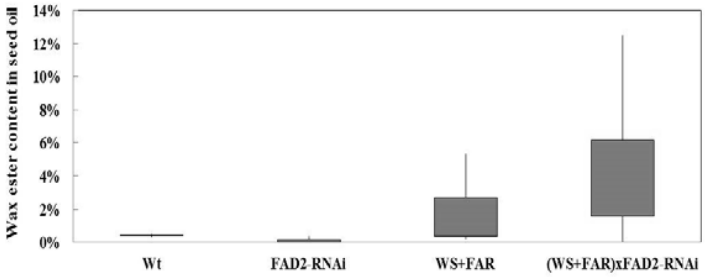


Figure 5.9 WE content in seed oil of WT, FAR+WS line, CaFAD2-RNAi line and of the cross combination ScFAR+ScWS+CaFAD2-RNAi: Wt = Wild type cv Galactica; FAD2-RNAi = transgenic line of CaFAD2-RNAi; WS+FAR = transgenic line of FAR+WS**;** (WS+FAR) x (FAD2-RNAi) = cross between FAR+WS transgenic line and CaFAD2-RNAi transgenic line.

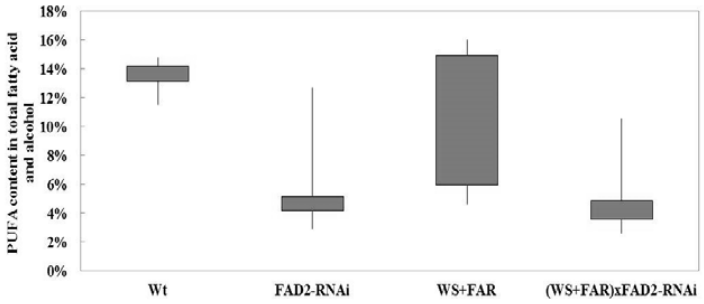


Figure 5.10 PUFA-content (C18:2+C18:3) in seed oil of WT, FAR+WS line, CaFAD2-RNAi line and of the cross combinating ScFAR+ScWS+CaFAD2-RNAi: the labels are as same as Figure 5.8.

5.3.6 Seed germination and seedling growth of the GM seeds containing WE

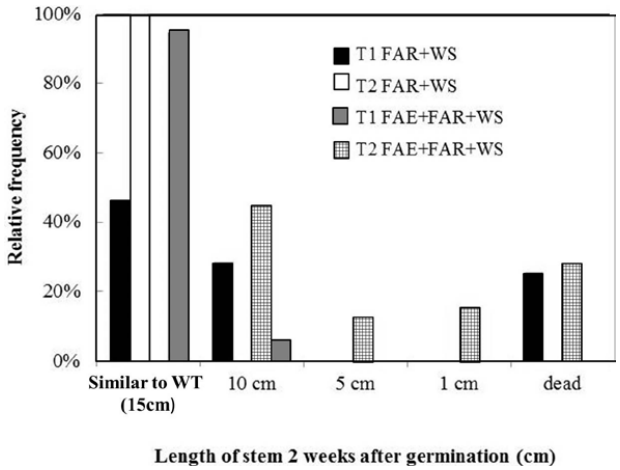


Figure 5.11 Seedling establishment and seedling vigor (%): According to the genetic background of the seeds used for the half seed analysis, the seedlings could be classified into 5 groups. WT were used as control but its data were not included into the figure. The seed lots taken were WT (16 seeds), T1 seeds of FAR+WS (35 seeds), and its T2 seeds (29 seeds), T1 seeds of FAE+FAR+WS (60 seeds) and its T2 seeds (20 seeds). The seedling growth situation was checked two weeks after the first seedlings emerged. The picture describes the phenotype distribution within each group. The X axis demonstrates the seedling growth phenotype, of which mark 'similar to WT (15cm) means the seedling with a growth similar as WT seedlings($10 < X \leq 15cm$); '10cm' is $5 < X \leq 10cm$; '5cm' is $1 < X \leq 5cm$; '1cm' is $X \leq 1$cm; 'dead' is without germination. The values of Y axis demonstrated the percentages of chipped seed with certain seedling growth phenotype within each group.

A low seedling vigor from high WE content families was observed during the subsequent generations. To investigate the relationship between the WE content in GM seed and the reduction in germination rate, T1 and T2 seeds of FAR+WS and FAE+FAR+WS, and WT seeds were randomly selected and chipped for half seed analysis and seedling growth evaluation. The small chopped cotyledon part (chip) was used for GC analysis, and the remainder was used for germination in soil. The growth of the seedlings, established from the chipped seeds, was monitored two weeks after sowing. All of the chipped WT seeds became seedlings successfully. The length of WT seedlings varied from 10 to 15 cm, which was used as standard. In comparison with WT, the lengths of chipped GM seeds were classified into five groups (Figure 5.11 and

Figure 5.12): GM seedlings which were as long as or more than 10 cm were classified into the group "*Similar to WT (15 cm)*"; those with a length less than 10 cm but more than 5 cm, were in the group marked as "*10 cm*"; those with length less than 5 cm were in group "*5 cm*"; those with only visible cotyledon expanding but less than 1 cm comprised in group "*1 cm*"; and the final group " *dead*" included those seeds that failed to germinate. As shown in Figure 5.10, most T2 seedlings belonged to the group "*similar to WT*"; the T1 seeds of FAE+FAR+WS showed a remarkable growth delay compared to the WT seeds. As shown in Figure 5.12, the T1 seeds of FAE+FAR+WS also showed the widest variation in WE content in comparison with other seed classes. The defect of germination and seedling growth was significantly correlated with the seed WE content with $p<0.05$. This demonstrates that the more WE accumulated in a seed, the higher the risk was of finding seedling growth delay or even germination failure.

In the experiment described above, no T3 seeds of FAR+WS have been included, because at the time of analyzing no T3 seeds were available yet. After acquiring the T3 seeds families from the selection described in 5.3.2, two families (W2T3B1 and W2T3B2) with a WE content of more than 20% were also tested for its seed germination rate by directly sowing into the soil (without mechanical treatment). Both seed families exhibited a germination rate lower than 60%, while for wild type control it was 100%.

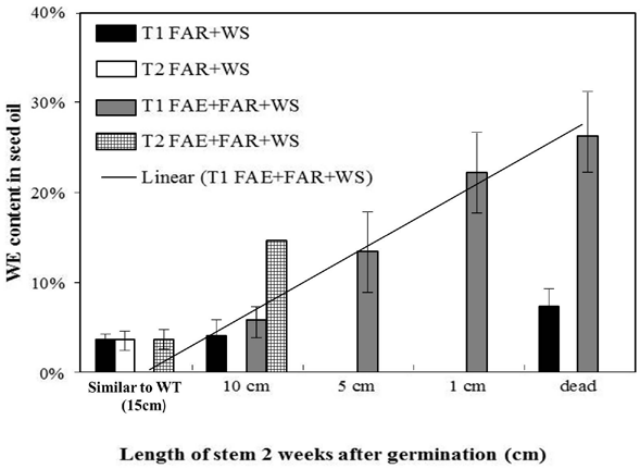


Figure 5.12 Relative frequency of lines in class of seedling vigor (%): The data shown in this figure were values derived from data also taken for Figure 5.11 (displayed as mean with SE bar), but correlated the seedling growth phenotype with the original seed WE content. Among the 5 seed-groups, only the T1seeds of FAE+FAR+WS indicated a wide range of WE content distribution as well as their phenotype. According to the Pearson correlation analysis, the WE content of seeds from T1 FAE+FAR+WS correlated with their negative seedling growth phenotype significantly, with an R=0.989. The seeds of T2 FAE+FAR+WS had a less wide distribution of their seedling growth phenotype. The chosen T1 and T2 chipped seeds of FAR+WS also had a narrow distribution of their WE content.

5.4 Discussion

The successful transformation of jojoba *FAR* and *WS* genes into *Arabidopsis* and rapeseed demonstrated the possibility of accumulating jojoba-type WE in transgenic plants (Lardizabal et al., 2000; Metz et al., 2000). Potentially, rapeseed could be developed into a crop for the commercial production of jojoba-type WE. However, such commercial production will greatly suffer from the (perceived) risk of contaminating *Brassica* food or feed oil-crops around the world by unintended crossing or by mixing of food and GM-rape further down in the processing chain. This contamination risk is realistic although not yet quantified (Wilkinson et al., 2003).

Crambe cannot cross with *Brassica* crops and identity preservation during the processing further down the chain is easy as crambe seeds are easily distinguished from rapeseed, making crambe an ideal alternative for jojoba WE production. By transforming *FAR*, *WS* and *FAE* genes driven by the soybean glycinin promoter into crambe, we have shown the feasibility of producing WE in GM crambe seeds. WE production in GM crambe seeds is directly related to one of the objectives of the ICON project which is using crambe as a production platform crop for different industrial oils (Stymne and Dyer, 2006). The objective of ICON was to achieve a relative WE concentration of at least 20% (WE to total oil), because with this amount of WE in seeds the cultivation of GM crambe could be economically viable or even attractive. The present results showed that transforming FAR+WS or FAE+FAR+WS vectors into crambe could fulfil this objective. The oil composition analysis of T1 to T3 seeds by GC demonstrated that the relative WE content of single seeds varied from very little (similar to WT) to higher than 50%. By selection and multiplication of lines and individual seeds with high WE content (based on family evaluation and half-seed analysis) the relative WE content in seeds was elevated greatly from the T1 to the T3 generation. The highest WE contents were found in one T3 line from a triple T-DNA insertion T0 plant of FAR+WS and one T2 line from a triple T-DNA insertion plant of FAE+FAR+WS. The seed oil compositional analysis by ESI-MS/MS showed that the highest absolute WE content detected in a single seed was 252.2 mg/g (WE/seed, w/w) which is equivalent to 65% of wax ester in the seed oil.

