Influence of *Bna.FAD2* Alleles on the Erucic Acid and Polyunsaturates Content in *Brassica napus* Oil

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Abstract

Brassica napus L. (rapeseed) is an economically important oilseed crop worldwide, having uses in both food and non-food sectors. Its industrial applications are linked to the natural occurrence of erucic acid (EA, C22:1), together with other fatty acids in its seeds. EA is a valuable fatty acid that could be derived into products such as erucamide, brassylic acid and pelargonic acid having a wide range of industrial applications such as plasticizers, slip additives, lubricants, pharmaceuticals, biodiesel and many more. EA biosynthesis is controlled by *Bna.FAE1s* (*FATTY ACID ELONGASES 1*) in *B. napus*. In addition, low levels of polyunsaturated fatty acids (PUFAs) are desirable in the oil for the industry as it increases the thermal stability of the oil. PUFAs biosynthesis is controlled by *Bna.FADs* (*FATTY ACID DESATURASES*) in *B. napus*. The present study was aimed to underpin the new loci affecting the EA biosynthesis by using the associative transcriptomics approach and to study the influence of *Bna.FAD2* family on the erucic acid (or very long chain fatty acids, VLCFAs) and polyunsaturated fatty acids levels. Although new loci influencing the erucic acid levels were not found from the present study but a unique specification – high erucic acid rapeseed in the low polyunsaturates (HELP) background was developed by introducing partially functional *Bna.FAD2* family from EMS mutagenized mutants to the high erucic acid rapeseed background. HELP lines showed the influence of partially functional *Bna.FAD2* alleles in the fatty acid compositions, ~8% increase in the erucic acid (60%) and VLCFAs (66%) levels as compared to the parental high erucic parents having functional *Bna.FAD2* family. Polyunsaturates content of less than 7% was found in these HELP lines. HELP oil is anticipated to be a valuable industrial oil that could contribute significantly to reduce the processing costs and serve as a renewable environment-friendly industrial resource with no toxicity.

Keywords – *Brassica napus*, rapeseed, oilseed rape, industrial rapeseed, renewable resource, biodiesel, lubricants, erucic acid, eicosenoic acid, PUFAs, VLCFAs, associative transcriptomics, EMS mutagenesis, *FAE1*, *FAD2*, HEAR, HELP.

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Supplementary file

Supplementary file in the CD is provided with this thesis. It contains four sequence files with extension '.ab1' named as

75HE92_A01_A8R_Cab.ab1,

CAB BnaA FAD2b_f.ab1,

CAB BnaC FAD2b_f.ab1 and

FAE1_C3_96_C3R_3_reference.ab1

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Declaration

Except where stated, I declare that this thesis is a presentation of the original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Some part of Chapter 3 presented within this thesis has previously been published in the following paper,

“Havlickova, L. , He, Z. , Wang, L. , Langer, S. , Harper, A. L., **Kaur, H**. , Broadley, M. R., Gegas, V. and Bancroft, I. (2018) Validation of an updated Associative Transcriptomics platform for the polyploid crop species *Brassica napus* by dissection of the genetic architecture of erucic acid and tocopherol isoform variation in seeds. *The Plant Journal*, 93: 181-192. doi:10.1111/tpj.13767.”

1. Introduction and Review of Literature

1.1 Introduction

Rapeseed (*Brassica napus* L.) is the world's second-largest oilseed crop after soybean (FAO, 2018) and the United Kingdom's third most valuable crop being cultivated in ~564 thousand hectares area (Defra, 2018) with a yield of 3.9 tonnes per hectare in 2017 (Defra, 2017). Rapeseed oil (commonly known as vegetable oil in the UK) is derived from the seeds of *B. napus* and is mainly consumed as food but it has a wide range of industrial perspectives such as lubricants, slip additives, biofuels, pharmaceuticals, jet fuels and plasticiser (Nieschlag and Wolff, 1971; Johnson and Fritz, 1989; Zanetti *et al.*, 2012; Iakovlieva *et al.*, 2017). Erucic acid (EA, C22:1) is one of the important fatty acids, naturally present in most members of the Brassicaceae family including rapeseed (Röbbelen, 1991; Zanetti *et al.*, 2012). In the past, various studies have shown the negative health effects of erucic acid in the oil (Thomasson and Boldingh, 1955; Beare, Gregory and Campbell, 1959; Charlton *et al.*, 1975; de Wildt and Speijers, 1984) and as a result, it was eliminated from the rapeseed to make it suitable for human consumption (Stefansson, Hougen and Downey, 1961). In order to meet the demands of the oleochemical industries, high erucic acid rapeseed (HEAR) cultivars, containing 45 to 50% erucic acid in their oil, are grown in the European Union (EU) and Canada. The United Kingdom is the largest producer of the high erucic acid rapeseed (Meakin, 2007; Knutsen *et al.*, 2016). In the EU, the renewable energy directive (RED) has promoted the use of the renewable energy resources (Renewable Energy Directive, 2017), leading to an increase in the production of the crops used for the biofuels. The main feedstock for biodiesel production in the EU is rapeseed with a share of 46% in 2016 (Flach, Lieberz and Rossetti, 2017). Understanding the genetic control of the erucic acid content is a major objective for the development of the rapeseed with higher erucic acid than the existing HEAR cultivars as it can lower the processing costs of the industry.

B. napus (AACC, 2n = 38) is a polyploid (amphidiploid) having two genomes – A and C, formed by the spontaneous hybridization of the two diploids, *B. rapa* (AA, 2n = 20)

and *B. oleracea* (CC, 2n = 18) (Nagaharu U., 1935; Palmer *et al.*, 1983; Parkin *et al.*, 1995). Genetic mapping studies show that the A and C genomes are intact and have not been rearranged in *B. napus* (Parkin *et al.*, 1995). These two genomes differ by only ~3.5% in transcript sequence (Trick *et al.*, 2009). *Brassica* crops are closely related to the most widely used model plant, *Arabidopsis thaliana* and both lineages diverged only ~20 million years ago (Yang *et al.*, 1999). Brassica species have undergone many segmental rearrangements compared to *A. thaliana* but the microstructure of the conserved genes show collinearity among them (Lagercrantz, 1998; O'Neill and Bancroft, 2000; Rana *et al.*, 2004; Parkin, 2005). The diploids, *B. rapa* and *B. oleracea* have triplicated genomic structures with respect to Arabidopsis like genome (Lagercrantz, 1998; Rana *et al.*, 2004; Cheng, Wu and Wang, 2014) and thus, six copies should be expected in the *B. napus* genome for a particular gene. But the spontaneous hybridization between these diploids to form *B. napus* was followed by an interspersed gene loss (O'Neill and Bancroft, 2000; Rana *et al.*, 2004; Parkin, 2005) and thus, the genes may not be present in six copies.

Studies in Arabidopsis show that the gene *FAE1* (*FATTY ACID ELONGASE 1*) is responsible for the conversion of oleic acid (C18:1) to eicosenoic acid (James and Dooner, 1990; Lemieux *et al.*, 1990; Kunst, Taylor and Underhill, 1992; James *et al.*, 1995; Millar and Kunst, 1997). In *B. napus, FAE1* corresponds to the rate-limiting enzyme, β-ketoacyl-CoA synthases (KCS) of the four-step elongation mechanism (Stumpf and Pollard, 1983; Cassagne *et al.*, 1987, 1994; Suneja *et al.*, 1991; Roscoe *et al.*, 2001). It is required for both elongation steps to produce the very long chain fatty acids (VLCFAs, fatty acids ≥ 20 carbons chain length) - eicosenoic acid (C20:1) and erucic acid in *B. napus* (Kondra and Stefansson, 1965). *B. napus* has two orthologues of *FAE1*, present in A and C genomes – *Bna.FAE1.A8* and *Bna.FAE1.C3,* acting in an additive manner (Harvey and Downey, 1964). There is another important gene family, *FAD2* (*FATTY ACID DESATURASE 2*), involved in the fatty acid biosynthesis and controls the formation of linoleic acid (C18:2) from oleic acid in *A. thaliana* (Miquel and Browse, 1992; Okuley *et al.*, 1994). In *B. napus*, four orthologues of *FAD2* are present – two each in A genome (*Bna.FAD2.A1* and *Bna.FAD2.A5*) and two in C genome (*Bna.FAD2.C1* and *Bna.FAD2.C5*) (Scheffler *et al.*, 1997). Linoleic acid is one of the polyunsaturated fatty acids (PUFAs) and high values of PUFAs are linked to low

thermal stability of the oil (Browse *et al.*, 1998; Durrett, Benning and Ohlrogge, 2008). These are removed during the purification of the erucic acid from the industrial rapeseed oil by distillation (Carlson *et al.*, 1977; Walp and Tomlinson, 2004) and therefore, low levels are desirable. There are many biochemical (oleic acid pool) and enzymatic barriers (*FAE1* activity and non-specificity of the *B. napus* lysophosphatidic acid acyltransferase for oleoyl-CoA) to increase the erucic acid content beyond 66% in the rapeseed oil (Lassner, Lardizabal and Metz, 1996). It has been possible to increase its level to 72% in the *B. napus* by using transgenic methods (Nath, Becker and Möllers, 2007; Nath, 2008) but the commercialization of the transgenic material is a difficult process involving various tight regulations. On the other hand, by using the non-transgenic methods, minimal progress has been achieved so far to increase the erucic acid content in *B. napus*. In the present study, we have used mutants developed by a non-transgenic approach, EMS (ethyl methane sulphonate) mutagenesis to introduce partially functional *Bna.FAD2s* to the high erucic varieties. It led to an increase in the very long chain fatty acids (and erucic acid) and a decrease in the polyunsaturated fatty acids.

1.2 Aims and Scope

The present study had two main objectives – first was to understand the control of the erucic acid content in the *B. napus* seeds oil by factors in addition to the known effects of *Bna.FAE1* loci. The second objective was to understand the influence of the partially functional *Bna.FAD2* family on the erucic acid content in the rapeseed oil. We hypothesised that the partially functional *Bna.FAD2* family (3 copies non-functional and 1 copy with mutations) would lower the polyunsaturated fatty acids and thereby, provide more oleic acid (substrate) for elongation to the very long chain fatty acids in *B. napus*. Associative transcriptomics (AT) approach was used, in a diversity panel of 383 *B. napus* genotypes, to search for any modifier loci in the *B. napus* transcriptome that may have an effect on the erucic acid content in addition to the known *Bna.FAE1* loci. For the second objective, a pilot experiment was conducted by cross-pollinating high erucic acid variety, Ningyou 7 and *Bna.fad2.C5* mutant, K0472 and high erucic acid rapeseed in low polyunsaturated fatty acids (HELP) background was developed.

The same approach was used to develop HELP lines in the background of another high erucic variety, Maplus. In addition, comparisons of *Bna.FAE1* alleles in the HELP lines developed from these two different high erucic acid cultivars were made. Finally, the quantitative effect of *Bna.fad2.C5* alleles on the very long chain fatty acids were tested by cross-pollinating Maplus with various mutants having different polyunsaturates content.

In the present study, the mutants developed by EMS (ethyl methane sulphonate) mutagenesis (Wells *et al.*, 2014) were used to introduce partially functional *Bna.FAD2* alleles in the high erucic acid varieties. It resulted in the development of a unique specification of rapeseed – high erucic acid rapeseed in low polyunsaturated fatty acids (HELP) background. HELP oil is anticipated to have a high potential for the industry due to its high erucic content (a valuable chemical) and low polyunsaturates content (high thermal stability).

1.3 Review of Literature

Genus *Brassica* belongs to the tribe *Brassiceae* and the family *Brassicaceae* including a diverse type of plants – grown for seed oil, vegetables, fodder and condiments. Genus *Brassica* comprises of many species but six of them are the most important ones – three of these are diploids (*B. rapa*, *B. nigra* and *B. oleracea*) and other three are allotetraploids (*B. napus, B. juncea* and *B. carinata*). Three genomes, named A, B and C, are present in these six species. Spontaneous hybridization between two of the three genomes formed the tetraploids and the depicted as U's triangle (Nagaharu U., 1935). The relationship between these genomes is shown in Figure 1.1.

B. napus is an allotetraploid (2n=38, AACC) comprising of A genome from *B. rapa* (2n=20, AA) and C genome from *B. oleracea* (2n=18, CC). *Brassicas* diverged from Arabidopsis only about 20 million years ago and thus, both of these are closely related to each other (Yang *et al.*, 1999). *Brassica* species experienced an extra whole genome triplication event in comparison to *A. thaliana* and thus, six-fold representation of the Arabidopsis genome is present in the tetraploids (Lagercrantz, 1998; Rana *et al.*, 2004; Cheng, Wu and Wang, 2014). So, each gene is expected to be present in the six copies in the *B. napus* genome but there was interspersed gene loss following polyploidy

(O'Neill and Bancroft, 2000; Rana *et al.*, 2004; Parkin, 2005) and thus, the genes may not be present in six copies in the *B. napus* genome.

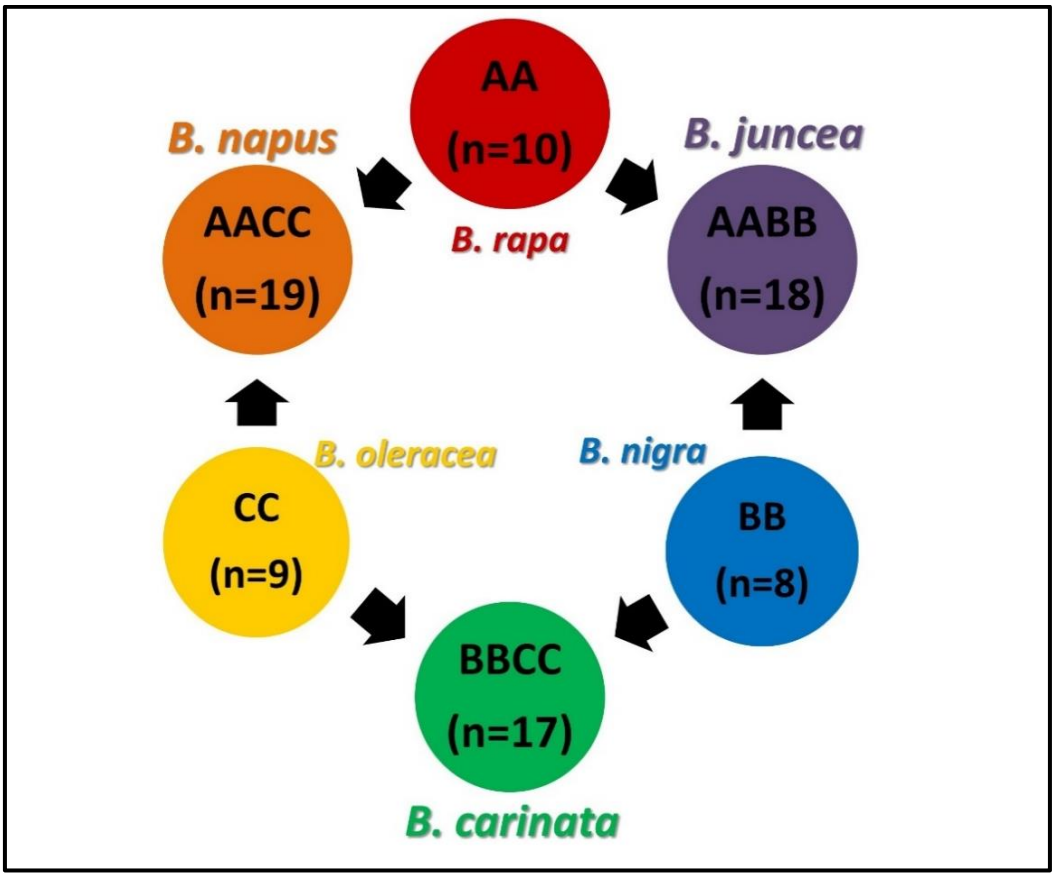

Figure 1.1 Relationship between the six *Brassica* species
Two of the three diploids (B. rapa, B. nigra and B. oleracea) combined to form one of the allotetraploid species (B. napus, B. juncea and B. carinata)

1.3.1 Fatty Acids, Types and Nomenclature

A fatty acid comprises of a long aliphatic chain (saturated or unsaturated) and a carboxylic group (COOH). Free fatty acids are usually not found in an organism and are mainly present in the forms such as triglycerides, galactolipids, phospholipids and cholesterol esters. Naturally occurring fatty acids usually have even numbered chain length of 4 to 28 Carbons (McNaught and Wilkinson, 1997). The fatty acids with no double bond in the aliphatic chain are known as saturated fatty acids (SAFAs) and the fatty acids with double bonds in the aliphatic chain are unsaturated fatty acids. Unsaturated fatty acids can be further classified into two classes – monounsaturated fatty acids (MUFAs) having one double bond in the carbon chain and polyunsaturated

fatty acids (PUFAs) having more than one double bond in the carbon chain. The fatty acids having 20 or more carbons in its chain are classified as very long chain fatty acids (VLCFAs). SAFAs are the most thermostable and PUFAs are the least thermostable among these fatty acids groups. Carbon-carbon double bond in the unsaturated fatty acids can form two isomers according to the position of two hydrogen atoms around the double bond - *cis* (same side) and *trans* (opposite sides) forms.

In a fatty acid chain, an aliphatic end is termed as the omega (ω) end and the carbon atom next to the carboxyl group is termed as alpha (α) carbon as shown in Figure 1.2. The fatty acids are usually named as 'C_L:D' where 'C_L' is the carbon length and 'D' is the number of the double bonds (if any). It is followed by the position of the first double bond which can be presented in two different ways – first is 'ω-x or n-x' where the double bond is counted from the methyl end (aliphatic end, ω) and the second is 'delta^x or Δ^x' where counting is from the carboxyl end (α). 'x' is the position of the carbon with a double bond from the respective ends. For example, the erucic acid can be represented as 'C22:1', '22:1', '22:1ω9', '22:1n9' or '*cis*Δ^{13} docosenoic acid' according to the different naming systems and its structure is shown in Figure 1.2.

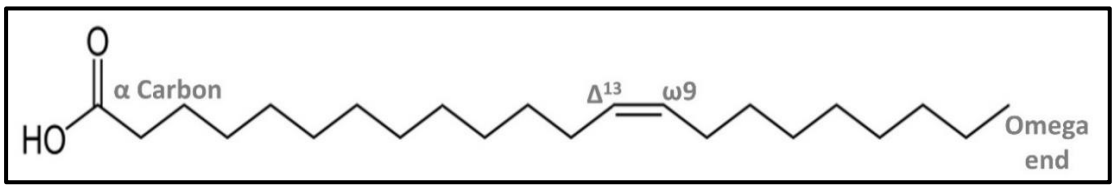

Figure 1.2 Linear structure of the erucic acid (C22:1)
Erucic acid linear form showing the positioning of the double bond from both ends – ω9 from the aliphatic end and Δ^{13} from the carboxylic end

1.3.2 Fatty Acid Compositions

In most of the higher plants, seed storage lipids are C16 and C18 fatty acids but in some of the families including Brassicaceae, very long chain fatty acids (fatty acids with carbon length ≥ 20) are present naturally (Röbbelen, 1991; James *et al.*, 1995; Zanetti *et al.*, 2012). The major fatty acids found in the rapeseed oil are – palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid

(C18:3), arachidic acid (C20:0), eicosenoic or gondoic acid (C20:1) and erucic acid (C22:1). The linear structures of some of these fatty acids are shown in Figure 1.3.

The economic value of an oilseed crop depends on its oil composition, so significant research has been conducted in the rapeseed to produce the designer oils with particular fatty acids. Some examples of the rapeseed with unique specifications are described here. Low erucic acid rapeseed (LEAR) varieties have low levels of the erucic acid in the oil. Canola is a double-low rapeseed with low erucic acid (less than 2%) and low glucosinolates (less than 20 μmol/g) in its seeds, first developed in the 1970s in Canada (Stefansson, Hougen and Downey, 1961; Stefansson and Kondra, 1975). High erucic acid rapeseed (HEAR) varieties, contain 45 to 50% erucic acid and low glucosinolates (less than 20 μmol/g) in the seeds, were first developed in Canada as well and have been commercialized in the last three decades (Scarth *et al.*, 1992; McVetty *et al.*, 2016). High oleic acid and low linolenic acid (HOLL) rapeseed varieties have 75 to 80% oleic acid and ~4% linolenic acid in their oil (Scarth *et al.*, 1988; Maher *et al.*, 2006). There are some other variations to the canola oil – high oleic acid and low linolenic acid canola oil (HOLLCO), low linolenic canola oil (LLCan, contains ~2% linolenic acid), high oleic canola oil (HOCAN), lauric acid canola (LTCAN) with ~40% lauric acid (C12:0) and 4.1% (C14:0) myristic acid and ɣ-linolenic acid canola (GLCAN) containing ~37% ɣ-linolenic acid (Bertrand, 2012). Lauric acid containing *B. napus* was developed for the industrial uses in surfactants and detergents (Voelker *et al.*, 1992). Industrial rapeseed oil having more than 45% erucic is known as high erucic rapeseed oil (HERO) (Piazza and Foglia, 2001). The concept of high erucic acid and low PUFA (HELP) lines has been proposed in a previous study but it was not possible to simultaneously increase the erucic acid and decrease the polyunsaturates in *B. napus* (Sasongko and Möllers, 2005).

1.3.3 Erucic Acid, Importance and Occurrence

Industrial uses of the rapeseed oil are of main interest due to the presence of erucic acid in its oil (Röbbelen, 1991). Erucic acid is a valuable monounsaturated fatty acid with a very long chain length of 22 carbons as shown in Figure 1.2. The double bond is present at the omega-9 or delta-13 position. High erucic oil has many industrially

favourable properties such as high lubricity, high stability and biodegradability. It can be used without processing or it can be derived into other products for use in the oleochemical industry. Fatty acids serve as a major source of fuel for heart and muscle cells and; these undergo mitochondrial β-oxidation in our cells. Various studies showed that on consumption, erucic acid was poorly oxidised and caused myocardial damage by the fatty acid depositions around the heart and kidneys due to its long chain length (Thomasson and Boldingh, 1955; Beare, Gregory and Campbell, 1959; Charlton *et al.*, 1975). Thus, it was considered an anti-nutritional component in the food and was eliminated by various breeding methods. Rapeseed oil was used as a lubricant in the steam engines as early as the 1700s (Gupta and Pratap, 2007) and high erucic acid cultivars are grown as 'green feedstock' for the oleochemical industry with an estimated demand of 100,000 to 120,000 tonnes worldwide. The United Kingdom is the main producer (~20,000 ha) of HEAR at the world level (Meakin, 2007; Knutsen *et al.*, 2016). It is also grown in Canada and other parts of Europe. Mainly winter-type HEAR is grown in Europe while spring-type HEAR is prevalent in Canada (McVetty *et al.*, 2016).

Erucic acid finds use in various industrial products such as lubricants, biofuels, slip additives, cosmetics, corrosion inhibitors, paints, pharmaceuticals, plastics, printing inks, slips, soaps, surfactants and many more (Nieschlag and Wolff, 1971; Johnson and Fritz, 1989; James *et al.*, 1995; Piazza and Foglia, 2001; Scarth and Tang, 2006; Zanetti *et al.*, 2012). Methyl and ethyl esters of rapeseed oil can be used as bio-additives for the jet fuel (Iakovlieva *et al.*, 2017). In addition, many patent applications have been filed for various novel uses of erucic acid. One of the examples of a patent (patent no. US 8,790,553 B2) is using the erucic acid containing dielectric fluid in an electrical equipment and it made this insulating liquid non-toxic and biodegradable without affecting the performance of the device. Some of the patents have been described in Molnar, 1974.

Erucic acid is naturally found in many species of the Brassicaceae family such as *B. napus*, *B. carinata*, *B. juncea, Crambe abyssinica, Eruca sativa, Sinapis alba, Camelina sativa* and many more (Zanetti *et al.*, 2012). *Brassica* vegetables contain traces of erucic acid as compared to the high amount present in their seeds.

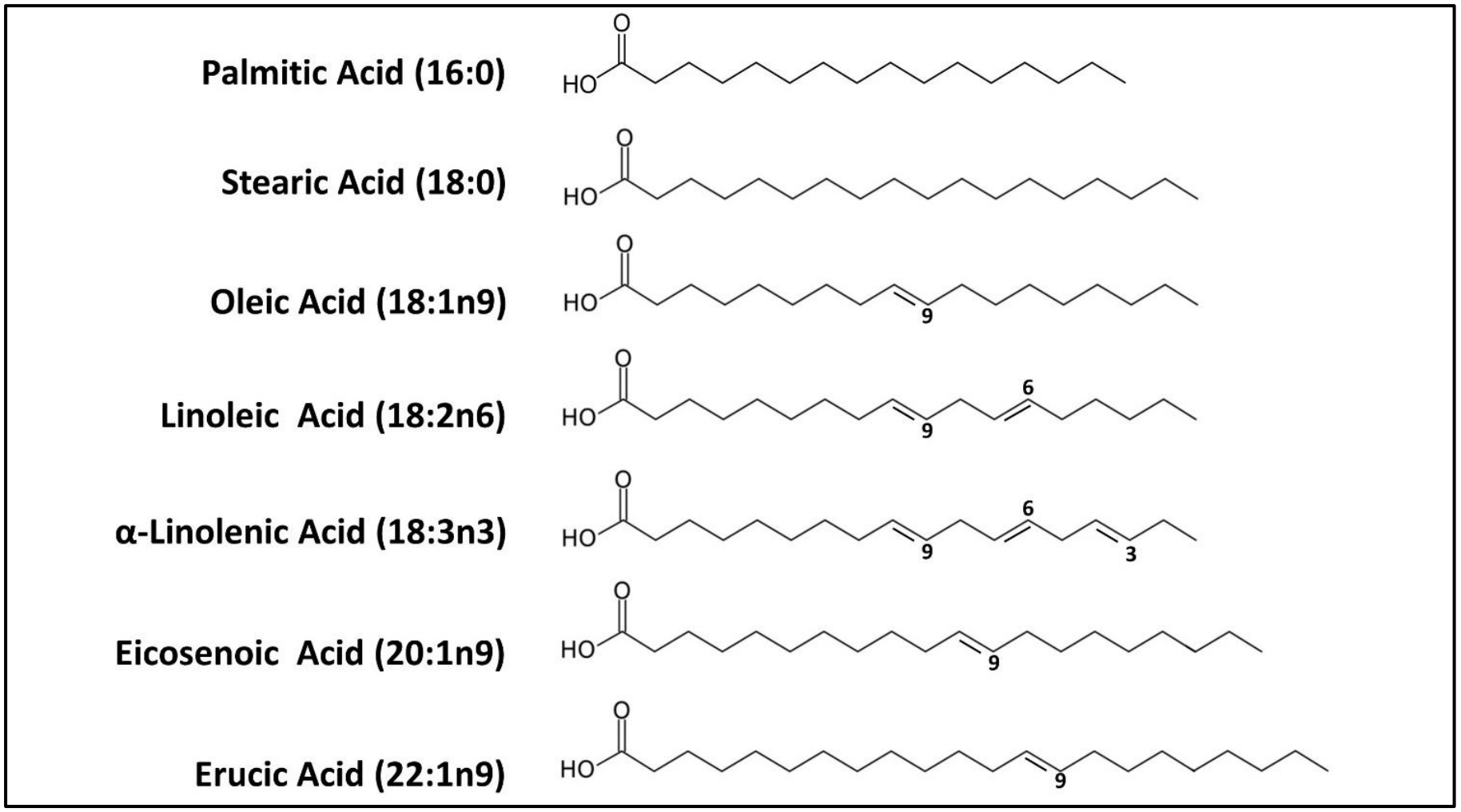

Figure 1.3 Linear structures of the major fatty acids found in the rapeseed oil

Double bonds are numbered from the omega (aliphatic) end in these linear structures of the fatty acids

Erucic acid is also present in some other plant families such as *Limanthes alba, Borago officinalis, Lupinus albus* and few more. Crambe oil has the highest erucic acid percentage (60%) in its seeds (Lalas *et al.*, 2012) but it has many negative attributes such as low oil content, high sulphur level and high glucosinolates in the meal as compared to rapeseed (Hebard, 2016). So, the main interest of erucic production is always in rapeseed due to its high and stable agronomic yields with many other positive attributes (Sanyal *et al.*, 2015).

1.3.4 Erucic Acid Derivatives

High erucic acid oil can be used without further processing or derived into various products having industrial applications. The main derivative of the erucic acid is erucamide ($C_{22}H_{43}NO$) and the principal source of its production is rapeseed oil. Rapeseed oil is distilled to remove the polyunsaturates (less than 2%) and C18s (less than 1%) to yield pure erucic acid (94 to 95%). Purified erucic acid is derived to erucamide by a reaction with ammonia at high temperature (200°C) and pressure (125 to 150 psi) (Molnar, 1974; Walp and Tomlinson, 2004). In addition, erucamide has a higher melting point and higher heat resistance than the derivative of oleic acid – oleamide and its market is expected to increase in the subsequent years (Zanetti *et al.*, 2012; Zion Research, 2016). It is used as an anti-sticking agent in lubricants; anti-static agent in polyethylene and polypropylene; dispersing agent in printing inks, carbon paper and metal decorating; waterproof and anti-fog agent in papermaking and textile industry; anti-bubbling agent in a boiler and; foam-stabilizer in sulfonate detergents. It also finds uses in the high-grade lube-oil. Other potential uses are in food packaging and surfactants (Molnar, 1974; Zion Research, 2016). The use of erucamide in slip agents is known to reduce the biofouling (Getachew *et al.*, 2016).

Another important derivative of erucic acid is the brassylic acid. It is produced by the oxidative ozonolysis of the erucic acid. The double bond in erucic acid is split to produce odd carbon numbered products – brassylic acid and pelargonic acid as shown in Figure 1.4 (Neischlag *et al.*, 1967; Van Dyne and Blasé, 1991). Brassylic acid ($C_{13}H_{24}O_4$, tridecanedioic acid) is a commercially important dicarboxylic acid. Esters of brassylic acid serve as low-temperature plasticizers for polyvinyl chloride (PVC),

lubricants for a wide temperature range, synthetic musk and various other uses (Neischlag *et al.*, 1967; Carlson *et al.*, 1977). Pelargonic acid ($C_9H_{18}O_2$, nonanoic acid) is used in combination with other chemicals as herbicide (Savage and Zomer, 1995). It also finds uses in therapeutics for treating seizures (Chang *et al.*, 2013). These two acids are also used in the production of nylon 9-9 and nylon 13-13 (Neischlag *et al.*, 1967; Van Dyne and Blasé, 1991).

Erucic acid can be converted to behenic acid ($C_{22}H_{44}O_2$, C22:0, docosanoic acid) by the catalytic dehydrogenation. It is used in cosmetics, hair conditioners, moisturizers, lubricating oils, paint removers (solvent evaporation retarder), anti-foaming agent, surfactants, textiles, plastic additives, detergents, recording materials and rubber production (Piazza and Foglia, 2001; Pennick *et al.*, 2012). There are various other derivatives of the erucic acid and are presented in Table 1.1 along with their industrial use (adapted from Caballero, 2006).

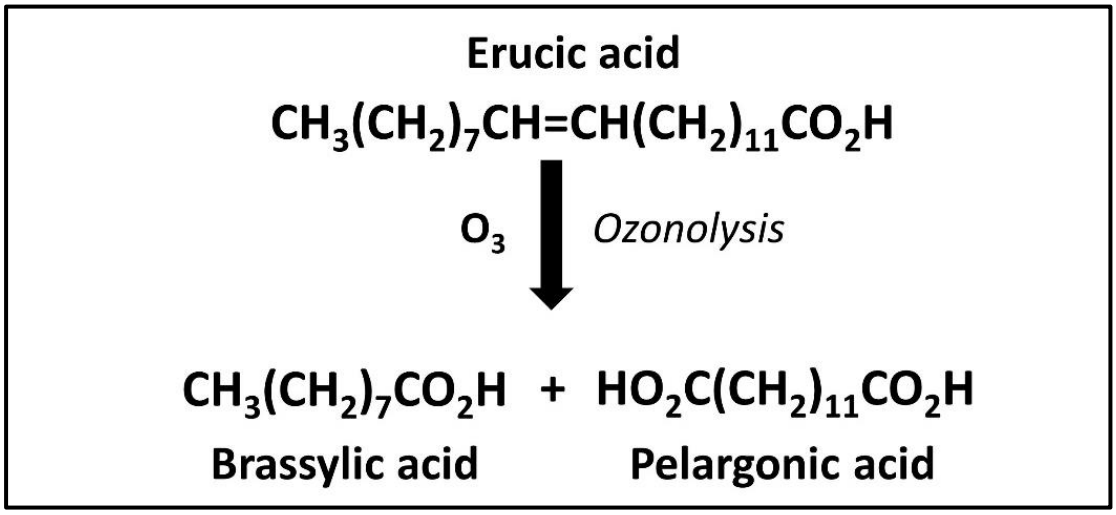

Figure 1.4 Ozonolysis of the erucic acid to yield brassylic acid and pelargonic acid

Table 1.1 The erucic acid derivatives and their industrial applications (adapted from Caballero, 2006)

Erucic acid derivative	Industrial application
Behenyl fumarate vinyl copolymer	Oil field chemical
Stearyl erucamide	Polymer additive
Behenyl trimethylammonium chloride	Personal care product
Brassidolide	Perfumery
Glyceryl trierucate	Pharmaceutical
Erucyl erucate	Cosmetics
Nylon 13-13	Apparel

1.3.5 Fatty Acids and TAG Biosynthesis Pathway

The fatty acid biosynthesis is well understood in the family Brassicaceae and its de novo synthesis begins with acetyl-CoA (Coenzyme A) carboxylation to malonyl-CoA (MCA) catalysed by acetyl-CoA carboxylase (ACCase) in plastids (Figure 1.5). Malonyl-CoA is converted to a malonyl-acyl carrier protein (ACP) by the ACP transferase. The first condensation begins between malonyl-ACP and acetyl-CoA with the help of ketoacyl synthase (KAS) III. This is followed by six cycles of condensation to yield palmitoyl-ACP (16:0 ACP), catalysed by KAS I. Palmitoyl-ACP is converted to stearoyl-ACP (18:0 ACP) by another condensation with malonyl-ACP by KAS II. Stearoyl-ACP is desaturated by Δ^9 desaturase to oleoyl-ACP (18:1 ACP). Palmitoyl-ACP, stearoyl-ACP and oleoyl-ACP are first converted to their respective fatty acids by fatty ACP thioesterases (FAT), then activated to the respective acyl-CoAs catalysed by acyl-CoA synthetase (ACS) and finally exported outside the plastids. These act as a substrate for the TAG (triacylglycerol) synthesizing enzymes and other glycerolipids of the endoplasmic reticulum (ER). These fatty acids are also elongated and desaturated in the cytosol, mainly in the ER (Schmid and Ohlrogge, 2002; Barker *et al.*, 2007; Bates, Stymne and Ohlrogge, 2013). A simplified flowchart for the fatty acid biosynthesis in the family Brassicaceae is summarized in Figure 1.5.

Oleoyl-CoA (18:1 CoA) can follow either of the two pathways – desaturation or elongation. Firstly, it may undergo two consecutive desaturation reactions (shown in the blue colour in Figure 1.5) to be converted to polyunsaturated fatty acids (C18:2 and C18:3) and the genes responsible are *FATTY ACID DESATURASES – FAD2* and *FAD3,* respectively (Arondel *et al.*, 1992; Miquel and Browse, 1992; Okuley *et al.*, 1994; Scheffler *et al.*, 1997). Oleoyl-CoA is first converted to oleoyl-PC (phosphatidyl choline) before desaturation reactions as shown in Figure 1.5. Secondly, it can be elongated (shown in the magenta colour, Figure 1.5) to eicosenoic acid (C20:1) and then erucic acid (C22:1) and; gene responsible is *FATTY ACID ELONGASE 1, FAE1* (James and Dooner, 1990; Lemieux *et al.*, 1990; Kunst, Taylor and Underhill, 1992; James *et al.*, 1995). Oleoyl-CoA is elongated to eicosenoyl-CoA and erucyl-CoA. Erucic acid can be further elongated to nervonic acid (C24:1) by elongases. Similarly, linoleic acid can be elongated to eicosadienoic acid (C20:2) and docosadienoic acid (C22:2) as depicted in Figure 1.5.

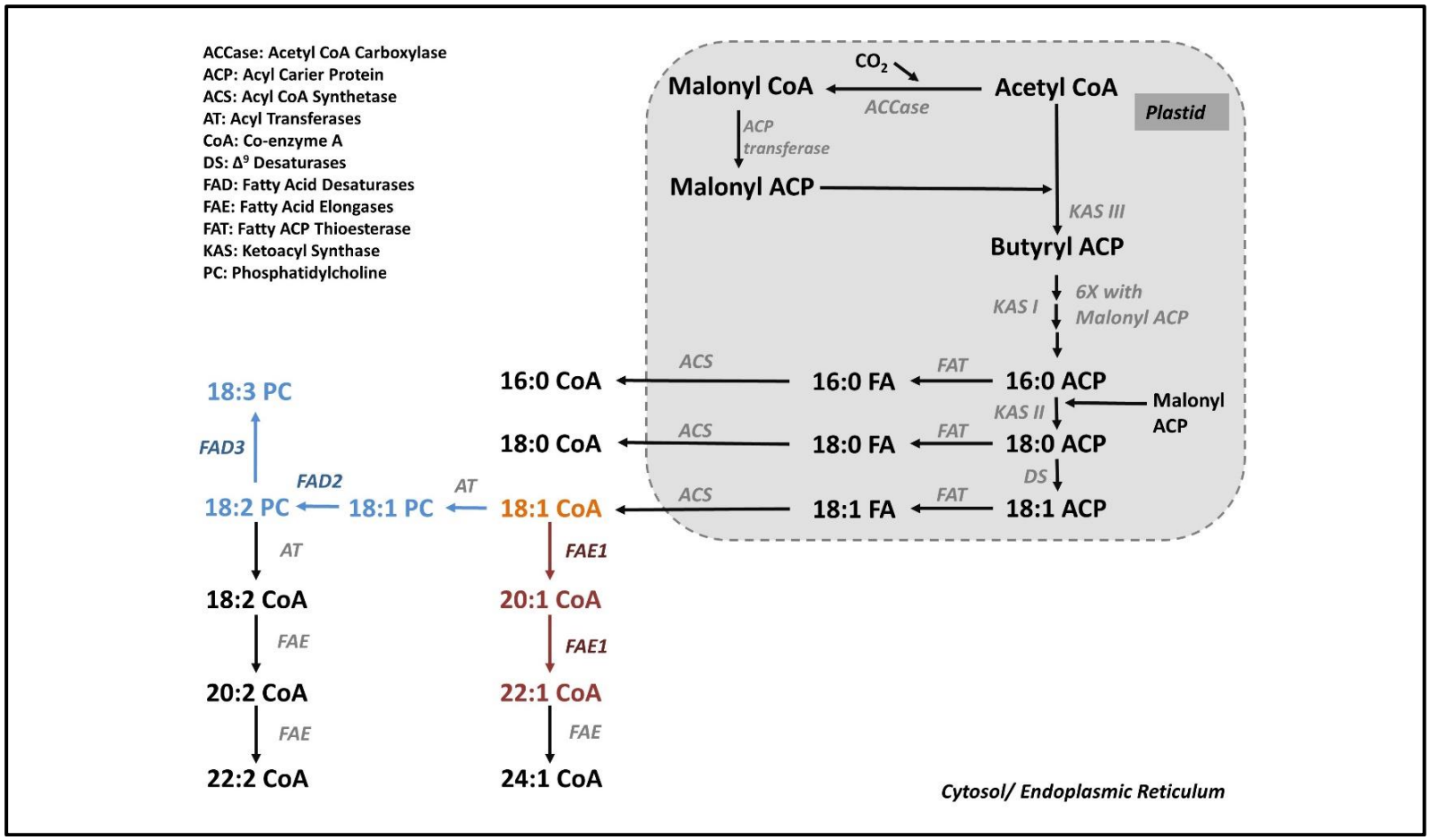

Figure 1.5 A simplified fatty acid biosynthesis pathway in the family Brassicaceae.

Malonyl-CoA is converted to a malonyl-ACP which condenses with acetyl-CoA to form butyryl ACP, followed by six cycles of condensation to yield palmitoyl-ACP. Palmitoyl-ACP is converted to stearoyl-ACP which is desaturated to oleoyl-ACP. ACPs are converted to their respective fatty acids, followed by activation to the respective acyl-CoAs and exported outside the plastids. Further details are provided in the text.

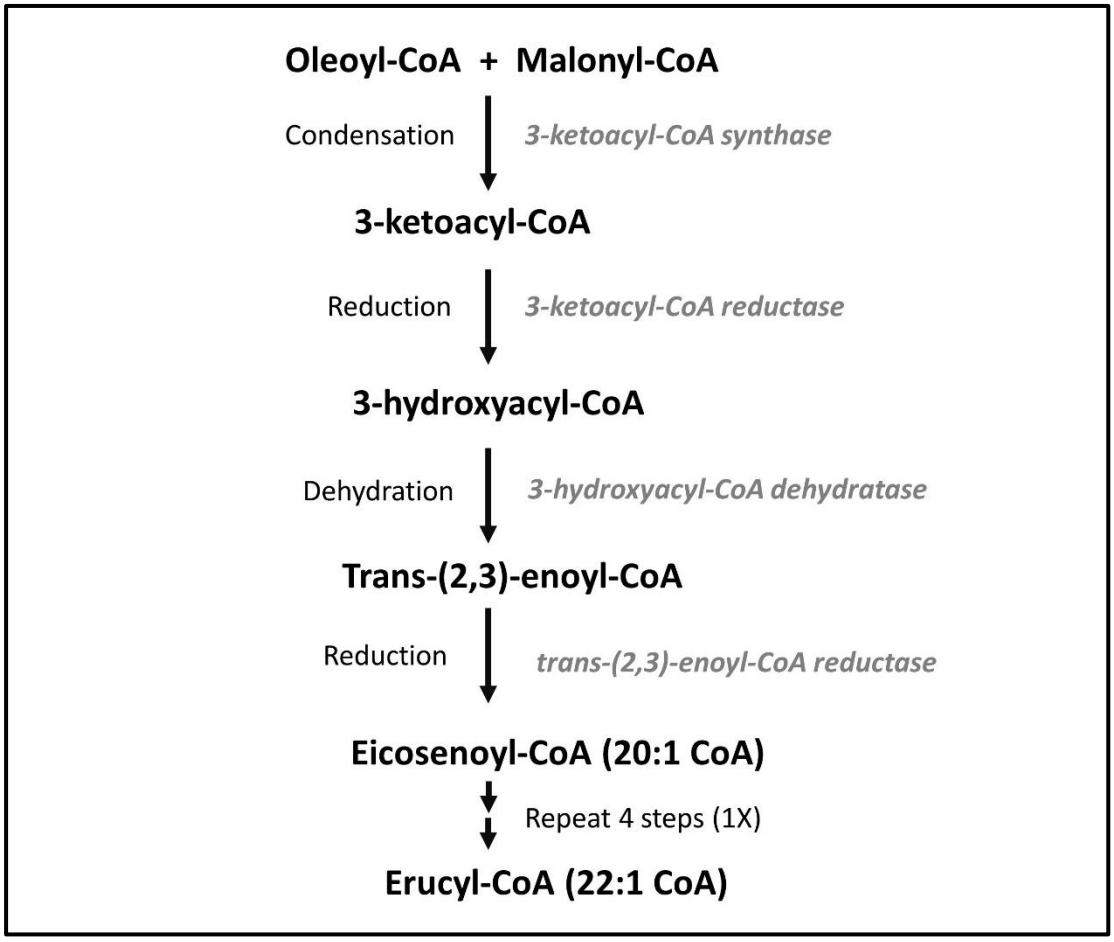

Figure 1.6 Four-step elongation pathway to produce erucic acid from oleic acid
Oleoyl-CoA (18:1 CoA) is converted to eicosenoyl-CoA (20:1 CoA) by the four steps reaction and these steps are repeated to form erucyl-CoA (22:1 CoA) from eicosenoyl-CoA

The elongation from oleic acid to erucic acid is sub-divided into two cycles of the four-step process catalyzed by four different enzymes (Figure 1.6): First, oleoyl-CoA and malonyl-CoA is condensed to form 3-ketoacyl-CoA by 3-ketoacyl-CoA synthase; second, 3-ketoacyl-CoA is reduced by 3-ketoacyl-CoA reductase to produce 3-hydroxyacyl-CoA; third, 3-hydroxyacyl-CoA is dehydrated to trans-(2,3)-enoyl-CoA by 3-hydroxyacyl-CoA dehydratase; and finally, trans-(2,3)-enoyl-CoA reductase reduces trans-(2,3)-enoyl-CoA to eicosenoyl-CoA. These four steps are repeated to form erucyl-CoA from eicosenoyl-CoA (Stumpf and Pollard, 1983; Cassagne *et al.*, 1987, 1994; Fehling and Mukherjee, 1991). These four enzymes involved in the elongation process are together known as the fatty acid elongases (Wettstein-Knowles, 1982).