The relative (WE per total oil) WE content analysis of T1 seeds displayed that the T-DNA copy number of T0 GM plants correlated with the WE content of the T1 bulked seeds. An increase in WE content was observed with an increasing T-DNA insertion number in the range from one to three, but decreasing again with more than three inserts. This could be due to an additive gene dosage effect at lower copy numbers, followed by gene silencing when too many gene copies are present. A gene-dosage effect was also seen when we compared seeds from homozygous offspring plants to heterozygous offspring plants in the T1 generation of a single T-DNA insertion line of FAR+WS (also used for the crossing with FAD2-RNAi). Here it was found that the seeds from a homozygous T1 plant had both their average and top WE content higher than the T1 seeds from a heterozygous plant. It suggests that in present research the T-DNA copy number is positively correlated to the WE content, probably due to a gene dosage effect. In previous researches, similar phenomena have also been found in other plant species (Hewelt et al., 1994; Guo et al., 1996; Osborn et al., 2003). However, it was also obvious that the lines with more T-DNA insertions (>3) did not have more WE, but instead even lower amounts. The largest insert number found was more than 12 with the FAR+WS transformations, but here no WE was detected in the T1 seeds at all. The reasons why high T-DNA insertion T1 families produce less or even no WE remain unclear. One hypothesis is that co-suppression (Napoli et al., 1990; Ketting and Plasterk, 2000) effects can be induced more easily by large numbers of T-DNA inserts. In the present research, the highest WE content seeds originated from triple T-DNA insertion T0 plants, it is unknown yet how many T-DNA copies the high wax content seeds have.

Another factor identified that could enhance the WE accumulation was CaFAD2-RNAi that also led to decrease of PUFA (Cheng et al., 2013). The F2-seed WE content assay showed that the combination of FAR+WS and CaFAD2-RNAi resulted in higher WE levels than mere FAR+WS. Blocking or reducing FAD2 activity could save more C18:1 acyl molecules from being taken up in PUFA biosynthesis and keep them available for the production of C22:1 acyl. C22:1 acyl molecules are preferred by FAR for producing C22:1-OH which later will become a part of WE (Okuley et al., 1994; Jadhav et al., 2005). On the other hand not only increasing the level of C18:1

substrate towards the C22:1 needed for the jojoba FAR and WE, is expected to increase the WE-production, but also the jojoba FAE is assumed to enhance the WE content in GM crambe seed by increasing the direct substrate levels for FAR and WE. The GC analysis on T1 seeds indicated, that indeed the WE content in the seed of FAE+FAR+WS was higher than in the seed of FAR+WS.. However, due to the issue of germination and seedling growth, most of the transformants of FAE+FAR+WS with the highest WE levels were lost due to low germination rates and seedling survival of seeds with very high WE. The GM crambe seed oil analysis also showed that the seeds of FAE+FAR+WS had more 24:0 and 24:1 fatty acid and fatty alcohol than the seeds of FAR+WS, so apparently the jojoba *FAE* not only leads to higher levels of C22:1 fatty acids and alcohols, but also promotes further elongation to C24-chain lengths more than the native crambe FAE.

Table 5.2 The comparison of fatty acids and fatty alcohols in Jojoba WE and GM crambe WE

	Fatty Alcohols(%)	Jojoba	Crambe (FAR+WS)	Crambe (FAE+FAR+WS)
Hexadecanol	C16:0	0.1	0.36	0.1
Octadecanol	C18:0	0.2	0.73	0.2
Octadec-9/11-enol	C18:1	1.1	1.1	0.5
Eicos-11-enol	C20:1	43.8	5.5	3.8
Docosanol	C22:0	1	9.3	6.9
Docos-13-enol	C22:1	44.9	46.0	28.9
Tetracos-15-enol	C24:1	8.9	4.0	18.0
Others		Trace	33.01	41.6

	Fatty Acid (%)	Jojoba	Crambe (FAR+WS)	Crambe (FAE+FAR+WS)
Hexadecanoic	C16:0	1.2	4.1	1.3
Hexadec-7/9-enoic	C16:1	0.3	4.9	3.9
Octadecanoic	C18:0	0.1	1.1	0.4
Octadec-9/11-enoic	C18:1	11.2	6.8	3.6
Eicosanoic	C20:0	0.1	2.8	3.1
Eicos-11-enoic	C20:1	71.3	25.3	24
Docos-13-enoic	C22:1	13.6	20.9	17.6
Tetraco-15-2noic	C24:1	1.3	2.2	11.3
Others		0.9	31.9	34.8

Note: The tables demonstrate the contents of main fatty alcohols and fatty acids in jojoba WE and the corresponding contents in GM crambe WE. The data of crambe was from the seeds with the highest WE content identified by ESI-MS/MS (Table 5.8). Table A shows the fatty alcohol and table B the fatty acid contents. The Jojoba WE composition data is from the study reported by Van Boven *et al.* 1997.

ESI-MS/MS analysis demonstrated that WE produced by GM crambe was a more complicated mixture with different types of WE molecules than the jojoba WE. As shown in Table 5.2, compared to jojoba WE, the WE mix from GM crambe had more WE molecules with fatty chain moieties longer than 20-carbons. For example, among the WE molecules from GM crambe seed, relatively more WE was found with of a fatty acid and alcohol both with length of 22 (22/22 WE) than in jojoba oil; The 22/22 WE proportion was in total about 16% for FAR+WS, and 8% for FAE+FAR+WS while only 2.1 % in jojoba oil. Further in the WEs from FAE+FAR+WS GM crambe seed, five times more 24/24 WE (with fatty acid/fatty alcohol pairs: 24:1/24:1+ 24:2/24:0)

was found than in jojoba oil (5.2% in FAE+FAR+WS GM crambe found here versus only 1.2% in jojoba).).

The fatty alcohols in jojoba WE contain about 45 % C22:1-OH, 44 % C20:1-OH, 9 % C24:1-OH, 1 % C22:0-OH and no C22:2-OH (Van Boven et al., 1997). GM crambe WE contained only 5% C20:1-OH (more than 8 times less than in jojoba WE).The level of C22:1-OH in crambe FAR+WS was the same as in jojoba (45 %) and the level of C24:1-OH (4 %) was half that in jojoba. In crambe FAE+FAR+WS much less C22:1-OH was found (only 29%) than in jojoba and much more C24:1-OH (18 %, which is twice that in jojoba). Remarkably, 8 % C22:2-OH was found in crambe WE while C22:2-OH does not occur in jojoba WE. The level of C22:0-OH in crambe WE (6 %) is six fold that in jojoba WE. In jojoba WE, the amount of C20:1 fatty acid can be up to 71% of the total fatty acids (Van Boven et al., 1997), while its concentration in GM crambe seed WE was relative low (25%). In general, the GM crambe WE had more very long chain fatty moieties (≥C22) than jojoba WE both with only FAR+WS and with the extra jojoba FAE in the FAE+FAR+WS.

The negative effects of the high WE levels on the germination of GM crambe and on seedling growth can be a serious constraint for selecting high WE producing lines. Especially, crambe seeds with the highest WE content will fail very often to germinate or to grow into healthy plants. For instance, the results of T1 seed WE content analysis (bulk seed and single seed chipping) indicated that the GM seeds of FAE+FAR+WS had more WE accumulated than those of FAR+WS. However, as shown in Figure 5.6 and 5.11, because many high WE content containing lines were lost during (half seed) seedling growth, in the further generation, the WE content in the seed oil of FAE+FAR+WS was lower than in the earlier generation as only surviving genotypes could contribute to the next generation and survival until seed maturation was negatively correlated with WE content. Cahoon and coworkers from Nebraska-Lincoln University found the same phenomenon in WE containing seeds of GM *Camelina sativa* (personal communication). The free fatty alcohols released from WE bio-decomposition, possibly are responsible for the growth delay or failure to germinate in seedlings with WE. An important physical characteristic of fatty alcohol is that it can act as a detergent. Detergents are generally very harmful to cell membranes, and this could be the reason for the observed difficulties in germination of seedling growth. According to the results shown in Figure 5.11, the seed with a WE content of more than 27% had a very high risk of germination failure. And the seedlings established from the seeds with WE content from 6% to 23% suffered from growth delay. The potential damage caused by this phenomenon can be very serious in later field trials, because the fraction of weak seedlings will be more vulnerable to biotic and/or abiotic stresses in the natural environment. The consequences can be that after sowing, seed germination and seedling growth will be too poor to establish enough plants in the field. So, if people want to establish a GM crambe field producing WE, the germination problem has to be solved in advance. Selecting the low WE content crambe seed for sowing as we did can be one option. But, it may damage the economical attractiveness of such a GM crop. Alternatively, another solution for this problem is to introduce new genes coding enzymes, which can digest the WE specifically at germination time by genetic modification. Recently, two genes responsible for conversion of fatty alcohol back into fatty acid have been identified from the germinating seeds of jojoba (Rajangam et al., 2013). Introducing these two genes into the GM crambe again with a promoter specific to the developing seedling cotyledon may solve the WE seed germination problem. Thus, retransformation of these two genes into WE producing lines based on hygromycin selection, or crossing the transformants containing these

two genes with WE producing lines are two ways available for combining all genes required and possibly solving the germination problem in high WE producing lines and seeds.

Chapter 6: General discussion

The results of the research described in the experimental Chapters 2 to 5 were obtained within three independent work packages (WPs) of the European Union 7th Frame Project "Industrial Crops producing added value Oil for Novel chemicals (ICON)". The achieved goals for WP1 "establish a high efficiency transformation protocol for *Crambe abyssinica* (crambe)" are presented in Chapter 2. It described the development of a protocol of *Agrobacterium*-mediated transformation for crambe with cotyledonary-nodes from 7-day-old seedlings as explants. This new protocol was deployed to produce genetically modified crambe for several other WPs. To be more ecofriendly and increase the public acceptance of GM crambe, the pMF1 marker-free system developed by Wageningen UR Plant Breeding was successfully deployed in the crambe transformation, which fulfilled the other target of WP1 (Chapter 3). The deliverables of WP3 'production of ultra-high (more than 66%) erucic acid containing oil in crambe' were also accomplished and described in Chapter 4. There were two vectors constructed to improve erucic acid content in genetically modified crambe seed (Chapter 4). One vector was pHan2 containing *Limnanthes douglasii* lysophosphatidate acyltransferase (*LdLPAT1*) and *Brassica napus* fatty acid elongase *(BnFAE1)*. The other one was pWatergate-3G containing *LdLPAT1*, *BnFAE1* and an RNA interference (RNAi) construct of the crambe endogenous fatty acid desaturase 2 gene (*CaFAD2-RNAi*). These genetic modifications improved the erucic acid content of crambe seed from about 60% to 79%. Although this was already a large increase, there is still room for further improvement. The potential competition effect of the endogenous LPAT2 on the exogenous LdLPAT1 is acknowledged as a suspected factor that restrains the erucic acid accumulation further. To reduce this competition, a highly preserved region of endogenous *LPAT2* mRNA (*CaLPAT2*) was selected as the silencing target and an RNAi vector, pHellsgate *CaLPAT2-RNAi* was constructed. The transformation of this vector into crambe and *Arabidopsis* indicated that the selected fragment of *CaLPAT2* was a good target to knock down the expression of this gene. In further research, the *CaLPAT2-RNAi* was assembled with *LdLPAT*, *BnFAE1* and *CaFAD2-RNAi* together into one construct for crambe transformation. But this research did not prove that this 4-gene combination could elevate the content of crambe erucic seed oil to a higher level than the pWatergate-3G did. For WP4 "produce jojoba like wax esters (WE) by genetically modified crambe", the feasibility of using crambe seed as a platform to produce novel WE was tested (Chapter 5). This was achieved by the transformation of two vectors into crambe. One vector was pBinGlyRed *ScWs + ScFAR* (pFWS3-2) containing *Simmondsia chinensis* (jojoba) *wax synthase* gene (*ScWS*) and *fatty acid reductase* gene (*ScWS*). The other vector was pBinGlyRed *ScWs + ScFAR + ScFAE* (pFWS3-3) containing *ScWS*, *ScFAR* and *fatty acid elongase* gene (*ScFAE*) from jojoba. In the best case, a single seed contained 25.2% WE of the seed dry weight and as much as 73% of the total seed oil. The pFWS3-3 transgenic crambe seeds had more nervonic acid deposition than the pFWS3-2 transgenic seeds. Therefore, all the targets of the ICON work-packages concerned by this thesis have been achieved. The results and their potential implications are discussed in more detail in this general discussion.

6.1 Observations and remarks

6.1.1 Avoiding chimerism

The phenomenon of chimerism has been frequently reported in plant transformation via tissue regeneration (Geier, 2012), which was also observed in the current research. In Chapter 2, to prove a successful transformation, the presence of the *Neomycin phosphotransferase II* (*NPTII*) gene and an activity of β-glucuronidase (GUS) were respectively verified at the molecular level by PCR and at histochemical level by GUS staining. In many cases, part of the transformed tissue showed a positive result in GUS staining, indicated by a blue color, whereas other parts of the tissue showed a negative result, indicated by white color (Figure 6.1A). This might be due to either very low expression of the B-glucuronidase in certain cells or due to chimerism, which was also thought to be the reason for partial coloring when cotyledonary explants were transformed (Chapter 2). Here a shoot had a negative result in amplification of *NPTII* fragment but a positive result in the GUS staining. Such possible chimeric cases had also been observed in some

pBinGlyRed (ASCI) T0 transformed tissue (Chapter 5). Under the fluorescence microscope, only a part of a regenerated shoot had DsRed fluorescence (Figure 6.1B).

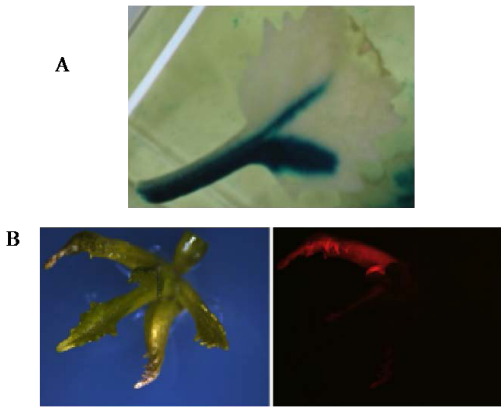


Figure 6.1 Examples of chimeric phenotypes found in different experiments: A chimeric leaf tissue from the transgenic candidate of pMF1 transformation verified by GUS staining; B a chimeric shoot regeneration cluster of pBinGlyRed ScFAR+ScWS identified by fluorescence microscope observation.

Furthermore, chimerism of T0 GM plants was clearly revealed in the test of the pMF1 marker-free system in the transformation of crambe (Chapter 3). The *in vitro* starting plantlets from two independent T0 transformants of pMF1-RbcSGFP-RbcSGUS were treated with dexamethasone (DEX). The axillary buds from each starting plantlet were isolated for further regeneration by which dozens of new plantlets were amplified from one single plantlet. Once those amplified plantlets were put into regeneration medium with the negative selection of 5-fluorouracil (5-FC), the non-marker free tissues would be eliminated, while only marker-free tissues should be kept. However, at the end of the experiment, the remaining tissues tested by PCR (for the presence of *NPTII*) and GUS staining indicated that in some tissues, besides the marker-free and non-marker-free shoot-regeneration clusters, there were also somatic wild type (WT) cells. These WT cells originated from about 0.5% axillary bud explants and no starting plantlet was pure WT. This clearly points to chimerism.

For the convenience to select GM plants in further generations and to analyze the actual effects from the engineering, it is quite important to be able to identify genuine transformants. Considering the importance of acquiring reliable transformed plants, the cases of escape and chimerism should be avoided as much as possible. For the T0 plant, a stringent selection with a selecting agent at high concentration and an increased time of selection are two options. In our case, compared to increasing the Kanamycin concentration, prolonged time of selection was preferred (Chapter 2). On the other hand, to stabilize the ideal phenotype, the selection among the offspring of these T0 GM plants is always needed. It is convenient to select pure GM germplasm from further generations. Going through meiosis is the most efficient way to get rid of chimerism.

6.1.2 Optimizing the copy-number of T-DNA insertion for a desired trait

It has been observed in many researches that a desired trait has a certain correlation with the copy-number of a gene. Such a gene can be endogenous or exogenous. For example in maize, a research in a ploidy series (from monoploid to tetraploid) showed that the expression levels of some genes had a large variation (Guo et al., 1996). In that research, the *thiol protease* gene had the highest expression level in monoploids and decreased in correspondence to the ploidy increment. And the expression level of a *MAD box gene* increased in correspondence to the ploidy increment; peaked in the triploids and decreased from triploid to tetraploid. For the exogenous gene introduced by GM, the expression also varied in correspondence to the copy-number of the T-DNA insert. The research of *GUS* gene transformation in potato showed that all the GM plants

with high GUS activity contained a single T-DNA insert (double copy in the homozygous), and the GM plants with low GUS activity contained multiple T-DNA inserts (Hobbs et al., 1993). In our GM crambe research, we also observed a relation between the GM trait and the number of T-DNA inserts. For example in Chapter 3, the expression level of *NPTII* and *CodA* in the plant with double T-DNA insert was higher than that of the single T-DNA insert transformant. Later in Chapter 5, the relationship between GM trait and the T-DNA insert number was best illustrated in producing wax ester. Southern Blotting determined the T-DNA insert number of 27 independent T0 transformants. The T1 seed oil analysis showed that within a certain range of increment in the T-DNA insert number of T0 plants, the average WE content in T1 seeds was enhanced correspondingly. However, once a certain T-DNA insert number had been reached, further increment lead to a decline of average WE content. The largest T-DNA insert number of T0 transformants in this research was more than 12 of which the T1 seeds had no WE accumulation at all (Chapter 5). Furthermore, in Chapter 4, the pHan2 transformation that was aimed at increasing the erucic acid and suppressing the oleic acid, by vectoring *LdLPAT* and *BnFAE1* driven by a seed specific promoter showed also that higher T-DNA insert number does not lead to higher erucic acid amounts. Surprisingly, the T1 seeds of the pHan2 transformant with sextuple T-DNA insertion, showed an opposite phenotype rather than the expected. The seeds from this family have very low erucic acid content but very high oleic content. Such reversed phenotype has also been noticed in the T1 seeds from pWatergate-3G transformation, in which two T1 seed families had the same seed oil profiles.

That enhancement of desired traits is corresponding with the increment of T-DNA insert number can be explained by a dosage effect of gene expression. On the other hand, co-suppression may explain that once the T-DNA insert number was larger than a certain level the WE content was lower (Chapter 5). This was consistent with other studies in which most co-suppression happened in large T-DNA insertion number transformants. For instance, as mentioned above, all GM potato lines with low GUS activity carried multiple T-DNA insertions (Hobbs et al., 1990). Research of introducing a *chimeric chalcone synthase* gene into Petunia showed that 66% co-suppression cases were from multiple T-DNA insertion events, while 34% were from single T-DNA insertion events (Napoli et al., 1990). Co-suppression was notable in Chapter 4 in which vectors pHan2, pWatergate-3G and pWatergate-4G were independently transformed in to crambe to elevate the C22:1 content and suppress C18:1 content in the GM seeds. Three phan2 transformants were verified by Southern blotting for their T-DNA insertion number. In these three independent T0 events, two were with double T-DNA insertion, and one with sextuple T-DNA insertion. The oil profile in the T1 seeds of the transformants showed higher C18:1 but lower C22:1 content than WT. Such reversed phenotype was also detected in pWatergate-3G transformants. It is reasonable to assume that the reversed phenotype is caused by the co-suppression of endogenous *CaFAE* from the highly expressed *BnFAE1* driven by a Napin promoter on the T-DNA. *FAE* (also called *KCS*) is the vital element of the fatty acid elongase complex. It is directly in charge of the conversion from C18:1 to C22:1 (Rossak et al., 2001; Mietkiewska et al., 2007; Rahman et al., 2008). The high C18:1 and low C22:1 oil profile suggested that this pathway was blocked in these seeds. Interestingly, in the propagation of the sextuple T-DNA insertion pHan2 family, we found that seeds in a few families showed recovered erucic acid content. Seeds of one T3 family showed increased erucic acid content, up to 72% in a single seed. This phenomenon suggested that the reduced T-DNA copy-number in segregating descendants might explain the recovery from co-suppression effect. Therefore, the selection of desired traits from the segregating offspring of transformants with large T-DNA copy-numbers insertion can be a good strategy to rescue valuable GM germplasm from co-suppression.