The *FAE1* encoded 3-ketoacyl-CoA synthase (KCS) act as the rate-limiting enzyme for the seed very long chain fatty acids production (Cassagne *et al.*, 1987, 1994; Suneja *et al.*, 1991; Millar and Kunst, 1997; Rossak, Smith and Kunst, 2001). KCS corresponds to

the *Bn-FAE1* gene in *B. napus* (Lühs and Friedt, 1994; Roscoe *et al.*, 2001) and the same enzyme is responsible for both the elongations steps to produce eicosenoic acid and erucic acid from oleic acid (Kondra and Stefansson, 1965).

Oils in the form of triacylglycerol (TAG) are the major storage lipids in plants and are a source for building blocks of membrane lipid biosynthesis. Their properties depend on the esterification at all the three positions: *sn-1*, *sn-2* and *sn-3* (*sn* is stereospecific numbering) of the glycerol backbone. TAG biosynthesis takes place by the Kennedy pathway and it involves *de novo* assembly of glycerol-3-phosphate and acyl-CoA to form TAG in the endoplasmic reticulum as shown in Figure 1.7 (in green colour). Different acyltransferases are responsible for the transfer of acyl groups from acyl thioesters to the glycerol moiety. This pathway involves four steps – First acylation at the *sn-1* position is carried out by glycerol-3-phosphate (G3P) acyltransferase (GPAT) to form lysophosphatidic acid (LPA) and the second acylation is catalysed by lysophosphatidic acid acyltransferase (LPAAT) at the *sn-2* position to form phosphatidic acid (PA). It is followed by the conversion of the phosphatidic acid to diacylglycerol (DAG) by the removal of a phosphate group (PO_4^-) by phosphatidic acid phosphatase (PAP). Finally, the third acylation at the *sn-3* is catalysed by 1, 2-diacylglycerol acyltransferase (DAGAT) leading to the formation of the triacylglycerol. In addition to this *de novo* synthesis of TAG molecules, a more complicated pathway also exists in plants. It involves the membrane lipid, phosphatidylcholine (PC) which act as a central intermediate in the flux of the fatty acids or DAG. Fatty acids flux through PC for TAG biosynthesis can occur by three mechanisms. First is 'Acyl editing' (also called as remodelling or retailoring) which is the exchange of acyl groups between polar lipids without net synthesis of lipids. PC-deacylation and lysophosphatidyl-choline (LPC) reacylation cycle exchanges the fatty acids on PC with the acyl-CoA pool (orange arrows in Figure 1.7) Second is by the PC headgroup exchange between from PC and DAG, catalysed by phospholipid diacylglycerol transferase (PDAT, in purple colour in Figure 1.7). Third is the use of PC derived DAG as a substrate for the TAG synthesis. (DAG(2) in blue colour in Figure 1.7). TAGs are stored in the oil bodies of the seeds until germination and then used as fuel for seedling growth (Frentzen, 1993, 1998; Graham, 2008; Bates *et al.*, 2009, 2012; Cagliari *et al.*, 2011; Bates, Stymne and Ohlrogge, 2013).

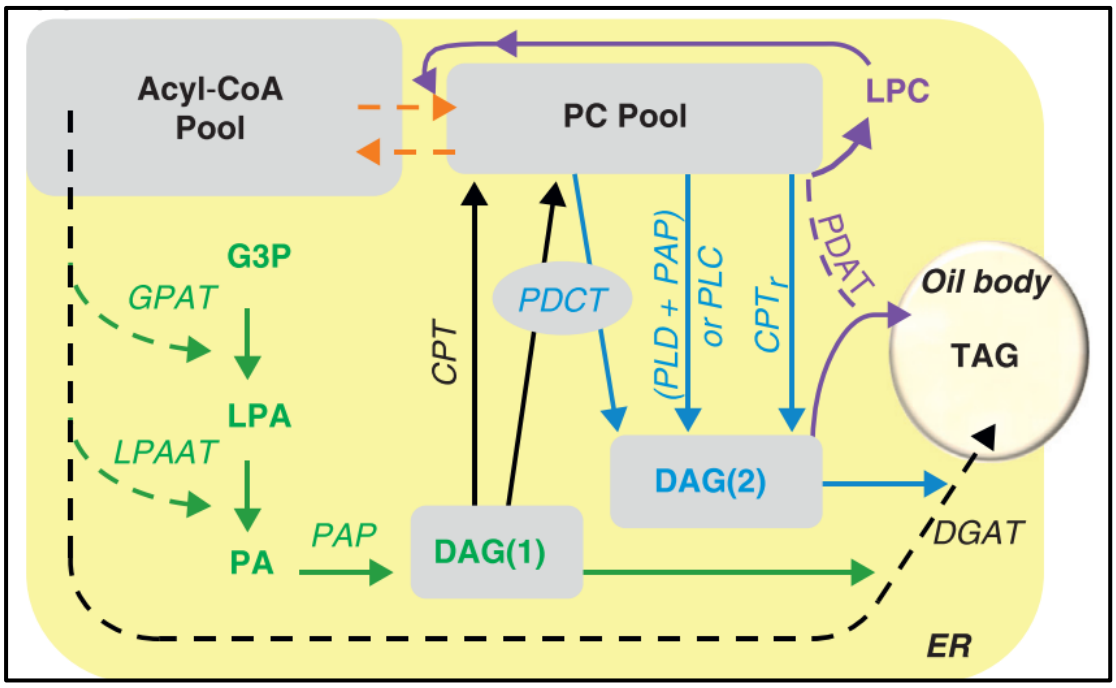

Figure 1.7 Triacylglycerol (TAG) biosynthesis in the endoplasmic reticulum (ER)
DAG(1) – de novo synthesized DAG, DAG(2) - PC-derived DAG. Green lines represents the de novo TAG synthesis, blue lines are PC-derived TAG synthesis, orange lines are acyl editing and purple lines show phospholipid: DAG acyltransferase (PDAT). Abbreviations - DAG – diacylglycerol, G3P –glycerol-3-phosphate, LPA –lysophosphatidic acid, LPC – lysophosphatidylcholine, PA –phosphatidic acid, PC- phospatidylcholine, TAG –triacylglycerol. GPAT - glycerol-3-phosphate acyltransferase; LPAAT - lysophosphatidic acid acyltransferase; PAP - phosphatidic acid phosphatase; DGAT - 1, 2-diacylglycerol acyltransferase; CPT – CDP choline:DAG cholinephosphotransferase; PAP - PA phosphatase; PDCT - PC:DAG cholinephosphotransferase; PLC - phospholipase C; PLD - phospholipase D (Figure and text adapted from Bates, Stymne and Ohlrogge, 2013)

1.3.6 Increasing the Erucic Acid and the Limitations

High erucic acid rapeseed cultivars contain 45 to 50% erucic acid in their oil. Many studies have been conducted to increase the erucic acid content in *B. napus* but there is a theoretical limit to increase it beyond ~66% in its seeds (Cao, Oo and Huang, 1990; Katavic *et al.*, 2001). In *B. napus*, lysophosphatidic acid acyltransferase (Bn-LPAAT) is highly specific for the substrate (C18 fatty acids) and has a poor affinity for the erucoyl and eicosenoyl moieties (Brockerhoff, 1971; Bernerth and Frentzen, 1990). So, erucic acid and eicosenoic acid are excluded from the *sn-2* position of the TAG molecule and this limits the highest possible erucic content in the seed oil to be ~66% (Kunst, Taylor and Underhill, 1992; Frentzen, 1993, 1998; Lassner, Lardizabal and Metz, 1996). To overcome this limit, *LPAAT* having broad specificity have been cloned and expressed

in rapeseed from various species such as *Escherichia coli, Limanthes douglasii* and *Saccharomyces cerevisiae* to incorporate VLCFAs in *sn-2* position but no significant increase in the erucic acid value was achieved (Coleman, 1990, 1992; Hanke *et al.*, 1995; Brough *et al.*, 1996; Lassner, Lardizabal and Metz, 1996; Zou *et al.*, 1997; Browse *et al.*, 1998; Frentzen, 1998). The activity of 3-ketoacyl-CoA synthase (KCS) was thought to be another limitation for increasing erucic acid content and thus, it was cloned and overexpressed from many plant species in the rapeseed but a minor increase in the erucic acid content was reported (James *et al.*, 1995; Katavic *et al.*, 2001). Combination of *Ld-LPAAT* from meadowfoam and *FAE1* from Arabidopsis was used in a study but a slight increase was reported in the erucic acid content. So, the acyl-CoA pool was thought to be another limitation (Han *et al.*, 2001). So, high erucic acid rapeseed was cross-pollinated to high oleic acid rapeseed (HOAR) to recombine the genes for high erucic acid and high oleic acid (and low polyunsaturates) to produce HELP lines but no significant change was reported in the erucic acid content (Sasongko and Möllers, 2005). In a study, transgenic HEAR (over-expressing *FAE1* and meadowforam *LPAAT*) was cross-pollinated to a non-transgenic high erucic and low PUFA rapeseed and showed EA ranging from 44 to 72% (Nath, Becker and Möllers, 2007; Nath, 2008). In another study, a HELP line was cross-pollinated to a line over-expressing the rapeseed *FAE1* and expressing *Ld-LPAAT*. The doubled haploid population was developed and erucic acid value of up to 59% was reported (Nath *et al.*, 2009). Other methods used for increasing the erucic acid were the co-suppression and antisense approaches to silence *FAD2* in *B. carinata* and increased level of erucic acid was found in the resulting genotypes (Jadhav *et al.*, 2005). Thus, there are three potential reasons for the theoretical limit for increasing EA value in rapeseed: (i) high specificity of lysophosphatidic acid acyltransferase (Bn-LPAAT); (ii) 3-ketoacyl-CoA synthase (KCS) activity and; (iii) oleoyl-CoA pool.

1.3.7 *FAD2* and *FAE1* Orthologues in *B. napus*

B. napus is closely related to the model plant *A. thaliana* but it has complex genetics due to its polyploid genome as compared to the simple genome of Arabidopsis. Comparative mapping with *A. thaliana* shows that the diploid genomes (*B. rapa, B. nigra* and *B. oleracea*) are palaeohexaploids (Lagercrantz and Lydiate, 1996; Rana *et*

al., 2004). There is a whole genome triplication (WGT) event in these diploids as compared to Arabidopsis (Cheng, Wu and Wang, 2014). Thus, in *B. napus,* made by the hybridization of *B. rapa* and *B. oleracea,* the genomics is even more complicated. Six-related genome segments of the Arabidopsis genome can be clearly seen in *B. napus* (Rana *et al.*, 2004). Many segmental rearrangements have occurred in the *Brassica* species but the conserved genes show collinearity in their sequences (Parkin, Sharpe and Lydiate, 2003). There was also extensive interspersed gene lose during the diploidization followed by polyploidy, so the gene families may not be always present in six copies in *B. napus* (Rana *et al.*, 2004).

In *A. thaliana, FAE1 (FATTY ACID ELONGASE 1)* is known to control the synthesis of the very long chain fatty acid, eicosenoic acid (James and Dooner, 1990; Lemieux *et al.*, 1990; Kunst, Taylor and Underhill, 1992; James *et al.*, 1995) and this gene corresponds to the rate-limiting enzyme, β-ketoacyl-CoA synthases (KCS) in the *B. napus* germplasm (Roscoe *et al.*, 2001). It is an intronless gene. The biosynthesis of erucic acid is controlled by two genes acting in an additive manner and showing no dominance in rapeseed (Harvey and Downey, 1964). The same gene controlling the erucic acid synthesis was discovered to control the eicosenoic acid synthesis as well in rapeseed (Kondra and Stefansson, 1965). Two major QTLs (quantitative trait loci) were found for the erucic acid control in *B. napus* in various studies, confirming the previously found results (Ecke, Uzunova and Weißleder, 1995; Howell, Lydiate and Marshall, 1996; Jourdren *et al.*, 1996; Thormann *et al.*, 1996; Qiu *et al.*, 2006). The two genes were found to be present on two separate chromosomes – one in the A genome at the chromosome A8 and another in the C genome at the chromosome C3. These were named as *Bna.FAE1.A8* (also called as *BN-FAE1.1, eru1, E1* and *BnaA.FAE1.a*) and *Bna.FAE1.C3* (also known as *BN-FAE1.2, eru2, E2* and *BnaC.FAE1.a*) in various studies (Howell, Lydiate and Marshall, 1996; Barret *et al.*, 1998; Fourmann *et al.*, 1998; Smooker *et al.*, 2011). These two copies show high sequence similarity to each other. Various mutations were discovered to differentiate high erucic acid rapeseed from low erucic acid rapeseed. The mutations and sequential differences in the copies of *FAE1* were also identified (Barret *et al.*, 1998; Fourmann *et al.*, 1998; Wu *et al.*, 2007, 2015; Rahman *et al.*, 2008; Wang *et al.*, 2008, 2010; Smooker *et al.*, 2011). In HEAR cultivars, both of these copies are known to be functional.

In *A. thaliana, FAD2 (FATTY ACID DESATURASES 2)* is responsible for the conversion of oleic acid to linoleic acid (Miquel and Browse, 1992; Okuley *et al.*, 1994). It is a delta-12 desaturase present in the endoplasmic reticulum and is also an intronless gene. In *B. napus*, four orthologues of *FAD2* are present – two each in A and C genomes (Scheffler *et al.*, 1997). These were mapped on chromosomes A1, A5, C1 and C5 (linkage groups - N1, N5, N11 and N15, respectively) by various studies. Major QTL was identified on the A5 chromosome and minor QTL was identified on the A1 chromosome (Scheffler *et al.*, 1997; Schierholt, Becker and Ecke, 2000; Hu *et al.*, 2006; Smooker *et al.*, 2011; Yang *et al.*, 2012; Wells *et al.*, 2014). According to the nomenclature suggested by Ostergaard and King, 2008, these were named as – *BnaA.FAD2.b, BnaA.FAD2.a, BnaC.FAD2.b* and *BnaC.FAD2.a* and are present on the chromosomes A1, A5, C1 and C5, respectively (Yang *et al.*, 2012). *BnaA.FAD2.b* is unlikely to be functional in *B. napus* due to various mutations (Yang *et al.*, 2012; Wells *et al.*, 2014). *BnaA.FAD2.a* was identified as a major QTL for controlling the linoleic acid content in *B. napus* and the mutations in this copy can make it non-functional (Schierholt, Becker and Ecke, 2000; Hu *et al.*, 2006; Yang *et al.*, 2012; Wells *et al.*, 2014). *BnaC.FAD2.b* was mapped on C1 chromosomes but the information about the mutations in this copy is not available (Smooker *et al.*, 2011; Yang *et al.*, 2012). *BnaC.FAD2.a* was targeted for mutations by Wells *et al.*, 2014 and generated a population with an allelic series of this copy showing very low levels of polyunsaturates and high levels of oleic acid.

1.3.8 HELP Development Using Transgenic Methods and the Challenges

High Erucic acid rapeseed in Low PUFA (HELP) is a unique specification of the rapeseed that could be of high value for the oleochemical industry. HELP lines were produced in a study by cross-pollinating high erucic acid rapeseed with high oleic acid rapeseed (low PUFAs) and the progeny was expected to have high erucic and low PUFA content. No major increase in the erucic acid content was found but low levels of PUFAs were found in the progeny (Sasongko and Möllers, 2005). *KCS* activity was thought to be a limiting factor in these lines. So, one HELP line (from Sasongko and Möllers, 2005) was cross-pollinated to a transgenic HEAR variety overexpressing the rapeseed *FAE1* and

expressing the *Ld-LPAAT* gene. The resulting progeny had large variation (45 to 72%) in the erucic acid content (Nath *et al.*, 2009).

Genetic modification (GM) technology is a powerful tool for studying various aspects of a gene regulation in an organism but it brings many governmental regulations and public investigations for growing the GM products. Many risk assessment measures such as genetic contamination, competition with pre-existing species, horizontal transfer and many more have to be considered for the release of genetically modified crops into the environment (Prakash *et al.*, 2011). There is a big gap between the amount of transgenic material developed and the GM material available in the marketplace (Rommens, 2010). Especially in the EU, there are tight and complicated regulations for field testing of the GM varieties. There is a safety assessment followed by approval from the European Food Safety Authority (EFSA) and the member states. Even if the GM product is approved by the European Commission, then the individual countries have the right to ban any transgenic crop (Meldolesi, 2009). So, from the commercialization point of view, it is always desirable to use non-GM technologies for the crop improvement. Non-transgenic approaches, such as mutation breeding, are more promising and less stringent ways for the development and release of new varieties (Konzak, Nilan and Kleinhofs, 1977). Antisense suppression of *FAD2* gene has been used in *B. carinata* to increase erucic acid levels (Jadhav *et al.*, 2005) but using these approaches in a polyploid like *B. napus* is very challenging (Wang *et al.*, 2008). To date, no non-transgenic methods have been reported to increase the erucic acid levels in *B. napus*.

1.3.9 Mutation Breeding and Marker-Assisted Selection

Selection of the spontaneous mutations for identifying suitable genotypes was used as one of the earliest methods in the plant breeding. In the early 1930s, it was discovered that mutations can be induced by radiations and chemicals in the crops and it was one of the major breakthroughs in the history of genetics. Molecular mutation breeding is an important plant breeding method to develop new varieties by generating variability through physical and chemical mutagenesis, followed by selections by genotyping of various traits (Shu, Forster and Nakagawa, 2011). TILLING (Targeting Induced Local Lesions IN Genomes) is a robust reverse genetic method for

the selection of existing and induced mutations by mutagenesis (McCallum *et al.*, 2000). One of the examples of the TILLING approach is the EMS mutagenesis followed by amplifying the exon from pooled DNA. PCR amplicon is melted, re-annealed and digested by a *Cel1 exonuclease* at heteroduplex loci. The mutations are then confirmed by PCR of specific amplicon followed by sequencing from the individual samples. EMS mutagenesis induces many point mutations in the genome of the plant and has an additional advantage of being a non-transgenic approach.

Marker-assisted selection (MAS) is the use of DNA markers for identifying and selecting genotypes for crop improvement in molecular plant breeding (Collard and Mackill, 2008). MAS based on single nucleotide polymorphism (SNP) serve as a powerful tool in the plant breeding experiments. These markers are tightly linked to the gene of interest and provide a rapid way for the transfer and selection of the traits in the desirable genotype. MAS has been found to serve as a quick and robust method in the breeding program (Mammadov *et al.*, 2012).

1.3.10 Genetic Association Studies

Genome-wide association studies (GWAS) is a powerful tool, utilizing the natural variation present in a diversity panel for its associations with a trait of interest. It associates the phenotypic variation with the sequence based variation in the diversity panel and provides markers linked to the trait for use in marker-assisted selections. Usually, single nucleotide polymorphism alleles are detected in the association studies. There can be a direct association where the SNP is directly related to the trait or it can be an indirect association where the SNP is in linkage disequilibrium (LD) with the trait (Lewis and Knight, 2012). LD is the non-random association of alleles at two or more loci in a population and the loci are said to be in linkage disequilibrium when a recombination is observed rarely between them. Genome-wide association studies often exploit historical recombination between loci of genetically diverse lines (diversity panel). It has been successfully used in many crops such as rice, sorghum, maize, barley and *A. thaliana* (Huang and Han, 2014). But polyploidy in many crops such as *B. napus*, makes it difficult for linking the markers to the traits due to the presence of more than one genome. Assembling the genome sequences of a polyploid crop poses a major difficulty. So, Harper *et al.*, 2012 developed associative

transcriptomics (AT) for the complex genomes that use mRNA (transcriptome) sequencing instead of whole-genome sequencing for the association studies in *B. napus*. Variation of both gene sequences (SNPs) and gene expression (gene expression markers, GEMs) can be detected and associated with the trait by using associative transcriptomics. Using AT, markers for erucic acid alleles (*Bna.FAE1.A8* and *Bna.FAE1.C3*) were mapped to the chromosomes A8 and C3 (Harper *et al.*, 2012; Havlickova *et al.*, 2018); the loci controlling the aliphatic glucosinolates biosynthesis (*HAG1*) were mapped on the chromosomes A9, C2 and C9 (Harper *et al.*, 2012) and; the loci involved in the tocopherols synthesis (*VTE4*) were mapped on the C2 chromosome (Havlickova *et al.*, 2018). Various other studies have shown the potential of associative transcriptomics to identify genes controlling the complex traits (Koprivova *et al.*, 2014; Alcock *et al.*, 2017; Miller *et al.*, 2018).

1.3.11 Glucosinolates

Glucosinolates are sulphur containing compounds naturally found in the *Brassicaceae* family and have a role in the secondary defence related metabolites (Booth and Gunstone, 2004; Alexander *et al.*, 2008; Halkier, 2016). These are biosynthesized from amino acids and have three moieties in their structure – β-thioglucose moiety, a sulfonated oxime moiety and a side chain derived from an amino acid (Ishida *et al.*, 2014). These can be of three types according to the structure of amino acid precursors – aliphatic, aromatic and indole glucosinolates. Glucosinolates from each group are synthesized by an independent metabolic pathway sharing a set of enzymes. Major glucosinolates present in the *B. napus* seeds are the aliphatic glucosinolates – progoitrin, gluconapin and glucobrassicanapin (Booth and Gunstone, 2004; Alexander *et al.*, 2008; Redovnikovic *et al.*, 2008; Ishida *et al.*, 2014; Halkier, 2016). After the extraction of the oil, leftover rapeseed meal is used as a feed for the livestock. So, it is important to measure the glucosinolates content in the rapeseed. In the USA and Canada, maximum glucosinolates level of 30 $\mu molg^{-1}$ are permitted and in the EU, levels of 25 $\mu molg^{-1}$ are allowed in the rapeseed meal (Alexander *et al.*, 2008). But recommended levels of glucosinolates levels by breeders are even less than 18 µmol per gram of the seeds (AHDB, 2018). So, low levels of glucosinolates are preferred in the seeds of *B. napus.*

2. General Materials and Methods

2.1 Plant Material

High erucic acid rapeseed (HEAR) varieties, Maplus (German winter-type oilseed rape, oil profile - ~12% C18:1, ~21% PUFA, ~50% C22:1, and glucosinolates = 24 µmol/g) and Ningyou 7 (Chinese semi-winter oilseed rape, oil profile – ~12% C18:1, ~21% PUFA, ~50% C22:1, and glucosinolates = 85 µmol/g) and; low erucic acid rapeseed (LEAR) variety, Cabriolet (winter-type oilseed rape; low glucosinolates; oil profile: ~76% C18:1, ~19% PUFAs, 0% C22:1, and glucosinolates = 20 µmol/g) were used. Winter-type oilseed rape (WOSR) has a requirement of vernalisation (cold period) in order to initiate the flowering, unlike the spring types while semi-winters OSR has a minimal requirement for vernalisation. In the fields, winter varieties are sown in the late summer to early autumn and have a long growth period. Thus, they have higher yields than the spring varieties. In the United Kingdom, spring oilseed is sown in March/April while winter oilseed is sown in August/September (Armitage, 2007). In John Innes Centre (Norwich, UK), Bancroft group, mutagenized around 33,000 seeds of *B. napus* var. Cabriolet with ethyl methane sulphonate (EMS) and developed a population, JBnaCAB_E with various mutations in the copy *Bna.fad2.C5* of the *Bna.FAD2* family (Wells *et al.*, 2014). An allelic series of mutants were developed with variations in the oleic acid and PUFA contents. For the present study, four mutants – K0047, K0472, M0830, and, M2444 with varying PUFA contents (4 to 7%) were used from JBnaCAB_E population.

2.2 Plant Growth Conditions and Cross-Pollination

Seeds were sown in the medium-grade compost (Scotts Levington F2+S) and kept in the glasshouse under long day conditions of 16-hour photoperiod and temperatures of 20°C/14°C for day/night. At four-leaf stage (after ~3 weeks of sowing), the plants were vernalised for 6 weeks with 8 hours photoperiod at 4°C.

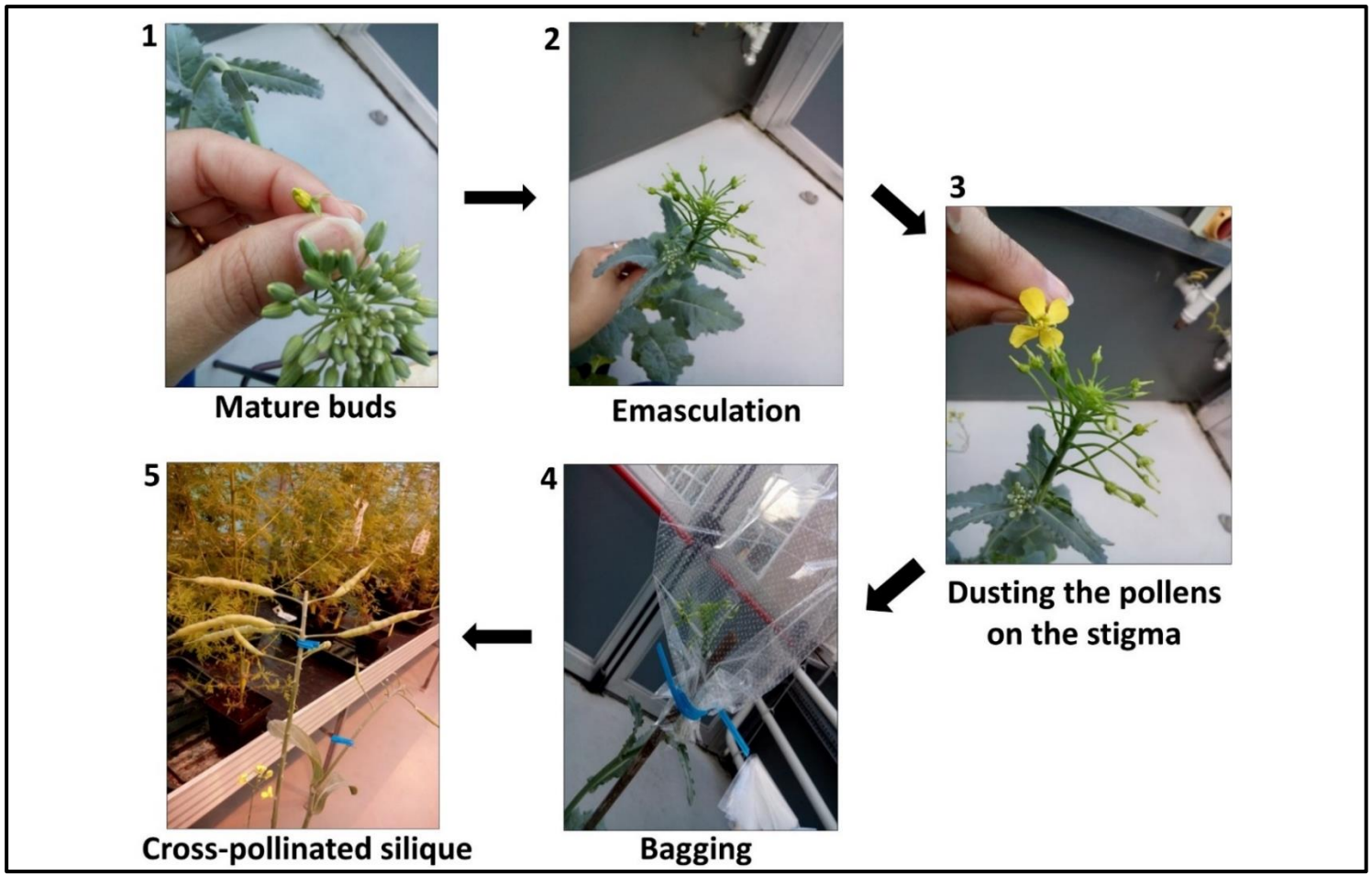

Figure 2.1 A method of the cross-pollination process in *B. napus*

(1) Selecting mature buds, (2) Emasculation (removing the anthers) and removing the immature buds, (3) Dusting the pollens to the stigma, (4) Bagging the cross-pollinated pistil to avoid any cross-contamination and, (5) Cross-pollinated silique

At the initiation of flowering, the plants were bagged for self-pollination and seeds were collected from the individual plants at the maturity. Seeds were stored at -20°C.

Cross-pollination was carried out between various cultivars and mutants. A general method used for the cross-pollination in *B. napus* is depicted in Figure 2.1. Briefly, mature buds were emasculated, pollens were dusted on the stigma and pistil was bagged. The bag was removed after the silique development and the seeds were collected at the maturity from the individual pods in separate bags. The seeds were stored at -20°C.

2.3 DNA Extraction

One young leaf per plant was sampled at the three-leaf stage and stored in an Eppendorf tube or collection micro-tube at -80°C until further use. Genomic DNA was extracted using the CTAB (cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980) with some modifications. Plant material was ground using the liquid nitrogen in a TissueLyser and 500 µl of 2x CTAB buffer (heated at 65°C) was added to the samples. Samples were incubated at 65°C for an hour, cooled down to the room temperature and 300 µl of chloroform and isoamyl alcohol solution in ratio 24:1 was added. Tubes were vortexed and then centrifuged at 14,000 rcf for 5 minutes. Approx. 500 µl of the supernatant was transferred to a new tube, followed by the addition of 1000 µl of ethanol and sodium acetate solution (ratio 24:1). The tubes were gently inverted to mix and the samples were incubated at the room temperature for 30 minutes for DNA precipitation. This was followed by the centrifugation at 14,000 rcf for 10 minutes and the removal of the supernatant into a new tube. DNA pellets were washed by 500 µl of 70% ethanol and centrifuged for 5 minutes at 14,000 rcf. The supernatant was discarded and the DNA pellet was re-suspended in 100 to 200 µl of water (distilled and autoclaved).

'DNeasy Plant 96 Qiagen Kit for 96 samples' was used for the automated isolation of the DNA. This method involves the use of MagAttract magnetic-particle technology and silica-based DNA purification for the extraction of the genomic DNA. Ninety-six samples were extracted at once using this the S-block provided in the kit according to

the manufacturer's instructions by using the BioSprint 96 workstation (https://www.qiagen.com/gb/).

2.4 *Bna.FAD2* and *Bna.FAE1* Primer Pairs

2.4.1 New Nomenclature

In the present study, we have used a different nomenclature than the previous studies for *Bna.FAD2 and Bna.FAE1* copies. The name of a copy is written as – 'species abbreviation, gene and chromosome number' separated by a dot (.). Functional copies are written in capitals and the non-functional copies are represented in the lower case. For the present study, the respective genotypes having the functional or non-functional copies are written as superscripts at the end of the name, in order to differentiate the respective loci present in various genotypes. Table 2.1 summarises the representation of the *FAD2* and *FAE1* in *B. napus.*

Table 2.1 *Bna.FAD2* and *Bna.FAE1* copies in *B. napus*

Gene	Copy	Functional Copy	Non-functional Copy
Bna.FAD2	*BnaC.FAD2.b*	*Bna.FAD2.C1*$^{NY7/Map}$	*Bna.fad2.C1*^{Cab}
	BnaA.FAD2.b	-	*Bna.fad2.A1*$^{NY7/Map/Cab}$
	BnaC.FAD2.a	*Bna.FAD2.C5*$^{NY7/Map/Cab}$	*Bna.fad2.C5*$^{M0830/K0472/K0047/M2444}$
	BnaA.FAD2.a	*Bna.FAD2.A5*$^{NY7/Map}$	*Bna.fad2.A5*^{Cab}
Bna.FAE1	*Bna.FAE1.A8*	*Bna.FAE1.A8*$^{NY7/Map}$	*Bna.fae1.A8*^{Cab}
	Bna.FAE1.C3	*Bna.FAE1.C3*$^{NY7/Map}$	*Bna.fae1.C3*^{Cab}

NY7 is Ningyou 7, Map is Maplus and Cab is Cabriolet;

Bna.fad2.C5 copy has mutations and is partially functional in mutants – M0830, K0472, K0047 and M2444.

2.4.2 *Bna.FAD2* Mutations

Four copies of *FAD2 – Bna.FAD2.A1, Bna.FAD2.A5, Bna.FAD2.C1* and *Bna.FAD2.C5,* are present in the *B. napus* genome (Scheffler *et al.*, 1997) and are present on the chromosomes A1, A5, C1 and C5, respectively.

Bna.FAD2.A1 (*BnaA.FAD2.b*) has a mutated open reading frame (ORF) with five insertions and deletions in the coding region (starting at 164th base pairs). It leads to the frameshift mutations and thus, an earlier truncation that leads to a non-functional gene or protein for this copy. So, it is unlikely to be functional in *B. napus* (Yang *et al.*, 2012; Wells *et al.*, 2014).

Bna.FAD2.A5 (*BnaA.FAD2.a*) is a functional copy and was identified as a major QTL for control of the linoleic acid content in *B. napus* (Schierholt, Becker and Ecke, 2000; Hu *et al.*, 2006; Yang *et al.*, 2012). Mutations in this copy led to a non-functional copy in various studies. In one study, one base pair mutation (C to T) led to a stop codon and thus, resulted in premature termination of the peptide, making this copy non-functional (Hu *et al.*, 2006). Another study found a four base pairs insertion leading to a frameshift mutation and thus, making it non-functional (Yang *et al.*, 2012). Cultivar Cabriolet has one base pair deletion in this copy, resulting in a frameshift mutation and thus, loss of function.

Bna.FAD2.C1 (*BnaC.FAD2.b*) was mapped on the C1 chromosome by various studies (Smooker *et al.*, 2011; Yang *et al.*, 2012) but the mutations in this copy are not studied in detail. It was not possible to amplify this copy in cultivar, Cabriolet with no expression in mRNAseq analysis and thus, this copy is not present (deletion) in this variety (Wells *et al.*, 2014).

Bna.FAD2.C5 (*BnaC.FAD2.a*) is a functional copy and was targeted for mutations by Wells *et al.*, 2014 and generated a Cabriolet population with an allelic series of this copy. Various mutations in this copy led to an increase in the oleic acid content and decreased the polyunsaturates in the mutants (Wells *et al.*, 2014). The mutants with an extreme effect of the mutation(s) or with a stop codon in this locus did not grow (*personal communications* with Ian Bancroft). The mutants used in the present study synthesise low levels of linoleic acid and this copy is inferred to be partially functional or hypomorphic.

2.4.3 *Bna.FAE1* Mutations

FAE1 is present in two copies in *B. napus*, one in each genome – *Bna.FAE1.A8* (also known as *BN-FAE1.1, e1, eru1* and *BnaA.FAE1.a*) is located on the A8 chromosome

and *Bna.FAE1.C3* (also known as *BN-FAE1.2, e2, eru2* and *BnaC.FAE1.a*) is located on the C3 chromosome (Harvey and Downey, 1964; Ecke, Uzunova and Weißleder, 1995; Howell, Lydiate and Marshall, 1996; Jourdren *et al.*, 1996; Thormann *et al.*, 1996; Barret *et al.*, 1998; Fourmann *et al.*, 1998; Qiu *et al.*, 2006; Wu *et al.*, 2007; Wang *et al.*, 2010, 2008; Smooker *et al.*, 2011).

For the present study, a point mutation of Cytosine (C) to Thymine (T) at 282nd amino acid (from the initiation codon) was found between the Ningyou 7 (high erucic variety) and Cabriolet (low erucic variety) for *Bna.FAE1.A8* sequences. It changed the amino acid serine (TCC) in Ningyou 7 to phenylalanine (TTC) in Cabriolet and thus, resulted in the loss of function, found in the previous studies as well (Roscoe *et al.*, 2001; Katavic *et al.*, 2002).

Two base pairs deletion at 1422nd and 1433rd position (from the start codon) in *Bna.FAE1.C3* copy results in the frame-shift mutations and thus, loss of function (Wang *et al.*, 2010) in Cabriolet in the present study as compared to the functional copy present in Ningyou 7. The deletions at other locations are also found in the *Bna.FAE1.C3* copy in other studies (Fourmann *et al.*, 1998; Rahman *et al.*, 2008; Wang *et al.*, 2010). Some of the *FAE1* primers (S-FAE1-A8_f S and FAE1-C3_r) used for the present study were sourced from Wang *et al.*, 2008. Maplus and Ningyou 7, had functional alleles for both copies of *FAE1*.

2.4.4 Primer Pairs for HELP Lines Selection

The sequences of the primer pairs and amplicons lengths used for amplifying *Bna.FAD2* and *Bna.FAE1* copies are given in Table 2.2. In the present study, markers for two copies of *FAD2* – *Bna.FAD2.C5* and *Bna.FAD2.A5* (*Bna.FAD2.C1* and *Bna.FAD2.A1* are non-functional or deleted from Cabriolet; details given in Section 2.4.2); and two *FAE1* copies – *Bna.FAE1.A8* and *Bna.FAE1.C3* were used for the development of HELP (high erucic and low PUFA) lines.

HELP lines construct had partially functional *Bna.FAD2s* (non-functional *Bna.fad2.A5* and mutated *Bna.fad2.C5*) and functional *Bna.FAE1s*. The polymorphism in the alleles of the *Bna.FAD2* and *Bna.FAE1* copies are depicted in Table 2.3 and Table 2.4.

Table 2.2 Primer pairs used for selecting HELP lines in the present study

Primers	Names	Oligo sequence (5' to 3')	Amplicons length, length (bp)
FAD2.Ca.1F	Sel 1+2 f	GTCTCCTCCCTCCAAAAAGT	*Bna.FAD2.C5,* 1212
FAD2.Ca.8R	FAD2grp1 3'UTR-r2	CAAGACGACCAGAGACAGC	
FAD2.Aa.2F	Sel 1+2 new	GTGTCTCCTCCCTCCAAA	*Bna.FAD2.A5*, 1133
FAD2.Aa.7R	FAD2 stop	CCTCATAACTTATTGTTGTACCAG	
FAE1.A8.F	S-FAE1-A8_f	TACTCATGCTACCTTCCAC	*Bna.FAE1.A8*, 1407
FAE1.A8.R	FAE-A8-990_R	CCTCTACATCGATCGGTGCT	
FAE1.C3.F	FAE-A8-869_F	GCCGCTATTTTGCTCTCCAA	*Bna.FAE1.C3*, 922
FAE1.C3.R	S-FAE1-C3_r	CCAATCAATTCGGGAGCCAC*	

*Designed from the region flanking *FAE1* (Wang *et al.*, 2008)
bp is base pairs.

Table 2.3 Polymorphism of *Bna.FAD2* and *Bna.FAE1* alleles

Mutation	*Bna.FAD2.A5*	*Bna.FAD2.C5*	*Bna.FAE1*.A8	*Bna.FAE1*.C3
Position*	158	231^	821	300
Functional allele	C	G	G	AA
Non-functional allele	Del C	A	A	Del AA

*Position provided is according to the reference sequence used and not the actual genomic position; ^Position of *Bna.FAD2.C5* mutation is true for K0472 only and is a partially functional allele (not non-functional) for this copy.

Table 2.4 Polymorphism of *Bna.FAD2.C5* copy in different mutants

Mutant	Position*	Functional allele	Partially functional allele
M0830	337	C	T
K0472	231	G	A
M2444	584	C	T
K0047	663	G	A

*Position is according to the reference sequence used and not the actual genomic position

2.5 PCR Amplification and Gel Electrophoresis

DNA amplification was carried out in the volumes of 25 µl with 50 ng of gDNA, 0.4 µM each of forward and reverse primer and 1x master mix (Thermo Scientific). PCR profiles varied according to the primer pairs used. For amplification of *Bna.FAD2* copies, the PCR profile used was – initial denaturation at 94°C for 5 minutes; 35 cycles each with 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 1 minute; final extension of 72°C for 10 minutes. For *Bna.FAE1* copies, the touchdown PCR profile

was used – initial denaturation at 94°C for 5 minutes; 15 cycles each with 94°C for 30 seconds, 63°C for 30 seconds (decrease by 1°C every cycle) and 72°C for 1 minute; and 30 cycles each with 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute; and final extension of 72°C for 15 minutes.

An agarose gel of 1.5% containing 3 µl ethidium bromide (per 100 ml of the gel) was used at 100 volts for 30 to 40 minutes for the separation of the PCR products and these were observed under UV gel viewer.

2.6 PCR Product Purification and Sequencing

PCR products were purified using the 'Mag-Bind® RXNPure Plus' according to the manufacturer's instructions. The purified products were sequenced using the Sanger sequencing from Beckman Coulter Genomics (http://www.beckmangenomics.com/) and Eurofins (https://www.eurofins.co.uk/). One of either forward or reverse primers was used for the sequencing reaction and the details for each copy is given in Table 2.5. The sequencing results were analysed with Mutation Surveyor® v5.0 software (https://softgenetics.com/mutationSurveyor.php) and the mutations were detected by comparing to the reference sequences as given in Table 2.5. The reference sequences are provided in the Supplementary file.

Table 2.5 Primers used for the sequencing reaction and the reference files

S. No.	Copy	Primer used for the sequencing reaction	Reference file used for the comparisons*
1	*Bna.FAD2.C5*	Forward	CAB BnaC FAD2b_f.ab1
2	*Bna.FAD2.A5*	Forward	CAB BnaA FAD2b_f.ab1
3	*Bna.FAE1.A8*	Reverse	75HE92_A01_A8R_Cab.ab1
4	*Bna.FAE1.C3*	Reverse	FAE1_C3_96_C3R_3_reference

*Reference files are provided in the Supplementary file

2.7 Fatty Acid Measurements

Gas chromatography (GC) was used for the analysis of the fatty acids and it involves derivatization of the fatty acids (have high boiling points and are difficult to evaporate)

to the fatty acid methyl esters to increase the flame ionization detector (FID) response (Zhang, Wang and Liu, 2015). The transesterification of the lipid extracts was carried out by using the acid method and the fatty acid methyl esters (FAMEs) were determined by the GC analysis. The fatty acids compositions could be analysed by the single seed method or bulk seeds method depending on the number of seeds used. Single seed method is very useful for observing the segregation (if any) of the fatty acids within the same seed batch but the bulk seeds method is more ideal for measuring the fatty acid profile of a particular line.

2.7.1 Single Seed Method

In the single seed method, the weight of one *Brassica* seed was recorded and it was transferred to a 1.8 ml screw-cap vial. Ten µl of 25 mg/ml of tripentadecanoin (C15:0, internal standard, ISTD) was added to each sample, followed by the addition of 500 µl of 1N HCl/MeOH (methanolic hydrochloric acid). The samples were sealed with Teflon-lined screw caps and vortexed briefly. These were incubated for 24 hours at 85°C to ensure complete derivatization. The samples were cooled down to the room temperature and 250 µl of 0.9% potassium chloride (w/v) was added to each sample. It was followed by the addition of 800 µl of hexane. Vials were vortexed briefly and the layers were allowed to separate for 10 minutes. Approximately 200 µl of the upper hexane layer was transferred to the blue-lined crimp cap vials and were stored at 4 to 10°C before the GC run.

2.7.2 Bulk Seeds Method

In the bulk seeds method, seeds were weighed (~30 mg), counted (~10) and transferred to a 2 ml snap-cap Eppendorf tube. Forty µl of 100 mg/ml tripentadecanoin (C15:0, ISTD) was added to each sample, followed by the addition of 400 µl of cold hexane: isopropyl alcohol (IPA) solution (ratio 3:2). Seeds were ground using the TissueLyser at 25 Hz for 1 minute at each side and incubated for 1 hour on the ice. These were centrifuged at 10,000 rpm for 5 minutes and the supernatant was transferred into a new tube. The pellet was dissolved another two times with 400 µl of cold hexane: IPA solution (ratio 3:2), vortexed, centrifuged and

transferred to the same tube (pooled supernatants). It was followed by the addition of 600 µl of 6.7% sodium sulphate (Na_2SO_4) to the pooled supernatants, vortexing and clarifying in a bench centrifuge for 30 seconds. The upper layer was removed into a new tube and dried in the Genevac evaporator using low BP settings for 30 to 60 minutes (lamp off). Dried samples were stored at 4°C overnight. These were reconstituted in 750 µl chloroform and $1/15^{th}$ part (50 µl) of the sample was transferred into 2 mL screw cap Supelco® glass vial. For each sample, 2 to 3 technical replicates were used. It was followed by the addition of 750 µl hexane and 500 µl 1N methanolic HCl to the samples, vortexing and capping with PTFE (polytetrafluoroethylene) silicon-lined screw caps. The samples were incubated at 85°C for 3 hours and then cooled down to the room temperature. It was followed by the addition of 250 µl of 0.9% potassium chloride. The tubes were vortexed and the two layers (polar and non-polar) were separated. Approx. 200 µl of the upper non-polar hexane layer was added to the tapered vial, capped with blue-lined crimp caps and stored at 4 to 10°C before the GC run.

2.7.3 Analysis and Calculations

The FAMEs were analysed on Thermo Scientific's Trace GC Ultra-FID. The GC column had the specifications: BPX forte 10 m x 0.1mm ID x 0.2 m film thicknesses; with a run time of ~5 minutes per sample.

Supelco® 37 component FAME mix was used as an external standard (Figure 2.2) and hexane was used as a blank for the GC run. External standard was used with each run. GC data was analysed with the Thermo Scientific's ChromQuest™ software (version 4.2.34) platform.

The fatty acids in each sample were identified by the comparison of the retention time with the external standard. Internal standard, tripentadecanoin (C15:0, it is not normally present in the lipid extracts) was added to each sample and the quantity of each fatty acid in the sample was determined using its quantity. The values used for the data analysis are depicted in Table 2.6. The fatty acids values are expressed as percentage by weight.

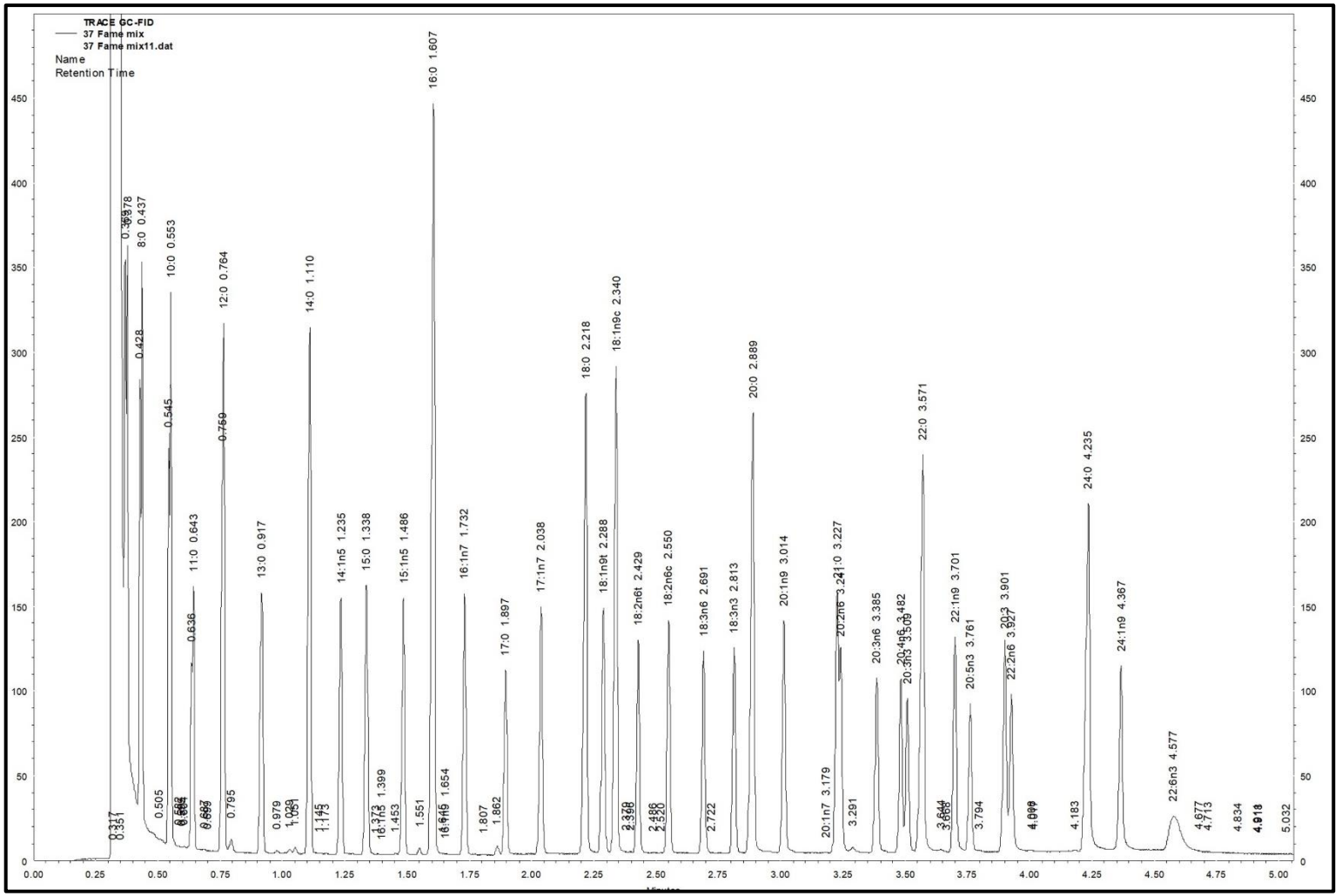

Figure 2.2 Gas chromatogram of the Supelco® 37 component FAME mix

Supelco 37® component mix – used as an analytical standard and the chromatogram shows the 37 peaks of various fatty acids

For the single seed method, ISTD amount is calculated as – 250 µg (10 µl of 25 mg/ml) of ISTD was added to the sample. It was diluted in 800 µl hexane, so 312.5 ng ((250/800) x 1000) was present in the injection. It was corrected with the conversion factor which is calculated using the following formula,

"[3 x M_r (C15:0 Free fatty acid)] / [M_r (C15:0 triacylglycerol)]"

Where Mr is the molecular weight. M_r (C15:0 Free fatty acid) is 242.4 g and M_r (C15:0 triacylglycerol) is 765.2 grams. So, the conversion factor (0.9503) is multiplied by the sample amount (312.5 ng) to get the ISTD amount per injection (296.9 ng).

Table 2.6 Values used for the fatty acid data analysis in ChromQuest™ software

S. No	Sample type	Sample amount per injection (µl)	ISTD amount (ng per injection)	Multiplier
1	Hexane	1	1	1
2	ISTD	1	200	1
3	Sample	1	296.9^{s}, 316.8^{b}	800^{d}

'^{s}' is used for single seed method; '^{b}' is used for bulk seeds method and; '^{d}' is the amount of solvent used to dissolve FAMEs

For bulk seeds method, ISTD amount is calculated as – 4000 µg (40 µl of 100 mg/ml) of ISTD was added to the sample and then 1/15th volume (266.67 µg) was used. It was diluted in 800 µl solvent (non-polar phase having 50 µl chloroform and 750 µl hexane), so 333.33 ng ((266.67/800) x 1000) sample was present in the injection which is corrected by multiplying with the conversion factor (0.9503). Thus, the ISTD amount per injection is 316.8 ng.ISTD amount of 200 ng was used per injection for 37 FAME mix. Sample injection for each sample was 1 µl and the multiplier is the amount of solvent used as depicted in Table 2.6.

2.8 Lipid Extraction for TLC Analysis and FAMEs Analysis

Lipid extraction was carried out using the modified protocol from Bligh and Dyer, 1959 at the Rothamsted Research, Harpenden, UK. One ml of 85°C isopropanol containing butylated hydroxytoluene (BHT) was added to 4-6 seeds of each sample and heated for 15 minutes. Heat quenched samples were homogenized in a homogenizer,

followed by rinsing the homogeniser with 2 ml of chloroform and 3 ml of methanol to recover the seed parts and lipids. This rinse was combined with the sample and then, 1.6 ml of water, 2 ml of chloroform and 2 ml of 0.88% potassium chloride were added for phase separation. These were mixed gently and the tubes were centrifuged for 2 minutes at 2000 rpm to separate the three layers. The bottom layer of the chloroform and lipid mixture was collected carefully in another tube and dried under N_2. The dried mixture was re-suspended in 500 µl of toluene containing 0.005% BHT before thin layer chromatography (TLC) analysis.

FAMEs were derived using this sample using the method described in Erp, Menard and Eastmond, 2014. Briefly, 10 µl of heptadecanoin (C17:0) standard (1 µg/µl, internal standard) was added to the 10 µl of the extracted sample in a glass vial. It was followed by the addition of 1 ml of 1N methanolic HCl and heating for 1.5 hours at 80°C for FAMEs derivatization. Then, 200 µl of hexane and 1.5 ml of 0.88% potassium chloride was added. The tubes were vortexed, followed by centrifugation for 2 minutes at 2000 rpm at the room temperature. Approx. 150 µl of the upper layer was transferred to a GC vial and the samples were analysed on the GC column. The total lipids content was calculated using these results.

2.9 Positional Distribution Analysis

2.9.1 TAG Extraction

This procedure was also performed at the Rothamsted Research, Harpenden, UK. Total lipids (~1500 µg) were loaded on the thin layer chromatography plate (20 x 20 cm, silica) and the mixture was separated using the solvent solution, hexane: diethyl ether: acetic acid (70:30:1, v/v). Triacylglycerol (TAG) was eluted from the TLC silica twice by washing with 5ml of chloroform and methanol solution (4:1, v/v). A phase separation was induced by adding 2 ml of methanol and 4 ml of 0.88% potassium chloride. The chloroform phase was collected and the aqueous phase was back extracted with 5 ml of chloroform. The chloroform phase was dried under nitrogen and re-suspended in 500 µl of toluene containing 0.005% BHT. The TAG was

derivatized to FAMEs by using 10 µl of this sample and analysed in GC (method described in Section 0). Samples were dried down using N_2.

2.9.2 The *sn-2* MAG Analysis

One mg of the TAG was re-suspended in 1 ml of diethyl ether, 0.8 ml of buffer (50 mM NaBr, 5mM $CaCl_2$, pH 7.6), and 200 µl of lipase (*Rhizomucor miehei* lipase; Sigma-Aldrich). It was vortexed for 40 minutes. This lipase cleaved the fatty acids from the positions *sn-1* and *sn-3*; and thus, leaving the fatty acid at the *sn-2* position. The reaction was stopped by the addition of 2 ml of chloroform and methanol solution (1:1, v/v). The chloroform layer was collected, dried down and re-suspended in 200 µl of chloroform. The mixture was separated in TLC in the solvent containing hexane: diethyl ether: acetic acid (35:70:1.5, v/v). MAGs (monoacylglycerols) and TAGs were scrapped from the plate and analysed by GC by the method described in Section 0.

3. Associative Transcriptomics (AT) Analysis of the Erucic Acid Content in *B. napus*

Contributions: Fatty acids from 404 accessions were analysed by Vasilis Gegas at Limagrain UK Ltd. RIPR panel was grown and RNA extraction of the panel was carried out by Sophia Cheng (Bancroft group) at the University of York, UK. The pipeline for the associative transcriptomics was developed by various members of the Bancroft group (University of York, UK) – Ian Bancroft, Andrea Harper, Zhesi He and Lenka Havlickova.

3.1 Hypothesis

There are 'modifier' loci (regulatory elements such as transcription factors, transporters) in the *B. napus* genome that works in addition to the known loci controlling the erucic acid content, *Bna.FAE1.A8* and *Bna.FAE1.C3*, to fine-tune the erucic acid content.

3.2 Test

Undertake associative transcriptomics (AT) studies across the Renewable Industrial Products from Rapeseed (RIPR) diversity panel (RIPR, 2014) to identify such modifier loci, using (i) the complete panel and (ii) the subsets of high and low erucic lines from the panel.

3.3 Materials and Methods

3.3.1 Diversity Panel and Fatty Acid Analysis

The RIPR diversity panel comprising of 383 *B. napus* accessions was used for the present study and the details of the panel are described in Havlickova *et al.*, 2018. Briefly, the panel was divided into 7 different groups – 169 winter oilseed rape, 123

spring oilseed rape, 27 swede, 11 semi-winter oilseed rape, 6 fodder, 3 kale and 44 unassigned crop types. The fatty acid compositions were measured in 404 accessions by the derivatization of the fatty acids to fatty acid methyl esters (FAMEs) by Vasilis Gegas at Limagrain UK Ltd. Briefly, ~30 mg of seeds were homogenized with 5 ml of heptane in a glass vial, followed by the addition of 500 µl of 2 M potassium hydroxide and incubation for 1 hour. It was neutralized with sodium hydrogen sulphate monohydrate and the upper layer was shifted to crimp-cap Chromacol 0.8 ml vials. These were analysed in a DANI Master gas chromatography fitted with an SGE-BPPX70 double column.

3.3.2 RNA Extraction and SNP Identification

Single Nucleotide Polymorphism (SNP) and Gene Expression Markers (GEM) data were used from 383 diversity panel of RIPR consortium (RIPR, 2014). Plants were grown in 4 replicates with the growth conditions described in Section 2.2 by Sophia Cheng, Bancroft group, University of York, UK. Second true leaf was harvested, frozen in liquid nitrogen and stored at -80°C. RNA was extracted by first grinding the leaf tissue in the liquid nitrogen and then using Omega Bio-Tek's E.Z.N.A. Plant RNA kit according to the manufacturer's instructions by Sophia Cheng, Bancroft group, University of York, UK. Associative transcriptomics methodology and pipeline was already developed in the Bancroft group by Ian Bancroft, Andrea Harper, Zhesi He and Lenka Havlickova. Illumina sequencing platform was used for transcriptome sequencing for all of the accessions (Higgins *et al.*, 2012; He *et al.*, 2017). The mRNA-seq data was mapped on the *Brassica* A and C pan-transcriptomes (He *et al.*, 2015) and the meta-analysis of alignments were used for SNP calling by using Maq v0.7.1 (Bancroft *et al.*, 2011). The markers were classified into two types, (i) the markers that can be assigned with confidence to a genomic position because these were mapped on the A or C genome using the Tapidor Ningyou 7 doubled haploid (TNDH) population and; (ii) the markers that cannot be assigned to a particular genomic position because these were not mapped to the TNDH population. Reads per kb per million aligned reads (RPKM) were used for quantifying and normalizing the transcript abundance. 'More than 0.4 RPKM' value was used as the significant expression detection for our analysis (Havlickova *et al.*, 2018).

3.3.3 Associative Transcriptomics Analysis

Methods outlined by Havlickova *et al.*, 2018 were used for the associative transcriptomics analysis using the statistical R package (https://www.r-project.org/). The Q matrix was created using a non-model based approach, population structure inference using kernel-PCA and optimisation (PSIKO) for AT analysis (Popescu *et al.*, 2014). The R package, GAPIT (Genome Association and Prediction Integrated Tool) used compressed mixed linear model (CMLM) for performing the genomic predictions (Lipka *et al.*, 2012) and it was used for AT analysis for the erucic acid data. For GEM associations, R script Regress was used to perform linear regression on RPKM data against the trait data. R script, Grapher was used for plotting SNPs and R script, Regress Plotter was used for plotting GEMs for the association study. In the association plots, all the markers, represented as dots, are assigned to a genomic position of the gene model in which a SNP or GEM is called. For SNP associations, the lighter dots (light red and grey) in the Manhattan plot represented the hemi-SNP markers for which genome of the polymorphism cannot be assigned because the true position can belong to the gene model or its homoeologue. Hemi-SNPs are the SNPs showing allelic polymorphism due to the presence of homoeologous sequences (Trick *et al.*, 2009). The darker dots (red and black) represented the simple SNP markers and hemi-SNP markers assigned with confidence to a genomic position (Havlickova *et al.*, 2018).

3.4 Results

3.4.1 Fatty Acid Analysis

The fatty acid compositions were analysed from the seeds of 404 accessions by Vasilis Gegas at Limagrain UK Ltd and the detailed results are provided in Appendix I. The amount of the erucic acid (C22:1) varied from 0 to 52% in these accessions (Figure 3.1) and shows the range of crop types used in the panel. The other fatty acids – C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:0 were also measured in the analysis.

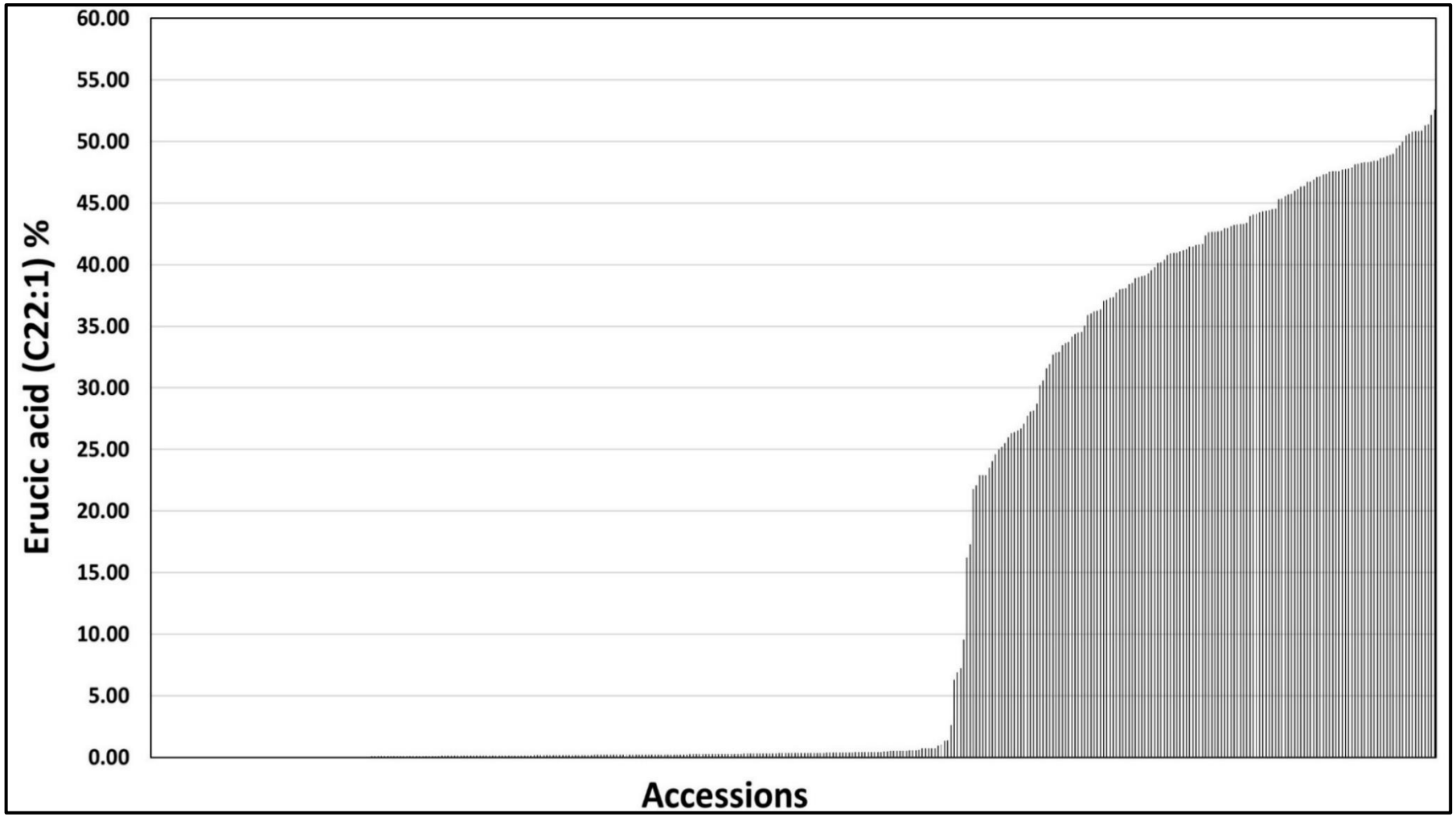

Figure 3.1 Range of erucic acid (C22:1) content in the seed oil across the *B. napus* accessions

The fatty acid compositions were analysed on the 404 B. napus accessions including the RIPR diversity panel and the erucic acid content ranged from 0 to 52%

3.4.2 AT Analysis of the Erucic Acid Content

The two loci, *Bna.FAE1.A8* and *Bna.FAE1.C3*, controlling the erucic acid content in the *B. napus* are already known (Harvey and Downey, 1964). Associative transcriptomics validated the position of these 2 loci (Harper *et al.*, 2012) and then it was re-analysed with a bigger panel through AT (Havlickova *et al.*, 2018). Functional genotypes were produced from the 100-base read length transcriptome data produced by using the Illumina HiSeq 2000 platform. SNPs were identified and the gene expression was quantified for 383 accessions under the RIPR panel. A total of 355,536 SNP markers were scored in the transcriptome data and 87% of these were hemi-SNPs.

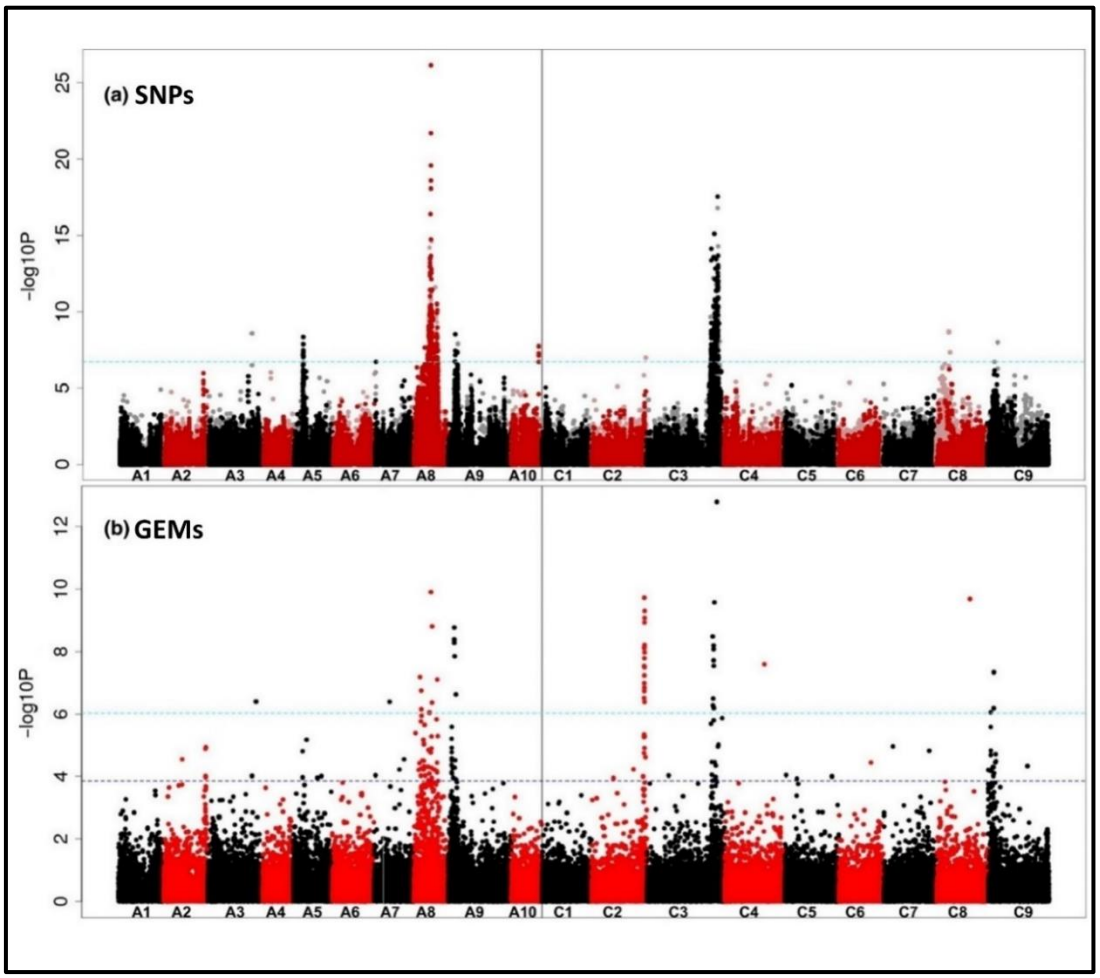

Figure 3.2 Association analysis of the erucic acid content (a) SNPs (b) GEMs
Associative transcriptomic analysis using 383 B. napus diversity panel. The trait significance value, -log10P value is plotted against 19 pseudo chromosomes of B. napus. The broken light blue horizontal line is the Bonferroni correction at 0.05 significance threshold and the broken dark blue horizontal line is 5% false discovery rate (adapted from Havlickova et al. 2018)

The erucic acid values were available for 404 accessions and the functional genotypes were available for 383 accessions, so AT analysis was conducted on 383 accessions as described in Havlickova *et al.*, 2018. Figure 3.2 shows the association signals for the SNPs (Figure 3.2a) and GEMs (Figure 3.2b) for the erucic acid content for the 383 diversity panel. The trait significance value, -log10P (vertical axis) was plotted against the gene order for 19 *B. napus* pseudo chromosomes (horizontal axis). The chromosomes of *B. napus* were marked as 'A1 to A10' and 'C1 to C9' and are shown in the alternate black and red colours in Figure 3.2. Association signals were detected on the chromosomes A5, A8, A9, A10 and C3 with a total of 318 genome-assigned SNP markers above the Bonferroni correction, i.e. -log10P value of 6.7, as depicted in Figure 3.2a. The main loci controlling the erucic acid content are present on the A8 and C3 chromosomes (Qiu *et al.*, 2006) and the association signals with a -log10P significance of more than 16 were observed in these two chromosomes. The genes corresponding to the orthologues of *FAE1* are not expressed in the leaves and thus, no scored markers were produced from them. But the known loci of these genes models, Cab035983.1 and Bo3g168810.1 (orthologues of *FAE1*, AT4G34520) were present near the centres of these association peaks, within ~42 kb (six genes) and ~56 kb (nine genes), respectively from the nearest significantly associated gene (Havlickova *et al.*, 2018).

In addition to these, SNP associations were found for a region of the genome on the A5 chromosome. A potential candidate, Cab033920.1, was pulled out from this peak. It is annotated as the *FATTY ACID HYDROXYLASE 1* (AT2G34770.1) with a role in the very long chain fatty acid (VLCFAs, fatty acids with ≥C20) biosynthesis (Nagano *et al.*, 2012). There was a well-defined peak in the chromosome A9 as well which was interpreted as a locus controlling the seed glucosinolates content (Howell, Sharpe and Lydiate, 2003; Harper *et al.*, 2012). Presence of the glucosinolates peaks in the erucic acid association data represents a co-selection in the modern low erucic acid rapeseed cultivars for producing double zero canola (low erucic-acid and low glucosinolates) quality seeds.

Gene expression markers data for the erucic acid content is depicted in the Figure 3.2b. The main loci controlling the erucic acid content were transcriptionally inactive in the leaves (tissues used for mRNA extraction) but both SNP and GEM association

peaks could be detected through markers in linkage disequilibrium (LD) on the A8 and C3 chromosomes. Low resolution was observed for the A8 signals and it may represent the effect of strong bottleneck breeding selection of low glucosinolates and zero seed erucic acid type rapeseed cultivars (Hasan *et al.*, 2008). Another reason could be the presence of other potential candidates that may contribute to the content of erucic acid in the seeds of *B. napus* as well (Havlickova *et al.*, 2018). There were signals on A2, A9, C2 and C9 chromosomes representing the loci involved in the glucosinolates biosynthesis in the seeds (Howell, Sharpe and Lydiate, 2003; Harper *et al.*, 2012) and these show co-selections in the modern cultivars for double zero rapeseed. Any other SNPs and GEMs (apart from these discussed peaks) detected in the AT were not found to be associated with the fatty acids control.

3.4.3 Assessment of the Candidate Gene

From the association analysis of the erucic acid content, a potential candidate gene-Cab033920.1 (AT2G34770.1), annotated as *FATTY ACID HYDROXYLASE 1 (FAH1)*, was identified on the chromosome A5 with a potential role in VLCFAs synthesis. AtFAH1 is localized in endoplasmic reticulum membrane and it is involved in the synthesis of 2-hydroxylated fatty acids, especially 2-hydroxylated VLCFAs in Arabidopsis. The 2-hydroxy VLCFAs are important for oxidative stress response (Nagano *et al.*, 2012). *A. thaliana* is used a model plant in plant research from many years and one way of validating a potential candidate is by using *Agrobacterium tumefaciens* transfer-DNA (T-DNA) induced insertion mutant collections in *A. thaliana* (O'Malley, Barragan and Ecker, 2015). Arabidopsis T-DNA line, SALK _140660 (NASC code: N640660) with a mutation in this gene was selected (http://signal.salk.edu/) and specific primer pairs (18F and 18R) were designed in the promoter region using the T-DNA primer design tool (http://signal.salk.edu/tdnaprimers.2.html). Forward (LP) and reverse primers (RP) sequences were 'TGTTTGGCAAGATAACCAACC' and 'TGGCAGAAGACCAATAATTCG', respectively. Twenty-three plants were grown, the DNA was extracted using the CTAB method (Section 2.3) and primer pairs were amplified using the methods described in Section 2.5 (same PCR profile as *Bna.FAD2s* was used).

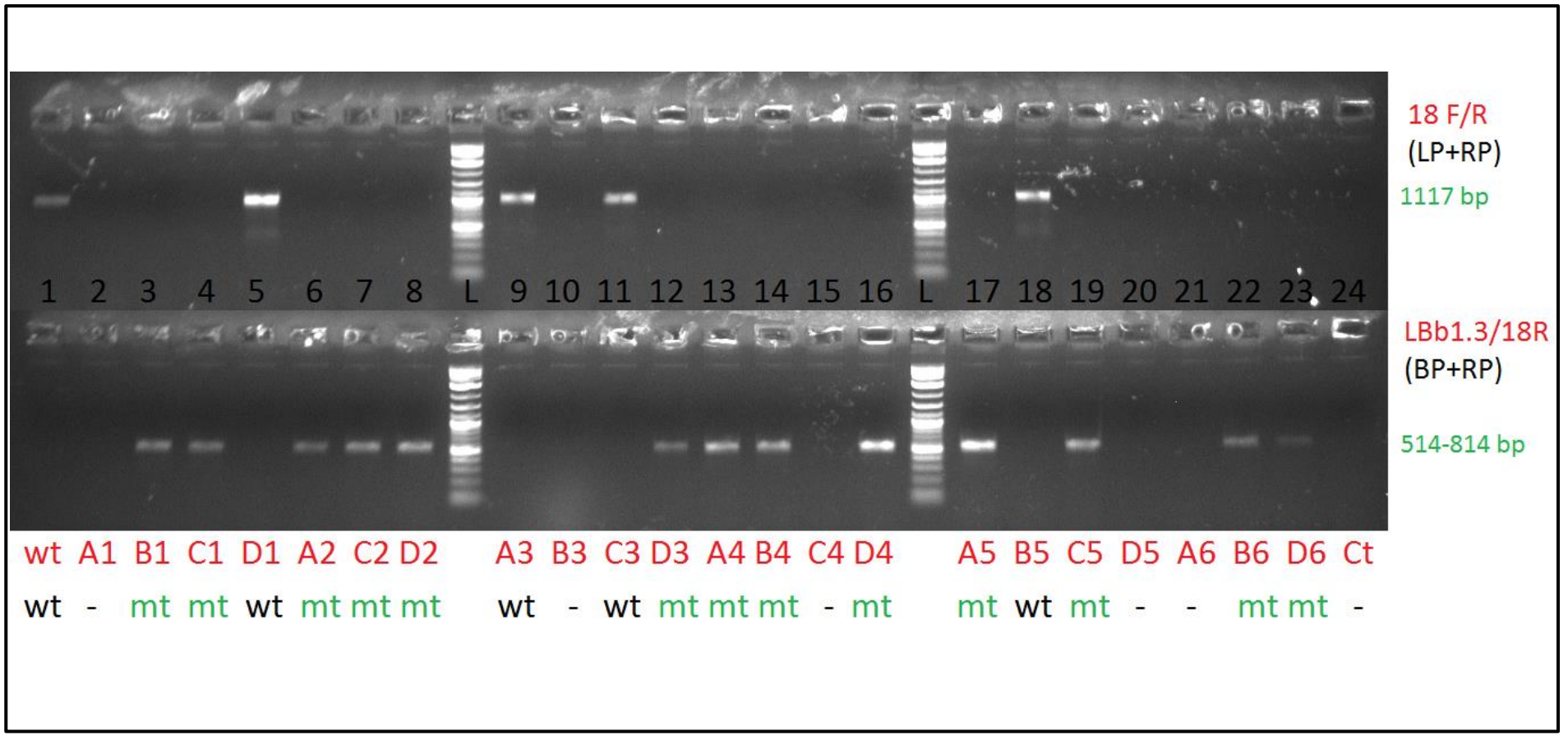

Figure 3.3 Detection of wild-type (*FAH1*) and mutants (*fah1*) in the Arabidopsis T-DNA lines of gene Cab033920.1 (orthologue of AT2G34770.1)

On the top gel, 18F and 18R primers amplified the gene with a band size of 1117 base pairs and show the plants with no insert in them (FAH1, wt). On the bottom gel, LBb1.3 and 18R primers amplified the insert and right end of the gene with a band size of 514 to 814 base pairs and show the mutant lines with an insert in the gene (fah1, mt). Ct is the control (blank). '-' represents the non-amplified bands. LP is the left primer, RP is the right primer and BP is the border primer of the insert.

Table 3.1 The fatty acids composition analysis of the Arabidopsis mutants

S. No.	Line	Type	14:0	16:0	16:1	18:1	18:2
1	B1	*fah1*	0.5	8.1	0.4	21.5	31.1
2	C1	*fah1*	0.3	8.4	0.4	20.9	30.7
3	A2	*fah1*	0.2	7.4	0.5	29.2	28.3
4	C2	*fah1*	0.1	7.7	0.2	20.6	31.3
5	D2	*fah1*	0.1	8.9	0.1	21.2	31.7
6	D3	*fah1*	0.3	7.8	0.3	20.9	31.7
7	A4	*fah1*	0.6	8.1	0.3	20.4	31.5
8	B4	*fah1*	0.3	7.7	0.3	20.1	31.5
9	D4	*fah1*	0.1	7.8	0.2	20.3	31.8
10	A5	*fah1*	0.8	7.8	0.3	19.8	31.6
11	C5	*fah1*	1.0	7.9	0.3	20.6	31.2
12	B6	*fah1*	1.6	9.3	0.3	20.4	30.7
13	D6	*fah1*	0.1	7.8	0.3	21.6	32.0
14	B5	*FAH1*	0.1	8.0	0.3	19.9	31.9
15	D1	*FAH1*	0.2	8.2	0.3	20.9	31.5
16	A3	*FAH1*	0.3	7.9	0.3	19.5	32.4
17	C3	*FAH1*	0.4	7.9	0.3	20.4	31.4
18	Col-0 a	*FAH1*	0.1	7.9	0.3	21.1	31.7
19	Col-0 b	*FAH1*	0.1	7.9	0.3	20.8	31.6
S. No.	**Sample Id**	**Type**	**18:3**	**20:0**	**20:1**	**22:0**	**22:1**
1	B1	*fah1*	16.6	1.8	18.3	0.3	1.3
2	C1	*fah1*	17.8	2.2	17.7	0.3	1.4
3	A2	*fah1*	15.3	2.2	15.5	0.3	1.2
4	C2	*fah1*	16.6	2.6	19.3	0.2	1.4
5	D2	*fah1*	18.0	2.0	16.7	0.1	1.2
6	D3	*fah1*	16.6	2.2	18.4	0.3	1.5
7	A4	*fah1*	17.0	2.2	18.0	0.4	1.4
8	B4	*fah1*	17.2	2.2	18.7	0.4	1.5
9	D4	*fah1*	16.9	2.2	18.8	0.3	1.5
10	A5	*fah1*	17.1	2.1	18.6	0.3	1.6
11	C5	*fah1*	16.7	2.1	18.6	0.3	1.4
12	B6	*fah1*	15.6	2.4	18.2	0.0	1.6
13	D6	*fah1*	16.1	2.1	18.4	0.3	1.4
14	B5	*FAH1*	17.4	2.2	18.3	0.4	1.5
15	D1	*FAH1*	17.6	1.9	17.8	0.3	1.3
16	A3	*FAH1*	17.5	2.4	17.8	0.3	1.6
17	C3	*FAH1*	17.3	2.1	18.4	0.3	1.5
18	Col-0 a	*FAH1*	16.8	2.0	18.4	0.3	1.3
19	Col-0 b	*FAH1*	17.0	2.1	18.5	0.3	1.4

'Col-0' is Columbia 0. 'Col-0 a' and 'Col-0 b' represents two biological replicates used for Col-0

With the forward and reverse primers, the product would be 1117 base pairs if no insertion was present in the gene, i.e., wild-type line. But if there was an insertion, i.e., mutant, then these primers would not amplify this product. So a primer, LBb1.3 (BP, sequence: ATTTTGCCGATTTCGGAAC, the sequence is complementary to the T-DNA insertion sequence) was used with the reverse primer in another PCR reaction and the mutants would amplify a product between 514 to 814 base pairs as shown in Figure 3.3. Thirteen plants were found to be homozygous mutants, 4 plants were homozygous wild-types and rest did not amplify as shown in Figure 3.3. No heterozygous plant was found in these tested plants.

The fatty acids were analysed on these lines along with the control Columbia-0 (Col-0) by using the method described in Section 2.7.2 and the results are shown in Table 3.1. In Arabidopsis, elongation of the fatty acids reaches till the eicosenoic acid (C20:1) and the erucic acid is found in very low amounts in the seeds (Li-Beisson *et al.*, 2013). There were no changes observed in the fatty acid compositions of the mutants as compared to the Columbia-0. As depicted in Table 3.1, no changes in the eicosenoic acid, oleic acid and other fatty acids in the mutants were found in comparison to the Col-0.

Thus, the candidate gene AT2G34770.1 did not affect the fatty acid compositions in the Arabidopsis seeds and thus, this analysis of the Arabidopsis orthologue provide no support for Cab033920.1 being involved in controlling the proportion of the very long chain fatty acids in the rapeseed. Testing more potential candidates may find new loci that may have an effect on the very long chain fatty acids in the rapeseed.

3.4.4 New Markers Development

From the AT analysis, no new loci were found to be involved in the control of the erucic acid synthesis. So, the SNP markers flanking the *FAE1* region were searched in the GWAS data that may act as modifier loci for controlling the erucic acid biosynthesis. The highest -$\log_{10}$P value for the erucic acid on the AT plot was found to be 25 on the A8 chromosome followed by a value of 16 on the C3 chromosome (Figure 3.2). So, the markers near the *FAE1* region on the A8 and C3 chromosomes were searched for the two types of SNPs, (i) one distinguishing Cabriolet (low erucic acid

rapeseed) from Maplus and Ningyou 7 (high erucic acid rapeseed); (ii) other differentiating the high erucic cultivars, Maplus (winter type) and Ningyou 7 (semi-winter type) from each other. Sequences of the respective CDS (cDNA sequence) model was obtained from the online *Brassica* database server and primers were designed with the Primer3 software (http://primer3.ut.ee/). Primers were re-checked with the genomic sequences. CDS models used for the primer designing are represented in Table 3.2 and their respective functions in the Arabidopsis information resource are also provided. In total, 14 primer pairs were designed as shown in Table 3.2. The primer sequence information and the information regarding base pair changes for designing the primers are given in Appendix II and III, respectively.

Table 3.2 Primers designed for the regions flanking *FAE1* region

Primer pair	CDS model	TAIR ID	Brief description of function
Chromosome A8			
EA.A8.1F/R	Cab035976.1	AT4G341640.1	Squalene synthase 1
EA.A8.2F/R	Cab035974.1	AT4G34670.1	Ribosomal protein S3Ae
EA.A8.3F/R	Cab035991.2	AT4G34450.1	Coatomer gamma-2 subunit, putative / gamma-2 coat protein
EA.A8.4F/R	Cab035992.1	AT4G34430.3	DNA-binding family protein
EA.A8.5F/R	Cab036061.1	AT4G33400.1	Vacuolar import/degradation, Vid27-related protein
EA.A8.6F/R	Cab033414.2	AT4G21660.1	Proline-rich spliceosome-associated (PSP) family protein
EA.A8.7F/R	Cab035852.1	AT4G20940.1	Leucine-rich receptor-like protein kinase family protein
EA.A8.8F/R	Cab035874.1	AT4G12590.1	Protein of unknown function DUF106, transmembrane
EA.A8.9F/R	Cab035955.1	AT4G34920.1	PLC-like phosphodiesterases superfamily protein
EA.A8.10F/R	Cab040805.1	AT4G29040.1	Regulatory particle AAA-ATPase 2A
Chromosome C3			
EA.C3.11F/R	Bo3g168710.1	AT4G34640.1	Squalene synthase 1
EA.C3.13F/R	Bo3g162450.1	AT4G21660.1	Proline-rich spliceosome-associated (PSP) family protein
EA.C3.15F/R	Bo3g164280.1	AT4G12590.1	Protein of unknown function DUF106, transmembrane
EA.C3.17F/R	Bo3g168860.1	AT4G34460.4	GTP binding protein beta 1

The details of the primer pairs are given in the Appendix II.