6.1.3 How to get seeds with high WE content still able to germinate

GM crambe after the transformation of jojoba gene vectors pFWS3-2 and pFWS3-3 accumulated jojoba WE as a novel compound in its seeds besides the normal TAG. The highest WE content we achieved in single seeds ranged up to 73% of the seed oil (Chapter 5). However, these high WE content seeds showed a poor germination vigor, which puts restrictions on their potential

application in field trials. This poor germination vigor of seeds was observed in the greenhouse, in which the environmental factors are controlled and relatively stable and suitable for plant growth. Therefore, using crambe as a platform to produce WE has been only a partial success in laboratory and greenhouse conditions till now. The germination vigor is expected to be even lower if plants are to be grown in the field. This problem must be solved before GM crambe can be grown in the field to produce WE.

Addressing this problem, there are two technical routes to solve the problem. One is to select seed families with moderate WE content that do not have such problem of germination and seedling growth. Clearly, a moderate level of WE will reduce the economic attractiveness of the GM crambe (Chapter 1). An alternative is to try and rescue crambe seeds with poor germination vigor but with high content of WE. Although WE is not the normal storage lipid in seeds of crambe (triacylglycerols), its basic structure is similar to triacylglycerol (TAG) (Lassner et al., 1999). Theoretically, the endogenous lipase in charge of breaking the ester bond of TAG can also break the ester bond between fatty acid and fatty alcohol. Subsequently, the free fatty alcohol that functions as a strong detergent can be very harmful to the cellular membrane. Lacking the proper enzymes that can digest the toxic free fatty alcohol is likely to be the main reason for the poor seed germination or seedling growth. Therefore, introducing genes that encode such enzymes to detoxify the fatty alcohol could be a solution. The capabilities of the jojoba genes *fatty alcohol oxidase* and *fatty aldehyde dehydrogenase* that catalyze the oxidation of free fatty alcohol into fatty acid in germinating seed have already been demonstrated (Rajangam et al., 2013). Transforming these two genes into the high WE production crambe GM germplasm would be the most promising approach to solve the problems of poor germination vigor. Another option could be crossing transformants containing these two detoxifying genes with high WE production crambe GM germplasm.

6.2 The technical route in engineering seed oil metabolism

Nowadays many plant studies are using GM to optimize or change the nutrient composition in seeds, tubers and other storage organs. In seeds, oil together with starch and protein are the most important storage compounds for germination and seedling growth. They are also very important renewable resources for the food, feed and chemical industry. Seed oil can have predominant industrial utilization and economical value if it contains specific lipid compounds, such as WE species, conjugate fatty acids, hydroxyl fatty acid and erucic acid (Cahoon et al., 2007; Vanhercke et al., 2013). The vegetable oil production annually is about 160 million metric tons. With the rising price of mineral oil, vegetable oil has obtained increasing attention and has been considered as an alternative for mineral oil in the future (Dyer et al., 2008; Carlsson et al., 2011). In comparison with mineral oil that is unsustainable, the renewability of vegetable oil is a huge advantage, however, the limited amount of desired production cannot yet meet the requirements of the industrial demand. Engineering of vegetable oil to enhance the desired traits for industrial demand is a very attractive proposition for both breeders and researchers. Metabolic engineering can be focused on oil biosynthesis and storage which are two crucial processes for the oil accumulation. Hereby, the essential genes involved in these two processes become the interesting targets.

6.2.1The biosynthesis pathway of seed oil: from sucrose to TAG

Like almost all the oil seed crops, the final compound of crambe seed oil is TAG (Gurr et al., 1974; Chapman and Ohlrogge, 2012). The structure of TAG is: a glycerol backbone with each of its three hydroxyl-positions (sn) connecting to a fatty acyl moiety by esterification bond. An overview of the TAG biosynthesis pathway is shown in Figure 6.2. In developing oil seeds the carbon of TAG is derived from sucrose that is produced by photosynthesis (Hill et al., 2003). In the cytosol of developing rapeseed, the conversion from sucrose to storage lipids involves the breaking down of the sucrose into glycolytic intermediates (hexose-P, phosphoenolpyruvate, pyruvate, and malate) (Nikolau et al., 2003). The glycolytic intermediates are subsequently imported into the plastid by transport proteins and used to synthesize acetyl-CoA via the pyruvate

dehydrogenase complex (Eastmond and Rawsthrone, 2000). The acetyl-CoA is converted to malonyl-CoA by ATP-dependent acetyl-CoA carboxylase (ACCase), from which two-carbon units are added to the growing acyl chain by fatty acid synthetase (Ohlrogge and Browse, 1995). The generation of malonyl-CoA in the stroma of the plastids is the first committed step of fatty acid biosynthesis (Rawsthrone, 2002). Subsequently, fatty acids are synthesized in a stepwise manner by adding two carbons (Kang and Rawsthorne, 1994). Following the processes of synthesis and desaturation, the hydrolysis catalyzed by acyl-CoA thioesterases releases acyl carrying proteins (16:0-, 18:0- and 18:1-ACP) that are transferred to the outer envelope of the plastid. Then the 16:0-, 18:0- and 18:1-ACP are reactivated to acyl-CoA by acyl-CoA synthetase and released to the cytosol (Koo et al., 2004). In the cytosol and endoplasmic reticulum (ER) this acyl-CoA can be further modified, e.g. acyl-CoA elongation, desaturation and hydroxylation (Ohlrogge and Browse, 1995; Hlousek-Radojcic et al., 1998; Abbadi et al., 2004). The acyl-CoA can also be used for the step-wise acylation of the glycerol backbone to directly synthesize TAG. In the first two steps of TAG assembly, glycerol-3P is acylated by glycerol-3P acyltransferase (GPAT) to form lysophosphatidic acid that is then acylated further by lysophosphatidate acyltransferase (Kim et al., 2005) to produce phosphatidic acid. This is followed by dephosphorylation of phosphatidic acid catalyzed by phosphatidate phosphatase (PAP) to release diacylglycerol (DAG) (Kocsis et al., 1996). The final acylation of DAG catalyzed by diacylglycerol acyltransferase (DGAT) generates TAG that is transferred to oil body for storage (Murphy and Vance, 1999). In addition to DGAT another enzyme, phospholipid: diacylglycerol acyltransferase has been found to be able to catalyze DAG acylation into TAG (Dahlqvist et al., 2000). But this enzyme uses phospholipids instead of acyl-CoA as the donor of fatty acid. Finally, the fraction of ER, where the TAG is assembled will differentiate into oil body (an organ of single-layer membrane with a structural protein called oleosin embedded) to store TAG. These stored TAG molecules will be preserved until seed germination. As described above the metabolism of lipids can be divided into biosynthesis and storage (Figure 6.2). And these two processes are the crucial targets for metabolic engineering of GM oil crop breeding.

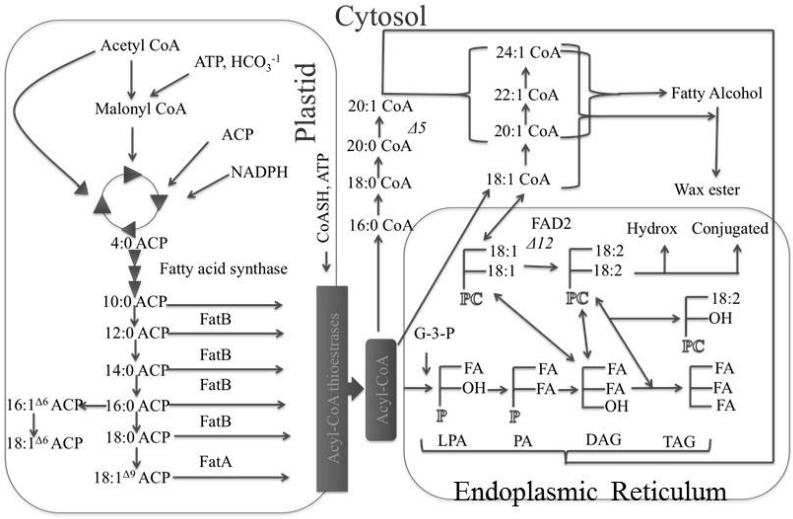


Figure 6.2 Fatty acid synthesis, modification, and assembly into triacylglycerols in plants: As described in the session "The biosynthesis pathway of seed oil: from sucrose to TAG" above, in the left part of the chart is the scheme of plastid fatty acid biosynthesis. Fatty acid synthesis is started by acetyl-CoA carboxylase that generates malonyl-CoA, and is terminated by acyl-ACP thioesterases (FatA and FatB), which release free fatty acids, allowing their export from the plastid and re-esterification to CoA at the plastid envelope. In the right part is the scheme of reactions for modifying fatty acids in the cytosol and their assembly into triacylglycerol and phosphatidylcholine (PC) in the endoplasmic reticulum. After activation to CoA, fatty acids produced in the plastid will be sequentially esterified directly to glycerol 3-phosphate (G-3-P) to produce lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG), and triacylglycerol (TAG). On the other hand, in most oilseeds the major flux of acyl chains are involved into the movements through phosphatidylcholine (PC) pools where modifications like further desaturation and hydroxylation takes place. In *Simmondsia chinensis* or GM plants with its genes transformed are wax esters accumulated in seeds.