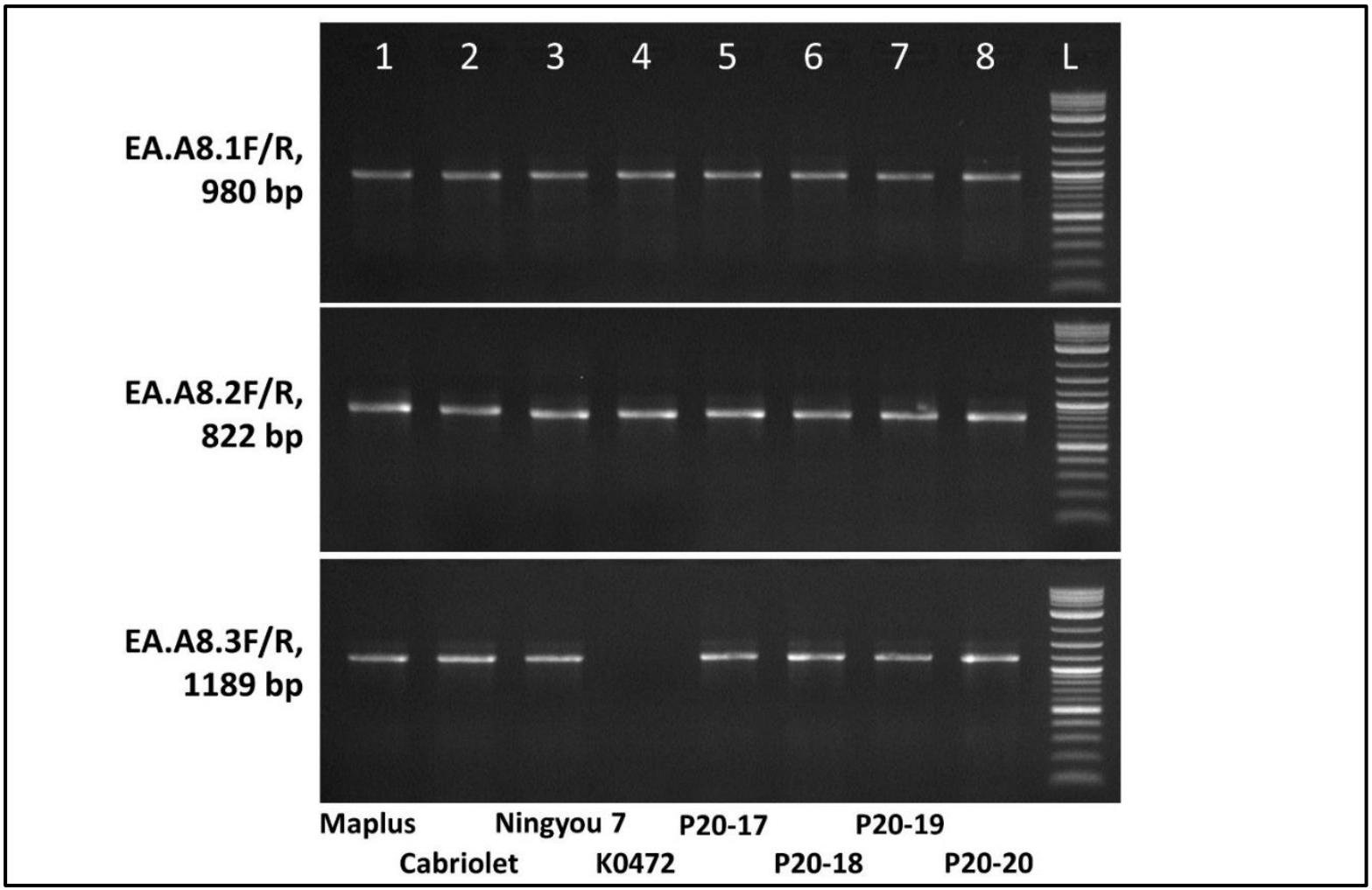

Figure 3.4 Newly designed primers amplifying regions flanking *Bna.FAE1* loci

Three of the 14 primer pairs are shown, used for PCR amplification of a panel of 8 genotypes – Maplus, Ningyou 7, Cabriolet, K0472 and 4 uniform PUFA lines

These primer pairs were used in the PCR reaction for a test panel of 8 genotypes – Maplus, Ningyou 7, Cabriolet, K0472 and 4 genotypes having uniform PUFA levels from the Section 5.4.1. From the 14 primer pairs used (three primer pairs are shown in Figure 3.4), 12 amplified the expected band size, 1 primer pair did not amplify (EA.C3.11F/R) and 1 primer pair amplified 2 bands (EA.A8.10F/R). So, 12 amplified products with the expected band size were sequenced from the Beckman Coulter Genomics (http://www.beckmangenomics.com/). Four primer pairs – EA.A8.4F/R, EA.A8.6F/R, EA.A8.7F/R and EA.A8.9F/R produced the expected results for differentiating the three cultivars – Maplus, Ningyou 7 and Cabriolet. Their results are shown in Table 3.3. Primer pairs, EA.A8.4F/R and EA.A8.9F/R distinguished between Maplus and Ningyou 7 with various SNPs. These primers were designed irrespective of the Cabriolet SNPs. Primer pairs, EA.A8.6F/R and EA.A8.7F/R differentiated the low erucic cultivar, Cabriolet from the high erucic cultivars, Maplus and Ningyou 7.

Table 3.3 Variants detection for regions flanking *Bna.FAE1* with new primers

S. No	Primer	Mutation	Maplus	Ningyou 7	Cabriolet	K0472	Fixed PUFA lines
1	**EA.A8.4F/R** Reference (Maplus)	(47)A>AT	A	AT	A	A	AT, A
		(76)G>GT	G	GT	G	G	GT, G
		(145)T>TC	T	TC	T	T	TC, T
		(256)G>GA	G	GA	G	G	GA, G
2	**EA.A8.6F/R** Reference (Cabriolet)	(64)TC>T	T	T	TC	TC	T, TC
		(251)G>C	C	C	G	G	C, G
		(274)C>T	T	T	C	C	T, C
		(205)Del T	Del T	Del T	T	T	Del T, T
		(360)TG>T	T	T	TG	TG	T, TG
		(392)T>C	C	C	T	T	C, T
		(401)TA>T	T	T	TA	TA	T, TA
3	**EA.A8.7F/R** Reference (Cabriolet)	(94)AG>A	A	A	AG	AG	A, AG
		(244)C>CT	CT	CT	C	C	CT, C
		(247)A>AG	AG	AG	A	A	AG, A
		(274)T>TA	TA	TA	T	T	TA, T
		(298)TC>T	T	T	TC	TC	T, TC
		(319)AG>A	A	A	AG	AG	A, AG
		(322)TC>T	T	T	TC	TC	T, TC
4	**EA.A8.9F/R** Reference (Maplus)	(305)CG>C	CG	C	CG	CG	CG, C
		(311)CT>C	CT	C	CT	CT	CT, C
		(337)GT>G	GT	G	GT	GT	GT, G

From these results, it was anticipated that these primers might be useful for differentiating (i) Maplus and Ningyou 7 *Bna.FAE1* alleles if any differences would be found in these two HEAR varieties and (ii) high erucic varieties from low erucic varieties. But the results of Chapter 5 later showed that the Maplus and Ningyou 7 *Bna.FAE1* alleles did not influence the erucic acid levels. So, there may be allelic differences in both alleles but this does not seem to affect the levels of erucic acid or VLCFAs in both cultivars.

Table 3.4 Enzymes involved in the VLCFAs biosynthesis from the TAIR database

TAIR ID	Enzyme involved
3-ketoacyl-CoA synthase	
AT4G34520.1	3- ketoacyl-CoA synthase 18, FAE1, Fatty acid elongation1, KCS18
AT1G01120.1	3- ketoacyl-CoA synthase 1, KCS1
AT1G04220.1	3- ketoacyl-CoA synthase 2, KCS2
AT1G25450.1	3- ketoacyl-CoA synthase 5, CER60, Eceriferum 60, KCS5
AT4G36830.1	HOS3-1
AT5G43760.1	3- ketoacyl-CoA synthase 20, KCS20
AT1G68530.1	3- ketoacyl-CoA synthase 6, ATCUT1, CER6, CUT1, Cuticular 1, Eceriferum 6, G2, KCS6
AT1G68530.2	3-ketoacyl-CoA synthase 6
AT2G16280.1	3- ketoacyl-CoA synthase 9, KCS9
AT2G04540.1	Beta-ketoacyl synthase
AT1G71160.1	3-ketoacyl-CoA synthase 7
AT4G34250.1	3-ketoacyl-CoA synthase 16
AT3G52160.1	3-ketoacyl-CoA synthase 15, KCS15
AT2G26250.1	3-ketoacyl-CoA synthase 10
AT2G28630.1	3-ketoacyl-CoA synthase 12
AT1G07720.1	3-ketoacyl-CoA synthase 3
AT2G15090.1	3-ketoacyl-CoA synthase 8
3-ketoacyl-CoA reductase	
AT1G67730.1	ATKCR1, beta-ketoacyl reductase 1, KCR1, YBR159
AT1G24470.1	ATKCR2, beta-ketoacyl reductase 2, KCR2
3-hydroxyacyl-CoA dehydratase	
AT4G14440.1	3-hydroxyacyl-CoA dehydratase 1, *A. thaliana* delta(3), delta(2)-enoyl-CoA isomerase 3, ATECI3, DELTA(3), DELTA(2)-ENOYL COA ISOMERASE 3, ECI3, HCD1
AT5G10480.3	PAS2, PASTICCINO 2, PEP, PEPINO
AT5G10480.1	PAS2, PASTICCINO 2, PEP, PEPINO
AT5G10480.2	PAS2, PASTICCINO 2, PEP, PEPINO
AT5G59770.1	Protein-tyrosine phosphatase-like, PTPLA
trans-(2,3)-enoyl-CoA reductase	
AT3G55360.1	ATTSC13, CER10, ECERIFERUM 10, ECR, ENOYL-COA REDUCTASE, GLASSY HAIR 6, GLH6, TSC13
AT5G16010.1	3-oxo-5-alpha-steroid 4-dehydrogenase family protein
Others	
AT5G46290.1	3-ketoacyl-acyl carrier protein synthase I

3.4.5 Compiling the Genes Related to VLCFA Biosynthesis

The elongation pathway for the biosynthesis of the erucic acid from oleic acid comprises two cycles of a four-step reaction and is well characterized (Section 1.3.4). Four enzymes are involved in this process – 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydratase and trans-(2, 3)-enoyl-CoA reductase (Lassner, Lardizabal and Metz, 1996). KCS is the main enzyme responsible for controlling the erucic acid content in *B. napus* (Rossak, Smith and Kunst, 2001). In the literature, *FAE1* is mostly used instead of KCS as *FAE1* is the corresponding orthologue in Arabidopsis. So, a list was compiled for the genes responsible for these enzymes and related genes for the very long chain fatty acids from the Arabidopsis database (TAIR, https://www.arabidopsis.org/) as shown in Table 3.4. Seventeen related genes were found for the main rate-limiting enzyme, KCS. Five related genes were found for 3-hydroxyacyl-CoA dehydratase; 2 related genes were found each for 3-ketoacyl-CoA reductase and trans-(2, 3)-enoyl-CoA reductase and; 1 other related gene was found, encoding related enzymes as shown in Table 3.4. Some genes found were similar to other GWAS conducted in *B. napus* (Qu *et al.*, 2017).

3.4.6 Splitting the Diversity Panel

Using the full diversity panel, already known loci controlling the erucic acid were detected and it was not possible to detect any modifier loci influencing the erucic acid synthesis using the full panel. So, the erucic acid data from the diversity panel was split into two subsets – first with genotypes having more than 30% erucic acid (121 accessions) and second with genotypes having less than 5% erucic acid (244 accessions) in their seeds. GWAS was conducted with these high and low erucic acid datasets. It could be observed that the association results were not very clear with the split panel as shown in Figure 3.5 and Figure 3.6. Only a few SNPs were found above the Bonferroni correction and no major peaks were found in the association analysis. The genes controlling the erucic acid or VLCFAs content were not detected in these association regions.

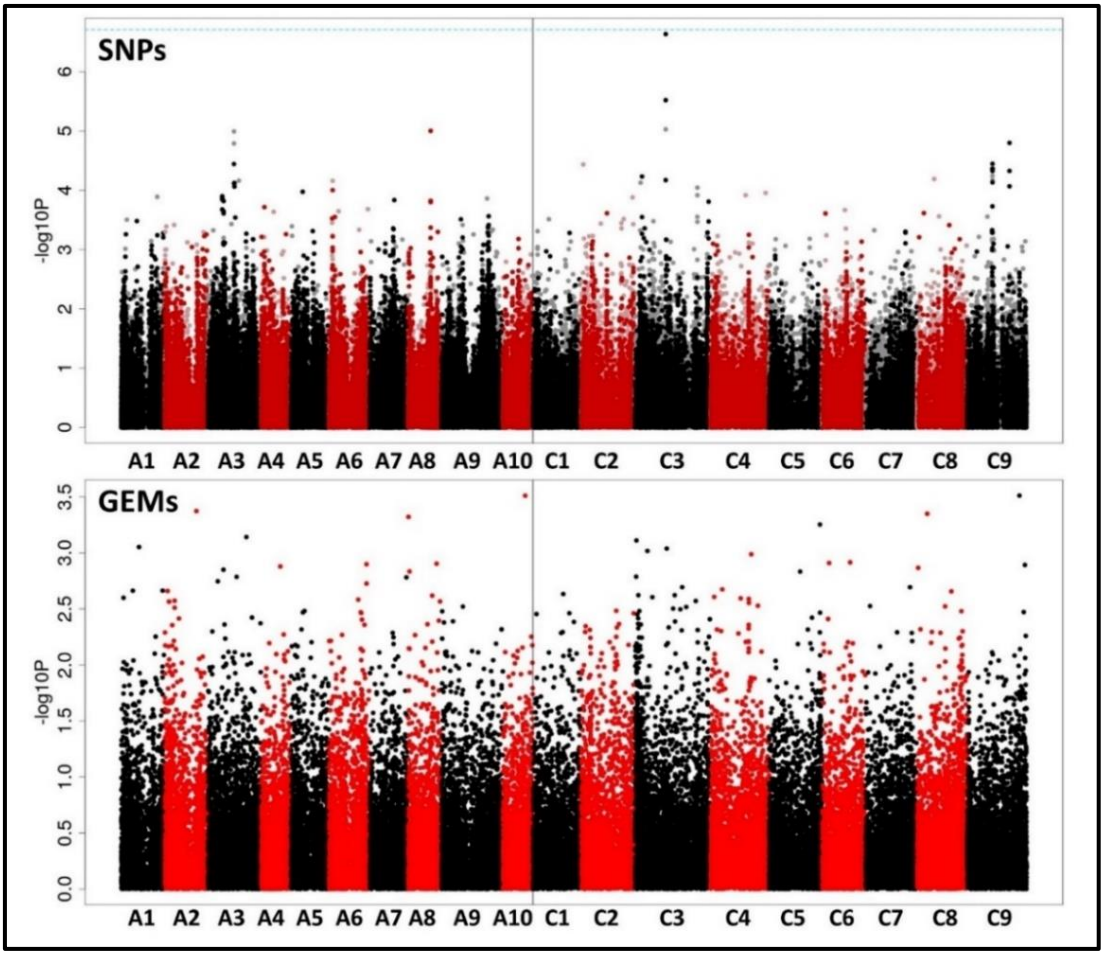

Figure 3.5 Association analysis of high erucic acid sub-set having more than 30% erucic acid (a) SNPs (b) GEMs

Associative transcriptomic analysis using 121 B. napus accessions from the RIPR diversity panel having more than 30% erucic acid in their oil. The trait significance value, -log10P value is plotted against 19 pseudo chromosomes of B. napus. The broken light blue horizontal line is the Bonferroni correction at 0.05 significance threshold

The list of genes involved in the very long chain fatty acid synthesis (Table 3.4) was also tested against these minor association peaks to see whether if these coincide in any of the association plots in Figure 3.5 and Figure 3.6. But none of these loci were found to be associated with the erucic content or very long chain fatty acid synthesis. The minor peaks such as in the chromosome C3 in the high erucic sub-set (Figure 3.5) did not correspond to any genes related to the fatty acid biosynthesis. Similarly, SNPs above the threshold were searched for the low erucic sub-set, such as in the chromosome A6 but no genes related to the fatty acid biosynthesis were found (Figure 3.6). So, testifying the panel for high and erucic acid data sub-sets did not provide any information for the genes involved in the elongation pathway of the

erucic acid biosynthesis. Searching the complete panel for more potential candidates for erucic acid control might help in exploring new modifier loci for the erucic content in the rapeseed.

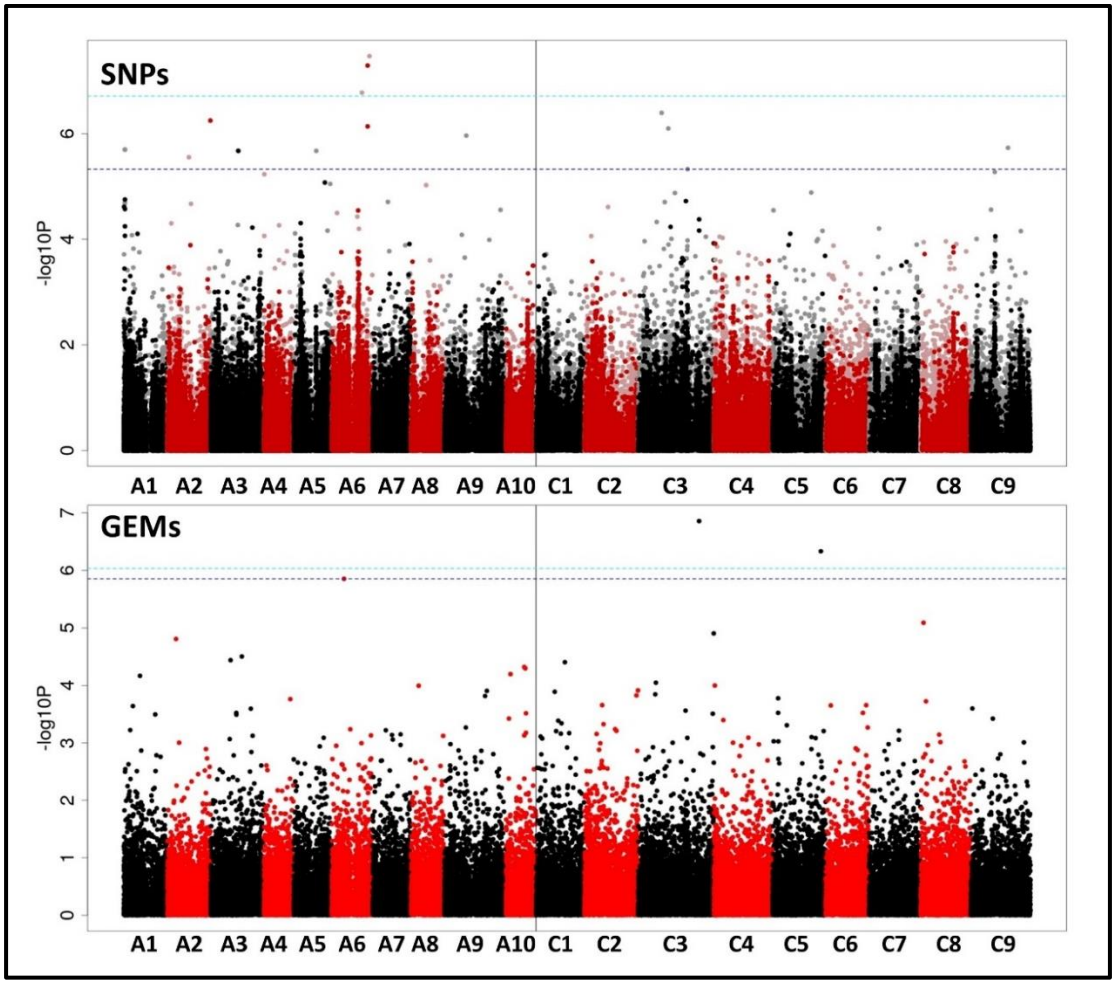

Figure 3.6 Association analysis of the low erucic acid sub-set having less than 5% erucic acid (a) SNPs (b) GEMs.

AT analysis using 244 B. napus accessions from the RIPR diversity panel having less than 2% EA. The trait significance value, -log10P value is plotted against 19 B. napus pseudo chromosomes. The broken light blue horizontal line is the Bonferroni correction at 0.05 significance threshold and the broken dark blue horizontal line is 5% false discovery rate

3.5 Summary

Associative transcriptomics is a powerful tool for identifying the markers related to the complex traits in the crops using the transcriptome information (Harper *et al.*, 2012). The fatty acid content was analysed for 404 accessions under the RIPR project

and genome-wide association study was conducted on the 383 *B. napus* diversity panel (RIPR, 2014) as functional genotypes were available for 383 accessions only. There are 355,536 SNP markers available in this diversity panel (Havlickova *et al.*, 2018). The erucic acid content in rapeseed is controlled by two genes acting in an additive manner (Harvey and Downey, 1964). These two genes can be represented as '*eru1* and *eru2'; 'e1 and e2'; 'BN-FAE1.1* and *BN-FAE1.2'* and; '*Bna.FAE1.A8* and *Bna.FAE1.C3'.* These are located on the A8 and C3 chromosomes in the rapeseed, respectively (Foisset *et al.*, 1996; Fourmann *et al.*, 1998; Qiu *et al.*, 2006). The major peaks found on the A8 and C3 chromosomes in the present study corresponded to these already known regions. There were various other minor association peaks detected in the Manhattan plot for the erucic acid such as on the chromosome A5. So, a potential candidate, Cab033920.1 (AT2G34770.1) on the A5 chromosome, involved in the very long chain fatty acid synthesis was tested by using the Arabidopsis knocked out T-DNA lines but no change was observed in the fatty acid composition of the mutants as compared to the control, Columbia-0. So, this loci did not provide any support for Cab033920.1 to control VLCFAs in *B. napus.*

The SNP markers flanking the *FAE1* region were searched in the AT plots; and top SNPs with high $-\log_{10}P$ value and near to the *FAE1* region were selected from the A8 and C3 chromosomes to distinguish SNPs between HEAR (Maplus and Ningyou 7) and LEAR (Cabriolet)and; within two high erucic acid cultivars, Maplus and Ningyou 7. Some of the primer pairs were found differentiating these combinations but the later study (Chapter 5) have shown that Maplus and Ningyou 7's *Bna.FAE1* alleles do not influence the erucic acid or VLCFAs levels in HELP lines. So, there were allelic differences in *Bna.FAE1* alleles of both cultivars but these did not influence their fatty acid compositions. The information about the related genes to the elongation enzymes was compiled from the Arabidopsis database, TAIR and these genes were searched in the minor association peaks but no new genes affecting the erucic acid were found. Finally, the erucic acid data was split into two subsets of high erucic acid (more than 30%) and low erucic acid accessions (less than 5%). GWAS was conducted on these datasets and weak associations were found at some of the chromosomes but no candidate corresponded to the erucic acid or fatty acid biosynthesis.

3.6 Conclusion

To summarize, the major genes controlling the erucic acid content, *Bna.FAE1.A8* and *Bna.FAE1.C3* corresponded to the major peaks in the association transcriptomics study. No modifier loci were found from the present study but there may be modifiers present for the erucic acid content in the rapeseed and could be found by testing more potential candidates from the minor association peaks or the regions flanking the *Bna.FAE1* region.

3.7 Publication

The following manuscript is published in *'The Plant Journal'* and contains a part of this chapter:

"Havlickova, L. , He, Z. , Wang, L. , Langer, S. , Harper, A. L., **Kaur, H**. , Broadley, M. R., Gegas, V. and Bancroft, I. (2018) Validation of an updated Associative Transcriptomics platform for the polyploid crop species *Brassica napus* by dissection of the genetic architecture of erucic acid and tocopherol isoform variation in seeds. *The Plant Journal*, 93: 181-192. doi:10.1111/tpj.13767."

4. Development of High Erucic and Low Polyunsaturates (HELP) Rapeseed Using Ningyou 7 *FAE1* Alleles

Contributions: Initial crosses between K0472 and Ningyou 7 were attempted by Caramel O'Neill (Bancroft group, John Innes Centre, Norwich, UK). The F_1 progeny was backcrossed to the Cabriolet (F_1B_1 progeny), followed by self-pollination. It was carried out by Lihong Sophia Cheng (Bancroft group, University of York, York, UK). Multiplication of $F_1B_1S_4$ generation (University of York) was managed by Natalia Stawniak and multiplication of $F_1B_1S_5$ generation (Czech Republic) was managed by Lenka Havlickova of the Bancroft group.

4.1 Hypothesis

In *B. napus,* partially functional *Bna.FAD2* (three non-functional copies and one mutated copy) would remove the draw on oleic acid (C18:1) into the desaturation pathway, thus, leaving more substrate (oleic acid) for the elongation to eicosenoic acid (C20:1) and erucic acid (C22:1) by *Bna.FAE1*. Desaturation pathway uses oleic acid in the form of oleoyl-PC while elongation pathway uses oleic acid in the form of oleoyl-CoA.

An overview of the fatty acid biosynthesis pathway showing oleic acid desaturation to polyunsaturated fatty acids (PUFAs, C18:2 and C18:3) and elongation to the very long chain fatty acids (VLCFAs, C20:1 and C22:1) is shown in Figure 4.1(a) and the hypothesis showing the influence of partially functional *Bna.FAD2* on the fatty acid biosynthesis pathway is depicted in Figure 4.1(b).

4.2 Test

Complete development of the genotype "4 x *Bna.fad2* and 2 x *Bna.FAE1*^{NY7}" was achieved by self-pollinated progeny derived from the cross "Cabriolet x (K0472 x

Ningyou 7)" accompanied by the marker-assisted selection of high erucic and low polyunsaturated (HELP) lines. The fatty acid profiles were analysed to see whether the very long chain fatty acids were increased relative to the high erucic acid cultivar-Ningyou 7 (NY7). Glucosinolates content was also measured in HELP lines.

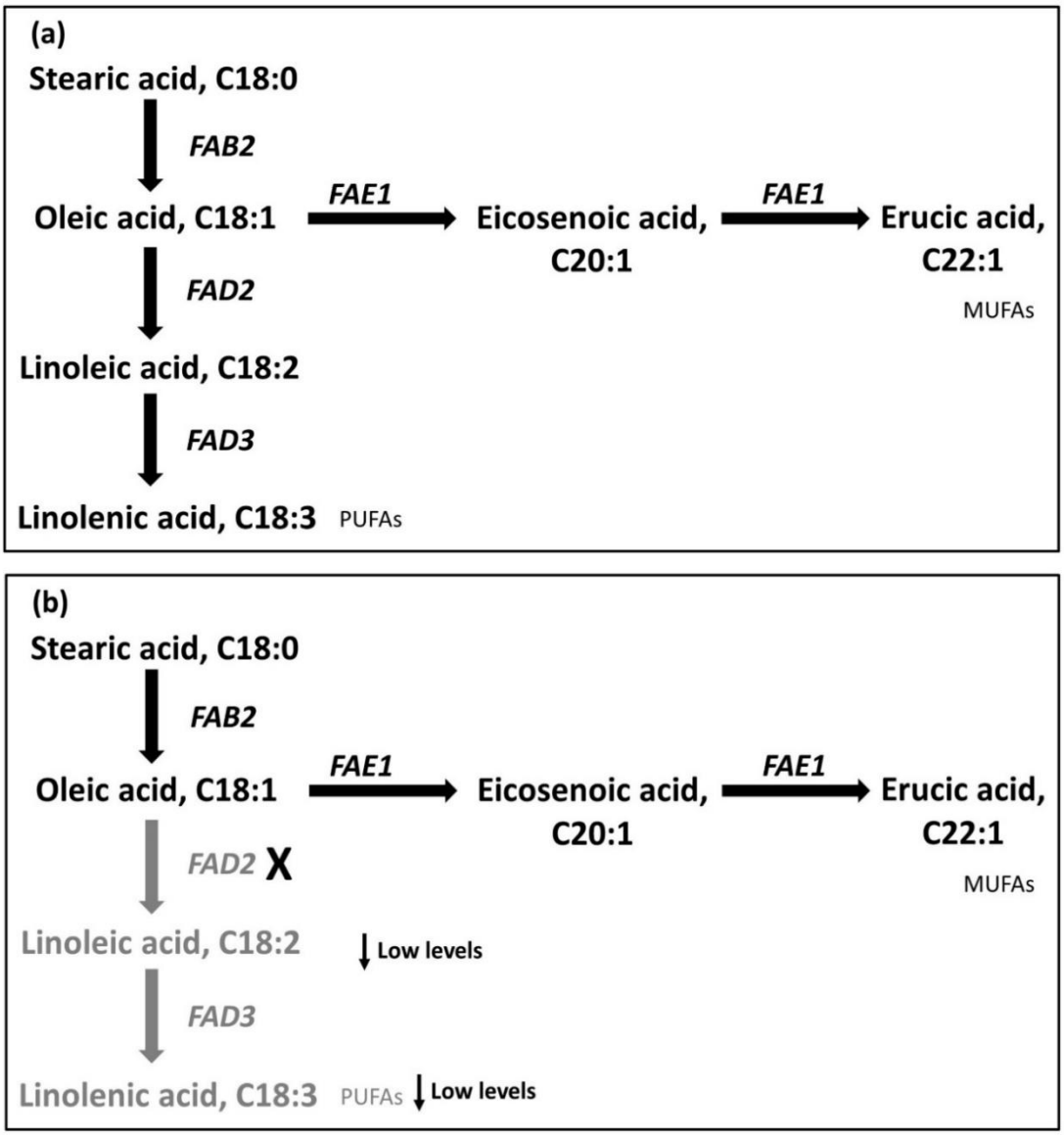

Figure 4.1 An overview of the fatty acid biosynthesis pathway (a) & hypothesis (b)
(a) A part of the fatty acid biosynthesis pathway in the family Brassicaceae showing the main terminal product, oleic acid going into desaturation (producing PUFAs) and elongation (producing MUFAs -VLCFAs) pathways. In the desaturation pathway, oleic acid is used in the form of oleoyl-PC by FADs while in the elongation pathway, FAE1 uses oleic acid in the form of oleoyl-CoA. (b) The hypothesis showing the effect of partially functional FAD2. It would remove the draw on oleic acid into the desaturation pathway and thus there will be more substrate available for the elongation to VLCFAs by FAE1

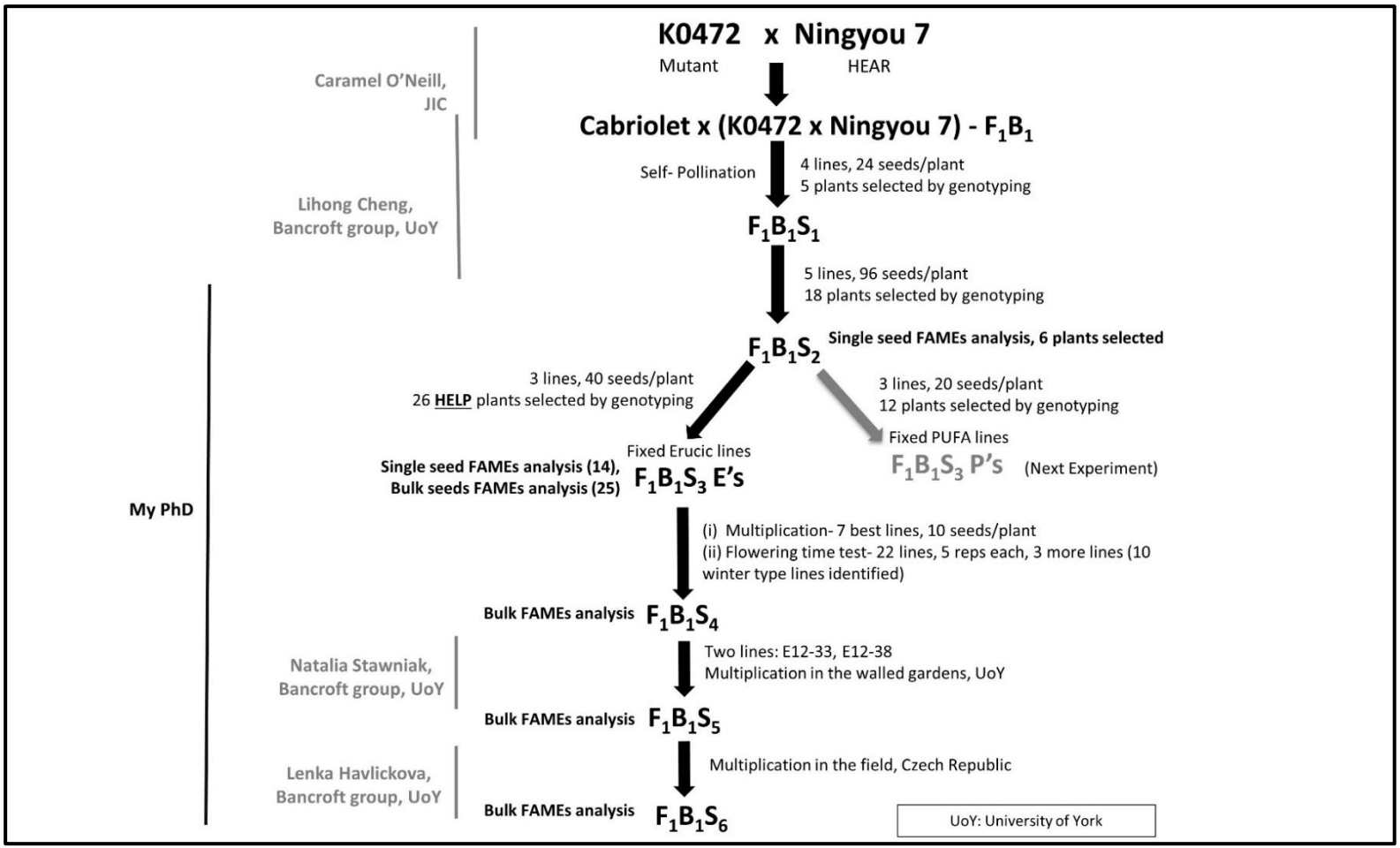

Figure 4.2 Development of HELP lines from the cross 'Cabriolet x (K0472 x Ningyou 7)'

Cabriolet EMS mutant, K0472 was cross-pollinated to the high erucic acid cultivar, NY7 to produce F_1 progeny which was backcrossed to Cabriolet to produce the F_1B_1 progeny and self-pollinated for 6 generations accompanied by various selections. On the left side, the contribution by various people is acknowledged.

4.3 Materials and Methods

Cabriolet EMS mutant, K0472 (oil profile: ~85% C18:1, ~6% PUFAs, 0% C22:1; Wells *et al.*, 2014) was cross-pollinated to the high erucic acid cultivar, Ningyou 7 (oil profile: ~12% C18:1, ~21% PUFAs, ~50% C22:1) to produce F_1 progeny at the John Innes Centre, Norwich by Caramel O'Neill. The F_1 progeny was backcrossed to the low erucic acid cultivar, Cabriolet (oil profile: ~76% C18:1, ~19% PUFAs, 0% C22:1) to produce the F_1B_1 progeny at the University of York (Sophia Cheng, Bancroft group). Cabriolet was used as the parental background for mutagenesis to produce mutant K0472. It was self-pollinated for 6 generations accompanied by *Bna.FAD2* and *Bna.FAE1* genotyping (Figure 4.2). I started my PhD with the selections of $F_1B_1S_1$ progeny.

Genotypic profiles of K0472, NY7, Cabriolet and HELP lines are shown in Table 4.1. Ningyou 7 had the functional copies of both *Bna.FAD2* and *Bna.FAE1* loci while K0472 had the non-functional copies of *Bna.FAE1,* a partially functional copy of *Bna.FAD2.C5* and a non-functional copy of *Bna.FAD2.A5.* Cabriolet had the similar but functional *Bna.FAD2.C5* copy to K0472 as shown in Table 4.1. The details of the selection process are provided in the following results section.

Table 4.1 *Bna.FAD2* and *Bna.FAE1* profiles of the lines used for the development of 'Cabriolet x (K0472 x Ningyou 7)' HELP lines

Genotype	*Bna.FAD2.A5*	*Bna.FAD2.C5*	*Bna.FAE1.A8*	*Bna.FAE1.C3*
Ningyou 7	Functional	Functional	Functional	Functional
K0472	Non-functional	Partially functional*	Non-functional	Non-functional
Cabriolet	Non-functional	Functional	Non-functional	Non-functional
HELP line	Non-functional	Partially functional*	Functional	Functional

*Partially functional alleles of *Bna.FAD2.C5* copy (Section 2.4.2). The details of the allelic polymorphism are given in Table 2.3 and Table 2.4

Table 4.2 *Bna.FAD2* and *Bna.FAE1* profiles of the selected F_1B_1 plants

Genotype	*Bna.FAD2.A5*	*Bna.FAD2.C5*	*Bna.FAE1.A8*	*Bna.FAE1.C3*
Cab x (K0472 x NY7) A3	Het	Het	Het	Het
Cab x (K0472 x NY7) A8	Het	Het	Het	Homo
Cab x (K0472 x NY7) A9	-	Het	Het	Het
Cab x (K0472 x NY7) A20	-	Het	Het	Het
Cab x (K0472 x NY7) C2	-	Het	Het	Het
HELP (objective)	-	-	Homo	Homo

'Het' is a heterozygous copy; 'Homo' is a homozygous functional copy; '-' is a homozygous non-functional (partially functional *Bna.FAD2.C5*) copy; Cab is Cabriolet &; NY7 is Ningyou 7

4.4 Results

4.4.1 F_1B_1 Generation

In the F_1B_1 progeny, 4 lines were randomly selected and 24 plants were grown from each line. DNA was extracted using the CTAB method (Section 2.3) and genotyped with *Bna.FAD2.A5, Bna.FAD2.C5, Bna.FAE1.A8* and *Bna.FAE1.C3* primer pairs (Table 2.5) from 96 plants. The selections were aimed at the HELP genotypic construct as depicted in Table 4.1 and Table 4.2. Heterozygous forms were selected for the copies where HELP genotypic construct was not available so that the selections for the homozygous functional and non-functional alleles could be made in the next segregating generation. Five plants were selected and their genotypes are shown in Table 4.2. In the selected lines, at least 3 copies were heterozygous and the 4th one was either homozygous (functional or non-functional) or heterozygous. These were self-pollinated and seeds were collected at the maturity.

4.4.2 $F_1B_1S_1$ Generation

From the selected 5 lines, 96 plants were grown for each line and DNA was extracted using the CTAB method described in Section 2.3. Primer pairs for *Bna.FAD2.A5, Bna.FAD2.C5, Bna.FAE1.A8* and *Bna.FAE1.C3* copies (Table 2.5) were amplified on these samples and the amplicon was sent for Sanger sequencing to the Beckman Coulter Genomics (http://www.beckmangenomics.com/). Eighteen plants of various allele combinations were selected for the $F_1B_1S_1$ progeny based on the genotypic profiles (Table 4.3). Seven of these lines (Table 4.3, S. No. 1 to 7) had functional copies of both *Bna.FAE1.A8* and *Bna.FAE1.C3*; non-functional copy of *Bna.fad2.A5* and; heterozygous copy of *Bna.FAD2.C5*. Four lines (Table 4.3, S. No. 8 to 11) had non-functional *Bna.fad2.A5*, partially functional *Bna.fad2.C5*, functional *Bna.FAE1.A8* and heterozygous *Bna.FAE1.C3* copies. Two lines (Table 4.3, S. No. 12 and 13) had *Bna.fad2.A5* copy non-functional, *Bna.fad2.C5* copy partially functional, *Bna.FAE1.C3* copy functional and *Bna.FAE1.A8* heterozygous. Three lines (Table 4.3, S. No. 14 to 16) had non-functional *Bna.fad2.A5*, partially functional *Bna.fad2.C5* and both copies of *Bna.FAE1* heterozygous. Two lines (Table 4.3, S. No. 17 and 18) had two of the

Bna.FAD2 copies homozygous functional and both *Bna.FAE1* copies non-functional. These 18 plants were self-pollinated and seeds were collected at the maturity.

4.4.3 $F_1B_1S_2$ Generation

4.4.3.1 Fatty Acid Analysis and Selections

FAMEs were analysed on 17 lines of $F_1B_1S_2$ seeds as one of the lines (S. No. 7, Table 4.3) did not produce sufficient seeds for the fatty acid analysis. The fatty acids were also measured for two controls, K0472 and Ningyou 7. The detailed results of the single seed fatty acid analysis of these lines are given in Appendix IV.

Table 4.3 *Bna.FAD2* and *Bna.FAE1* profiles of the selected $F_1B_1S_1$ plants of the cross 'Cabriolet x (K0472 x Ningyou 7)'

Code	Genotype	*Bna.FAD2. A5*	*Bna.FAD2. C5*	*Bna.FAE1. A8*	*Bna.FAE1. C3*
6	Cab x (K0472xNY7)A9-68	-	Het	Homo	Homo
9	Cab x (K0472xNY7)A20-24	-	Het	Homo	Homo
12	Cab x (K0472xNY7)A20-54	-	Het	Homo	Homo
13	Cab x (K0472xNY7)A20-73	-	Het	Homo	Homo
16	Cab x (K0472xNY7)C2-20	-	Het	Homo	Homo
17	Cab x (K0472xNY7)C2-54	-	Het	Homo	Homo
18	Cab x (K0472xNY7)C2-60	-	Het	Homo	Homo
1	Cab x (K0472xNY7)A3-42	-	-	Homo	Het
11	Cab x (K0472xNY7)A20-48	-	-	Homo	Het
19	Cab x (K0472xNY7)C2-78	-	-	Homo	Het
20	Cab x (K0472xNY7)C2-88	-	-	Homo	Het
3	Cab x (K0472xNY7)A3-92	-	-	Het	Homo
7	Cab x (K0472xNY7)A20-10	-	-	Het	Homo
10	Cab x (K0472xNY7)A20-35	-	-	Het	Het
14	Cab x (K0472xNY7)A20-94	-	-	Het	Het
15	Cab x (K0472xNY7)C2-16	-	-	Het	Het
4	Cab x (K0472xNY7)A8-24	Homo	Homo	-	-
8	Cab x (K0472xNY7)A20-22	Homo	Homo	-	-
2	Ningyou 7	Homo	Homo	Homo	Homo
5	K0472	-	-	-	-

'Het' is a heterozygous copy; 'Homo' is a homozygous functional copy; '-' is a homozygous non-functional (partially functional for *Bna.FAD2.C5*) copy; Cab is Cabriolet and; NY7 is Ningyou 7. The Code described here was used for the single seed FAMEs analysis. Genotypes in bold were the selected ones later on.

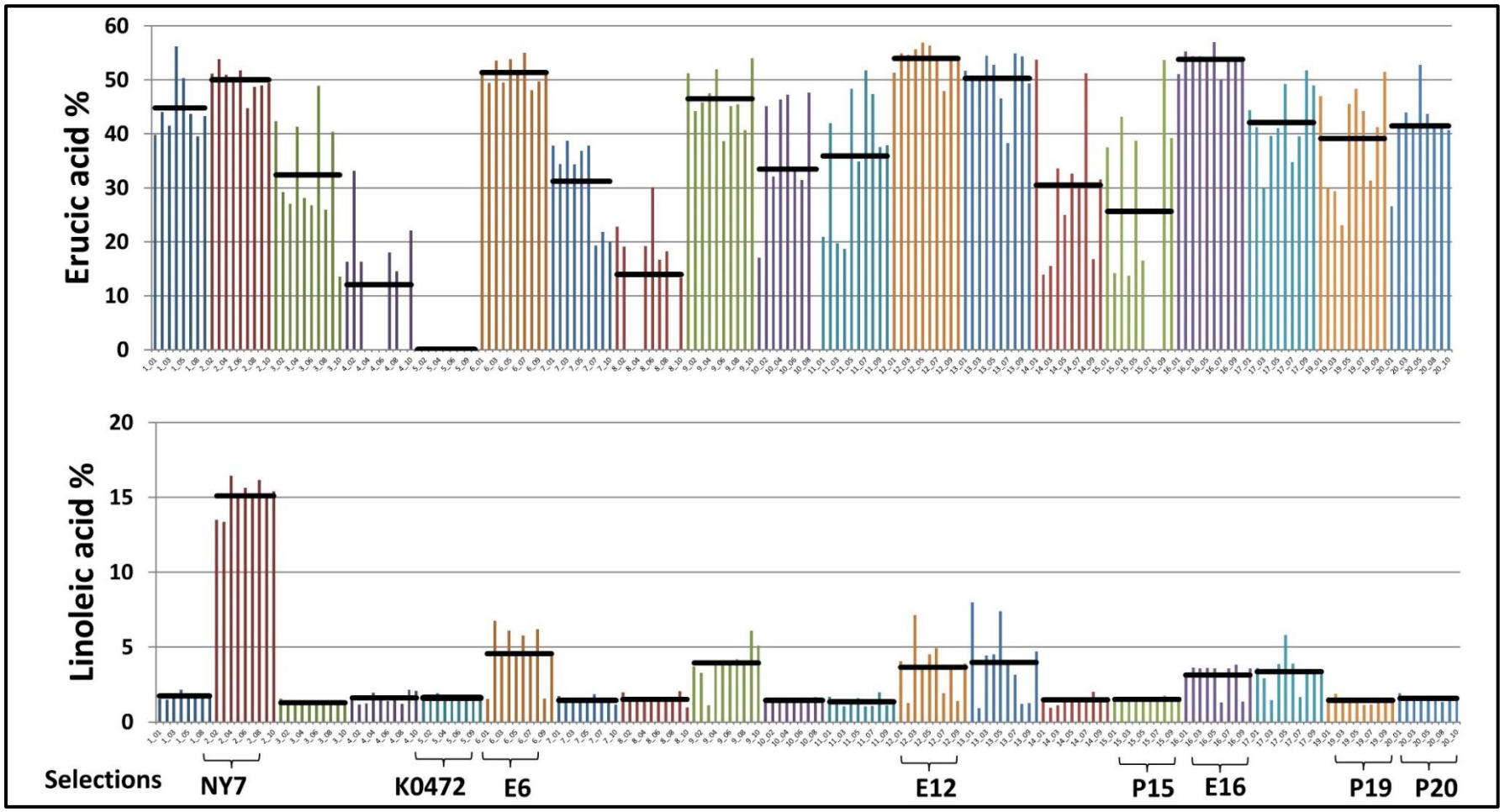

Figure 4.3 Erucic acid (C22:1) and linoleic acid (C18:2) percentages of $F_1B_1S_2$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)'

FAMEs were analysed from the 10 replicates each of 17 lines with 2 controls, K0472 and Ningyou 7 (using single seed method). Each vertical bar represents the fatty acid percentage of one seed and horizontal black bar on top of each group shows the mean value of the group.

Table 4.4 Mean fatty acid percentages (10 biological replicates) of $F_1B_1S_2$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)'

Code	Genotype	16:0	16:1	18:0	18:1	18:2	18:3	18:2+ 18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	VLCFA$^+$
1	Cab x (K0472 x NY7) A3-42	2.5	0.3	0.7	32.3	1.7	3.5	5.3	0.6	12.3	0.1	0.3	44.8	0.1	0.6	57.7
2	Ningyou 7	3.1	0.2	0.8	10.6	15.1	12.1	27.2	0.6	4.7	0.5	0.8	50.0	0.3	1.2	55.9
3	Cab x (K0472 x NY7) A3-92	2.6	0.2	1.0	40.9	1.3	2.9	4.2	0.6	16.9	0.1	0.3	32.4	0.1	0.7	50.0
4	Cab x (K0472 x NY7) A8-24	3.1	0.3	1.6	64.1	1.6	3.4	5.0	0.6	12.3	0.1	0.3	12.0	0.2	0.5	24.8
5	K0472	3.7	0.3	1.2	87.9	1.6	3.0	4.6	0.5	1.3	0.0	0.3	0.0	0.2	0.1	1.4
E6	Cab x (K0472 x NY7) A9-68	2.6	0.2	0.7	24.7	4.6	6.0	10.6	0.6	8.1	0.2	0.4	51.4	0.1	0.5	60.0
7	Cab x (K0472 x NY7) A20-10	2.8	0.2	1.4	41.9	1.5	3.7	5.2	0.7	15.2	0.1	0.3	31.2	0.1	0.8	47.2
8	Cab x (K0472 x NY7) A20-22	3.3	0.4	1.4	58.6	1.5	4.0	5.5	0.7	15.1	0.1	0.4	13.9	0.2	0.6	29.6
9	Cab x (K0472 x NY7) A20-24	2.8	0.3	1.0	28.9	3.9	5.3	9.2	0.9	8.5	0.1	0.6	46.5	0.3	1.0	56.0
10	Cab x (K0472 x NY7) A20-35	2.5	0.2	1.2	43.1	1.4	3.3	4.7	0.8	12.9	0.1	0.4	33.5	0.1	0.6	46.9
11	Cab x (K0472 x NY7) A20-48	3.1	0.4	1.4	37.9	1.4	3.8	5.1	1.0	13.3	0.1	0.6	35.9	0.3	0.9	50.1
E12	Cab x (K0472 x NY7) A20-54	2.7	0.3	0.7	24.6	3.6	6.3	10.0	0.7	5.3	0.1	0.6	53.9	0.2	0.8	60.1
13	Cab x (K0472 x NY7) A20-73	2.9	0.3	0.9	25.3	4.0	6.9	10.8	0.8	6.3	0.1	0.8	50.3	0.3	1.2	57.8
14	Cab x (K0472 x NY7) A20-94	2.8	0.3	1.2	41.5	1.5	3.6	5.1	0.7	16.8	0.1	0.4	30.5	0.1	0.7	47.9
P15	Cab x (K0472 x NY7) C2-16	3.0	0.4	1.1	49.7	1.5	3.4	4.9	0.6	13.7	0.1	0.3	25.7	0.1	0.5	39.9
E16	Cab x (K0472 x NY7) C2-20	2.6	0.3	0.9	25.8	3.1	5.2	8.3	0.8	6.1	0.1	0.6	53.8	0.1	0.7	60.6
17	Cab x (K0472 x NY7) C2-54	2.9	0.3	0.9	29.8	3.4	5.1	8.5	0.6	13.7	0.1	0.3	42.1	0.1	0.8	56.5
P19	Cab x (K0472 x NY7) C2-78	2.7	0.3	0.9	35.1	1.5	3.0	4.5	0.6	15.6	0.1	0.3	39.2	0.1	0.7	55.4
P20	Cab x (K0472 x NY7) C2-88	2.7	0.4	1.1	33.7	1.6	3.1	4.7	0.7	14.0	0.1	0.4	41.5	0.1	0.7	56.2

Detailed single seed values are given in Appendix IV;

'+' VLCFA = 20:1+22:1:24:1; Cab is Cabriolet and; NY7 is Ningyou 7

Selected genotypes are shown in **bold**

The fatty acids were analysed on 10 seeds of each line by the single seed method described in Section 2.7.1. But some samples evaporated during the incubation step at 85°C and thus, less than 10 replicates were analysed by the gas chromatography for those lines. Erucic acid and linoleic acid (C18:2) percentages of 17 lines and 2 controls (NY7 and K0472) are shown in Figure 4.3 and the mean percentages of the fatty acid values (mean of the single seed values) are given in Table 4.4. Six candidate lines were selected from the fatty acid analysis as shown in Figure 4.3.

Three lines – E6, E12 and E16 had high erucic content of more than 50% in their seeds. It was consistent in their 10 replicates as shown in Figure 4.3. This uniformity in the erucic content corresponds to their homozygous functional copies, *Bna.FAE1.A8* and *Bna.FAE1.C3* (Table 4.3) of the gene *FAE1* (converts oleic acid to eicosenoic acid and erucic acid). In addition, these lines had varying amount of PUFA, linoleic acid in their seeds which is due to one heterozygous copy, *Bna.FAD2.C5* of the *Bna.FAD2* family (controls the conversion of oleic acid to linoleic acid). Highest erucic acid level of 54% (mean value) was found in the genotypes – E12 and E16 (Table 4.4), but in the individual seeds, the erucic levels of up to 57% was found (Appendix IV). There was approx. 7% increase in the erucic acid values as compared to the high erucic variety, Ningyou 7. PUFA levels in these were found to be lower (8 to 10%) as compared to NY7 (27%). Oleic acid values of ~25% were found in these 3 selected plants which were higher in comparison to NY7.

Another 3 lines – P15, P19 and P20 were also selected as shown in Figure 4.3. These had a uniform amount of linoleic acid in their seeds which corresponded to the non-functional copy, *Bna.fad2.A5* and partially functional copy, *Bna.fad2.C5* of the *Bna.FAD2* family (Table 4.3). Less than 5% PUFAs were found in these three lines. The variable erucic acid content was also observed in these lines (Figure 4.3). Genotype P15 had non-functional *Bna.fae1.A8* copy and heterozygous *Bna.FAE1.C3* copy while genotypes P19 and P20 had functional *Bna.FAE1.A8* copy and heterozygous *Bna.FAE1.C3* copy. Their PUFA levels were similar to one of the parents, K0472 (Table 4.4). In comparison to high erucic cultivar, NY7, there was approximately 22% decrease in the PUFA content in these lines. As the acid method used for the fatty acid analysis was a destructive method, so the seeds with desirable oil content could not

be recovered. So, these lines were genotyped to select the lines with the desirable HELP construct.

4.4.3.2 Genotyping and Selections

Forty plants each of the genotypes E6, E12 and E16 were grown in the P-40 trays (Figure 4.4) filled with the medium-grade compost (Scotts Levington F2+S) in the glasshouse conditions.

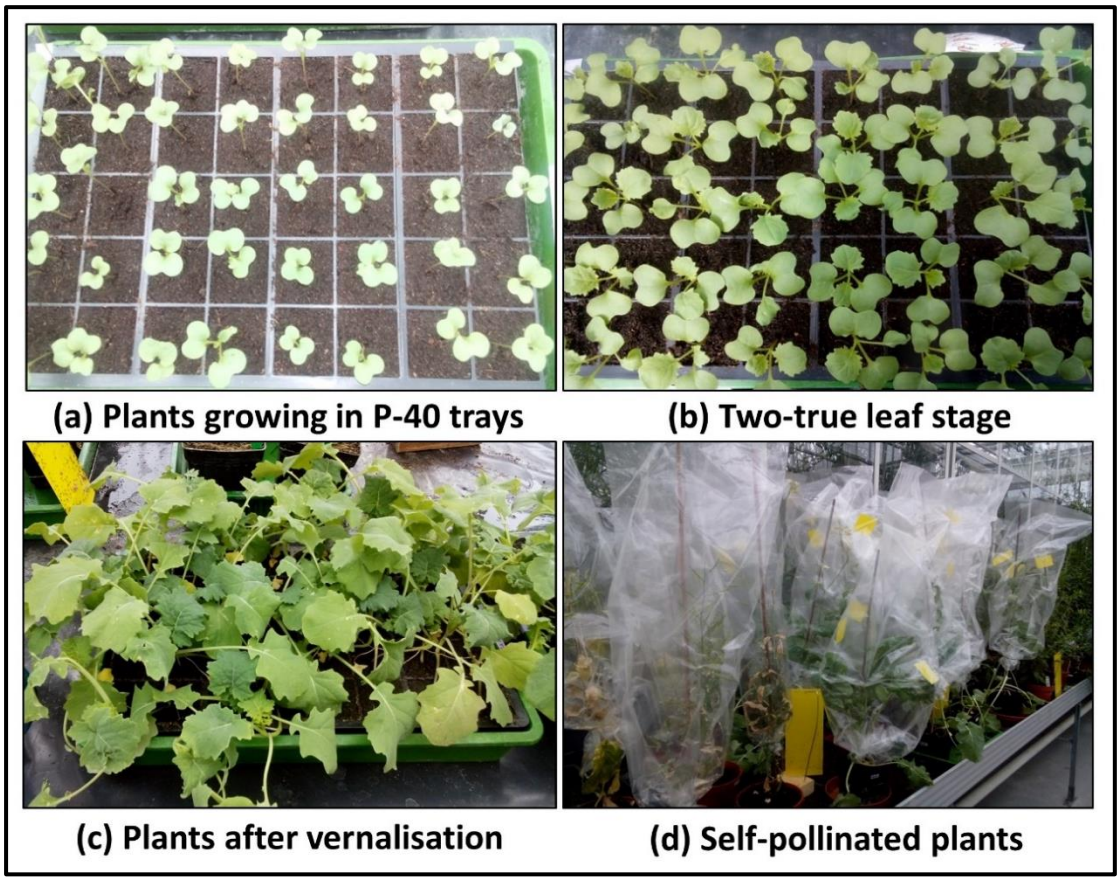

Figure 4.4 Various stages during the growth of $F_1B_1S_2$ plants ('Cabriolet x (K0472 x Ningyou 7)') in the glasshouse

(a) Seeds were sown in the P-40 tray (tray with 40 pots) producing two cotyledonary leaves, (b) Two true leaf stage of the plant growth. The plants were sampled at the three-leaf stage for DNA extraction, (c) Plants were taken out of vernalisation after 6 weeks and, (d) Plants were re-potted to larger pots after vernalisation and these were bagged for self-pollination in the glasshouse. The plants were harvested and thrashed to get seeds at the maturity

DNA extractions were made using the CTAB method (Section 2.3) and *Bna.FAD2.A5, Bna.FAD2.C5, Bna.FAE1.A8* and *Bna.FAE1.C3* copies were amplified, followed by genotyping of 120 individual plants (few amplicons shown in Figure 4.5). These were

sent for Sanger sequencing to the Beckman Coulter Genomics and mutations were screened using the Mutation Surveyor® (https://softgenetics.com/mutationSurveyor.php). *Bna.FAE1.A8, Bna.FAE1.C3* and *Bna.fad2.A5* were already according to the HELP genotypic construct in these lines. So, it was confirmed in the progeny with the sequencing results. Copy *Bna.FAD2.C5* segregated in the progeny and 26 lines with the non-functional copy were selected as shown in Table 4.5. These were bagged for self-pollination (Figure 4.4) and seeds were collected at the maturity. These 26 selected plants were the high erucic and low polyunsaturates (HELP) rapeseed lines with a genotypic construct "4 x *Bna.fad2* and 2 x *Bna.FAE1*^{NY7}".

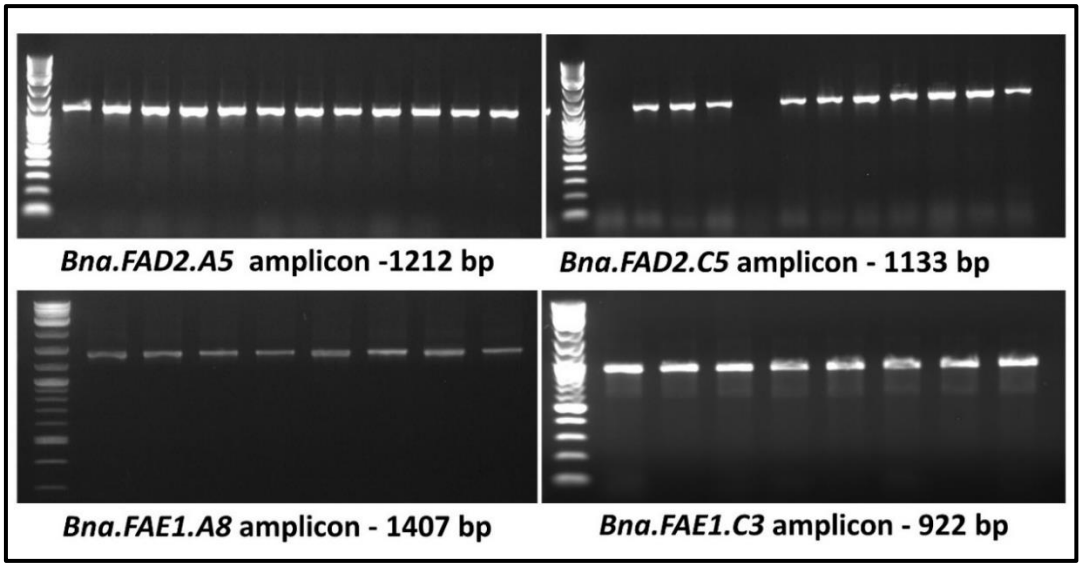

Figure 4.5 DNA amplicons of *Bna.FAD2* and *Bna.FAE1* copies of $F_1B_1S_2$ plants of the cross 'Cabriolet x (K0472 x Ningyou 7)'

PCR amplification of Bna.FAD2.A5, Bna.FAD2.C5, Bna.FAE1.A8 and Bna.FAE1.C3 copies of the $F_1B_1S_2$ generation. Band size (bp) is mentioned next to the amplicon. Bna.FAD2 and Bna.FAE1 copies of 120 plants were amplified by PCR and only a few amplicons are shown

Table 4.5 Selected HELP lines of $F_1B_1S_2$ progeny ($F_1B_1S_3$ seeds) of the cross 'Cabriolet x (K0472 x Ningyou 7)'

S. No.	Parental Line	$F_1B_1S_2$ progeny	Number of plants
1	E6	E6-5, E6-10, E6-15, E6-16, E6-19, E6-24, E6-27, E6-31, E6-34, E6-39	10
2	E12	E12-2, E12-21, E12-26, E12-30, E12-33, E12-34, E12-38	7
3	E16	E16-6, E16-8, E16-10, E16-11, E16-20, E16-28, E16-31, E16-38, E16-39	9

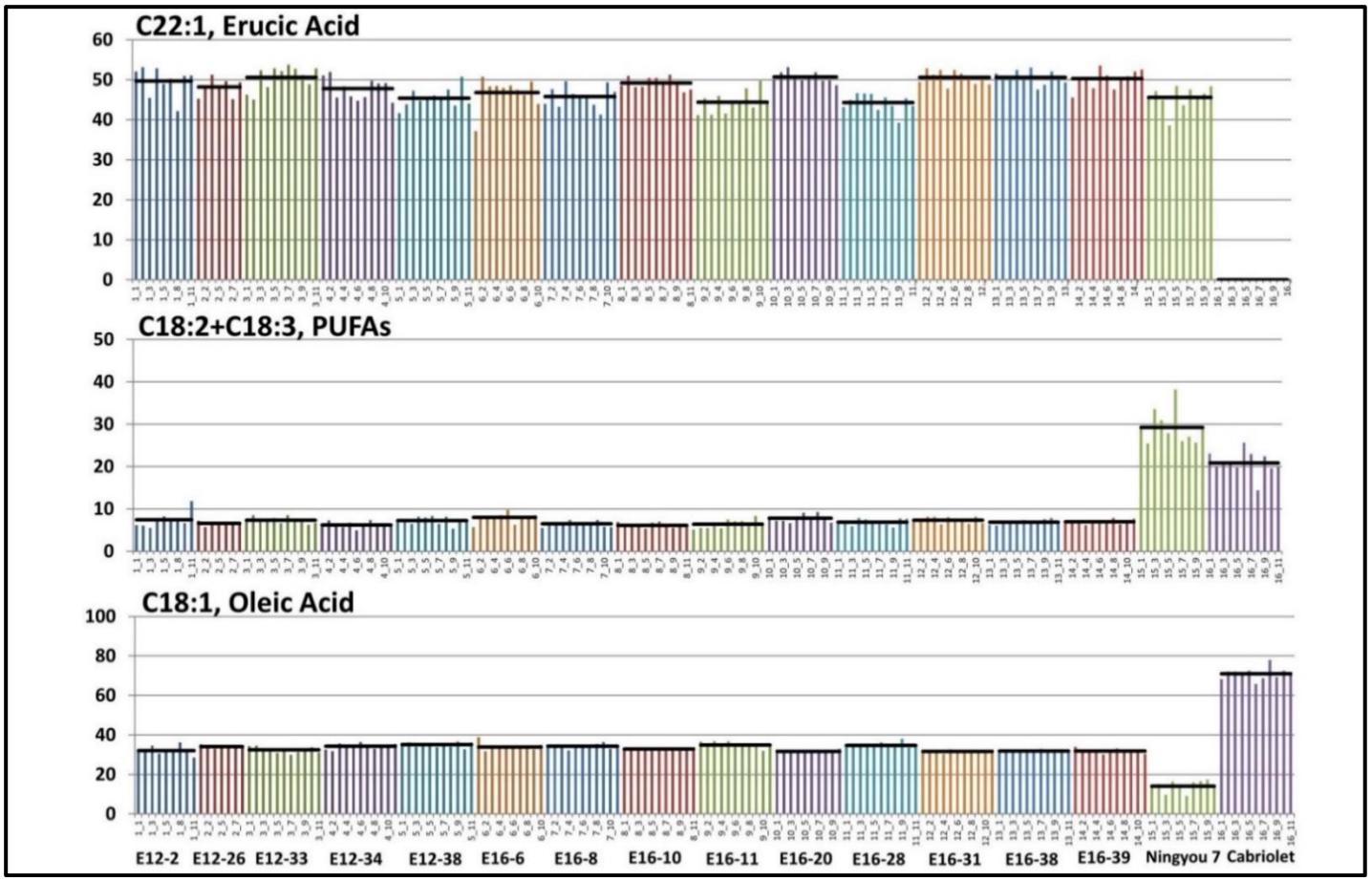

Figure 4.6 Erucic acid, PUFAs and oleic acid percentages of HELP lines ($F_1B_1S_3$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)') and controls

The fatty acids were measured by the single seed method using 11 replicates each from 14 HELP lines ($F_1B_1S_3$ seeds) and controls, Ningyou 7 and Cabriolet. Erucic acid, PUFAs (linoleic acid and linolenic acid) and oleic acid percentages are shown. The bar on the top of each group shows the mean values and each vertical bar represents the fatty acid percentage measured from a single seed.

4.4.4 $F_1B_1S_3$ Generation

4.4.4.1 Single Seed Fatty Acids Analysis

During the growth of $F_1B_1S_2$ lines in the glasshouse, there was thrips (minute insects of order Thysanoptera that causes damage to the plant parts while feeding on them, damage to plants is shown in Appendix V) infestation in the glasshouse that resulted in a high damage to the HELP lines and thus, sufficient seeds were not harvested from the thrips infested lines. Enough quantities were not available for the fatty acid analysis of some lines. In addition, some lines matured late. So, the fatty acids were analysed on 14 HELP lines along with the controls NY7 and Cabriolet by the method described in Section 2.7.1. There were analysed in 11 replicates each. The results of erucic acid, PUFAs and oleic acid content are shown in Figure 4.6.

Table 4.6 Mean fatty acid percentages (11 biological replicates) of HELP lines ($F_1B_1S_3$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)') and controls

S. No.	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
1	E12-2	2.9	0.4	0.6	32.1	2.1	5.4	0.5	5.0	0.4	49.6	0.9
2	E12-26	2.8	0.2	0.7	34.2	2.0	4.5	0.6	5.5	0.5	48.1	0.8
3	**E12-33**	2.8	0.3	0.5	32.4	1.8	5.5	0.5	4.3	0.3	**50.5**	0.9
4	E12-34	3.1	0.4	0.6	34.2	1.8	4.3	0.5	6.1	0.3	47.8	0.8
5	E12-38	2.8	0.3	0.7	35.1	2.1	5.1	0.6	7.0	0.3	45.4	0.7
6	E16-6	3.0	0.5	0.7	33.9	2.2	5.7	0.5	5.5	0.4	46.8	0.8
7	E16-8	3.0	0.4	0.7	34.2	1.9	4.5	0.5	8.1	0.3	45.7	0.6
8	E16-10	3.0	0.4	0.7	32.9	1.8	4.3	0.6	6.2	0.3	49.2	0.6
9	E16-11	3.0	0.4	0.7	35.0	1.9	4.5	0.5	8.6	0.3	44.4	0.7
10	**E16-20**	3.0	0.5	0.6	31.7	2.1	5.7	0.6	3.9	0.5	**50.7**	0.8
11	E16-28	3.1	0.4	0.8	34.8	2.0	4.8	0.6	8.2	0.3	44.3	0.6
12	**E16-31**	2.8	0.4	0.6	31.7	2.0	5.3	0.5	4.9	0.3	**50.6**	0.7
13	**E16-38**	2.9	0.4	0.7	31.8	2.0	4.9	0.6	5.2	0.3	**50.6**	0.7
14	**E16-39**	2.9	0.4	0.7	31.8	1.9	5.0	0.6	5.4	0.3	**50.2**	0.7
15	NY7	3.9	0.3	0.8	15.5	17.5	11.3	0.5	4.8	0.6	43.8	1.0
16	Cabriolet	4.9	0.3	0.8	71.1	10.4	10.4	0.4	1.4	0.2	0.0	0.1

Lines in **bold** were selected for multiplication

It could be observed that in each HELP line, erucic acid and PUFA contents were consistent in the individual seeds (Figure 4.6). The detailed single seed analysis results

are provided in Appendix VI. In the individual seeds of HELP lines, up to 8% increase in erucic acid was found as compared to the high erucic cultivar, Ningyou 7. PUFAs content was found to be between 5 to 7% in the HELP lines as compared to 30% in NY7. A significant increase of 17 to 20% was found in the oleic acid levels in the HELP lines in comparison to NY7. The mean values of the single seed fatty acid data are given in Table 4.6. Mean erucic acid content was also higher and mean PUFAs were lower in the HELP lines as compared to NY7. Mean oleic acid in HELP lines was higher as compared to NY7.

4.4.4.2 Selection and Multiplication

Based on the fatty acid results, HELP lines were selected for multiplication in the next generation. Five genotypes – E12-33, E16-20, E16-31, E16-38 and E16-39 (shown in bold in Table 4.6) were selected. These had approx. 6% (average) more erucic acid than the parental variety, Ningyou 7. Another two genotypes, E12-38 and E16-28 were also selected having similar erucic content as NY7 (Table 4.6). So, these 7 HELP lines were sown in 10 replicates each with the high erucic acid cultivar, NY7 in the glasshouse. These were self-pollinated and seeds were harvested at the maturity from the individual plants.

4.4.4.3 Bulk Seeds Fatty Acid Analysis

Single seed method for the fatty acid analysis is a powerful method for observing the segregation in the individual seeds in a progeny. But for the commercial applications, it is preferable to bulk up the seeds for the fatty acids measurements. So, bulk seeds FAMEs method (Section 2.7.2) was tested on the HEAR line, Maplus and then used for the fatty acid composition analysis of 22 HELP lines along with 3 controls lines – Maplus, Ningyou 7 and Cabriolet. Two technical replications were used for each sample and the results are shown in Table 4.7. Up to 58% erucic levels were found in many HELP lines (E6-5, E6-39 and E16-20) as compared to 50% in the HEAR cultivars – Maplus and NY7. So, a similar increase of 8% in erucic acid was found as measured by the single seed method. The comparison of the single and bulk seeds methods of the fatty acid analysis of these lines is provided in Appendix VII. It could be observed

that the bulk seed method gives more real values of the fatty acid percentages than calculating mean values from the single seed measurements. PUFAs levels were reduced to 4-5% in the HELP lines as compared to 22-24% in the high erucic acid cultivars. Highest levels of very long chain fatty acids were more than 65% in the HELP lines. Saturated fatty acid values of HELP lines were found to be slightly lower (~2%) than the HEAR cultivars. This change in the fatty acid composition of HELP lines is the effect of partially functional *Bna.FAD2* family. It decreased the PUFAs, increased the VLCFAs pool and also increased the oleic acid content in the HELP lines.

4.4.4.4 Flowering Time Test on HELP Lines

In Europe, winter varieties of rapeseed (WOSR, winter oilseed rape) are preferred due to their higher yield over the spring varieties (Röbbelen, 1991; McVetty *et al.*, 2016). These vary in their time period for vernalisation (cold-period).

WOSR requires a period of vernalisation for initiation of flowering and have a longer growth period. Spring varieties have no such requirement of vernalisation and semi-winter types require very small or no vernalisation period. Parent, Ningyou 7 was a semi-winter type while Cabriolet was a winter-type. So, the flowering time test was conducted on HELP lines to know their flowering behaviour – spring, winter or semi-winter type.

Twenty-two HELP lines (fatty acid profiles known) with five replicates each (named A to E) and 3 HELP lines (fatty acid profile unknown due to insufficient seeds) with one replicate each were grown with the controls – Ningyou 7, Maplus and Cabriolet. These plants were not vernalised and the flowering time was reported (Figure 4.7). Eleven HELP lines flowered at nearly the same time as NY7 (Table 4.8), so these lines were categorized as semi-winter types. Ten lines did not flower even after 77 days after sowing, so these lines were considered as winter types as depicted in Table 4.8. Four HELP lines segregated for their flowering behaviour. The semi-winter HELP lines and NY7 were self-pollinated and the seeds were harvested at the maturity. These had very poor yield and only a few seeds were harvested. Winter type HELP lines were vernalised along with winter type controls – Maplus and Cabriolet. These were self-pollinated on the flowering stage and the seeds were harvested at the maturity.

Table 4.7 The fatty acid percentages (mean of 2 technial replicates) of HELP lines ($F_1B_1S_3$ seeds of cross 'Cabriolet x (K0472 x NY7)') and controls

S. No	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA[+]	VLCFA[+]	SAFA[+]
1	E6-10	0.0	1.9	0.3	0.5	26.1	1.5	3.3	0.5	9.0	0.0	0.3	55.9	0.0	0.7	4.9	65.6	3.2
2	E6-15	0.0	1.8	0.2	0.6	26.8	1.5	3.0	0.6	8.8	0.0	0.3	55.8	0.0	0.6	4.4	65.2	3.3
3	E6-19	0.0	2.0	0.1	0.6	26.1	1.7	3.7	0.6	8.0	0.0	0.4	56.2	0.0	0.7	5.4	64.9	3.5
4	E6-24	0.1	2.1	0.3	0.5	26.6	1.6	3.3	0.5	8.5	0.0	0.3	55.4	0.0	0.7	5.0	64.6	3.6
5	E6-31	0.0	1.8	0.2	0.5	25.9	1.6	3.4	0.5	7.7	0.0	0.4	57.2	0.0	0.7	5.0	65.7	3.2
6	E6-34	0.0	2.0	0.2	0.5	26.1	1.8	3.8	0.5	8.3	0.0	0.3	55.6	0.0	0.7	5.6	64.6	3.4
7	E6-39	0.0	1.8	0.2	0.5	25.7	1.6	3.6	0.5	7.1	0.0	0.4	57.7	0.0	0.7	5.3	65.6	3.2
8	E6-5	0.0	1.8	0.2	0.5	26.0	1.4	3.6	0.5	6.6	0.0	0.4	57.9	0.0	0.9	5.0	65.4	3.3
9	E12-2	0.0	2.2	0.1	0.6	26.6	1.5	3.5	0.6	6.2	0.0	0.4	57.1	0.0	1.1	5.0	64.4	3.8
10	E12-26	0.0	2.1	0.0	0.7	28.7	1.5	3.2	0.7	7.2	0.0	0.6	54.2	0.0	1.1	4.7	62.4	4.2
11	E12-33	0.0	2.0	0.2	0.6	27.9	1.3	3.1	0.5	7.6	0.0	0.4	55.2	0.0	1.2	4.4	64.0	3.5
12	E12-34	0.0	2.1	0.2	0.5	26.8	1.4	3.0	0.6	7.0	0.0	0.5	56.7	0.1	1.1	4.4	64.8	3.8
13	E12-38	0.0	1.8	0.2	0.6	28.1	1.3	2.9	0.6	8.8	0.0	0.4	54.1	0.1	1.0	4.2	64.0	3.6
14	E16-10	0.0	2.3	0.3	0.7	27.8	1.3	2.8	0.6	9.3	0.0	0.4	53.7	0.1	0.8	4.1	63.8	4.0
15	E16-11	0.0	2.2	0.2	0.7	30.1	1.3	2.9	0.6	10.6	0.0	0.3	50.2	0.0	0.9	4.2	61.7	3.8
16	E16-20	0.0	2.1	0.3	0.6	25.8	1.4	3.5	0.6	6.5	0.0	0.5	57.5	0.1	1.0	5.0	65.0	4.0
17	E16-28	0.0	2.2	0.2	0.7	28.2	1.4	3.2	0.6	8.8	0.0	0.4	53.3	0.1	0.9	4.6	63.0	4.0
18	E16-31	0.0	2.1	0.2	0.6	26.7	1.4	3.3	0.6	7.4	0.0	0.4	56.5	0.1	0.9	4.7	64.8	3.7
19	E16-38	0.0	2.1	0.3	0.7	26.2	1.4	3.4	0.6	7.5	0.0	0.5	56.3	0.0	1.0	4.8	64.8	3.9
20	E16-39	0.0	2.1	0.2	0.7	27.5	1.4	3.1	0.6	8.9	0.0	0.4	54.3	0.1	0.9	4.4	64.1	3.8
21	E16-6	0.0	2.1	0.3	0.7	26.8	1.4	3.2	0.6	8.0	0.0	0.4	55.6	0.1	0.9	4.6	64.5	3.8
22	E16-8	0.0	2.2	0.2	0.7	28.6	1.4	2.9	0.6	10.0	0.0	0.4	52.1	0.0	0.9	4.3	63.0	3.8
23	Maplus	0.1	4.0	0.3	0.7	9.2	14.4	9.6	0.6	8.2	0.5	0.6	50.9	0.2	0.9	23.9	59.9	6.1
24	NY7	0.1	3.0	0.1	0.8	13.5	13.6	8.6	0.6	6.1	0.4	0.8	50.8	0.3	1.3	22.2	58.2	5.5
25	Cab	0.1	4.6	0.3	0.9	73.3	9.1	8.9	0.4	1.6	0.0	0.3	0.0	0.2	0.2	18.0	1.8	6.6

[+]PUFA=18:2+18:3; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0; NY7 is Ningyou 7 and; Cab is Cabriolet

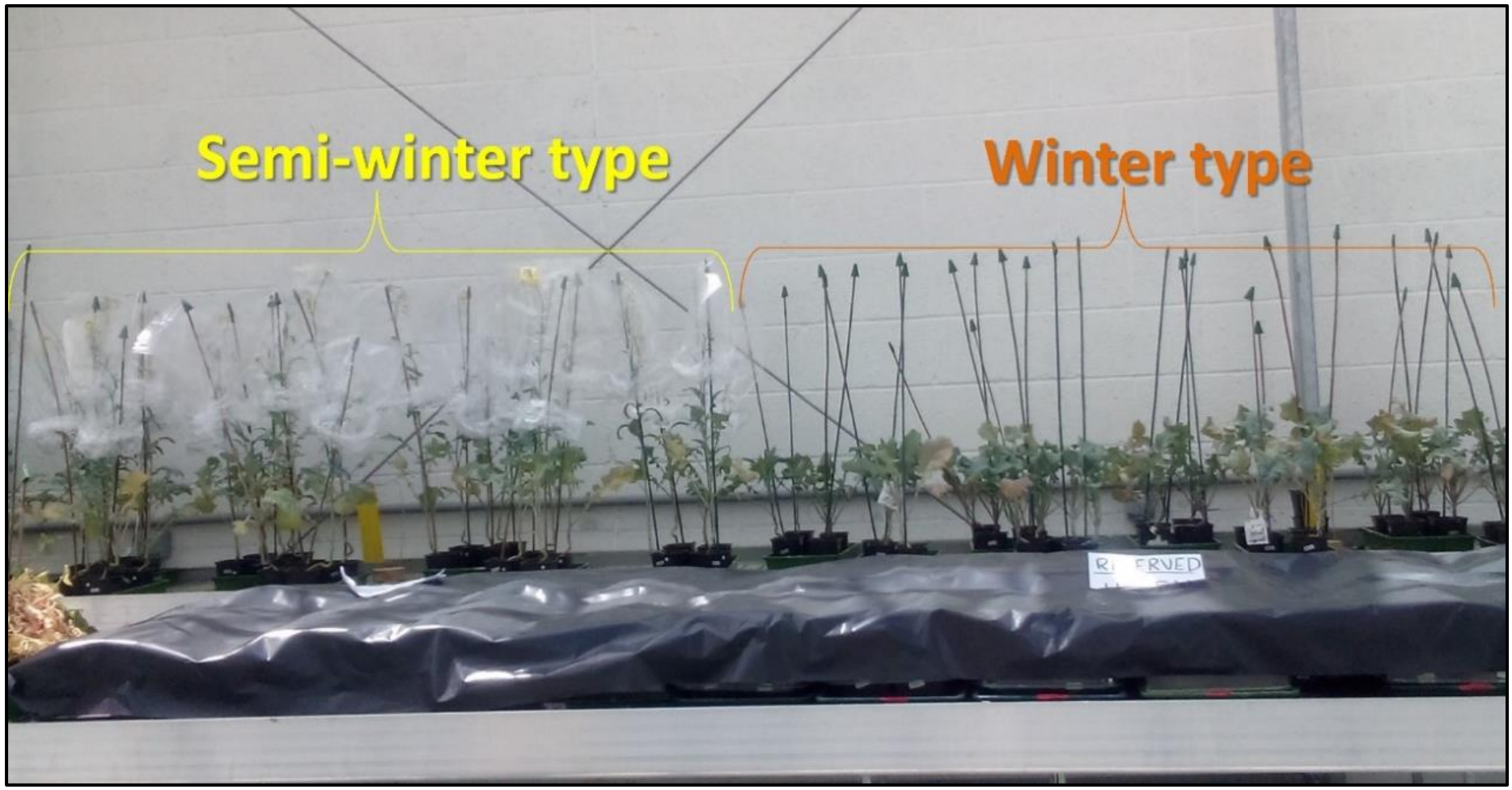

Figure 4.7 Flowering time test on the HELP lines ($F_1B_1S_3$ progeny of the cross 'Cabriolet x (K0472 x Ningyou 7)') in the glasshouse

Semi-winter type are shown on the left and flowered without vernalisation. These were bagged for self-pollination. Winter-type are shown on the right and did not flower without vernalisation after 77 days of sowing

Table 4.8 Flowering-type test of HELP lines ($F_1B_1S_3$ progeny of the cross 'Cabriolet x (K0472 x Ningyou 7)') and controls

S. No	Semi-winter type	Winter type	Mixed
1	E6-5 (A-E)[+]	E6-15 (A-E)	E6-24 (D,E)*; (A,B,C)**
2	E6-10 (A-E)	E6-19 (A-E)	E6-31 (D)*; (A,B,C,E)**
3	E16-6 (A-E)	E12-2 (A-E)	E6-34 (C,D,E)*; (A,B)**
4	E16-8 (A-E)	E12-26 (A-E)	E6-39 (A,C,D,E)*; (B)**
5	E16-10 (A-E)	E12-33 (A-E)	
6	E16-11 (A-E)	E12-34 (A-E)	
7	E16-20 (A-E)	E12-38 (A-E)	
8	E16-28 (A-E)	E6-16 (A-B)	
9	E16-31 (A-E)	E12-21 (A-B)	
10	E16-38 (A-E)	E12-30 (A-B)	
11	E16-39 (A-E)		
Controls	Ningyou 7	Maplus, Cabriolet	

[+]A-E is used for coding 5 plants from 'A to E'; *semi-winter type and; **winter type

4.4.5 $F_1B_1S_4$ generation

4.4.5.1 Fatty Acid Analysis

The fatty acid profiles were analysed from 29 winter type HELP lines harvested from the flowering time experiment (Section 4.4.4.4) and 18 winter type HELP lines multiplied in the Section 4.4.4.2 by the bulk seeds method (Section 2.7.2). The fatty acids were analysed in two different batches. Cultivars – Cabriolet, Ningyou 7 and Maplus were used as the controls for the fatty acid analysis. Three technical replicates were used for each sample and the results are shown in Table 4.9. One example of the outputs of the gas chromatography for a HELP line is shown in Figure 4.8.

HELP lines showed the same trend of high erucic acid content and low PUFAs in the seeds as observed in the previous generation. Erucic acid levels of many HELP lines were 8 to 9% higher than the high erucic parent NY7. The value ranged from 50 to 58.5% for the individual HELP lines as compared to 46 to 51% for high erucic cultivars. Figure 4.9 shows the frequency histogram for the VLCFAs and PUFAs of HELP lines. It could be observed that most of these HELP lines had VLCFAs between 62 to 63% and PUFA content between 4.5 to 5%. PUFA levels were uniform and lower than 6.5% in HELP lines. HELP lines had linoleic acid (C18:2) lower than 2% and linolenic acid (C18:3) lower than 5%.