6.2.2 Use of metabolic engineering to reformulate the lipid biosynthesis system in developing seed

The first report of changing seed oil composition by GM is by introducing the *LPAT* gene from meadowfoam (*Limnanthes alba*) into rapeseed (Lassner et al., 1995). Quite similar as the *LdLPAT* we used in the research of Chapter 4, this exogenous LPAT allows the C22:1 acyl to attach to the sn-2 position of TAG. The biosynthesis and storage of erucic acid has been illustrated in Figure 6.2. Erucic acid is from twice the elongation of oleic CoA (C18:1 CoA), which produces C20:1 CoA firstly and then C22:1 CoA. In storage, the accessible erucic CoA is incorporated into TAG molecule, and into an oil body. In the pioneer research, the meadowfoam *LPAT* was working on the storage process, aiming at enhancing erucic acid storage of the rapeseed oil. In our research the improvement of erucic acid content in crambe was achieved by enhancing the biosynthesis (Chapter 4). The biosynthesis was enhanced by expressing *BnFAE1* present in the construct pHan2, pWatergate-3G and by *CaFAD2-RNAi* (to prevent C18:1 CoA from being desaturated into C18:2 CoA) present in pWatergate-3G and by CaLPAT2-RNAi present in pWatergate-4G. The pHellsgate CaLPAT-RNAi construct tested in Chapter 4 is also supposed to enhance erucic acid storage.

In addition to increasing erucic acid content, using GM crambe seeds to produce WE was also a challenging work. In this research, the genes used in transformation were all targeting the biosynthesis of WE. The combination of exogenous genes in transformation has been shown in Table 1.5 (Chapter 1). The *ScFAE* and *CaFAD2-RNAi* were concerned with erucic and nervonic biosynthesis; *ScFAR* was in charge of fatty alcohol production and *ScWS* was in charge of esterification between fatty acid and alcohol to form WE. But, no gene involved in the storage process of WE was transformed into crambe. Because of this omission it is not clear whether the WE in GM crambe seed is stored in oil bodies, although the WE in jojoba seeds is stored in wax bodies (Miwa, 1971). Considering that the molecule of WE is similar to the un-polarized long carbon chain of TAG, we assume that the WE is possibly accumulated in the oil body for stable deposition. If this is the case then there would be the possibility to improve the storage of WE by improving the efficiency of storage of WE in the oil body. This can be achieved by locating the enzymes ScFAE and ScWS to the adjacent region where the oil body will grow out from the ER system. This idea has been partially proven by Heilmann and colleagues (Heilmann et al., 2012). In that research, *Mus musculus* genes *FAR* and *WS* without the target signal part were separately fused with *Brassica napus* oleosin gene. Transformed *Arabidopsis* of these two fusion genes driven by the seed specific napin promoter showed that more WE was deposited in the seed oil than in the transformant of *WS* and *FAR* with the original target signal part. Another option to further improve WE content is to combine crambe diacylglycerol acyltransferase RNAi (CaDGAT-RNAi) with the genes of WE biosynthesis. This is because the role of DGAT is catalyzing the final step of TAG assembling and determining the amount and composition of the seed oil (Lung and Weselake, 2006). Knocking down DGAT can reduce the production of TAG (Katavic et al., 1995). If the loading capacity of an oil body in a cell is limited, then the suppression of TAG will save the deposition room for WE. On the other hand, it is also possible due to feedback control. The incorporation of CaDGAT-RNAi will allow more fatty acid used as carbon resource to produce WE. Together with DGAT, phospholipid: diacylglycerol acyltransferase (PDAT) can also catalyze the final acylation of diacylglycerol to generate TAG in developing plant seed. It has been reported that when the endogenous *DGAT* were knocked down in *Arabidopsis*, the expression level of *PDGT* was coincidently raised, and an *Arabidopsis* double mutant of *DGAT* and *PDGT* had less TAG than the respective single mutant (Zhang et al., 2009). It is reasonable to speculate that the combination of *CaDGAT*-RNAi and *CaPDGT*-RNAi can efficiently block TAG production.

The lipid biosynthesis pathway in developing seeds is a system of complexity and formularization. In WT developing seeds the organic carbon atoms fixed by photosynthesis are transferred from sugar, step by step, into oil. In the mature seeds, the lipid compositions of seed oil were relatively stable, e.g. the crambe seed oil composition described in Chapter 1(Table 1.3).

The lipid metabolic engineering researchers are trying to introduce exogenous genes (driven by specific promoters like napin and glycinin) into the genome. By manipulating the carbon flux transition, researchers expect to produce or increase the desired lipid compounds and/or suppress the undesired ones. However, much research in which one or two vital genes were introduced able to modulate seed oil biosynthesis just gained moderate success (Lassner et al., 1995; Han et al., 2001; Mietkiewska et al., 2007; Lardizabal et al., 2008). Such unexpected results are mostly due to the fact that the oil biosynthesis pathway is not yet fully understood (Cahoon et al., 2007). For example, after transforming the gene coding oleate 12-hydroxylase from castor bean into *Arabidopsis*, the seed of GM *Arabidopsis* accumulated 20% ricinoleic (0% in WT) (Lu et al., 2006), while originally there is 90% in castor beans (Broun and Somerville, 1997). Another similar case is using GM plant to produce calendic acid which is originally produced by *Calendula officinalis* (pot marigold). The pot marigold FAD2 conjugase is in charge of the final step of biosynthesis of calendic acid from C18:1 fatty acid. The GM soybean with the exogenous pot marigold *FAD2 conjugase* had 20% calendic acid contained in the seed oil (0% in WT), while it is about 55% in the seeds of the original species (Cahoon et al., 2001). The accumulation level of these unusual fatty acids (ricinoleic acids and calendic acid) in the GM seeds was much lower than that in the original species because such fatty acids are integrated into phosphatidylcholine but not TAG (Cahoon et al., 2006). The lipid biosynthesis pathway in the species that the gene originates from has special kind of PDATs. These enzymes can transmit the unusual fatty acid moieties from PC into DAG to form TAG (Dahlqvist et al., 2000; Li et al., 2010). In the mentioned two researches, the genes that are involved in the biosynthesis process of fatty acids have been transformed, but the crucial PDAT that is involved and probably essential in the storage process was absent. The scientific research history of GM in improving the erucic acid content of plants also demonstrated a progress in which more and more genes were found and added to promote erucic acid accumulation (Lassner et al., 1995; Wilmer et al., 2000; Han et al., 2001; Jadhav et al., 2005; Mietkiewska et al., 2007; Rahman et al., 2008). Meanwhile, history indicates the importance of the cooperation between biosynthesis and storage. For example, the combination of *LdLPAT+Bn-fae1* with alleles of low PUFA in *Brassica napus* L. (null mutant of *FAD2*) had more erucic acid accumulation than the transformant of *Ld-LPAT+Bn-fae1* to WT (Nath et al., 2009). This is also the reason why we combined *CaFAD2-RNAi* with *LdLPAT* and *BnFAE1* in the vector pWatergate-3G (Chapter 4). Accordingly the strategy of comprehensive genetic modification is preferred. Hence, a 4G vector of *LdLPAT+BnFAE1+CaFAD2-RNAi+CaLPAT-RNAi* was constructed and transformed into crambe for higher erucic acid crambe GM breeding. As shown in Figure 6.2, the seed lipid metabolism is a network system. An attempt to change a certain lipid compound (like erucic acid) production by GM mostly requires the modification of several relevant genes. These genes encode the enzymes with vital functions in the target lipid compound accumulation (in terms of biosynthesis and storage). In this complex network of carbon flux transition, these interesting enzymes are just like the valves of an engine that can deploy organic carbon to hydrocarbon subtracts, whereas each valve has limited effect on the whole pathway and the final product. If just one of them is engineered, the effect would be too minor to change the whole homeostasis. To achieve the major changes of the homeostasis, it is better to engineer multiple genes. With turning on/off these valves as much as possible significant carbon flux can be integrated into the accumulation of desired compounds and as a result the adjustment will be more efficient. While the priority of the most crucial "valves" should be taken into account since these "valves" have different contributions.

6.3 Potential beneficial genes for crambe GM breeding

Our research has highlighted the potential of transgenic crambe. Metabolic engineering of crambe seed oil is playing a leading role. In the biosynthesis pathway of crambe seed oil, several functional endogenous enzymes and complexes can be essential to determine the seed oil composition and hereby meaningful to increase the value of crambe seed oil. Several of them are the targets of the present research, and the main stream of the pathway is shown in Figure 6.2. Additionally, there are also interesting genes from other species, such as *Limnanthes douglasii LPAT* and jojoba *FAR* and *WS*. Their potential function in changing the value of crambe seed oil

makes also them interesting for the crambe GM breeding. As a prospect of further GM work on crambe, we provided a list of such genes in which the functions are also integrated (Table 6.1).