Table 4.9 The fatty acid percentages (mean of 3 technical replicates) of HELP lines ($F_1B_1S_4$ seeds of cross 'Cabriolet x (K0472 x NY7)') and controls

S. No.	Genotype	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1	PUFA*	VLCFA*	SAFA*
	Seeds from flowering time experiment (Section 4.4.4.4)																	
1	E6-15-B	0.0	2.2	0.3	0.7	28.1	1.8	3.8	0.5	9.3	0.0	0.3	52.6	0.0	0.5	5.6	62.4	3.7
2	E6-15-D	0.0	2.1	0.2	0.6	26.8	1.8	4.0	0.5	8.5	0.0	0.3	54.4	0.0	0.6	5.9	63.5	3.6
3	E6-19-B	0.0	2.2	0.3	0.7	27.7	2.1	4.4	0.5	9.0	0.0	0.4	52.1	0.0	0.7	6.4	61.8	3.8
4	E6-19-D	0.0	2.1	0.2	0.6	26.3	1.7	4.2	0.5	7.9	0.0	0.4	55.4	0.0	0.6	5.9	63.9	3.6
5	E6-24-B	0.0	2.2	0.2	0.7	29.0	1.5	3.4	0.5	11.3	0.0	0.3	50.4	0.0	0.6	4.9	62.3	3.6
6	E6-24-C	0.0	2.2	0.3	0.7	27.7	1.8	3.8	0.6	8.6	0.0	0.4	53.5	0.0	0.6	5.6	62.7	3.8
7	E6-31-A	0.0	2.0	0.3	0.6	26.6	1.9	4.2	0.6	7.1	0.0	0.4	55.6	0.0	0.7	6.1	63.4	3.6
8	E6-31-E	0.0	2.1	0.2	0.6	26.4	1.7	4.3	0.5	7.3	0.0	0.4	55.7	0.0	0.6	6.0	63.6	3.7
9	E6-34-A	0.0	2.3	0.2	0.6	27.0	1.7	4.5	0.5	9.8	0.0	0.3	52.5	0.0	0.6	6.2	62.8	3.7
10	E6-34-B	0.0	2.3	0.3	0.6	26.8	1.8	4.6	0.5	9.3	0.0	0.3	53.0	0.0	0.6	6.4	63.0	3.6
11	E12-2-B	0.0	2.2	0.2	0.6	28.0	1.6	4.0	0.5	7.6	0.0	0.4	54.1	0.0	0.9	5.6	62.6	3.7
12	E12-21-A	0.0	2.4	0.2	0.7	29.9	1.6	3.6	0.6	7.5	0.0	0.5	51.9	0.0	1.0	5.2	60.4	4.2
13	E12-26-B	0.0	2.5	0.1	0.8	28.6	1.8	4.2	0.7	7.4	0.0	0.5	52.4	0.0	0.9	6.0	60.8	4.5
14	E12-26-D	0.0	2.4	0.3	0.8	30.0	1.7	3.8	0.6	7.3	0.0	0.5	51.4	0.0	1.1	5.5	59.8	4.4
15	E12-33-A	0.1	2.5	0.3	0.7	29.6	1.6	3.4	0.6	8.9	0.0	0.3	51.2	0.0	0.9	4.9	61.0	4.2
16	E12-33-B	0.0	2.3	0.3	0.7	29.4	1.4	3.4	0.6	8.1	0.0	0.5	52.3	0.0	0.9	4.9	61.4	4.1
17	E12-33-C	0.0	2.3	0.3	0.6	28.2	1.6	4.0	0.5	6.9	0.0	0.4	54.1	0.0	1.1	5.6	62.1	3.8
18	E12-33-D	0.0	2.3	0.2	0.8	29.3	1.5	3.3	0.7	8.5	0.0	0.4	52.2	0.0	0.9	4.8	61.6	4.1
19	E12-33-E	0.0	2.4	0.2	0.6	28.7	1.5	3.6	0.5	8.4	0.0	0.4	52.6	0.1	1.0	5.1	61.9	4.0
20	E12-34-A	0.0	2.6	0.4	0.6	26.8	1.8	4.7	0.6	5.8	0.0	0.5	55.1	0.0	1.1	6.5	62.1	4.2
21	E12-34-B	0.0	2.6	0.4	0.7	28.8	1.6	3.7	0.5	8.8	0.0	0.3	51.7	0.0	0.9	5.3	61.4	4.1
22	E12-34-C	0.0	2.4	0.3	0.6	27.4	1.5	3.7	0.6	6.4	0.0	0.5	55.5	0.0	1.0	5.2	62.9	4.1
23	E12-34-D	0.0	2.6	0.4	0.6	28.4	1.7	3.9	0.6	7.4	0.0	0.4	53.0	0.0	1.0	5.6	61.3	4.3
24	E12-34-E	0.0	2.4	0.3	0.5	26.5	1.4	3.7	0.5	5.5	0.0	0.5	57.6	0.0	1.0	5.1	64.1	4.0
25	E12-38-A	0.0	2.4	0.2	0.7	30.2	1.3	3.2	0.5	9.0	0.0	0.4	51.2	0.1	0.9	4.5	61.1	4.0
26	E12-38-B	0.0	2.3	0.2	0.6	28.9	1.5	3.5	0.5	8.0	0.0	0.4	53.1	0.1	0.9	4.9	62.0	3.9

*PUFA=18:2+18:3; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0

Continued from Table 4.9

S. No.	Genotype	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1	PUFA*	VLCFA*	SAFA*
27	E12-38-C	0.0	2.2	0.2	0.6	28.5	1.4	3.5	0.5	7.8	0.0	0.4	53.9	0.0	0.9	4.9	62.6	3.7
28	E12-38-D	0.0	2.2	0.3	0.7	28.3	1.7	3.9	0.5	8.0	0.0	0.4	53.1	0.0	0.9	5.6	62.0	3.8
29	E12-38-E	0.0	2.2	0.2	0.7	27.7	1.6	3.8	0.5	7.8	0.0	0.4	54.3	0.0	0.9	5.3	63.0	3.8
30	Maplus	0.0	4.4	0.3	0.8	10.7	15.5	10.2	0.6	7.3	0.4	0.6	48.5	0.1	0.7	25.7	56.5	6.3
31	Ningyou 7	0.0	3.2	0.2	0.8	12.0	14.2	11.8	0.5	6.0	0.4	0.8	48.7	0.3	1.1	26.0	55.7	5.6
32	Cabriolet	0.0	4.6	0.3	1.1	73.5	9.0	9.6	0.4	1.2	0.0	0.3	0.0	0.0	0.0	18.5	1.2	6.4
	Seeds from HELP lines multiplication (Section 4.4.4.2)																	
33	E12-33-1	0.0	2.2	0.3	0.5	26.3	1.3	3.7	0.5	5.5	0.0	0.5	58.1	0.1	1.1	4.9	64.7	3.8
34	E12-33-2	0.0	2.3	0.3	0.6	27.4	1.4	3.7	0.8	5.9	0.0	0.7	55.8	0.0	1.1	5.1	62.8	4.4
35	E12-33-3	0.0	2.3	0.3	0.6	26.3	1.3	3.7	0.5	5.0	0.0	0.5	58.5	0.1	1.0	5.0	64.5	4.0
36	E12-33-4	0.0	2.2	0.2	0.7	27.7	1.3	3.5	0.7	6.9	0.0	0.5	55.0	0.2	1.1	4.8	63.0	4.3
37	E12-33-5	0.0	2.2	0.3	0.6	27.2	1.3	3.3	0.5	6.2	0.0	0.5	56.9	0.0	1.0	4.7	64.1	3.8
38	E12-33-6	0.0	2.1	0.2	0.6	26.7	1.2	3.8	0.6	5.9	0.0	0.5	57.3	0.1	1.0	5.0	64.3	3.8
39	E12-33-7	0.0	2.4	0.3	0.6	26.7	1.3	3.6	0.5	6.2	0.0	0.5	57.1	0.0	0.9	4.9	64.2	3.9
40	E12-33-8	0.0	2.1	0.2	0.5	26.7	1.3	3.5	0.5	5.8	0.0	0.5	57.8	0.1	1.0	4.8	64.6	3.7
41	E12-33-9	0.0	2.4	0.2	0.6	27.7	1.5	4.2	0.5	6.4	0.0	0.5	54.9	0.0	1.1	5.7	62.4	4.1
42	E12-33-10	0.0	2.2	0.3	1.0	30.7	1.1	2.7	0.7	9.8	0.0	0.5	50.0	0.1	0.9	3.8	60.7	4.5
43	E12-33-11	0.1	2.7	0.4	1.5	28.1	1.3	3.1	0.5	6.8	0.1	0.4	54.0	0.1	0.9	4.4	61.8	5.2
44	E12-38-2	0.0	2.4	0.3	0.9	30.1	1.3	3.1	0.6	8.6	0.0	0.4	51.7	0.0	0.6	4.4	60.8	4.4
45	E12-38-3	0.0	2.2	0.3	0.7	28.2	1.3	3.6	0.6	6.9	0.0	0.4	55.0	0.0	0.8	4.9	62.7	4.0
46	E12-38-4	0.0	2.3	0.3	0.7	27.7	1.4	3.8	0.6	7.0	0.0	0.5	54.7	0.1	0.8	5.2	62.5	4.2
47	E12-38-6	0.0	2.7	0.3	0.7	28.7	1.3	3.4	0.6	7.7	0.0	0.5	53.3	0.0	0.8	4.7	61.8	4.5
48	E12-38-7	0.0	2.4	0.2	1.0	30.4	1.2	3.0	0.7	9.5	0.0	0.5	50.4	0.0	0.6	4.2	60.5	4.7
49	E12-38-9	0.0	2.3	0.1	1.0	30.5	1.3	3.2	0.7	9.8	0.0	0.4	50.2	0.0	0.6	4.5	60.6	4.4
50	E12-38-10	0.0	2.2	0.3	0.8	28.5	1.2	3.2	0.7	7.5	0.0	0.5	54.4	0.0	0.7	4.5	62.6	4.2
51	Maplus	0.0	4.2	0.3	0.7	9.4	15.0	10.7	0.6	6.6	0.4	0.5	50.8	0.0	0.7	25.7	58.2	6.0
52	Ningyou 7	0.0	3.4	0.2	1.0	12.6	13.6	11.6	0.6	8.6	0.5	0.6	46.3	0.2	0.8	25.2	55.8	5.7

*PUFA=18:2+18:3; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0

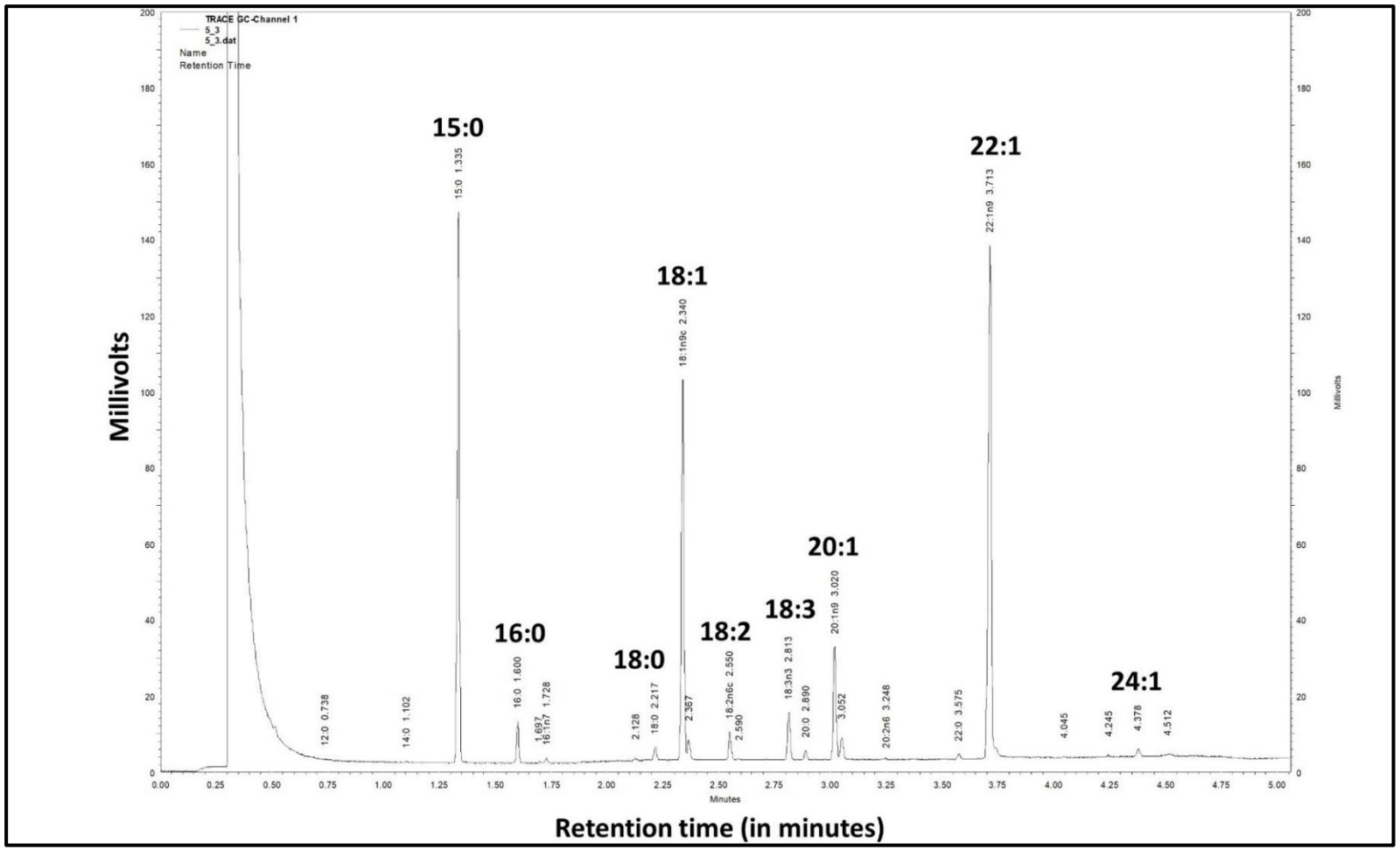

Figure 4.8 Gas chromatogram showing peaks of various fatty acids present in a HELP line

Gas chromatography output showing the retention time on the x-axis and peaks of the various fatty acids. The height of the peak corresponds to the amount of the fatty acid. The peak corresponding to 15:0 shows the internal standard used for the GC analysis

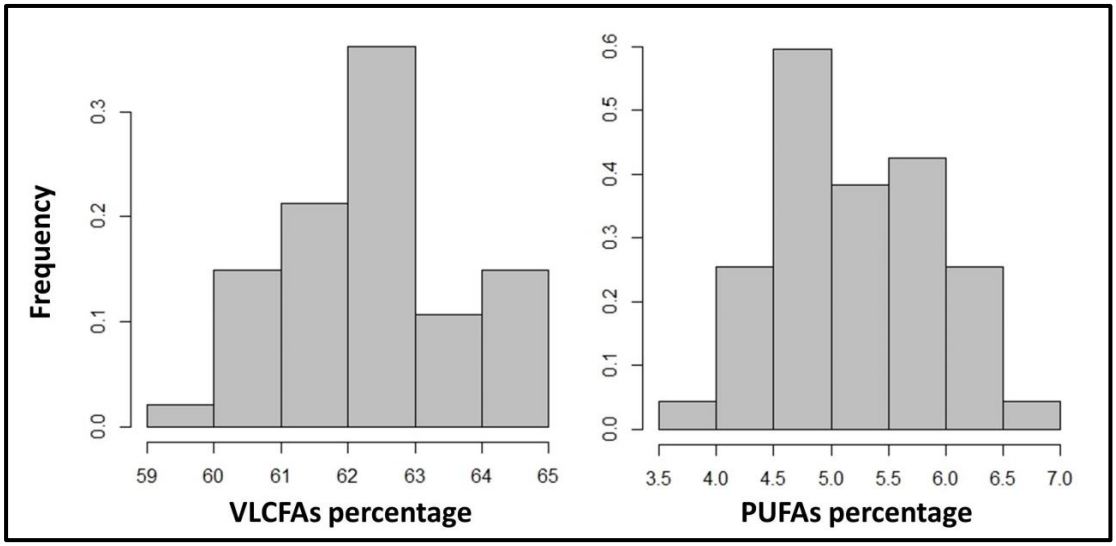

Figure 4.9 Histograms of VLCFAs and PUFAs percentage of HELP lines ($F_1B_1S_4$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)')

The frequency histogram for the VLCFAs (left) and PUFAs (right) of HELP lines. HELP lines had VLCFAs between 59 to 65% while PUFAs between 3.5 to 7%

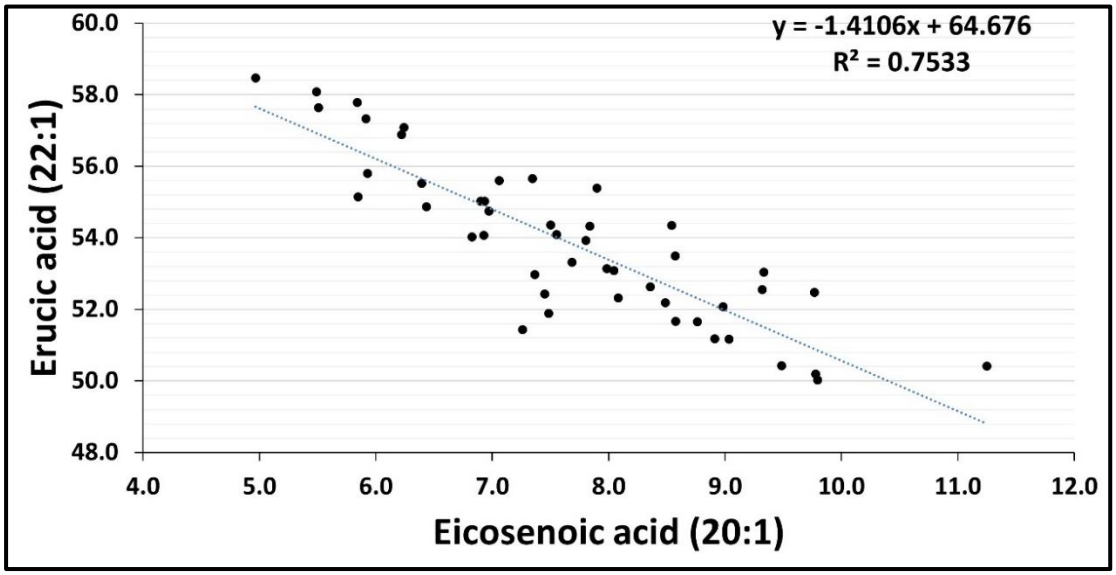

Figure 4.10 Scatter-plot and fitted regression line of erucic acid and eicosenoic levels in HELP lines ($F_1B_1S_4$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)')

The regression plot of erucic acid (C22:1) and eicosenoic acid (C20:1) values of HELP lines. An R^2 value of 0.7533 was observed, showing a decrease in the erucic acid levels with an increase in eicosenoic acid levels and vice-versa

The non-uniformity in the EA levels in HELP lines could be due to same enzymes used for the elongation steps of both eicosenoic acid (C20:1) and erucic acid in *B. napus* (Kondra and Stefansson, 1965; Jonsson, 1977). In many HELP lines, *Bna.FAE1* elongated oleic acid to eicosenoic acid but then the elongation did not continue to the erucic acid, thus making the levels of erucic acid and eicosenoic acid non-uniform

in HELP genotypes. Figure 4.10 shows the regression plot of erucic acid and eicosenoic acid levels in HELP lines and an R^2 value of 0.7533 was reported. It could be observed that a decrease in eicosenoic acid levels caused an increase in erucic acid levels and thereby, consistent levels of very long chain fatty acids (sum of C20:1, C22:1 and C24:1) were found in the HELP lines. Similar results of negative correlation were observed between eicosenoic acid and erucic acid levels by Sasongko and Möllers, 2005. Moreover, the studies have shown the epistasis effect for the inheritance of eicosenoic acid in rapeseed and thus, its levels are hard to control among the generations (Coonrod *et al.*, 2008).

The high VLCFAs values found in the HELP lines were very close to the maximum theoretical limit of 66% possible in the rapeseed (Cao, Oo and Huang, 1990) by using the non-transgenic methods. This was due to the low affinity of *Bn-LPAAT* to add the erucic acid to the central position of the TAG molecule (Bernerth and Frentzen, 1990). Due to partially functional *Bna.FAD2* copies, oleic acid was not used in the desaturation pathway and thus, higher levels (26 to 30%) were found in the HELP lines as compared to the high erucic acid varieties (11 to 12%).

4.4.5.2 Glucosinolates Analysis

Glucosinolates content was measured in 10 HELP lines and parents – Ningyou 7, K0472 and Cabriolet with two technical replicates each as shown in Table 4.10. Parental variety, NY7 had high total seed glucosinolates (83 $\mu molg^{-1}$) while other parents, K0472 (12 $\mu molg^{-1}$) and Cabriolet (19 $\mu molg^{-1}$) had a lower amount of total seed glucosinolates. So, a range of values of the total glucosinolates (54 to 113 µmol/g) was found in the HELP lines.

4.4.5.3 Multiplication of HELP Lines

Winter type HELP lines having sufficient seeds, E12-33 (1 to 10) and E12-38 (2, 4, 7, 9 and 10) were grown for the multiplication in the glasshouse and transplanted in the outside plots (replicated field conditions) at the University of York (walled gardens) in the 2016-17 season as shown in Figure 4.11 (maintained by Natalia Stawniak, Bancroft group). The individual plants were bagged for self-pollination and the seeds were harvested at the maturity.

Table 4.10 Glucosinolates measurements (µmol/g) of HELP lines ($F_1B_1S_4$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)') and controls

S. No.	Genotype	Aliphatic GLS	Indole GLS	Aromatic GLS	Total GLS
1	E12-33-3	54.30	0.20	0.87	55.37
2	E12-33-7	57.65	0.27	1.32	59.24
3	E12-38-10	56.82	0.53	1.96	59.31
4	E12-33-8	58.65	0.22	1.24	60.12
5	E12-33-6	64.34	0.29	1.14	65.77
6	E12-33-1	65.40	0.24	1.27	66.90
7	E6-31-A	65.53	0.37	1.25	67.16
8	E6-31-E	96.89	0.33	1.24	98.47
9	E12-34-E	99.47	0.54	3.37	103.37
10	E6-19-D	113.13	0.69	1.30	115.12
11	Ningyou 7	82.86	0.28	1.38	84.52
12	K0472	12.39	0.44	0.14	12.84
13	Cabriolet	19.19	0.97	0.33	20.16

GLS is glucosinolates. The values represented are mean of two technical replicates.

Figure 4.11 HELP plants ($F_1B_1S_4$ progeny) growing in the replicated field plots

HELP lines ($F_1B_1S_4$ plants of the cross 'Cabriolet x (K0472 x Ningyou 7)') were grown in the replicated field conditions at the walled gardens, University of York in the season 2016-17.

4.4.6 $F_1B_1S_5$ Generation

4.4.6.1 Fatty Acid Analysis

HELP lines were harvested from the walled gardens (University of York, UK) and the fatty acid profiles of 9 HELP lines were measured with control NY7 using the bulk seeds method described in Section 2.7.2. Three technical replicates were used for each sample and the detailed results are depicted in Table 4.11.

Table 4.11 The fatty acid percentages (mean of 3 technical replicates) of the HELP lines ($F_1B_1S_5$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)') and control

S. No.	Genotype	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1
1	E12-33-1-1	0.0	2.0	0.3	0.6	26.1	1.3	3.9	0.5	5.2
2	E12-33-2-1	0.0	2.2	0.3	0.6	25.4	1.5	4.9	0.5	4.7
3	E12-33-2-2	0.0	2.1	0.3	0.6	25.6	1.5	4.6	0.5	4.8
4	E12-33-2-3	0.0	1.9	0.3	0.5	24.0	1.6	5.1	0.5	4.0
5	E12-33-3-1	0.0	2.2	0.3	0.6	25.5	1.2	3.8	0.5	4.5
6	E12-33-10-1	0.0	2.0	0.3	0.6	25.9	1.1	3.7	0.6	4.9
7	E12-33-10-2	0.0	1.9	0.3	0.5	25.4	1.4	4.4	0.5	4.9
8	E12-33-10-3	0.1	2.0	0.3	0.6	26.3	1.2	3.7	0.6	5.4
9	E12-33-10-4	0.0	2.2	0.3	0.7	26.5	1.5	4.2	0.5	5.8
10	Ningyou 7	0.0	3.4	0.2	1.0	12.6	13.6	11.6	0.6	8.6
S. No.	**Genotype**	**20:2**	**22:0**	**22:1**	**24:0**	**24:1**	**PUFA**	**VLCFA**	**SAFA**	**MUFA**
1	E12-33-1-1	0.0	0.5	58.5	0.1	1.0	5.2	64.8	3.7	91.1
2	E12-33-2-1	0.0	0.6	58.1	0.1	1.1	6.5	63.9	3.9	89.6
3	E12-33-2-2	0.0	0.5	58.4	0.0	1.3	6.0	64.4	3.7	90.4
4	E12-33-2-3	0.0	0.6	60.0	0.2	1.4	6.7	65.4	3.7	89.7
5	E12-33-3-1	0.0	0.5	59.4	0.1	1.2	5.0	65.2	4.0	90.9
6	E12-33-10-1	0.0	0.5	59.3	0.0	1.1	4.8	65.3	3.7	91.5
7	E12-33-10-2	0.0	0.5	58.9	0.1	1.2	5.9	65.0	3.5	90.7
8	E12-33-10-3	0.0	0.5	58.2	0.0	1.0	5.0	64.6	3.8	91.2
9	E12-33-10-4	0.0	0.5	56.9	0.0	1.0	5.7	63.7	3.8	90.5
10	Ningyou 7	0.5	0.6	46.3	0.2	0.8	25.2	55.8	5.7	68.5

PUFA=18:2+18:3; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0 and; MUFA= 16:1+18:1+20:1+22:1+24:1

These plants were grown in the replicated field conditions and the fatty acid profiles were more consistent than the previously observed results of the HELP lines. In the

HELP lines, erucic acid levels were between 57 to 60%, eicosenoic acid content was between 4 to 6%, PUFA levels were less than 7%, oleic acid values were between 24 to 26% and the very long chain fatty acid content was 64 to 65% in the HELP lines as depicted in Table 4.11. HELP, E12-33-1-1 and E12-33-3-1 lines had the similar fatty acid compositions as the parental lines, E12-33-1 and E12-33-3, respectively. HELP lines, E12-33-2-1 to 3, were the self-pollinated progeny of E12-33-2 and; VLCFAs and erucic acid values were higher in the progeny. Similarly, HELP lines, E12-33-10-1 to 4, were the self-pollinated progeny of E12-33-10 with higher values of EA and VLCFAs.

The fatty acid compositions of 19 HELP lines in this generation were also measured at the Biorenewables Development Centre (BDC), University of York, UK by Raymond Sloan and the results are shown in Table 4.12.

Table 4.12 The fatty acid percentages of the HELP lines ($F_1B_1S_5$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)') measured at the BDC, University of York

Genotype	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
E12-33-3-4	2.6	0.0	0.6	25.9	1.4	3.5	3.4	1.7	0.6	60.4
E12-33-4-2	1.6	0.0	0.6	26.2	1.4	3.5	3.0	1.6	0.7	61.4
E12-33-8-2	1.7	0.0	0.5	26.1	1.4	3.5	2.9	1.6	0.6	61.6
E12-33-8-1	1.4	0.0	0.6	26.6	1.3	3.2	3.9	1.3	0.7	61.0
E12-33-4-3	3.9	0.0	0.5	26.0	1.1	3.0	3.5	1.4	0.6	60.0
E12-33-9-1	1.9	0.3	0.6	26.5	1.5	3.7	2.9	1.5	0.7	60.4
E12-33-1-5	3.1	1.1	0.6	27.1	1.7	4.0	3.8	1.7	0.6	56.5
E12-33-5-1	1.7	0.0	0.6	26.9	1.5	3.5	3.5	1.7	0.6	60.0
E12-33-1-3	2.0	0.7	0.5	25.9	1.4	3.6	2.7	1.5	0.6	61.0
E12-33-1-4	1.4	0.0	0.5	26.6	1.4	3.6	3.2	1.6	0.6	61.1
E12-33-3-5	2.2	0.8	0.5	25.9	1.4	3.5	3.0	1.5	0.7	60.4
E12-33-4-4	1.7	0.0	0.6	27.0	1.3	3.2	3.8	1.6	0.6	60.1
E12-38-2-1	1.6	0.1	0.6	28.3	1.4	3.3	3.8	1.6	0.6	58.7
E12-38-10-2	1.6	0.2	0.6	27.0	1.5	3.6	3.9	1.6	0.6	59.5
E12-38-10-1	2.9	0.0	0.6	27.8	1.5	3.6	0.6	1.6	0.5	60.8
E12-38-9-2	1.7	0.2	0.6	27.2	1.5	3.5	4.3	1.7	0.6	58.8
E12-38-7-1	1.6	0.2	0.6	29.0	1.5	3.8	0.6	1.8	0.6	60.2
E12-38-10-4	3.0	0.0	0.6	26.3	1.6	3.8	3.3	1.6	0.0	59.9
E12-38-9-1	2.1	0.4	0.7	32.0	1.5	3.7	3.2	1.4	0.6	54.4

It could be observed that eicosenoic acid levels were very low in these lines (less than 2%) and higher erucic levels were observed as compared to levels in Table 4.11. The

differences within the same batches of HELP lines could be due to the different methods and different instruments used for the fatty acid analysis. In most of the HELP lines, the erucic acid levels were ~60% and highest value of more than 61% erucic acid was found in many HELP lines. Polyunsaturates were less than 6% in the HELP lines with the linoleic acid content of less than 2% and linolenic acid content of less than 4%. Oleic acid values ranged from 26 to 32% were observed in the HELP lines (Table 4.12).

4.4.6.2 Glucosinolates analysis

Glucosinolates content were measured in 6 HELP lines and two technical replicates were used for each sample. The results are depicted in Table 4.13. The levels of 30 to 48 µmol per gram of seeds were reported and the content was lower than the previous generations but only 6 samples were measured, so the conclusion could not be made about the segregation of the glucosinolates in this generation. The genes controlling the glucosinolates and erucic acid synthesis are present on the different chromosomes in *B. napus*. So, the glucosinolates could be measured for more samples to select the lines with lower levels.

Table 4.13 Glucosinolates measurements (µmol/g) of the HELP lines ($F_1B_1S_5$ seeds of the cross 'Cabriolet x (K0472 x Ningyou 7)')

Genotype	Aliphatic GLS	Indole GLS	Aromatic GLS	Total GLS
E12-33-2-3	29.69	0.14	0.81	30.64
E12-33-1-1	34.71	0.30	0.89	35.89
E12-33-10-1	35.58	0.20	0.80	36.58
E12-33-10-3	42.11	0.42	1.13	43.66
E12-33-10-2	44.71	0.33	1.26	46.31
E12-33-3-1	46.69	0.21	0.97	47.87

GLS is glucosinolates. The values represented are mean of two technical replicates.

4.4.6.3 Multiplication

HELP lines were multiplied in a field at the Opava, Czech Republic in the season 2017-18 (Figure 4.12) and it was managed by Lenka Havlickova, Bancroft group (University of York, UK). A good yield of HELP seeds (17 kilograms) was harvested and these would be used for the measurement of the industrial characteristics of the HELP oil for its future perspectives.

Figure 4.12 HELP plants growing at a field in the Czech Republic
The HELP lines developed by the cross 'Cabriolet x (K0472 x Ningyou 7)' were multiplied in a field at the Opava, Czech Republic in the season 2017-18.

4.5 Summary

A *Bna.FAD2* mutant, K0472 (Cabriolet mutant) was cross-pollinated to the high erucic cultivar, Ningyou 7 in order to combine the genes of low polyunsaturates content (partially functional *Bna.FAD2* family) and high erucic content (functional *Bna.FAE1*) in the progeny. The F_1 progeny was backcrossed to the low erucic cultivar, Cabriolet (parental background for mutagenesis) to produce the F_1B_1 progeny and followed by genotypic selections and fatty acid analysis. The selected lines were self-pollinated for 6 generations. High erucic and low polyunsaturated rapeseed (HELP) lines were selected at the $F_1B_1S_2$ progeny ($F_1B_1S_3$ seeds) with a genotypic construct "4 x *Bna.fad2* and 2 x *Bna.FAE1*^{NY7}". The seeds were multiplied and the fatty acids were measured at each generation to check the stability of the fatty acids compositions of the HELP oil. Winter type rapeseed is considered more suitable for cultivation in the UK climate (Röbbelen, 1991). So, the flowering time test was conducted on the HELP lines and the winter-type HELP lines were identified. The fatty acid compositions of HELP lines showed the reduced PUFA (C18:2 and C18:3) content (less than 6%) and increased erucic content (Up to 61%) as compared to high erucic parent, NY7. Total very long

chain fatty acids content (C20:1, C22:1 and C24:1) of 63 to 65% was reported in the HELP oil. An increase of 12 to 14% in oleic acid (C18:1) and slightly lower saturated fatty acids were also observed in the HELP as compared to NY7. HELP oil had only 3 to 4% SAFAs content.

Bna.FAD2 family is responsible for controlling the PUFA content in *B. napus* (Scheffler *et al.*, 1997). HELP lines had partially functional *Bna.FAD2* copies and thus, a lesser amount of oleic acid went into the desaturation pathway and reduced the PUFA levels by more than 18% as compared to NY7 (functional *Bna.FAD2* copies). Oleic acid pool in *B. napus* is considered as a major bottleneck for increasing the erucic acid beyond a certain level (Han *et al.*, 2001; Sasongko and Möllers, 2005). Due to the partially functional *Bna.FAD2* copies, oleic acid levels became more than double in the HELP lines as compared to high erucic cultivars. High levels of erucic acid, eicosenoic acid and oleic acid made the total mono-unsaturated fatty acids levels up to 93% in HELP lines. In the HELP lines, eicosenoic acid (C20:1) levels were non-uniform and thus, making the erucic levels non-uniform as well. It was probably due to the same enzymes controlling the synthesis of erucic acid and eicosenoic acid in *B. napus* (Kondra and Stefansson, 1965; Jonsson, 1977). In addition, eicosenoic acid levels are known to have epistasis effects and thus, making it hard to control its level in the generations (Coonrod *et al.*, 2008). The present study showed a negative correlation between the erucic acid and eicosenoic acid levels in the HELP lines as observed by Sasongko and Möllers, 2005.

The leftover high protein meal left after the extraction of the oil from rapeseed is used as a feedstock for animals. So, it is very important to know the glucosinolates levels in the rapeseed as glucosinolates are considered as anti-nutritional factors in the meal (Alexander *et al.*, 2008). So, glucosinolates content was analysed in the HELP lines and a range of values was reported. This is due to the high glucosinolates present in the parent, Ningyou 7 and low glucosinolates content in the other parental genotypes, K0472 and Cabriolet. The genes controlling the erucic acid and glucosinolates synthesis are unlinked and are present on the different chromosomes in *B. napus*. So, it would be very easy to select lines with low glucosinolates content by measuring its levels in more HELP lines. HELP lines were multiplied in a field at the Czech Republic

in the season 2017-18 and the industrial characteristics would be measured from these seeds. The fatty acid content was measured but are not presented in the thesis.

4.6 Conclusion

The influence of introducing partially functional *Bna.FAD2* removed the draw on oleic acid (C18:1) into the desaturation pathway and hence decreased the PUFA contents (C18:2 and C18:3) in the HELP lines. This increased the oleic acid levels and thus, more oleic acid went into the elongation pathway, increasing the very long chain fatty acids pool (eicosenoic acid, C20:1 and erucic acid, C22:1) by *Bna.FAE1*. Hence, these results support our hypothesis.

5. Development of High Erucic and Low Polyunsaturates (HELP) Rapeseed Using Maplus *FAE1* Alleles

5.1 Hypothesis

Maplus *Bna.FAE1* alleles may result in a greater proportion of the very long chain fatty acids than Ningyou 7 *Bna.FAE1* alleles.

5.2 Test

Substituting the Ningyou 7's (Chinese semi-winter high erucic acid variety) *FAE1* alleles, $Bna.FAE1^{NY7}$ with Maplus (German winter-type high erucic acid variety) *FAE1* alleles, $Bna.FAE1^{Map}$ followed by assessing the amount of the very long chain fatty acids accumulated. This was achieved by crossing the fixed low polyunsaturates lines (selected progenies of P15, P19 and P20 lines; Section 4.4.3.1) onto Maplus, self-pollination and marker-assisted selection to have lines with genotypic construct *"*4 x *Bna.fad2* and 2 x $Bna.FAE1^{Map}$*".* The fatty acid compositions were analysed and compared to see whether very long chain fatty acids increased relative to the lines containing *"*4 x *Bna.fad2* and 2 x $Bna.FAE1^{NY7}$*".*

5.3 Materials and Methods

Maplus (oil profile: ~12% C18:1, ~21% PUFAs, ~50% C22:1) is a commercial open-pollinated high erucic acid variety of rapeseed sourced from the breeding company, NPZ-Lembke, Germany (https://www.npz.de/). It was used as the female parent for the cross-pollination. In the $F_1B_1S_2$ progeny of the cross 'Cabriolet x (K0472 x Ningyou 7)', genotypes P15, P19 and P20 had a non-functional *Bna.fad2.A5* copy and a partially functional *Bna.fad2.C5* copy. Thus, the uniform level of the polyunsaturates was present in their seeds (Section 4.4.3.1 and Figure 4.3). These were called fixed PUFA

lines and their genotypic profiles are depicted in Table 5.1. Polymorphism details of these copies are given in Table 2.3. Genotype, P15 had *Bna.FAE1.A8* and *Bna.FAE1.C3* copies heterozygous while genotypes, P19 and P20 had heterozygous *Bna.FAE1.C3* copy and functional *Bna.FAE1.A8* copy.

Table 5.1 *Bna.FAD2* and *Bna.FAE1* profile of the fixed PUFA lines ($F_1B_1S_2$ progeny of the cross 'Cabriolet x (K0472 x Ningyou 7)')

Code	Genotype	*Bna.FAD2. A5*	*Bna.FAD2. C5*	*Bna.FAE1. A8*	*Bna.FAE1. C3*
P15	Cab x (K0472xNY7) C2-16	-	-	Het	Het
P19	Cab x (K0472xNY7) C2-78	-	-	Homo	Het
P20	Cab x (K0472xNY7) C2-88	-	-	Homo	Het

'Homo' is a homozygous functional copy, 'Het' is a heterozygous copy, '-' is a homozygous non-functional copy (partially functional *Bna.FAD2.C5*), Cab is Cabriolet and NY7 is Ningyou 7.

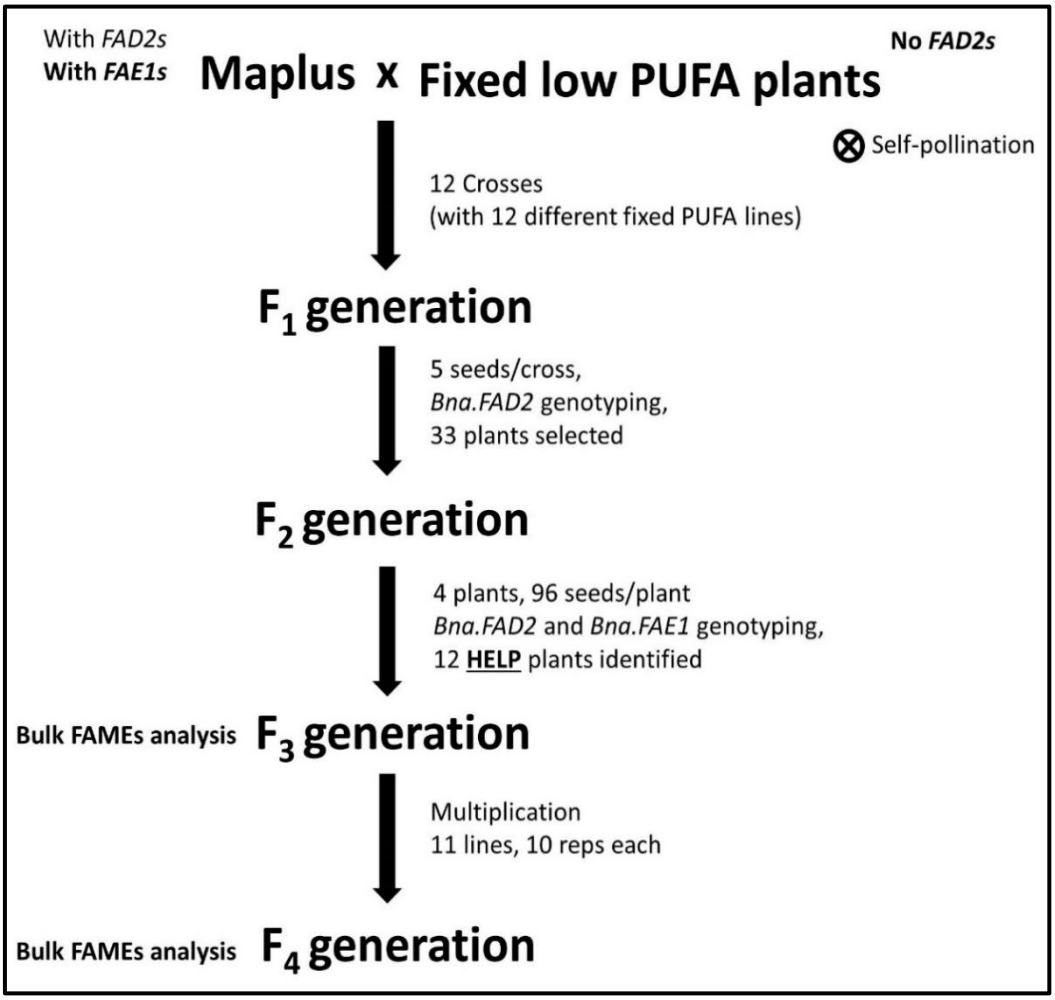

Figure 5.1 Summary of the development of "4 x *Bna.fad2* and 2 x *Bna.FAE1*^{Map}"
Maplus was cross-pollinated to the fixed polyunsaturated fatty acid lines from the $F_1B_1S_3$ progeny of the cross, 'Cabriolet x (K0472 x Ningyou 7)'. It was followed by the phenotypic and genotypic selections until the F_4 generation

Selections were made in the progeny $F_1B_1S_3$ for *Bna.FAE1* loci to be used in the cross-pollination with Maplus. The summary of the development of high erucic and low polyunsaturates (HELP) lines using Maplus background and fixed PUFA lines is summarized in Figure 5.1. Maplus was cross-pollinated to the fixed PUFA lines, followed by various selections for 4 generations. HELP lines were multiplied and the fatty acid compositions were analysed using the bulk seeds method (Section 2.7.2).

5.4 Results

5.4.1 Selections and Cross-Pollinations

From the genotypes P15, P19 and P20; twenty plants were grown for each; DNA was extracted using the CTAB method (Section 2.3). Progenies were numbered as 'P15-1 to 20', 'P19-1 to 20' and 'P20-1 to 20'. These lines had *Bna.FAD2* family already according to the HELP construct, so it was aimed to identify two types of lines according to *Bna.FAE1* profile – one was Cabriolet type (non-functional *Bna.FAE1s*) and other was Ningyou 7 type (functional *Bna.FAE1s*). Former could serve as the control lines for Cabriolet type alleles. Both *Bna.FAE1* primer pairs were used for genotyping (few amplicons are shown in Figure 5.2) of these 60 lines.

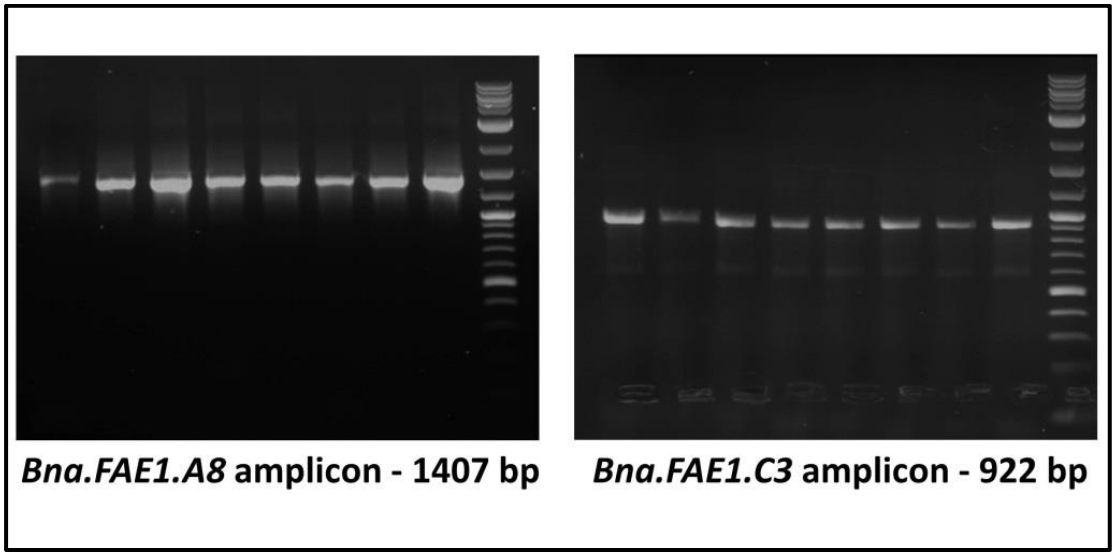

Figure 5.2 PCR amplicons for screening *Bna.FAE1* copies in the fixed PUFA lines
PCR amplification of both copies of Bna.FAE1 loci in the fixed PUFA lines ($F_1B_1S_3$ progeny) of the cross, 'Cabriolet x (K0472 x Ningyou 7)'

In the P15 progeny, 13 plants had heterozygous and 7 plants had the functional copies of *Bna.FAE1.A8.* For *Bna.FAE1.C3* locus, 11 plants were heterozygous, 5 plants were non-functional and 4 plants were functional. So, three plants were selected from the P15 progeny with a combination of non-functional *Bna.fae1.C3* and heterozygous *Bna.FAE1.A8* loci as depicted in Table 5.2. Homozygous non-functional alleles could be selected for *Bna.FAE1.A8* locus in the F_1 progeny after the cross-pollination with Maplus. In the P19 progeny, three plants were found with both copies, *Bna.FAE1.A8* and *Bna.FAE1.C3*, functional (Table 5.2). In the P20 progeny, six plants had functional alleles of both *Bna.FAE1* copies as shown in Table 5.2. Thus, the progeny of P19 and P20 had the HELP genotypic profile of functional *Bna.FAE1s* and partially functional *Bna.FAD2s*. Therefore, in total, 12 plants were selected for the cross-pollination with Maplus. Extra crosses were also attempted to get the sufficient seeds for some of the combinations where pods failed to form any seeds after cross-pollination. Individual pods were harvested at the maturity in the separate bags for each cross-pollinated plant, labelled and stored. These 12 selected plants were self-pollinated as well and seeds were harvested at the maturity.

Table 5.2 Selected plants from the progeny of the fixed PUFA lines (from the cross 'Cabriolet x (K0472 x Ningyou 7)') and their *Bna.FAD2* and *Bna.FAE1* profiles

Selected lines	*Bna.FAD2.A5*	*Bna.FAD2.C5*	*Bna.FAE1.A8*	*Bna.FAE1.C3*
P15-7, P15-12, P15-19	-	-	Het	-
P19-4, P19-12, P19-15	-	-	Homo	Homo
P20-2, P20-5, P20-6, P20-7, P20-10, P20-12	-	-	Homo	Homo

'Homo' is a homozygous functional copy, 'Het' is a heterozygous copy and '-' is a homozygous non-functional copy (partially functional copy for *Bna.FAD2.C5*)

5.4.2 F_1 Generation

For each of the 12 crosses described above, one seed each from 5 different pods (named 'A to E') of the same F_1 plant was grown in order to get the maximum number of cross-pollinated plants. Due to the poor germination of some plants, these were planted again from the different pods. Then, there was vernalisation cabinet

breakdown once and the temperature went down to less than 0°C. Many plants were not able to recover from the cold shock and hence, did not grow. Thirty-three F_1 plants were able to grow and DNA was extracted using the CTAB method (Section 2.3). *Bna.FAD2* and *Bna.FAE1* profiles of Maplus and the progenies of P15, P19 and P20 were already known and so, F_1 genotype was predicted. For instance, for both *Bna.FAD2.A5* and *Bna.FAD2.C5* copies; Maplus had functional alleles while P15, P19 and P20 progenies had non-functional (or partially functional) alleles, so F_1 plants should be heterozygous for these copies. Thus, both *Bna.FAD2* primer pairs were amplified (Table 2.5) and followed by Sanger sequencing from the Beckman Coulter Genomics (http://www.beckmangenomics.com/) in these 33 plants to screen the successful crosses. All the plants were found to be heterozygous for these 2 copies and thus, all the cross-pollination were successful. These were self-pollinated followed by harvesting at the maturity.

5.4.3 F_2 Generation

Four lines, 'Maplus x P19-4A', 'Maplus x P19-12B', 'Maplus x P20-2C' and 'Maplus x P20-6D' were randomly selected and 96 plants were grown from each line. DNA was extracted using 'DNeasy Plant 96 Qiagen kit'. These were genotyped for *Bna.FAD2* and *Bna.FAE1* primer pairs (Table 2.5) and sequenced from the Beckman Coulter Genomics (http://www.beckmangenomics.com/).

Both Maplus and HELP progeny of P19 and P20 genotypes had homozygous functional alleles of *Bna.FAE1*, so the lines resulting from the cross between them were expected to have the functional copies. It was confirmed by the sequencing results as shown in Table 5.3. It was aimed to select plants with partially functional *Bna.fad2.C5* and non-functional *Bna.fad2.A5* copies (HELP genotypic construct). Two plants from the cross 'Maplus x P19-4A'; four plants from the cross 'Maplus x P19-12B'; three plants from the cross 'Maplus x P20-2C' and; three plants from the cross 'Maplus x P20-6D' had desirable HELP genotypic construct as depicted in Table 5.3. Eight additional plants with one of the *Bna.FAD2* copy heterozygous and all other copies according to the HELP genotype were also selected (Table 5.3). These plants were self-pollinated and harvested at the maturity. These HELP plants were expected to be winter type as Maplus was winter-type as well.

Table 5.3 *Bna.FAE1* and *Bna.FAD2* profiles of the selected plants in the F_2 progeny

Codes and Cross	*Bna.FAE1.A8*	*Bna.FAE1.C3*	*Bna.FAD2.A5*	*Bna.FAD2.C5*
Maplus x P19-4A				
5-10, 5-86	Homo	Homo	-	-
5-15	Homo	Homo	-	Het
5-35	Homo	Homo	Het	-
Maplus x P19-12B				
6-5, 6-15, 6-30, 6-47	Homo	Homo	-	-
6-75	Homo	Homo	-	Het
6-83	Homo	Homo	Het	-
Maplus x P20-2C				
7-13, 7-16, 7-54	Homo	Homo	-	-
7-5, 7-47	Homo	Homo	Het	-
Maplus x P20-6D				
8-20, 8-32, 8-85	Homo	Homo	-	-
8-17	Homo	Homo	Het	-
8-11	Homo	Homo	-	Het

Codes in **bold s**hows the HELP lines; codes not in bold had 3 copies according to the HELP construct and one heterozygous copy; 'Homo' is a homozygous functional copy; 'Het' is a heterozygous copy and; '-' is a homozygous non-functional copy (partially functional copy for *Bna.FAD2.C5*). HELP lines were developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]'

5.4.4 F_3 Generation

The fatty acid compositions were analysed using the bulk seeds method (Section 2.7.2) from 9 HELP lines with sufficient seeds and high erucic varieties, Maplus and Ningyou 7. Three technical replicates were used for each sample and the results are depicted in the following Table 5.4. The erucic acid (C22:1) content ranged from 44 to 59% in the HELP lines as compared to 49-51% in the high erucic varieties, Maplus and NY7. Eicosenoic acid (C20:1) content varied from 6-14% in HELP lines as compared to ~8% in the high erucic controls. Similarly, oleic acid (C18:1) varied from 25 to 31% in the HELP lines in comparison to 12-13% found in Maplus and NY7. Total polyunsaturated fatty acids (sum of C18:2, C18:3 and C20:2) in HELP lines were found to be less than 6% as compared to 21-23% in the high erucic varieties. The partially functional *Bna.FAD2* family is responsible for the low polyunsaturates in the HELP lines and thus, supporting our hypothesis of the previous chapter (Section 4.1). It also increased the oleic acid and very long chain fatty acids pool in the HELP lines. Total

monounsaturated fatty acids content of up to 93% were found in the HELP lines as compared to 71-73% in high erucic varieties. Saturated fatty acids showed a decrease of 1 to 2% in the HELP lines as compared to the high erucic acid cultivars.

Table 5.4 The fatty acid percentages (mean of 3 technical replicates) of HELP lines (F_3 seeds) and controls

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1
1	5-10	0.0	2.1	0.3	0.6	26.9	1.6	3.6	0.6	8.0
2	5-86	0.0	2.6	0.3	0.9	26.9	1.8	4.6	0.7	13.6
3	6-15	0.0	1.9	0.2	0.5	24.9	1.7	3.9	0.6	5.8
4	6-47	0.0	2.2	0.3	0.5	26.6	1.4	3.6	0.5	7.7
5	6-5	0.0	2.1	0.3	0.6	28.2	1.4	2.9	0.5	9.4
6	7-16	0.0	2.4	0.3	0.5	25.4	2.0	4.3	0.6	8.8
7	7-54	0.0	2.2	0.2	0.7	28.2	1.4	1.8	0.7	10.4
8	8-20	0.0	2.4	0.3	0.7	31.0	1.8	3.4	0.5	14.6
9	8-85	0.0	2.4	0.3	0.7	28.8	1.4	2.9	0.6	10.3
10	Maplus	0.0	3.6	0.2	0.8	12.3	12.4	8.2	0.7	8.7
11	NY7	0.0	3.1	0.2	0.8	13.0	13.5	8.9	0.7	7.5
S. No.	**Line**	**20:2**	**22:0**	**22:1**	**24:0**	**24:1**	**PUFA**	**VLCFA**	**SAFA**	**MUFA**
1	5-10	0.0	0.4	54.5	0.1	1.2	5.2	63.8	3.9	90.9
2	5-86	0.1	0.3	47.5	0.1	0.6	6.5	61.7	4.6	88.9
3	6-15	0.0	0.5	58.9	0.2	1.0	5.6	65.7	3.6	90.8
4	6-47	0.0	0.4	55.6	0.1	1.1	5.0	64.4	3.7	91.3
5	6-5	0.0	0.3	52.0	0.0	2.2	4.3	63.6	3.6	92.1
6	7-16	0.0	0.4	54.4	0.0	0.8	6.2	64.1	4.0	89.8
7	7-54	0.0	0.4	53.1	0.2	0.8	3.2	64.2	4.1	92.7
8	8-20	0.0	0.2	44.3	0.0	0.7	5.2	59.5	3.9	90.8
9	8-85	0.0	0.4	51.1	0.1	1.0	4.4	62.4	4.2	91.4
10	Maplus	0.4	0.6	51.1	0.1	0.8	21.0	60.7	5.7	73.3
11	NY7	0.5	0.9	48.9	0.4	1.7	22.9	58.1	5.9	71.3

PUFA=18:2+18:3+20:2; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0; MUFA = 16:1+18:1+20:1+22:1+24:1 and; NY7 is Ningyou 7. HELP lines were developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]'.

Comparing the two HELP lines, 6-15 and 8-20, showing extreme differences in some of the fatty acid contents explains the reason for the non-uniformity in the fatty acid compositions within the HELP lines. HELP 8-20 had 44% erucic acid while HELP 6-15 had 59% erucic acid but they had opposite trend in the eicosenoic acid levels. Former had a higher amount (~15%) while later had a lower amount (~6%) of the eicosenoic acid. In *B. napus* seeds, the same enzyme (*FAE1*) controls the synthesis of eicosenoic

acid and erucic acid (Kondra and Stefansson, 1965; Jonsson, 1977). So, it is hard to control the amount of eicosenoic acid and erucic acid. In addition, HELP 8-20 had 6% more oleic acid than HELP 6-15, showing that lesser amount of oleic acid went into elongation pathway in the HELP 8-20 as compared to HELP 6-15 (Table 5.4). Although these two HELP lines were very dissimilar in various fatty acid levels but had a similar amount of overall monounsaturated fatty acids and polyunsaturated fatty acids.

In *B. napus,* lysophosphatidic acid acyltransferase (*Bn-LPAAT*) catalyses the second acylation at the *sn-2* position during the formation of triacylglycerol (Frentzen, 1993, 1998; Bates, Stymne and Ohlrogge, 2013). *Bn-LPAAT* has poor affinity for the erucoyl moieties; thus, erucic acid is excluded from the *sn-2* position and preventing trierucin synthesis in *B. napus*. This limits the highest level of erucic acid to 66% in *B. napus* (Brockerhoff, 1971; Cao, Oo and Huang, 1990; Frentzen, 1993; Katavic *et al.*, 2001). Total very long chain fatty acids observed in some HELP lines are very close to this level. Thus, the larger influence of the partially functional *Bna.FAD2* family could be observed on the polyunsaturates, oleic acid and overall monounsaturates in the HELP lines but in this progeny, only slight changes in the erucic acid and total very long chain fatty acids could be observed. So, these HELP lines were multiplied for the next generation to see the changes in the fatty acid compositions.

5.4.5 F_4 Generation

5.4.5.1 Multiplication and Fatty Acid Analysis

Eleven HELP lines (5-86 flowered very late and it was not multiplied) were grown in 10 replications each with control Maplus in the glasshouse (Figure 5.3). These were self-pollinated and seeds were harvested at the maturity.

Bulk seeds method (Section 2.7.2) was used for the fatty acid analysis on 96 HELP lines. Some HELP plants did not grow or flowered and thus, no seeds were available. Ten biological replicates of Maplus and two biological replicates each of NY7, Cabriolet and K0472 were also analysed for the fatty acid compositions. Three technical replicates were used for each sample for the fatty acid analysis. The detailed fatty acid results are given in Appendix VIII. Mean values of the saturated fatty acids, polyunsaturated fatty acids, oleic acid, erucic acid and very long chain fatty acids of

each HELP line and controls are presented in Table 5.5. As observed in the earlier generation, saturated fatty acids (SAFAs) were 1 to 2% lower in the HELP lines as compared to the high and low erucic controls (Appendix VIII). Polyunsaturated fatty acids (PUFAs) ranged from 4 to 6.5% in the HELP lines as compared to ~25% found in the high erucic cultivars (Table 5.5). Partially functional *Bna.FAD2* family in the HELP lines are responsible for lower levels of PUFAs as compared to the functional copies present in the high erucic cultivars. Very low amount of oleic acid went into the desaturation pathway and thus, reducing the PUFA levels by more than 18% in HELP lines. It could also be observed that the mean values of all the groups of the HELP lines had very low PUFA content than the other controls (Table 5.5).

Figure 5.3 HELP lines (F_4 seeds) growing in the glasshouse after vernalisation
HELP lines were developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]

Due to the partially functional *Bna.FAD2* family (three copies non-functional and one copy partially functional), oleic acid levels became more than double in the HELP lines as compared to the high erucic cultivars as depicted in Appendix VIII and Table 5.5. This led to an increase in the erucic acid levels as well. Highest levels of up to 60.6% erucic level were found in a HELP line (6-15-2) in comparison to 49% found in the high erucic commercial cultivars. Mean values of the very long chain fatty acids are shown

in Table 5.5 and it could be observed that the VLCFAs levels are more than 60% in the HELP lines and it is higher than the high erucic controls. This generation had higher values of erucic acid and VLCFAs as compared to the previous generation. The total content of monounsaturated fatty acids of up to 93% was found in many HELP lines (Table 5.5).

Table 5.5 'Mean ± Standard Deviation' values of the fatty acids percentages of the HELP lines (F_4 seeds) and controls.

Group	SAFA	PUFA	OA	EA	VLCFA	MUFA
Cabriolet	6.5 ± 0.5	18.3 ± 0.7	73.3 ± 1.0	0.0 ± 0.1	1.6 ± 0.2	75.2 ± 1.2
K0472	6.7 ± 0.1	7.0 ± 0.5	83.7 ± 0.4	0.2 ± 0.1	2.2 ± 0.0	86.3 ± 0.5
Maplus	6.4 ± 0.6	25.4 ± 1.2	12.2 ± 1.8	46.1 ± 2.3	55.7 ± 2.1	68.2 ± 1.5
NY7	5.6 ± 0.1	24.3 ± 0.3	13.0 ± 0.2	48.6 ± 0.7	56.9 ± 0.7	70.1 ± 0.5
5-10	4.6 ± 0.4	5.2 ± 0.8	27.9 ± 1.3	49.8 ± 2.8	62.1 ± 1.5	90.2 ± 0.9
6-15	3.8 ± 0.3	5.7 ± 1.1	25.6 ± 1.6	56.8 ± 2.3	64.6 ± 0.9	90.4 ± 1.2
6-30	4.2 ± 0.3	5.4 ± 1.2	28.0 ± 2.1	51.5 ± 2.5	62.1 ± 1.5	90.4 ± 1.2
6-47	4.1 ± 0.4	3.8 ± 0.4	28.7 ± 1.6	53.4 ± 3.3	63.2 ± 1.6	92.1 ± 0.4
6-5	3.7 ± 0.2	3.6 ± 0.2	28.9 ± 0.8	51.6 ± 1.9	63.5 ± 0.8	92.7 ± 0.2
7-13	4.8 ± 0.4	5.9 ± 1.4	28.1 ± 2.6	49.2 ± 4.2	60.9 ± 1.8	89.4 ± 1.2
7-16	4.3 ± 0.3	5.7 ± 1.2	26.8 ± 1.9	53.1 ± 2.9	62.9 ± 1.2	90.0 ± 1.2
7-54	4.4 ± 0.4	4.5 ± 0.4	28.6 ± 1.3	51.0 ± 2.8	62.2 ± 1.6	91.1 ± 0.7
8-20	4.3 ± 0.3	5.4 ± 0.6	29.3 ± 1.5	48.1 ± 2.7	60.7 ± 1.5	90.3 ± 0.8
8-32	4.6 ± 0.4	6.6 ± 0.9	25.7 ± 1.3	52.9 ± 3.0	62.8 ± 1.2	88.8 ± 1.1
8-85	4.3 ± 0.8	5.9 ± 1.1	27.4 ± 1.8	52.1 ± 3.4	62.0 ± 2.2	89.8 ± 1.3

OA is oleic acid (18:1) and EA is erucic acid (22:1). The detailed fatty acid analysis is given in Appendix VIII. PUFA=18:2+18:3+20:2; VLCFA=20:1+22:1+24:1; SAFA =14:0+16:0+18:0+20:0+22:0+24:0 and; MUFA = 16:1+18:1+20:1+22:1+24:1. HELP lines were developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]'.

Figure 5.4 shows the scattered plot with a fitted line between the erucic acid and eicosenoic acid levels in the HELP lines. A high R-squared (R^2) value of 0.8467 was reported and it showed a high negative correlation between the erucic acid and eicosenoic acid levels of the HELP lines. Thus, it made the total very long chain fatty acids pool uniform in the HELP lines. Another scatter-plot between the very long chain fatty acids and oleic acid is shown in Figure 5.5. It showed an R-squared value of 0.4569 and also showed a negative correlation between VLCFA and oleic acid levels. Thus, in HELP lines, if low levels of oleic acid went into elongation pathway and thus,

increased the oleic acid content but decreased the VLCFA content and vice versa. So, erucic acid level depends on the level of oleic acid going into the elongation pathway and also on the amount of eicosenoic acid converting to the erucic acid.

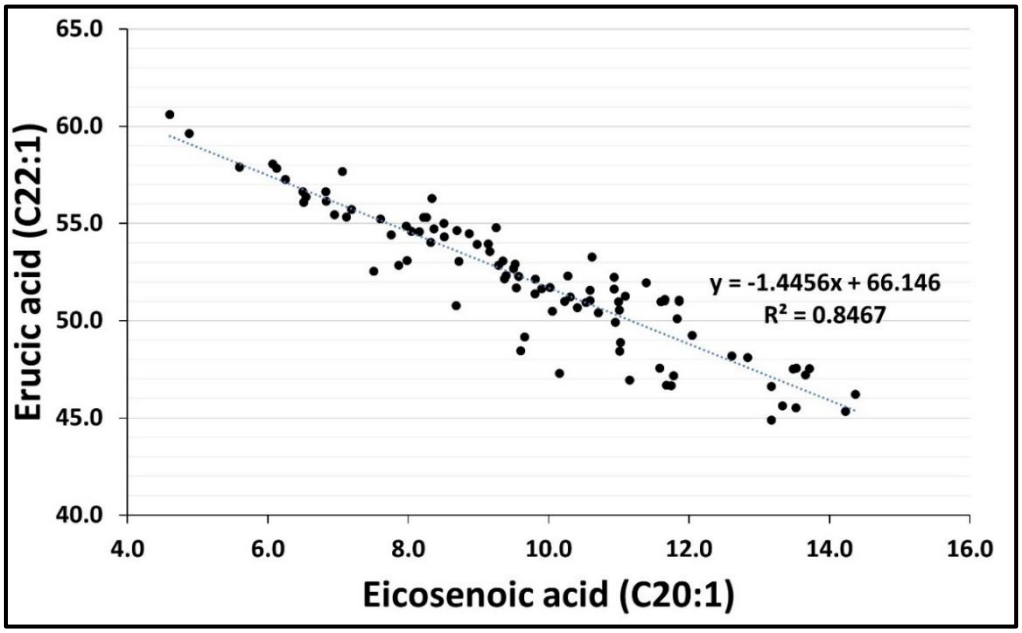

Figure 5.4 Scatter-plot with fitted regression line between the erucic acid and eicosenoic levels of the HELP lines

A high R^2 value of 0.8467 was observed and the fitted line shows a negative correlation between the erucic acid and eicosenoic acid levels of the HELP lines (developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]')

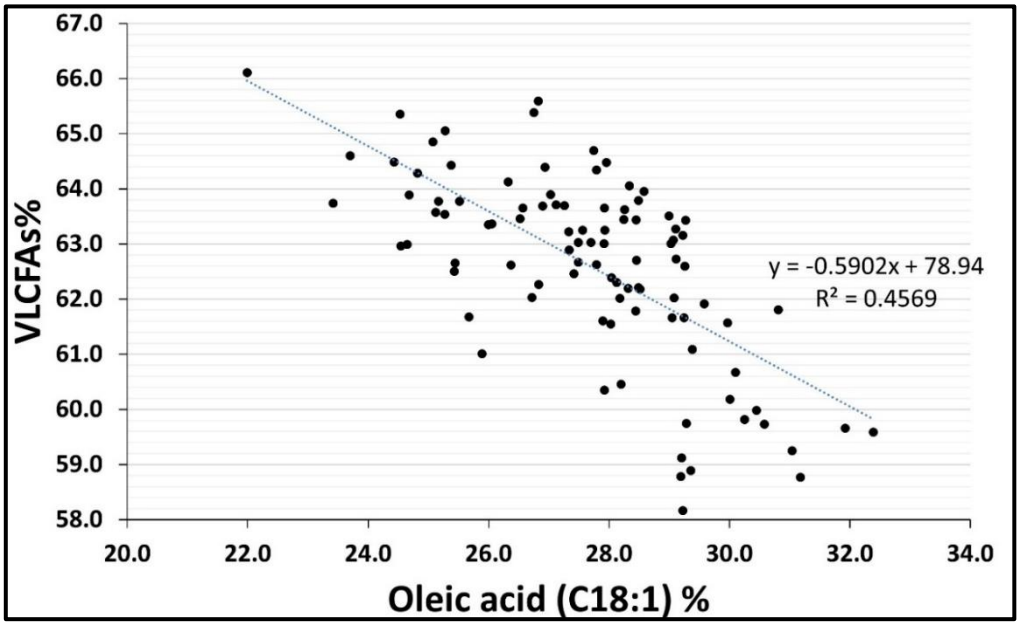

Figure 5.5 Scatter-plot with fitted regression line between VLCFAs and oleic acid levels of the HELP lines

An R^2 value of 0.4569 was observed and the fitted regression line shows a negative correlation between VLCFA and oleic acid levels of the HELP lines (developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]')

5.4.5.2 Glucosinolates Analysis

Glucosinolates content was analysed on the randomly selected 26 HELP lines along with the control lines – K0472, Cabriolet, Maplus and Ningyou 7. Two biological replicates were used for each genotype and the results are shown in Table 5.6. Total glucosinolates content of HELP lines ranged from 6 to 75 µmol per gram of the seeds. Maplus, Cabriolet and K0472 had lower total glucosinolates content while Ningyou 7 had a higher value in their seeds. HELP parents, progenies of P19 and P20, were produced by cross-pollinating K0472, Cabriolet and Ningyou 7. This could be the reason for finding a range of glucosinolates content in these HELP lines. The low glucosinolates lines could be selected from these HELP lines for the next generation.

Table 5.6 Glucosinolates analysis (µmol/g) results of controls and F_4 HELP seeds (developed from the cross, 'Maplus x [Cabriolet x (K0472 x Ningyou 7)]')

S. No.	Genotype	GLS ± SD*	S. No.	Genotype	GLS ± SD*
1	K0472	12.8 ± 8.4	16	6-15-10	29.4 ± 16.8
2	Cabriolet	20.2 ± 8.5	17	7-54-8	29.5 ± 16.6
3	Maplus	23.9 ± 10.8	18	6-5-5	31.1 ± 15.2
4	NY7	94.9 ± 14.7	19	6-15-6	33.6 ± 13.7
5	6-47-4	5.9 ± 3.4	20	6-15-1	35.1 ± 14.3
6	6-47-7	13.2 ± 7.4	21	8-85-3	36.1 ±3.1
7	6-15-8	14.4 ± 6.8	22	7-16-8	43.0 ± 1.3
8	6-15-2	15.1 ± 5.9	23	8-32-3	43.7 ± 17.5
9	7-54-5	15.9 ± 8.2	24	6-30-7	45.0 ± 25.1
10	6-30-2	18.2 ± 9.1	25	8-20-8	57.4 ± 4.3
11	5-10-3	21.9 ± 8.9	26	7-16-10	58.2 ± 2.0
12	8-32-5	22.2 ± 7.4	27	6-5-8	58.3 ± 21.3
13	6-15-9	22.9 ± 7.6	28	8-20-7	64.3 ± 7.3
14	8-85-4	24.4 ± 1.0	29	7-13-1	67.3 ± 2.7
15	5-10-1	26.5 ± 12.5	30	7-13-3	75.4 ± 3.7

*GLS is the total glucosinolates and SD is the standard deviation. The values represented are the means of two technical replicates.

5.4.6 Comparison of Maplus and Ningyou 7's HELP

Maplus is a German winter oilseed rape (WOSR) with low glucosinolates content while Ningyou 7 is a Chinese semi-winter oilseed rape with high glucosinolates in its seed. Both of them are high erucic acid varieties with similar fatty acid compositions with approx. 50% erucic acid, 21% polyunsaturated fatty acids and 12% oleic acid in their

oils. Due to winter type, Maplus has strong vernalisation requirements of about 8 weeks but Ningyou 7 does not have such requirements for flowering. Maplus and Ningyou 7's plants are shown in Figure 5.6 and it could be observed that NY7 flowered and matured very early due to its semi-winter behaviour while Maplus took long to flower and mature after same days of sowing. In addition, the winter types of rapeseed are known to be more productive than the spring forms and in Europe, mainly winter types of rapeseed are cultivated (Downey and Röbbelen, 1989; McVetty *et al.*, 2016). So, Maplus based HELP would be potentially more suitable for cultivation in Europe. Sixty-three lines of Ningyou 7 HELP and ninety-six lines of Maplus HELP were available for analysis. Their fatty acid data did not meet the assumptions (normal data and equal variances) of one-way ANOVA (analysis of variance) for polyunsaturates, oleic acid and very long chain fatty acids levels. So, these were compared using a non-parametric test, Kruskal-Wallis test. Mean, standard deviation and p-values of various fatty acids of both Maplus HELP and Ningyou 7 HELP are shown in Table 5.7. No significant differences were found between Maplus HELP and NY7 HELP in the PUFAs, oleic acid and VLCFAs values.

Figure 5.6 Ningyou 7 plant (left) and Maplus plant (right) growing in the glasshouse
Both high erucic varieties were photographed at the same number of days after sowing

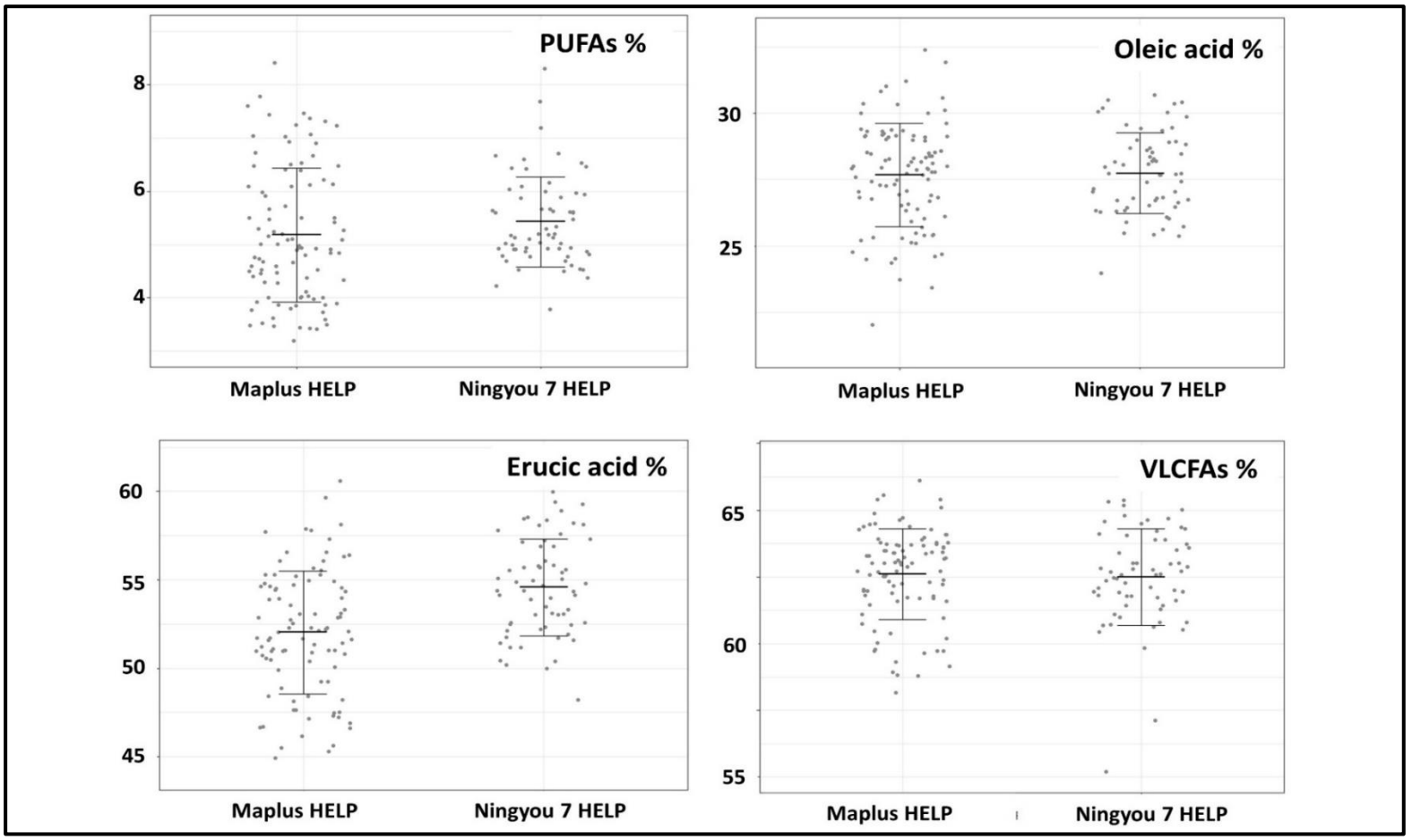

Figure 5.7 Scatterplot of various fatty acids of Maplus HELP and Ningyou 7 HELP lines

PUFAs, oleic acid, erucic acid and VLCFAs content with their mean and standard error values are shown for Maplus HELP and Ningyou 7 HELP. Each dot in the graph represents the mean value of one genotype for the respective fatty acid. The mean value of each HELP group is represented as dark horizontal line (in the middle) with standard error bars on both sides.

Table 5.7 Mean values and p-values of comparison of Maplus HELP and Ningyou 7 HELP lines

Fatty acids	Mean ± Standard Deviation		p-value (Kruskal-Wallis test)
	Maplus HELP	Ningyou 7 HELP	
PUFAs	5.18 ± 1.26	5.43 ± 0.84	0.0568
OA	27.67 ± 1.95	27.67 ± 1.53	0.9593
VLCFAs	62.61 ± 1.70	62.49 ± 1.81	0.5575

PUFAs=polyunsaturated fatty acids; OA=oleic acid and; VLCFAs=very long chain fatty acids

The scatterplot of Maplus HELP and Ningyou 7 HELP for various fatty acids are shown in Figure 5.7. No significant differences could be observed from the scatterplots also. As there was a larger number of observations for Maplus HELP than the NY7 HELP, so the range of values is larger for the former one. But these average over the same values for most of the fatty acids. There is ~2% difference in the erucic acid mean values but it could be attributed to a larger sample size of Maplus HELP.

In Section 3.4.4, new markers were designed to differentiate the regions flanking *FAE1*. Two of these markers differentiated between the Maplus and Ningyou 7 regions flanking *FAE1* (Table 3.3) but these allelic differences does not influence the VLCFAs content of the Maplus and Ningyou 7 based HELP lines.

5.5 Summary

Bna.FAE1 family in *B. napus* is responsible for the elongation of oleic acid (C18:1) to the very long chain fatty acids, eicosenoic acid (C20:1) and erucic acid (C22:1) (Harvey and Downey, 1964; Kondra and Stefansson, 1965). The desaturation of oleic acid to linoleic acid (C18:2) is catalysed by *Bna.FAD2* family (Scheffler *et al.*, 1997). Stable polyunsaturated fatty acids, but varying erucic acid lines – P15, P19 and P20 (fixed PUFA lines) from the $F_1B_1S_2$ progeny of the cross, 'Cabriolet x (K0472 x Ningyou 7)', had partially functional *Bna.FAD2* family (3 copies non-functional and 1 copy partially functional) and varying profile of *Bna.FAE1* loci. Nine high erucic and low PUFA (HELP) lines were selected from the lines, P19 and P20 (functional *Bna.FAE1s*) and; three Cabriolet type lines (non-functional *Bna.FAE1s*) were selected from the line, P15. There were cross-pollinated to a high erucic acid rapeseed cultivar, Maplus and HELP lines with functional *Bna.FAE1s* and partially functional *Bna.FAD2s* were selected in

the F_2 plants (F_3 seeds). The seeds were multiplied in the next generation and the fatty acid compositions were measured in both F_3 and F_4 seeds. HELP lines had higher very long chain fatty acids pool, higher oleic acid, lower saturated fatty acids and lower polyunsaturates as compared to the high erucic acid varieties, Maplus and Ningyou 7.

Due to the partially functional *Bna.FAD2* family in the HELP lines, very low amount of oleic acid went into desaturation pathway and thus, lowering the value of PUFAs to less than 6%. Due to the same reason, oleic acid values were more than double in the HELP lines as compared to the high erucic acid varieties. A total monounsaturated fatty acid content of up to 93% was found in the HELP lines. Saturated fatty acids decreased slightly (1 to 2%) in the HELP lines as compared to the high erucic cultivars. Erucic acid showed a large variation ranging from 44 to 59%. The large variation or non-uniformity of erucic acid could be due to either or both reasons described following. Firstly, the same enzyme is responsible for the elongation of oleic acid to eicosenoic acid and then elongation of eicosenoic acid to erucic acid (Kondra and Stefansson, 1965). The non-uniformity in the erucic acid values could be due to the varying amount of eicosenoic acid accumulated in the oil of HELP lines. This reason could be supported by the uniform levels of very long chain fatty acids (sum of C20:1, C22:1 and C24:1) found in the HELP lines and by a negative correlation observed between the eicosenoic acid and erucic acid in this study. In addition, previous studies have shown the complicated genetic effect of eicosenoic acid control in *Brassicas* but the specific modifiers for eicosenoic acid control are not known. The partial dominance and epistasis in eicosenoic acid control make it difficult to control its levels between the generations (Jonsson, 1977; Mahmood *et al.*, 2003; Coonrod *et al.*, 2008). Thus, a variable amount of eicosenoic and erucic acid were found in the HELP lines. Secondly, oleic acid is converted to very long chain fatty acids and a varying amount of oleic acid went into the elongation pathway in different HELP lines. The higher amount of oleic acid going into the elongation pathways made the VLCFAs levels high and vice versa. This was shown by a negative correlation found in the oleic acid and VLCFAs in the present study. HEAR varieties with stable eicosenoic acid and erucic acid have not been reported in the literature.

Glucosinolates content was also analysed in few HELP lines as low glucosinolates are required for using the leftover rapeseed meal after oil extraction for feeding livestock

(Alexander *et al.*, 2008). A range of content was reported in the HELP lines. The loci controlling the glucosinolates are unlinked to the loci controlling VLCFAs, so the lines having low glucosinolates HELP lines could be selected easily.

In Chapter 4, a pilot experiment was conducted to know the influence of partially functional *Bna.FAD2* family on the very long chain fatty acid levels by cross-pollinating Ningyou 7 and K0472 but Ningyou 7 is a semi-winter type and it may have low suitability for the cultivation of HELP lines in Europe developed in the Ningyou 7 background. The lower yields were reported from Ningyou 7 based HELP in the field conditions in the season 2017-18 as compared to standard winter-type rapeseed varieties. Hence, new HELP lines were developed in the Maplus background which is a winter type oilseed rape and grown as a HEAR cultivar in Europe. Maplus HELP and Ningyou 7 HELP were compared for various fatty acids and no significant differences were found between the two types. Thus, there may be allelic differences present in the *Bna.FAE1* loci of both high erucic cultivars but these differences do not influence their VLCFAs levels.

5.6 Conclusion

Ningyou 7's *Bna.FAE1*^{NY7} alleles were substituted with Maplus alleles *Bna.FAE1*^{Map} and Maplus HELP lines with the genotypic construct *"*4 x *Bna.fad2* and 2 x *Bna.FAE1*^{Map}*"* was developed. Maplus HELP line was compared with Ningyou 7 HELP, *"*4 x *Bna.fad2* and 2 x *Bna.FAE1*^{NY7}*"* HELP to see the differences in the fatty acid compositions. No significant differences were found between the proportions of very long chain fatty acids, polyunsaturated fatty acids and oleic acid. So, Maplus *Bna.FAE1* alleles did not result in a higher proportion of the very long chain fatty acids than Ningyou 7 *Bna.FAE1* alleles. Hence, the results indicate that *Bna.FAE1* alleles, from both of these HEAR cultivars, produce same amount of VLCFAs and the sequential differences in these alleles do not influence their expression.

6. Quantitative Effects of *Bna.fad2.C5* Alleles in the High Erucic and Low Polyunsaturates (HELP) Rapeseed

6.1 Hypothesis

Quantitatively reducing the polyunsaturates content would quantitatively increase the very long chain fatty acids content, rather than being a threshold effect before any change was observed in the high erucic and low polyunsaturates (HELP) rapeseed lines.

6.2 Test

To establish an allelic series of "2 x *Bna.FAE1*^{Map}" in low polyunsaturated background involving *Bna.fad2* alleles of various effect from the different mutant lines. This was achieved by crossing *Bna.fad2.*C5 mutant lines – M0830 (4.6% PUFAs), K0472 (5.9% PUFAs), K0047 (6.4% PUFAs) and M2444 (6.9% PUFAs) onto Maplus, followed by self-pollination and marker-assisted selection in order to develop the genotypic construct, "4 x *Bna.fad2*$^{M0830/K0472/K0047/M2444}$ and 2 x *Bna.FAE1*^{Map}".

6.3 Materials and Methods

Maplus is a German winter-type oilseed rape with high erucic acid and low glucosinolates in its seeds (oil profile: ~50% C22:1, ~21% PUFAs and 12% C18:1). It is widely grown in Europe as high erucic acid rapeseed (HEAR) variety and was sourced from the breeding company, NPZ-Lembke, Germany (https://www.npz.de/). Maplus plants growing in the glasshouse are shown in Figure 6.1 and it was used as the female parent for the cross-pollination. Wells *et al.*, 2014 studied the genetic basis of polyunsaturated fatty acids content in the rapeseed oil and used the varieties, Cabriolet and Tapidor. For *Bna.FAD2* family, Cabriolet had three non-functional copies

– *Bna.fad2.C1*, *Bna.fad2.A1* and *Bna.fad2.A5* and; one functional copy – *Bna.FAD2.C5*. Various mutations in these copies are explained in detail in Section 2.4.2. Mutagenesis was used to target this one functional copy and an ethyl methane sulphonate (EMS) mutated population, JBnaCAB_E was developed using Cabriolet. Various mutants with an allelic series of *Bna.fad2.C5* were developed with varying proportions of oleic acid and PUFAs (Wells *et al.*, 2014). These mutants were high oleic acid and low PUFA (HOLP) rapeseed lines. Four mutants – M0830, K0472, M2444 and K0047 were used in the present study. Their fatty acid profiles are depicted in Table 6.1 (only a few fatty acids are shown) and their polyunsaturates (C18:2 and C18:3) content varied from 4.6 to 6.9%. The mutants differed in the position of the mutations in the copy *Bna.fad2.C5* and the mutations are depicted in Table 6.2.

Figure 6.1 Maplus plants in various growth stages in the glasshouse

Table 6.1 The fatty acid compositions of the *Bna.fad2.C5* mutants

Mutant	18:1	18:2	18:3	PUFA	20:1	22:0	22:1	24:1
M0830	86.4	1.8	2.8	4.6	1.5	0.3	0.0	0.2
K0472	84.7	2.3	3.6	5.9	1.5	0.3	0.1	0.2
M2444	84.0	2.5	4.4	6.9	1.4	0.3	0.1	0.1
K0047	83.3	2.3	4.1	6.4	1.7	0.4	0.1	0.2

PUFA=18:2+18:3 and this Table is adapted from Wells *et al.*, 2014

Table 6.2 Mutations in the copy *Bna.fad2.C5* in various mutants

Mutant	M0830	K0472	M2444	K0047
SNP Mutation (Cabriolet > mutant)	C > T	G > A	C > T	G > A
Position relative to first coding base	776	284	637	716
Amino acid number	259	95	213	239
Original amino acid	Pro	Cys	Pro	Gly
Mutated amino acid	Leu	Tyr	Ser	Asp

This Table is adapted from Wells *et al.*, 2014

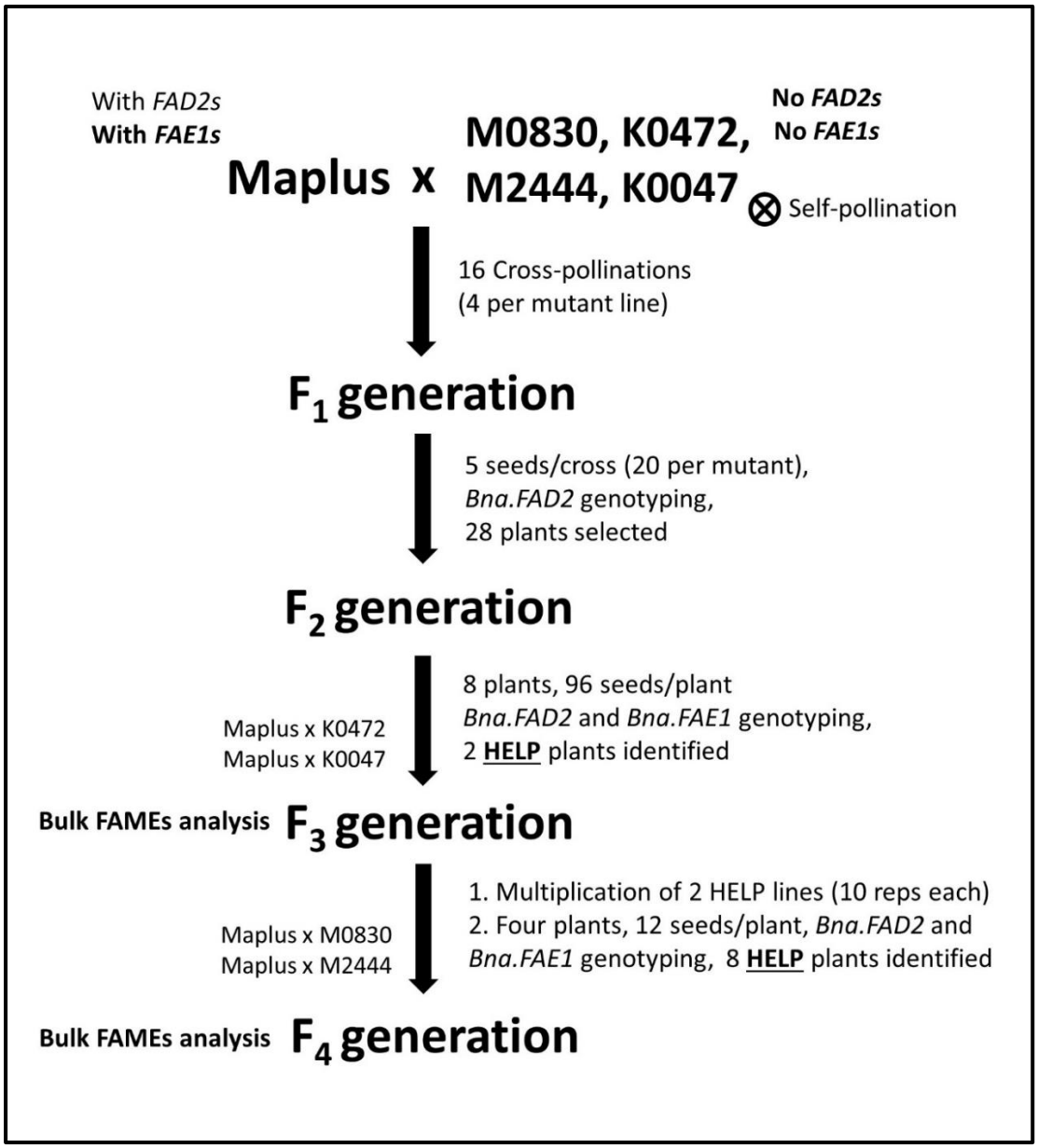

Figure 6.2 Summary of the development of 'Maplus x Mutant' HELP lines

Maplus was cross-pollinated to four Bna.fad2.C5 mutants – M0830, K0472, M2444 and K0047 to produce the HELP with the genotypic constructs, "4 x Bna.fad2$^{M0830/K0472/M2444/K0047}$ and 2 x Bna.FAE1^{Map}". It was followed by self-pollination accompanied by various genotypic and phenotypic selections up to F_4 progeny

6.4 Results

6.4.1 Cross-Pollination

Bna.fad2.C5 mutants – M0830, K0472, M2444 and K0047 were grown in the glasshouse in four replicates (numbered 1 to 4). Each mutant was cross-pollinated to the Maplus plants in four replicates. Extra cross-pollinations were attempted for the plants where pods failed to develop seeds. Individual pods (numbered A to Z) were harvested in the separate bags for each cross-pollinated plant. The mutant lines were self-pollinated and seeds were harvested at the maturity. The summary of the cross-pollination, selection and self-pollination for the development of the high erucic acid and low polyunsaturated fatty acids lines is depicted in Figure 6.2.