Table 6.1 A list of genes possibly interesting for crambe transformation to alter/modify oil content and/or composition

Name of the enzyme and/complex	Source	Function
Phosphoenolpyruvate carboxylase	Soy bean	Adds bicarbonate to phoshoenolpyruvate to form oxaloacetate (the precursor of amino acid) (Sebei et al., 2006)
Acetyl-CoA carboxylase	Palm/maize/rapeseed	Catalyze the production of malonyl-CoA (The first step of fatty acid biosynthesis) (Roesler et al., 1997)
Photosynthesis system II	Various	Produce NADPH for the use of ACCs
Fatty acid elongase/ 3-ketoacyl-CoA synthase	*Crambe abyssinica*	Catalyze the elongation of fatty acid chain from 18:1 CoA to 20:1 CoA, and then 22:1 CoA
Fatty acid desaturase 2	Various	Desaturate oleic acid into linoleic acid
Divergent forms of the plant Δ12-oleic-acid desaturase (FAD2)	*Calendula officinalis*	Modifies a Δ9-double bond to produce the conjugated trans-Δ8,trans-Δ10-double bonds found in calendic acid (18:3Δ8trans,10trans,12cis)
Divergent forms of the plant Δ12-oleic-acid desaturase (FAD2)	*Calendula officinalis*	Modifies a Δ9-double bond to produce the conjugated trans-Δ8,trans-Δ10-double bonds found in calendic acid (18:3Δ8trans,10trans,12cis)
ω-3 Desaturase	*Caenorhabditis elegans*	Modifies a Δ15-double bond to produce ω-3 fatty acid (a-linolenic) (Abbadi et al., 2004)
Δ-6 Desaturase	*Phaeodactylum tricornutum*	Modifies a Δ6-double bond to ω-3 fatty acid to produce (stearidonic acid)
Δ-6 Elongase	*Borago officinalis*	Modifies stearidonic acid with two-carbon unite to produce 20:4 $^{\Delta 8,11,14,17}$
Δ-5 Desaturase	*Physcomitrella patens*	Modifies 20:4 $^{\Delta 8,11,14,17}$ a double bond to produce 20:5$^{\Delta 5,8,11,14,17}$
Glycerolphosphate acyltransferase	Rapeseed/sunflower	Produce lysophosphatidic acid (Jain et al., 2000)
Lysophosphatidate acyltransferase 2	*Limnanthes douglasii/alba*	Acylate lyso-phosphatidic acid into phosphatidic acid
Phosphatidate phosphatase	Rapeseed	Dephosphorylate phosphatidic acid into diacylglycerol (Carman and Han, 2006)
Diacylglycerol acyltransferas1	*Vernicia fordii*	Produce TAG with eleostearic acid integrated
Diacylglycerol acyltransferas2	*Vernicia fordii* /rapeseed	Produce TAG with eleostearic acid integrated
Phospholipid:diacylglycerol acyltransferase	*Arabidopsis*	Produce TAG from DAG acyl transfer
Fatty acid reductase	Jojoba/mouse/avian	Transform fatty acid into fatty alcohol
Wax ester synthase	Jojoba/mouse/avian	Catalyze esterification to form wax ester
Oleate 12-hydroxylase	Castor bean	Hydroxy fatty acid accumulation (Lu et al., 2006)

6.4 Conclusions and prospective

In this research, the GM crambe erucic acid content has been improved into a level that is very remarkable and economically attractive. This indicates a promising possibility of using GM crambe to replace high erucic acid rapeseed for erucic acid production. The following steps should be selection and stabilization of the trait of the high erucic acid content in further generations of these lines and then to put the chosen families into field trials to test their agricultural value. The present result also demonstrates a success in obtaining a remarkable yield

of WE in seeds of transgenic crambe. However, the germination and seedling growth problems still need to be resolved. On the other hand this result leads to the possibility of using crambe as a platform to produce specific oils that are demanded by the chemical industry. Furthermore, analogous to the success of using marker-free systems in strawberry, apple and potato, we have successfully used the pMF1 marker-free system to produce transgenic crambe. This system offers a possibility to generate marker-free crambe, which may bring more public acceptance about the GM crambe. The feasibility of marker-free system in crambe GM will be verified by loading genes with real biological functions.

As being part of the ICON project, we have performed a pioneer research to explore the potential value of GM crambe. According to the obtained results presented here a proposition on how future work in relation to crambe GM breeding is suggested. We propose to build a single gene genetic modification library of crambe, in which each of the lines will only have one modified gene that is involved in oil metabolism. This library will consist of many different GM crambe lines with various GM germplasm. The performance of the artificially modified gene in each GM line should be strong and stable, for which homozygous GM lines will be preferred. Till now, there are three protocols of crambe transformation including the one described in Chapter 2. The successful transformation frequencies of these protocols are all higher than 1%. The genes *NPTII* and *HPT (hygromycin neomycin transferase)* can be used as selectable markers of antibiotic based selection for gene transformation. Although building the proposed library will be a big project, it is quite meaningful and accomplishable. The establishment of the library will simplify the breeding research. For instance when aiming at high erucic, low erucic acid or low PUFA, researchers will only need to do crossing between different GM lines from the library until all the genetic modification natures wanted are assembled in one single plant. For the convenience of verifying the successful hybrid, it is better to link the transgenes with different visible markers, e.g. GFP, RFP and GUS. Using the marker-free system to construct this library is encouraged, because it can also restrict the environmental contamination and earn more public acceptance; and this marker-free system will allow retransformation with the same selection marker constantly.

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Summary

Crambe abyssinica (crambe) is an allohexaploid (2n=6x=90) crop species, and belongs to the *Brassicaceae* family and *Crambe* genus. It is an erect annual herb of which the center of origin is the Mediterranean region, and it is also prevalent across Asia and Western Europe. The oil content of crambe seed is about 35-60% (35% most common), and the protein content is about 20-40%. As oil seed crop, it has a wide potential as industrial raw material. In the present research, a robust transformation protocol of *Agrobacterium*-mediated transformation for crambe with cotyledonary-nodes from 7-day-old seedlings as explants was developed. This transformation protocol has a duration of around 20 weeks and a success frequency of about 4%. With this transformation method a series of experiments have been carried out to genetically modify crambe seed oil content.

To be more eco-friendly and increase the possibility of public acceptance to GM crambe, the vector pMF1-RbscGFP-RbscGUS (pJS-M14) that belongs to the marker-free genetic modification system developed by Wageningen UR Plant Breeding, was used in crambe transformation. The T-DNA region of the vector has a *Recombinase R-LBD* gene fusion, a *CodA* and *NPTII* gene fusion and a *GFP* gene between two recombination sites, while a *GUS* gene is located outside of the recombination sites. After transformation, the application of dexamethasone (DEX) can activate the recombinase, which will remove the two gene fusions and *GFP* mentioned above. Marker-free plants, generated by recombination, can be obtained through negative selection on 5-fluorocytocine (5-FC), because the *CodA* part encodes an enzyme that can convert 5-FC into the toxic compound 5-fluorouracil (5-FU). Among the T0 lines obtained using pJS-M14, one with a single T-DNA insertion and another with a double insertion were chosen for producing marker-free plants by DEX-induced recombination and 5-FC selection. A series of DEX concentrations (0, 5, 15, 25 μM) was tested in order to identify the optimal concentration in generating marker-free plants. In contrast to earlier experiments with other crops, DEX treatment of crambe was administered through the rooting medium instead of by submergence. DEX treatment was continued into the subsequent axillary bud regeneration phase followed by negative selection on 5-FC containing regeneration medium. Marker-free (MF) plants were indeed obtained. The application of DEX was essential for production of marker-free plants but no significant difference was found among the different concentrations used. Although the 5-FC selection clearly reduced the number of non-MF regenerants, still numerous non-recombined plants survived and also chimerism was observed, both for transgenic and wild type cells in the original transgenic lines as well as for marker-free and non marker-free cells after DEX and 5-FC selection. But, by the PCR and GUS staining test, the marker-free regenerated shoots were isolated successfully.

Later transgene experiments were performed to increase Crambe seed oil's erucic acid content. Crambe has a high erucic acid (C22:1) content of about 60% in its seed oil. Increasing it beyond 66% is impossible by classical breeding as the crambe enzyme lysophosphatidic acyltransferase 2 (LPAT2) building fatty acids into the middle (sn-2) position of triglycerides does not use longer chain (> C18) fatty acids as substrate in the developing seed. An RNA-interference (RNAi) construct was developed targeting a conserved sequence of *LPAT2* of crambe as well as of other *Brassicaceae* species, under control of the 35S promoter. Transformed *Arabidopsis thaliana* harboring this construct had an efficiently suppressed LPAT2 expression in leaves and the expected phenotype (shorter pods and a high proportion of seed abortion), in comparison with the wild type. The transformed crambe containing the same RNAi construct showed a highly reduced

LPAT2 expression level both in leaves and developing seeds as well, but no seed abortion or difference in seed size. Oil composition analysis of the transgenic crambe seed oil showed that it contained a higher erucic acid content (max 66.3%) and corresponding lower polyunsaturated fatty acid (PUFA) content (min 10.6%), which both were significantly different from the wild type. To increase crambe seed oil erucic content further, the LPAT2-RNAi was integrated with three different genes into one vector under control of the seed specific Napin promoter (termed pWatergate-4G (LdLPAT+BnFAE1+CaFAD2-RNAi+CaLPAT-RNAi)): 1) *Limnanthes douglasii* LPAT (LdLPAT) that can incorporate erucic acid on sn-2, 2) *B. napus* fatty acid elongase (BnFAE1) that is in charge of prolonging oleic acid into erucic acid, and 3) an RNAi targeted at native fatty acid desaturase 2 (FAD2-RNAi) that is in charge of desaturating oleic acid into linoleic acid and linolenic acid. Simultaneously, vector pHan2 (LdLPAT+BnFAE1) and vector pWatergate-3G (LdLPAT+BnFAE1+CaFAD2-RNAi) were also transformed into crambe respectively to monitor the effect of the different genes in varying combinations. T1 seed oil composition analysis showed that the highest erucic content measured was up to 79%, while 43% tri-erucin content in seed oil was found. The seeds of GM plants containing pWatergate-4G and pWatergate-3G accumulated less PUFA in the seed oil composition, compared to seeds of wild type, pHan2 and pHellsgate CaLAPT2-RNAi transformants. On the other hand, super low erucic acid content in some seeds of pHan2, pWatergate-3G and pWatergate-4G were also frequently detected during the assay. The lowest erucic acid level of individual seeds observed was less than 1% when there was 85% oleic acid, which indicated that the elongation from C18:1 to C22:1 was almost blocked. This phenotype implied that the exogenous BnFAE1 introduced by the transgene might cause co-suppression against the endogenous one. These results indicate that due to the modulation of the transgenes, more C18:1 could be channeled to C22:1 and less C18:1 was converted to poly-unsaturated fatty acids (PUFA, C18:2+C18:3 mainly); but the unexpected native CaFAE co-suppression could reverse the phenotype into the opposite way completely. Therefore, it suggests that the best way to obtain increased erucic acid content would be by combining LdLPAT+CaLPAT2-RNAi+CaFAD2-RNAi and leaving out BnFAE1 as FAE overexpression can lead to co-suppression of CaFAE thus negating the desired effect.

Finally, transgene crambe was used as a platform producing high value industrial oil Jojoba wax ester (WE). The key genes (*fatty acid reductase* (*ScFAR*), *wax ester synthase* (*ScWS*) and *fatty acid elongase* (*ScFAE*)) in charge of Jojoba wax ester biosynthesis in its seed were constructed into two vectors pFWS3-2 (*ScFAR*+*ScWS*) and pFWS3-3 (*ScFAR*+*ScWS*+*ScFAE*) under control of the seed specific promoter soybean glycinin. The oil composition of T1 seeds indicated that by the integration of the jojoba genes, WE could be accumulated in addition to TAG. The pFW3-3 transformants accumulated significantly more C24:1 fatty chain moieties than the FW3-2 transformants. The selection for high WE producing lines was performed during the offspring propagation. From T1 to T3, WE content of the GM seeds' increased gradually by the selection. The highest WE content was 25mg/g (WE/seed). Furthermore, the interaction between transgene seed's WE and PUFA biosynthesis was studied, by crossing pFWS3-2 transformants with the CaFAD2-RNAi transformants. The F2 seed oil composition indicated that the inhibition of FAD2 led to an increased WE content when it was combined with the pFWS3-2 transgene. During seed propagation, the low germination vigor of seeds with high WE content was noticed. A series of seed germination experiments showed systematically that the more WE accumulated in seed, the higher the chance that the seed germination failed and that the seedling showed delayed growth or even death at seedling stage. The free fatty alcohols released from WE bio-decomposition during seed germination, possibly are responsible for the growth delay or failure to germinate in seedlings with WE. An important physical characteristic of fatty alcohol is that it can act as a detergent. Detergents are generally very harmful to cell membranes, and this could be the reason for the observed difficulties in germination of seedling growth. The possible solution to this issue can be either selecting moderate WE content Crambe seeds, which do not have such germination problem, or expressing functional genes which can detoxify fatty alcohol during seed germination.

Samenvatting (Summary in Dutch)

Crambe abyssinica (crambe) is een allohexaploid gewas en behoort tot de familie der kruisbloemigen (*Brassicaceae)* en het geslacht *Crambe*. Het is een opgaand, eenjarig kruid met als brongebied het Middellandse Zee gebied. De plant komt daarnaast ook voor in grote delen van Azië en West-Europa. Het oliegehalte van crambe zaad kan variëren tussen 35 en 60 procent (meestal rond de 35%) en het eiwitgehalte ligt doorgaans tussen de 20 en 40 procent. Als oliehoudend zaadgewas heeft het grote potentie als industrieel uitgangsmateriaal. In het onderzoek beschreven in dit proefschrift werd een solide transformatie protocol ontwikkeld voor crambe gebaseerd op *Agrobacterium tumefaciens* en uitgaande van de cotylknopen van 7-dagen-oude zaailingen. Dit transformatieprotocol duurt in totaal ongeveer 20 weken en kent een frequentie van 4%. Met behulp van dit protocol zijn een serie experimenten uitgevoerd om via genetisch modificatie (GM) het gehalte en de samenstelling van crambe zaadolie te veranderen.

Om tegemoet te komen aan de eisen voor introductie in het milieu die de EU stelt aan GM gewassen en om de kans op acceptatie door het publiek van GM crambe te vergroten is het 'merker-vrije' genetische modificatie vector systeem dat door Wageningen UR Plant Breeding is ontwikkeld in crambe toegepast en uitgetest. Hiervoor is de vector pMF1-RbscGFP-RbscGUS (pJS-M14) gebruikt. Het T-DNA gebied van deze vector bevat een *Recombinase R-LBD* genfusie, een *codA-nptII* genfusie plus het *gfp* reportergen tussen twee recombinatie plekken terwijl een *gus* reportergen buiten die recombinatie plekken aanwezig is. Na transformatie kan een behandeling met dexamethason (DEX) het recombinase-enzym activeren, waarna alles tussen de recombinatie plekken, dus de twee genfusies plus het *gfp* reportergen, verwijderd wordt. Merker-vrije planten, gegenereerd door excisie na recombinatie, kunnen geselecteerd worden door groei op medium met 5-fluorocytosine (5-FC). Deze op zich niet-toxische stof wordt o.i.v. het enzym dat gecodeerd wordt door het *codA* deel van de genfusie (cytosine deaminase) omgezet in het toxische 5-fluorouracil (5-FU), indien dat gen nog aanwezig is. Van de T0 lijnen die verkregen werden na gebruik van pJS-M14 zijn twee lijnen uitgekozen om met DEX te behandelen om recombinatie te induceren en op 5-FC te groeien om de merker-vrije individuen te kunnen oppikken. Eén lijn had één T-DNA insertie en de ander had twee T-DNA kopieën geïntegreerd. Een serie DEX concentraties is getest om de optimale concentratie te bepalen om merker-vrije crambe plantjes te krijgen. In tegenstelling tot eerdere experimenten met andere gewassen is het crambe plantmateriaal niet ondergedompeld in vloeibaar medium met DEX maar is de DEX toegediend via het groeimedium voor opname door de wortels. De DEX behandeling is voortgezet in de volgende fase, die van regeneratie vanuit axillaire knoppen na snijden. Daarna volgde de negatieve selectie op 5-FC. Op deze manier zijn merker-vrije crambe planten verkregen. Toepassing van de DEX behandeling bleek essentieel hiervoor, maar er werden geen significante verschillen gevonden tussen de verschillende DEX concentraties. De 5-FC verminderde duidelijk het aantal niet-merker-vrije regeneranten, maar toch wisten vele niet-gerecombineerde plantjes de selectie te overleven. Chimerisme werd ook waargenomen, zowel van transgene en wild-type cellen in de originele GM lijnen als van merker-vrije en niet merker-vrije cellen na DEX behandeling en 5-FC selectie. Toch konden uiteindelijk merker-vrije, geregenereerde scheuten met succes geïsoleerd worden, zoals bevestigd werd door PCR en GUS kleuring.

Verschillende transformatie experimenten zijn uitgevoerd om het gehalte erucazuur in de zaadolie van crambe te verhogen. Crambe zaadolie kent een hoog gehalte aan erucazuur (C22:1) van ongeveer 60%. Verhogingen boven de 66% zijn niet mogelijk door klassieke veredeling omdat het eigen crambe enzym lysofosfatidyl acyltransferase 2 (LPAT2), verantwoordelijk voor het

inbouwen van vetzuren in de middelste (sn-2) positie van glycerol bij de productie van triglyceriden (olie), geen vetzuren langer dan C18 als substraat in ontwikkelend zaad kan gebruiken. Een RNA-interference (RNAi) construct is ontworpen gericht tegen een geconserveerd deel van het *LPAT2* gen van zowel crambe als andere *Brassicaceae* onder controle van de 35S promotor. Met dit construct getransformeerde *Arabidopsis thaliana* planten vertoonden een efficiënte onderdrukking van *LPAT2* expressie in bladeren en een verandering in fenotype bekend van mutanten; namelijk kortere vruchten en veel zaadabortie, vergeleken met het wild type. GM crambe planten, getransformeerd met ditzelfde construct, werden gekenmerkt door een sterk verlaagde expressie van *LPAT2* in bladeren en ontwikkelende zaden, maar niet door zaadabortie of verschillen in zaadgrootte. De oliesamenstelling van de GM crambe gaf een verhoging van het erucazuur gehalte te zien (maximaal 66.3%) en een corresponderend lager gehalte aan meervoudig onverzadigde vetzuren (PUFA, minimaal 10.6%), beide significant verschillend van het wild type. Om het erucazuur gehalte in crambe zaadolie verder te verhogen werd het *LPAT*-RNAi met drie andere genen gecombineerd in één vector, onder controle van de zaad-specifieke Napin promotor. Deze combi-vector, genaamd pWatergate-4G, bevat de volgende genen: het *LPAT2* gen van *Limnanthes douglasii*, dat erucazuur op de sn-2 positie kan incorporeren (*LdLPAT2)*; het vetzuur elongase gen van *Brassica napus*, dat oliezuur (C18:1) kan verlengen tot erucazuur (*BnFAE1*); een RNAi gericht tegen het native vetzuur desaturase van crambe, dat het enkelvoudig onverzadigde oliezuur omzet in meervoudig onverzadigde vetzuren (C18:2 en C18:3, *CaFAD2*-RNAi) plus het eerder genoemde *LPAT*-RNAi. Naast de transformaties met pWatergate-4G werden transformaties gedaan met de vectoren pHan2 (*LdLPAT+BnFAE1*) en pWatergate-3G (*LdLPAT+BnFAE1+CaFAD2*-RNAi) om het effect van de verschillende genen in diverse combinaties te bestuderen. In de analyse van de oliesamenstelling van T1 zaden is een hoogste gehalte van erucazuur gemeten van 79% met een gehalte van tri-erucine (dus met een erucazuur molecuul aan alle drie de sn posities van glycerol) van 43%. De zaden van de GM planten verkregen met pWatergate-4G of pWatergate-3G laten minder PUFA zien in de olie in vergelijking met de zaden van wildtypes, van pHan2 getransformeerden of van planten getransformeerd met een construct waarin de *CaLPAT2*-RNAi sequentie aanwezig was. Daarnaast zijn er uit deze transformaties met pHan2, pWatergate-3G & -4G ook lijnen verkregen die een superlaag gehalte aan erucazuur in zaadolie hadden. Het laagste niveau van erucazuur in individuele zaden was minder dan 1% terwijl de olie 85% oliezuur bevatte, wat aangeeft dat de elongatie van C18:1 naar C22:1 hier bijna volledig is geblokkeerd. Dit fenotype duidt er op dat de als transgen toegevoegde *BnFAE1* de expressie van het endogene *CaFAE1* via co-suppressie uitschakelt. In de andere lijnen heeft de introductie van de verschillende gencombinaties er wel toe geleid dat meer C18:1 is omgezet in C22:1 en minder in meervoudig onverzadigde vetzuren (PUFA oftewel C18:2 en C18:3). Samen suggereren deze resultaten dat de beste strategie om het erucazuur gehalte in crambe zaadolie te verhogen is door de endogene activiteiten van crambe *LPAT2* en *FAD2* uit te schakelen en de *LPAT2* van *Limnanthes douglasii* toe te voegen, kortom een construct met *LdLPAT2+CaLPAT2*-RNAi+*CaFAD2*-RNAi zonder de aanwezigheid van *BnFAE1* om cosuppressie te vermijden. De eigen crambe *FAE1* is ruim voldoende om de benodigde elongatie van C18:1 naar C22:1 te leveren.

Tot slot is GM crambe gebruikt als productieplatform voor hoogwaardige, industriële wasesters van het type jojoba wasesters. De jojoba sleutelgenen die zorgen voor de vorming van die esters in jojoba zaad, zijn vetzuur reductase (*ScFAR*) en wasester synthase (*ScWS)* en vetzuur elongase (*ScFAE*). Deze genen zijn in twee combinaties in vectoren (pFWS3-2; *ScFar+ScWS* en pFWS3-3; *SCFAR+SCWS+SCFAE*) gezet onder controle van de zaad-specifieke glycinine promotor uit soja. De oliesamenstelling in T1 zaden gaf aan dat de introductie van de jojoba genen tot vorming en

ophoping van wasesters (WE) leidde. De transformanten met pFWS3-3 gaven significant meer C24:1 vetzuren in de esters dan de transformanten met pFWS3-2. In volgende generaties werd geselecteerd voor hoog WE producerende nakomelingen. Van T1 tot T3 kon het WE gehalte in de GM zaden door die selectie geleidelijk verhoogd worden. Het hoogste waargenomen WE gehalte was 25 mg/g (WE/zaad). Door pFWS3-2 transformanten te kruisen met GM crambe waarin de FAD2 activiteit was geremd door *CaFAD2*-RNAi (minder PUFA) werd bekeken of het verminderde gebruik van C18:1 als substraat voor PUFA kon leiden tot een verhoging van het WE gehalte. Dit bleek het geval in de F2 die bestudeerd werd. Tijdens de kruisingen en zaadvermeerderingen bleek dat de lijnen met een hoog WE gehalte slechter kiemden. Een serie experimenten toonde aan dat naar mate er meer WE accumuleerde in de zaden, de kans dat zij niet of slecht kiemden toenam en dat zaailingen een verminderde groei vertoonden of zelfs dood gingen in dit stadium. De reden hiervoor wordt gezocht in de vette alcoholen, die bij kieming vrijkomen door afbraak van de WE. Een belangrijke eigenschap van dergelijke langketenige vette alcoholen is dat zij kunnen functioneren als detergentia en daarmee een desastreus effect hebben op de celmembranen in de zich ontwikkelende zaailingen. Dit zou een mogelijke verklaring kunnen zijn voor de waargenomen moeilijkheden in de zaailingfase. Een oplossing voor dit probleem zou kunnen zijn om te selecteren voor crambe lijnen met matig hoge WE gehaltes die de genoemde problemen niet hebben of door extra genen in te brengen die de vette alcoholen kunnen detoxificeren of onschadelijk maken tijdens de zaadkieming.

Acknowledgements

Five years ago, I found a chance of being a PhD candidate whose task it would be to focus on plant lipid metabolism manipulation research at Wageningen UR Plant Breeding. I took this chance without any hesitation. Now I am very happy and proud that I finished the task and can graduate on the results achieved within it with a PhD degree in my hand as the proof of it. This is a significant success in my life. To achieve it, I have been working hard for quite a long time and during this period, there were many people who were helping me, working with me, and encouraging me. This significant achievement would have been impossible for me to accomplish without them. Here I want to express my sincere gratitude to all of them.

Firstly I want to thank my dear co-promoters Dr. Robert van Loo and Dr. Frans Krens. The cooperation with you was the most important factor for me to work at Wageningen UR Plant Breeding happily and efficiently. You are the watchmen who were always preventing me from messing-up. With any trouble I met, my first thought and action would be going to your offices or to calling in your help. And you always provided me with support, either directly or by pointing out the persons whom I could approach. During my work for my PhD degree, every Thursday morning half past eight, we had meetings in Frans' office, to discuss the progress of my work. Although normally Frans would have liked to keep these sessions for about one hour, they would always be more than one and a half hour, full of enthusiastic discussions. With these regular meetings you did not only supervise my research progress, but also improved my English oral speaking abilities greatly. I will always miss our Thursday morning meetings. I want to express my special appreciation to Frans, thank you for the effort you made on supervising my thesis and article writing, thank you for the attention and patience. I also want to say special thanks to Robert, thank you for bringing me into a new project (Quinoa) after I finished my PhD work, which is very meaningful to me as I just started a new job as junior scientist in Jiangsu Academy of Agricultural Sciences.

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It has been my great pleasure to work in Wageningen University. With its very nice academic atmosphere and facility support, I had a very enjoyable experience here. Within these five years, I joint many courses, workshops, seminars, PhD days and many other activities. All of them are good and useful for my culture and mastering my career. I like the great buildings located around the campus, like Atlas, Forum, the sports center and Radix. I also like the big greenhouses from Unifarm a lot. It is a wonderful place inside this steel and glass construct. I appreciated the experience to spend time in it and deal with plants, even when after a long day it would become very exhausting.

It was my great pleasure to spend my five years in the Netherlands, a beautiful country of gardening, although there are a lot of cloudy or even rainy days, and the winter is so long and gray. But all of this can never disturb its beauty. With its charming cities and landscape nature, there is no doubt that in this country the great artists like Vincent van Gogh and Rembrandt van Rijn thrived. I have been in a lot of countries around the world and I would say that the Dutch cities are the most beautiful ones. Especially Wageningen, this small town is so quiet and peaceful. Located beside the river Rhine and close to an area with forest, this makes it a wonderful place for living. I will always look for chances to come back Wageningen again, for either business or holiday.

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Curriculum vitae

Weicong Qi was born on 7th of February 1981 in Hebei province, China. He finished his BSc in the field of biology at Hebei Agricultural University in 2005, and then obtained a master degree in genetics in 2008 at Nanjing Agricultural University. From March 2009 to August 2013, he conducted his PhD research at Wageningen UR Plant Breeding, Wageningen University & Research Centre. He worked on lipid metabolism manipulation in *Crambe abyssinica* under the supervision of Drs. E.N. van Loo and F. A. Krens. At present Weicong has a position as junior scientist in the institute of Agro-biotech, Jiangsu Academy of Agricultural Sciences, Nanjing City, Jiangsu Province, China, working on maize breeding.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Weicong Qi
Date: 27 August 2014
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Using Genetic Transformation to produce Erucic Acid and Novel Wax Esters in *Crambe abyssinica*	Jan 05, 2009
► **Writing or rewriting a project proposal**	
Production and modification of Erucic Acid and Novel Wax Esters in *Crambe abyssinica*	Oct 2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme 3 'Metabolism and Adaptation', Leiden University	Feb 19, 2010
EPS theme 3 'Metabolism and Adaptation', Wageningen University	Feb 10, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meeting "Experimental Plant Sciences", Lunteren	Apr 19-20, 2010
ALW meeting "Experimental Plant Sciences", Lunteren	Apr 04-05, 2011
ALW meeting "Experimental Plant Sciences", Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Symposium 'Photosynthesis: from femto to Peta and from nano to Global', Wageningen University	Nov 05, 2009
Plant Breeding Seminar Series	Feb 2009-2013
Workshop: Plant facility	Sep 07, 2012
Minisymposium" How to write a world class paper"	Apr 19, 2011
Minisymposium" Plant Breeding in the Genomics Era"	Nov 25, 2011
Plant Breeding Research Day (Arnhem)	Feb 28, 2012
Conference Next Generation Plant Breeding (Ede, The Netherlands)	Nov 12-13, 2012
Invited seminar by Anne Endler 'Dissecting Cellulose Production in Arabidopsis'	Jun 15, 2010
Invited seminar by BGI: Genomics in China	Apr 01, 2010
Plant Sciences seminars by Andries Koops: 'Bioscience strategy on plant-based raw materials for a biobased economy' and Luisa Trindade: 'Targeted breeding for a Biobased Economy'	Dec 14, 2010
► **Seminar plus**	
► **International symposia and congresses**	
5th European Symposium on Plant Lipids, Gdansk	Jul 08-13, 2011
► **Presentations**	
Poster in ALW Lunteren meeting	Apr 04-05, 2011
Poster in 5th European Symposium on Plant Lipids, Gdansk	Jul 08-13, 2011
► **IAB interview**	
Meeting with a member of the International Advisory Board	Feb 18, 2011
► **Excursions**	
Subtotal Scientific Exposure	*11.4 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Spring School 'RNAi'	Apr 14-16, 2010
Postgraduate course 'Basic statistics'	Jun 22-23, 27-29, 2011
Postgraduate course 'Bio-energy Production from Crop Plant and Algea'	Nov 21-23, 2012
► **Journal club**	
member of literature discussion group at PBR	2009-2012
► **Individual research training**	
Training in Bangquan's laboratory, Life science college, Huibei University, China	Oct-Nov 2009
Subtotal In-Depth Studies	*9.3 credits**

4) Personal development	date
► **Skill training courses**	
Moral dilemmas in Daily Scientific Practices	Feb 15-18, 2011
Project and Time management	Oct-Dec 2011
Mobilising your Scientific Network	Sep 18 & 25, 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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