6.4.2 F_1 Generation

Five seeds from different pods of the same F_1 plant were sown for each of the 12 crosses (4 crosses of each mutant). Thus, for each 'Maplus x mutant' combination, 20 plants were grown for the F_1 progeny as shown in Figure 6.3. There was poor germination for the crosses, 'Maplus x M0830' and 'Maplus x K0472' (Figure 6.3). So, these were grown again from different pods of the same F_1 plant. In addition, there was vernalisation cabinet breakdown and many plants did not survive the cold shock. From the crosses, 'Maplus x M0830', 'Maplus x K0472', 'Maplus x M2444' and 'Maplus x K0047', 9, 9, 18 and 19 F_1 plants were able to survive and grow, respectively. DNA was extracted from these 55 plants using the CTAB method (Section 2.3). *Bna.FAD2* and *Bna.FAE1* profiles of the Maplus and mutants were already known. So, F_1 progeny's profile could be predicted from the parental alleles. For *Bna.FAD2.A5* and *Bna.FAD2.C5* copies, Maplus had functional alleles while mutants had non-functional (or partially functional) alleles, so F_1 progeny would have heterozygous alleles. Both of these *Bna.FAD2* primer pairs were amplified in these plants to screen successful crosses (few amplicons shown in Figure 6.4). PCR products were sequenced from Beckman Coulter Genomics and the mutations were analysed using the Mutation Surveyor®. All the cross-pollinations but 4 plants of 'Maplus x K0047' cross were

successful. Seven plants from successful crosses of each 'Maplus x mutant' type were self-pollinated and the seeds were harvested at the maturity.

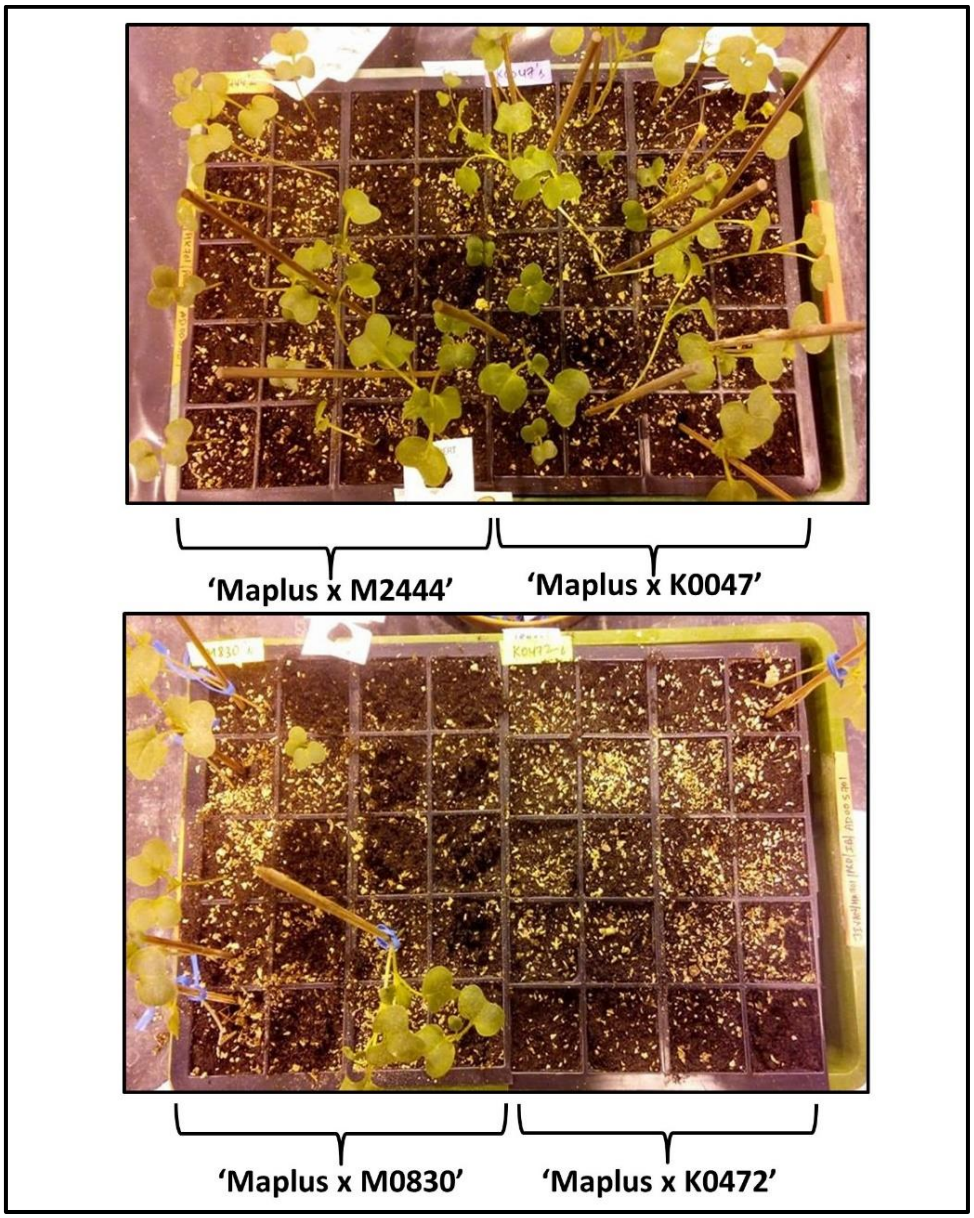

Figure 6.3 The F_1 progeny of 'Maplus x mutant' growing in the P-40 trays
Twenty plants of each Maplus x M2444, Maplus x K0047, Maplus x M0830 and Maplus x K0472 crosses growing in the P-40 trays in the glasshouse at ~2 weeks after sowing

6.4.3 F_2 Generation

Two lines were randomly selected from each combination of crosses ('Maplus x M0830', 'Maplus x K0472', 'Maplus x M2444' and 'Maplus x K0047') and 96 seeds per line were sown in the glasshouse. DNA was extracted using 'DNeasy Plant 96 Qiagen

Kit for 96 samples' (Figure 6.4) from 768 samples and genotyped for both *Bna.FAD2s and Bna.FAE1s* primer pairs as shown in Figure 6.5 (only a few amplicons are shown here). The F_2 plants were segregating for the two of the *Bna.FAD2* copies and both *Bna.FAE1* copies. It would have been a very rare chance to get the desired combination of four alleles in the F_2 generation when all four were segregating. It was possible to select one plant each from the crosses, 'Maplus x K0472' and 'Maplus x K0047' having the desired construct of HELP lines but HELP plants were not found in the other two crosses. So, two plants 2-91 and 4-87 were selected from the crosses, 'Maplus x K0472' and 'Maplus x K0047', respectively.

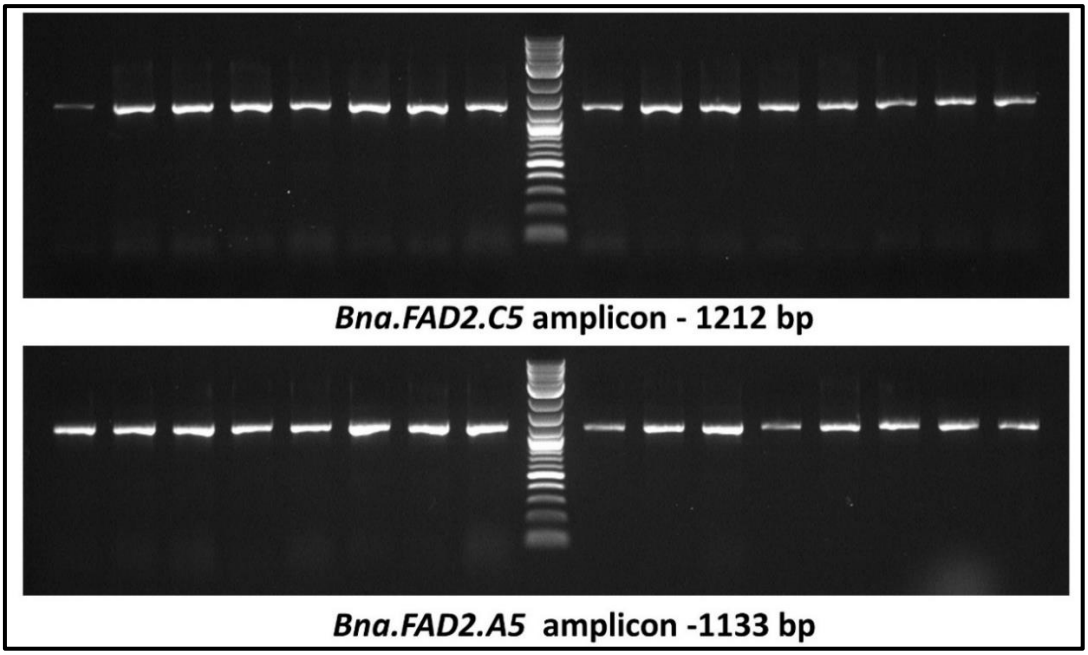

Figure 6.4 PCR amplification of *Bna.FAD2.C5* and *Bna.FAD2.A5* copies in F_1 progeny of the cross 'Maplus x Mutant'

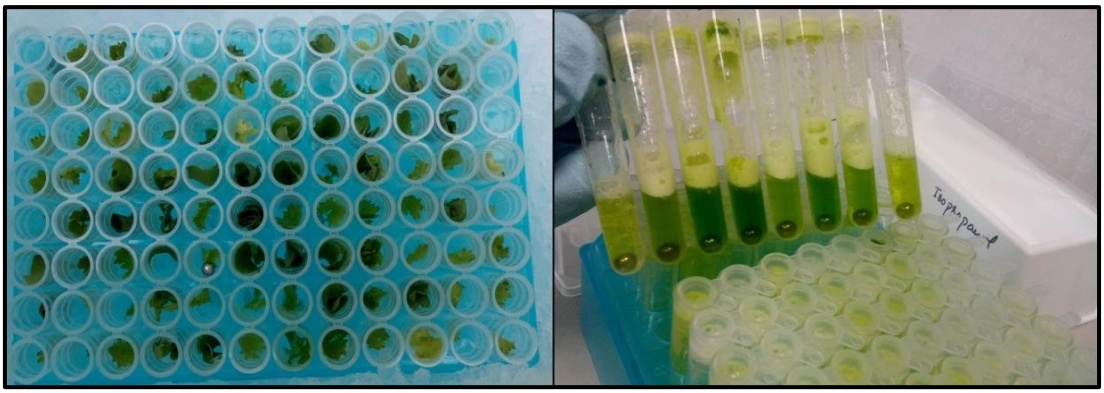

Figure 6.5 Sample preparation step for 'DNeasy Plant 96 Qiagen Kit'

Leaves were samples in a 96 well-microplate and one 3 mm tungsten bead was added to each well with the buffer for the automated DNA extraction in the BioSprint 96 workstation

Table 6.3 Selected plants in the F_2 progeny of the cross 'Maplus x Mutant' and their *Bna.FAD2* and *Bna.FAE1* profiles

Cross Description	Line Code	*Bna.FAE1.A8*	*Bna.FAE1.C3*	*Bna.FAD2.A5*	*Bna.FAD2.C5*
HELP profile		Homo	Homo	-	-
Mutation position[+] (bp)		821	300-301	158	337/231/584 /663[#]
Maplus x M0830	1-26[3]	Homo	Homo	Het	-
	10-66[3]	Homo	Homo	-	Het
	1-79[2]	Homo	Het	-	Het
	1-87[2]	Het	Homo	-	Het
	1-93[2]	Het	Homo	-	Het
	10-7[2]	Het	Homo	-	Het
Maplus x K0472	**2-91**	Homo	Homo	-	-
	2-10[3]	Homo	Homo	-	Het
	11-68[3]	Het	Homo	-	-
	11-82[3]	Homo	Homo	Het	-
	2-52[2]	Homo	Homo	Het	Het
	2-60[2]	Homo	Homo	Het	Het
Maplus x M2444	3-63[3]	Het	Homo	-	-
	12-93[3]	Het	Homo	-	-
	3-26[2]	Het	Het	-	-
	3-36[2]	Homo	Homo	Het	Het
	3-48[2]	Homo	Het	-	Het
	12-50[2]	Het	Homo	-	Het
Maplus x K0047	**4-87**	Homo	Homo	-	-
	4-77[3]	Homo	Homo	Het	-
	13-15[3]	Homo	Homo	-	Het
	4-6[2]	Het	Het	-	-
	4-43[2]	Het	Homo	Het	-
	4-68[2]	Homo	Homo	Het	Het
	13-52[2]	Het	Homo	Het	-

[+]Mutation position is according to the reference sequence and is not the actual gene position; [#]Different mutation positions for each mutant – '337' for M0830, '231' for K0472, '584' for M2444 and '663' for K0047; Codes in **bold** are the **HELP** lines; '[3]' represents three copies according to the HELP construct, '[2]' represents two copies according to the HELP construct; 'Homo' is a homozygous functional copy; 'Het' is a heterozygous copy and; '-' is a homozygous non-functional copy (partially functional copy for *Bna.FAD2.C5*).

In addition, genotypes having three copies according to the HELP construct but had one heterozygous copy (segregation in the next progeny) were also selected from each cross combination as shown in Table 6.3 (with subscript 3). In addition, 2 to 4 plants from each cross-type were selected with 2 copies heterozygous and 2 according to the HELP profile as depicted in Table 6.3 (with subscript 2).

6.4.4 F_3 Generation

6.4.4.1 Fatty Acid Analysis

The fatty acid compositions were analysed on the two HELP lines, 2-91 and 4-87 and control, Maplus with 3 technical replicates each using the bulk seeds method (Section 2.7.2). The results are depicted in Table 6.4. Both HELP lines had very low polyunsaturated fatty acids content as compared to 21% found in the high erucic acid variety, Maplus. Lower PUFA content of ~5% was found in 'Maplus x K0472' HELP 2-91 as compared to ~8% in 'Maplus x K0047' HELP 4-87. A slight increase in the erucic acid and very long chain fatty acids levels were observed in the HELP lines as compared to the control, Maplus. Oleic acid level increased to 25-27% in the HELP lines in comparison to ~12% found in Maplus. Saturated fatty acid levels were reduced by ~2% in the HELP lines as compared to HEAR. Total monounsaturated fatty acids content of ~88% and ~91% were found in these HELP lines.

Table 6.4 The fatty acid percentages (mean of 3 technical replicates) of the HELP lines (F_3 seeds of the cross 'Maplus x Mutant') and Maplus

Line	**14:0**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**	**20:1**
2-91	0.0	2.2	0.3	0.7	27.1	1.7	3.2	0.6	8.4
4-87	0.0	2.3	0.2	0.7	25.2	2.9	4.8	0.6	9.1
Maplus	0.0	3.6	0.2	0.8	12.3	12.4	8.2	0.7	8.7
Line	**20:2**	**22:0**	**22:1**	**24:0**	**24:1**	**PUFA**	**VLCFA**	**SAFA**	**MUFA**
2-91	0.0	0.4	53.7	0.1	1.4	4.9	63.5	4.2	90.9
4-87	0.0	0.4	52.6	0.1	0.9	7.8	62.7	4.1	88.1
Maplus	0.4	0.6	51.1	0.1	0.8	21.0	60.7	5.7	73.3

Line 2-91 is a 'Maplus x K0472' HELP; Line 4-87 is a 'Maplus x K0047' HELP; PUFA = 18:2+ 18:3+ 20:2; VLCFA =20:1+22:1+24:1; SAFA = 14:0+16:0+18:0+20:0+22:0+24:0 and; MUFA= 16:1+18:1+ 20:1+ 22:1+24:1

6.4.4.2 Multiplication

The F_3 seeds of 'HELP 2-91' and 'HELP 4-87' were multiplied in the glasshouse with 10 replications each with controls – Maplus and Ningyou 7 as shown in Figure 6.6. The plants were self-pollinated and seeds were harvested from the individual plants at the maturity.

Figure 6.6 Multiplication of HELP lines, 2-91 and 4-87 in the glasshouse
HELP lines from the crosses, 'Maplus x K0472' and 'Maplus x K0047' (F_3 progeny) were multiplied in 10 replicates each with the controls

6.4.4.3 Selections

In addition to the multiplication of the HELP lines, genotyping was carried out to select HELP lines from each cross-type. One line, having one heterozygous copy and other three according to the HELP profile, from each cross type ('Maplus x M0830', 'Maplus x K0472', 'Maplus x M2444' and 'Maplus x K0047') was selected for the next generation. Twelve plants were sown for each line and genomic DNA was extracted using the CTAB method. *Bna.FAD2* and *Bna.FAE1* copies were amplified and sequenced from the Eurofins (https://www.eurofins.co.uk/).

HELP lines were not found in the F_3 progeny of crosses, 'Maplus x K0472' and 'Maplus x K0047' but there was already one plant identified in the previous generation of these crosses. One HELP plant, '1-26-8' was identified in the cross 'Maplus x M0830' and two HELP plants, '3-63-4 and 3-63-5' were identified in the cross 'Maplus x M2444' (Figure 6.7). But these plants did not develop any pods and hence no seeds were produced as shown in Figure 6.8. It could happen due to the temperature fluctuations during the vernalisation period and thus, plants didn't have enough low-temperature period to initiate flowering later on. So, the selection process for HELP lines was repeated.

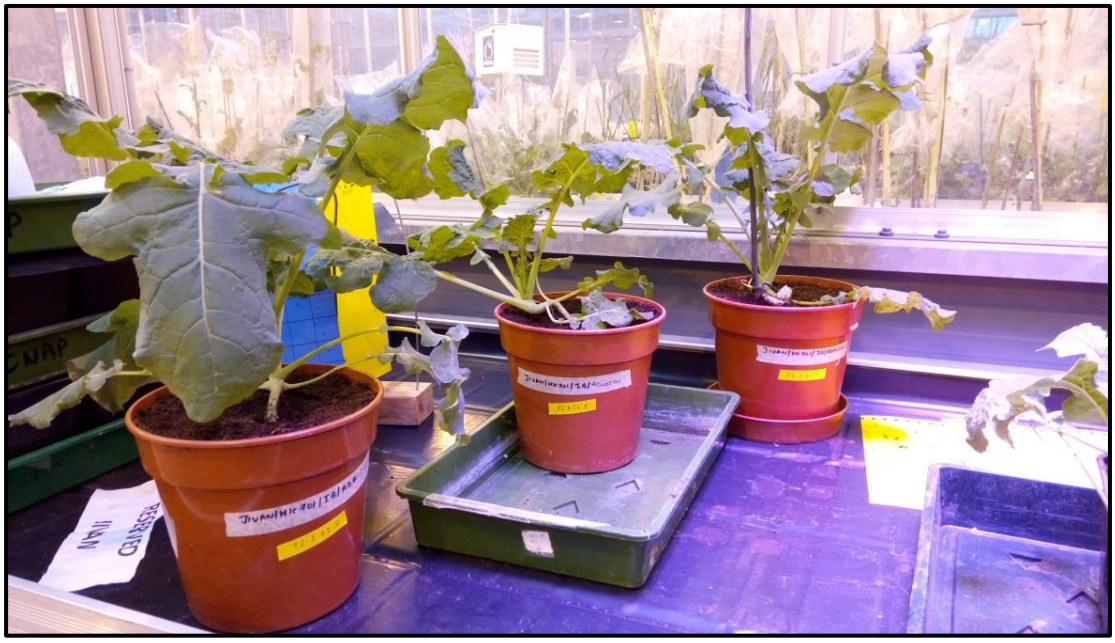

Figure 6.7 HELP lines – 1-26-8, 3-63-4 and 3-63-5 growing in the glasshouse
One HELP plant, 1-26-8 (in the middle) was identified in the cross 'Maplus x M0830' and 2 HELP plants, 3-63-4 and 3-63-5 (on the left and right) were identified in the cross 'Maplus x M2444' (F_3 plants)

Figure 6.8 HELP plants not producing any pods in the F_3 progeny
Selected HELP plants did not develop any pods and thus no seeds were produced as well

Two HELP lines, 1-26 and 10-66 from the cross, 'Maplus x M0830' and two HELP lines, 3-63 and 12-93 from the cross, 'Maplus x M2444' were selected. From each of these lines, 24 individual plants were grown. Control genotypes – Maplus, Cabriolet, M0830, M2444, K0472 and K0047 were also sown with these genotypes. DNA was extracted using the CTAB method and these were genotyped for *Bna.FAD2* and *Bna.FAE1* copies. PCR amplicons were sequenced from the Eurofins (https://www.eurofins.co.uk/) and mutations were detected using Mutation Surveyor®. Three HELP lines, 1-26-5, 1-26-19 and 1-26-24 were selected from the cross, 'Maplus x M0830'. Four HELP lines, 3-63-7, 3-63-10, 3-63-21 and 12-93-16 were selected from the cross, 'Maplus x M2444'. These were self-pollinated and seeds were harvested at the maturity.

6.4.5 F_4 Generation

6.4.5.1 Fatty Acid Analysis

The fatty acid compositions were analysed on 44 different samples including F_4 HELP lines from 'Maplus x K0472', 'Maplus x K0047', 'Maplus x M0830' and 'Maplus x M2444'; *Bna.fad2.C5* mutants – K0472, K0047, M0830 and M2444 and; high erucic acid variety, Maplus. Two biological replicates were used for each mutant and ten biological replicates were used for the variety, Maplus. Three technical replicates were used for each sample and the results are shown in Table 6.5.

In 'Maplus x K0472' HELP, 10 genotypes were analysed for the fatty acid content (Table 6.5). A mean value of 54.4% was found for the erucic acid content as compared to 46.1% in the HEAR variety, Maplus. In the HELP genotypes, erucic acid ranged from 50 to 58%. PUFA content of these HELP lines declined by 20% as compared to the parental HEAR. PUFA levels ranged from 4 to 6.7% in the individual HELP lines in comparison to 24-28% found in the biological replicates of Maplus. Mean value of the very long chain fatty acids were found to be 7.7% higher than Maplus and these ranged from 60 to 65% in these HELP lines. Total MUFA content in these individual HELP genotypes was found to be more than 90% in comparison to 68% (mean value) in Maplus. Mean value of the saturated fatty acids in HELP lines was 2.3% lower than the high erucic acid parent. Oleic acid levels increased by 14.6% in comparison to Maplus and decreased by 57% as compared to the mutant K0472 in the HELP lines.

Table 6.5 The fatty acid percentages (mean of 3 technical replicates) of the HELP lines (F_4 seeds of the cross 'Maplus x Mutant') and controls

S. No.	Genotypes	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
	Maplus x K0472																		
1	2-91-1	0.0	3.1	0.0	0.6	28.8	2.3	4.0	0.4	8.1	0.0	0.0	51.8	0.0	0.8	6.3	60.8	4.2	89.5
2	2-91-2	0.0	2.3	0.2	0.6	25.0	1.9	4.0	0.6	7.1	0.0	0.4	56.9	0.1	0.9	5.8	64.9	4.1	90.1
3	2-91-3	0.0	2.0	0.2	0.6	27.1	1.3	2.9	0.6	8.6	0.0	0.4	55.4	0.1	0.8	4.2	64.8	3.6	92.1
4	2-91-4	0.0	2.4	0.2	0.6	25.6	1.7	3.9	0.6	7.5	0.0	0.4	56.0	0.0	0.8	5.7	64.4	4.0	90.3
5	2-91-5	0.0	2.2	0.2	0.6	23.9	2.1	4.6	0.7	6.1	0.0	0.5	58.0	0.1	1.0	6.7	65.1	4.1	89.2
6	2-91-6	0.0	2.2	0.2	0.7	26.7	1.4	3.0	0.6	7.2	0.0	0.4	56.8	0.0	0.9	4.4	64.9	3.8	91.8
7	2-91-7	0.0	2.4	0.2	0.6	27.8	2.3	3.6	0.5	10.5	0.0	0.3	50.7	0.0	1.0	5.9	62.2	3.8	90.3
8	2-91-8	0.0	2.7	0.4	0.6	26.7	1.8	4.4	0.6	6.0	0.0	0.3	55.5	0.1	0.9	6.1	62.4	4.3	89.6
9	2-91-9	0.0	2.2	0.2	0.8	27.8	1.3	2.7	0.7	9.2	0.0	0.4	53.9	0.0	0.8	4.0	63.9	4.1	91.9
10	2-91-10	0.0	3.2	0.4	0.8	29.0	1.9	3.3	0.7	10.6	0.0	0.4	48.9	0.0	0.7	5.2	60.2	5.2	89.6
	Maplus x K0047																		
11	4-87-1	0.0	2.3	0.2	0.7	23.1	3.5	5.6	0.7	7.7	0.0	0.5	54.7	0.2	0.9	9.1	63.3	4.3	86.6
12	4-87-2	0.0	2.1	0.2	0.8	25.6	2.6	4.5	0.6	9.3	0.0	0.4	53.1	0.0	0.8	7.1	63.2	3.9	89.0
13	4-87-3	0.0	2.1	0.2	0.8	24.6	2.5	4.6	0.7	7.6	0.0	0.5	55.6	0.0	0.8	7.1	64.0	4.1	88.9
14	4-87-4	0.0	2.2	0.2	0.8	23.7	3.3	5.3	0.8	9.5	0.0	0.4	53.1	0.0	0.7	8.5	63.3	4.3	87.2
15	4-87-5	0.0	2.1	0.2	0.7	24.8	2.4	4.5	0.7	7.7	0.0	0.5	55.6	0.0	0.9	6.9	64.1	4.0	89.1
16	4-87-6	0.0	2.2	0.2	1.0	27.0	2.2	4.1	0.7	10.9	0.0	0.4	50.5	0.1	0.8	6.3	62.1	4.4	89.4
17	4-87-7	0.0	2.2	0.3	0.8	24.3	3.0	5.0	0.7	8.8	0.0	0.5	53.7	0.1	0.8	7.9	63.2	4.3	87.8
18	4-87-8	0.0	2.2	0.2	0.8	24.5	3.0	4.8	0.7	9.2	0.0	0.4	53.3	0.1	0.8	7.8	63.3	4.1	88.1
19	4-87-9	0.0	2.2	0.3	0.7	24.0	2.7	4.9	0.7	7.1	0.0	0.5	56.0	0.2	0.9	7.6	63.9	4.3	88.2
20	4-87-10	0.0	2.1	0.2	0.8	26.9	2.0	4.0	0.6	9.9	0.0	0.4	52.1	0.1	0.8	6.0	62.7	4.1	89.9
	Maplus x M0830																		
21	1-26-5	0.0	2.7	0.0	0.3	31.0	1.6	3.5	0.3	8.7	0.0	0.0	51.4	0.0	0.7	5.1	60.7	3.2	91.7
22	1-26-19	0.0	2.6	0.3	0.7	28.2	1.7	3.9	0.5	6.0	0.0	0.4	54.4	0.2	1.1	5.6	61.5	4.3	90.1
23	1-26-24	0.0	2.5	0.3	0.6	29.6	1.5	3.2	0.5	8.1	0.0	0.3	52.4	0.0	0.8	4.7	61.4	4.0	91.3

Table 6.5 Continued

S. No.	Genotypes	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
	Maplus x M2444																		
24	3-63-7	0.0	2.9	0.4	0.7	25.0	2.6	5.9	0.7	6.2	0.0	0.5	53.9	0.1	1.1	8.5	61.2	4.9	86.7
25	3-63-10	0.0	3.7	0.5	0.7	22.7	4.5	7.6	0.7	5.8	0.0	0.7	51.7	0.3	1.2	12.1	58.7	6.0	81.9
26	3-63-21	0.0	3.4	0.4	0.7	25.4	3.1	5.9	0.7	6.6	0.0	0.5	52.3	0.0	1.0	9.1	59.9	5.2	85.7
27	12-93-16	0.0	2.5	0.4	0.7	27.0	2.0	4.4	0.6	7.2	0.0	0.4	53.9	0.0	0.8	6.5	62.0	4.2	89.4
	Mutants																		
28	K0472-1	0.0	3.7	0.4	1.2	84.0	2.0	4.6	0.7	2.0	0.0	0.6	0.2	0.5	0.0	6.6	2.2	6.7	86.7
29	K0472-2	0.0	3.4	0.3	1.2	83.4	2.3	5.1	0.8	2.1	0.0	0.7	0.1	0.5	0.0	7.4	2.2	6.6	86.0
30	K0047-1	0.0	4.4	0.4	1.0	78.6	3.5	8.1	0.8	2.1	0.0	0.7	0.0	0.5	0.1	11.5	2.2	7.3	81.2
31	K0047-2	0.0	3.4	0.3	1.6	85.5	2.1	4.2	0.8	1.4	0.0	0.7	0.0	0.2	0.0	6.2	1.4	6.6	87.2
32	M0830-1	0.0	3.4	0.1	1.2	85.4	2.0	4.4	1.0	2.0	0.0	0.5	0.0	0.0	0.0	6.4	2.0	6.0	87.5
33	M0830-2	0.0	3.6	0.4	1.1	84.1	2.1	5.0	0.9	2.1	0.0	0.7	0.0	0.0	0.0	7.0	2.1	6.3	86.7
34	M2444-1	0.0	3.8	0.4	1.0	81.7	2.7	6.5	0.7	2.1	0.0	0.6	0.0	0.3	0.2	9.2	2.3	6.4	84.3
35	M2444-2	0.0	3.8	0.3	1.2	83.1	2.5	5.5	0.8	1.8	0.0	0.6	0.1	0.3	0.0	8.0	1.9	6.7	85.3
	Maplus																		
36	Maplus-1	0.0	4.2	0.3	0.9	11.5	13.7	9.9	0.7	10.9	0.6	0.5	46.4	0.0	0.4	24.2	57.7	6.3	69.5
37	Maplus-2	0.0	4.3	0.3	0.9	11.1	14.2	10.1	0.7	10.5	0.6	0.5	46.3	0.1	0.5	24.9	57.3	6.5	68.7
38	Maplus-3	0.0	4.9	0.4	0.7	10.2	17.3	9.9	0.5	7.2	0.7	0.5	46.8	0.1	0.9	27.8	54.9	6.7	65.4
39	Maplus-4	0.2	4.1	0.2	0.8	14.7	16.2	9.0	0.7	9.0	0.5	0.5	42.7	0.2	1.0	25.7	52.8	6.6	67.6
40	Maplus-5	0.0	3.9	0.2	0.8	11.1	16.3	9.0	0.6	8.4	0.6	0.6	47.5	0.2	0.9	25.8	56.9	6.0	68.2
41	Maplus-6	0.0	3.5	0.2	0.7	11.9	15.6	8.3	0.6	8.1	0.5	0.6	49.1	0.2	0.8	24.4	57.9	5.6	70.0
42	Maplus-7	0.0	3.8	0.2	0.7	11.0	16.6	9.1	0.5	7.1	0.5	0.6	48.9	0.1	0.9	26.2	56.9	5.6	68.1
43	Maplus-9	0.0	4.2	0.2	1.0	15.3	16.2	7.3	0.7	9.5	0.5	0.6	43.5	0.2	0.8	24.0	53.8	6.7	69.3
44	Maplus-10	0.0	5.1	0.3	0.8	13.1	17.9	7.4	0.7	8.6	0.6	0.6	43.8	0.2	0.8	25.9	53.1	7.5	66.6
	Maplus (Mean)	0.0	4.2	0.3	0.8	12.2	16.0	8.9	0.7	8.8	0.6	0.6	46.1	0.2	0.8	25.4	55.7	6.4	68.2

PUFA =18:2+18:3+20:2; VLCFA =20:1+22:1+24:1; SAFA= 14:0+16:0+18:0+20:0+22:0+24:0; MUFA=16:1+18:1+20:1+22:1+24:1

In ‘Maplus x K0047’ HELP, 10 genotypes were analysed for the fatty acid compositions (Table 6.5). Erucic acid levels ranged from 50 to 56% in the HELP lines and on average, 7.6% increase was found in comparison to HEAR, Maplus. Low PUFA levels were found in the HELP oil as compared to Maplus and an average reduction of 18% was found. PUFA levels similar to the parent, K0047 were reported in the HELP lines and the value ranged from 6 to 9%. VLCFAs showed a similar increase of 7.6% like the erucic acid content as compared to the HEAR. VLCFAs ranged from 62 to 64% in the individual HELP lines as compared to 53 to 58% found in the Maplus replicates. Saturated fatty acids content decreased by 2.2% in the HELP lines in comparison to Maplus and ~4% were found in the HELP lines. Total MUFAs content ranged from 86 to 90% in the HELP lines, showing an average 20% increase as compared to parental HEAR. For the oleic acid content, an increase of 12.7% as compared to Maplus and a reduction of ~57% compared to mutant K0047 were found in the HELP lines.

The fatty acid compositions of 3 HELP lines of the cross, ‘Maplus x M0830’ were measured (Table 6.5). An increase of 6.6% and 5.5% (mean values) were observed in the erucic acid and very long chain fatty acids values as compared to Maplus, respectively. Erucic acid values of 51 to 54% and VLCFAs values of approx. 61% were found in the HELP lines. PUFA levels of 5.1% were found on average in the HELP lines as compared to 25.4% (mean) in Maplus. Slightly lower amount of PUFAs was found in these HELP lines as compared to parent, M0830. On average, saturated fatty acids were declined by 2.6% in the HELP lines as compared to Maplus. On the average, oleic acid content increased by 17.4% as compared to Maplus and a decreased by 55% as compared to M0830 in the HELP lines.

In ‘Maplus x M2444’ HELP, 4 lines were analysed for the fatty acid compositions. An increment of 6.8% and ~5% were found in the erucic acid and very long chain fatty acid values, respectively in the HELP lines as compared to the HEAR, Maplus. Erucic acid levels ranged from 52 to 54% and VLCFAs ranged from 59 to 62% in the individual HELP lines. On average, a decrease of 16.4% was observed in the PUFA levels of the HELP lines as compared to Maplus. Similar quantities of PUFAs were reported in the HELP lines like the parental mutant, M2444. About 18% increase in the overall monounsaturated fatty acids content was found in the HELP lines as compared to Maplus. In the oleic acid values, an increase of 13% as compared to Maplus and a

decrease of 57.4% as compared to mutant, M2444 was found in the HELP lines. A reduction of ~1% was found in the saturated fatty acids in the HELP lines as compared to Maplus.

Thus, a general trend of higher erucic acid, higher very long chain fatty acids, higher oleic acid and lower saturated fatty acids were found in the HELP lines as compared to the high erucic acid rapeseed due to the partially functional *Bna.FAD2* family in the HELP lines. The different mutations in the *Bna.fad2.C5* copy show the gradation of effects on the PUFA content in the HELP lines.

6.4.5.2 Glucosinolates Analysis

Glucosinolates content was measured in 24 HELP lines and 6 parental controls. Two biological replicates were used and the detailed results are shown in Table 6.6. In the HELP lines, glucosinolates content ranged from 4 to 57 $\mu molg^{-1}$ and in the controls, it varied from 13 to 51 $\mu molg^{-1}$. Maplus had a total glucosinolates content of 26 $\mu molg^{-1}$, averaged from three biological replicates (17, 22 and 40 $\mu molg^{-1}$). In 'Maplus x M0830' HELP, two lines were measured and the glucosinolates content was 33 and 48 $\mu molg^{-1}$. The parent line, M0830 had the glucosinolates content of 51 $\mu molg^{-1}$. In 'Maplus x K0472' HELP, glucosinolates content in 9 HELP lines ranged from 8 to 46 $\mu molg^{-1}$ and the parental line, K0472 had only 13 $\mu molg^{-1}$ glucosinolates in its seeds. In 'Maplus x M2444' HELP, glucosinolates content ranged from 28 to 57 $\mu molg^{-1}$ in the three HELP lines. The parental line, M2444 had glucosinolates content of 18.5 $\mu molg^{-1}$. In 'Maplus x K0047' HELP, 10 HELP lines were measured for the glucosinolates content and lowest levels ranging from 4 to 12 $\mu molg^{-1}$ were observed. These had very low levels of a specific glucosinolate, progoitrin in their seeds. Parental line, K0047 had glucosinolates content of 24 $\mu molg^{-1}$. It could be observed from the glucosinolates content that the values in the HELP progenies did not correspond to the either of the parents. Many factors in addition to the genetics, determine the amount and type of glucosinolates accumulated in the rapeseed such as biotic and abiotic stresses (Jensen *et al.*, 1996; Martínez-Ballesta, Moreno and Carvajal, 2013). Thus, the levels of glucosinolates of HELP lines may have changed according to some stress such as an attack by some pests in the glasshouse.

Table 6.6 Glucosinolates content measured (mean of 2 biological replicates) in the HELP lines (F_4 seeds of the cross 'Maplus x Mutant') and controls

Line	GIB	PRO	GAL	GNL	GNA	4HG	GBN	GBS	4MG	GST	Neo	GLS
Maplus x M0830												
1-26-19	0.0	21.8	0.0	1.1	5.2	0.2	4.3	0.1	0.0	0.2	0.2	32.9
1-26-24	0.0	31.2	0.0	1.4	7.7	0.2	6.4	0.1	0.0	0.4	0.3	47.7
Maplus x K0472												
2-91-2	0.1	12.6	0.2	0.6	4.1	0.1	3.2	0.1	0.0	0.1	0.3	21.5
2-91-3	0.1	11.5	0.2	0.5	5.8	0.1	1.6	0.1	0.0	0.2	0.1	20.3
2-91-4	0.2	8.8	0.2	0.2	4.5	0.2	1.0	0.1	0.0	0.1	0.3	15.8
2-91-5	0.2	19.6	0.4	0.9	8.0	0.2	3.4	0.1	0.0	0.2	0.2	33.2
2-91-6	0.2	4.5	0.1	0.3	2.1	0.2	0.7	0.1	0.0	0.0	0.1	8.3
2-91-7	0.2	20.7	0.3	1.7	6.2	0.2	3.3	0.2	0.0	0.2	0.7	33.8
2-91-8	0.0	24.7	0.0	3.2	2.7	0.2	12.3	0.0	0.0	0.2	0.4	43.7
2-91-9	0.2	4.0	0.1	0.3	2.5	0.3	0.7	0.2	0.0	0.0	0.1	8.3
2-91-10	0.0	24.4	0.0	2.9	4.7	0.1	12.7	0.0	0.0	0.8	0.4	46.0
Maplus x M2444												
3-63-7	0.0	17.6	0.0	1.2	5.6	0.1	4.4	0.1	0.0	0.4	0.4	29.7
3-63-4	0.0	34.1	0.0	2.3	5.4	0.0	14.7	0.0	0.0	0.0	0.2	56.7
12-93-16	0.0	15.1	0.0	1.3	6.4	0.0	4.3	0.0	0.0	1.0	0.3	28.4
Maplus x K0047												
4-87-1	0.1	0.8	0.0	0.0	3.3	0.2	0.5	0.1	0.0	0.3	0.1	5.5
4-87-2	0.2	0.8	0.1	0.0	5.0	0.2	0.5	0.1	0.0	0.0	0.2	7.1
4-87-3	0.1	0.6	0.0	0.0	2.4	0.2	0.2	0.1	0.0	0.0	0.2	3.8
4-87-4	0.2	0.6	0.0	0.0	4.0	0.2	0.4	0.2	0.0	0.0	0.2	5.8
4-87-5	0.2	1.0	0.0	0.0	8.5	0.2	0.9	0.0	0.0	0.1	0.1	11.0
4-87-6	0.2	1.1	0.0	0.0	8.4	0.2	1.5	0.1	0.0	0.1	0.1	11.6
4-87-7	0.2	0.8	0.0	0.0	5.2	0.2	0.6	0.1	0.0	0.1	0.1	7.4
4-87-8	0.1	0.7	0.0	0.0	5.3	0.2	0.3	0.0	0.0	0.1	0.0	6.8
4-87-9	0.1	0.6	0.0	0.0	2.4	0.2	0.3	0.0	0.0	0.1	0.1	3.8
4-87-10	0.1	0.5	0.0	0.0	5.7	0.2	0.3	0.1	0.0	0.1	0.0	7.0
Controls												
Cab	0.1	11.1	0.4	0.6	4.7	0.3	1.9	0.3	0.0	0.3	0.4	20.2
M0830	0.0	34.5	0.0	2.8	6.4	0.0	6.1	0.0	0.0	0.7	0.4	50.9
K0472	0.1	5.4	0.3	0.2	5.0	0.2	1.2	0.0	0.0	0.1	0.2	12.8
M2444	0.0	10.9	0.0	0.9	4.1	0.0	1.7	0.1	0.0	0.5	0.4	18.5
K0047	0.1	13.2	0.2	0.5	6.3	0.1	2.0	0.1	0.0	1.0	0.2	23.6
Maplus	0.1	13.3	0.6	0.5	5.4	0.1	5.6	0.2	0.0	0.1	0.2	26.2

GIB = Glucoiberin; PRO = Progoitrin; GAL = Glucoalyssin; GNL = Gluconapoleiferin; GNA = Gluconapin; 4HG= 4-Hydroxyglucobrassicin; GBN = Glucobrassicanapin; GBS = Glucobrassicin; 4MG = 4-methoxyglucobrassicin; GST = Gluconasturtin; Neo = Neoglucobrassicin; GLS = total amount of glucosinolates and; Cab is Cabriolet

HELP lines from the cross 'Maplus x K0047' have been found to have very low levels of progoitrin (2-hydroxyl but-3-enyl glucosinolates) which is known to have toxic effects on mammals in addition to the insects (Greer and Deeney, 1959). These lines had lowest levels of total glucosinolates among the HELP lines and had progoitrin content of less than 1 $\mu molg^{-1}$.

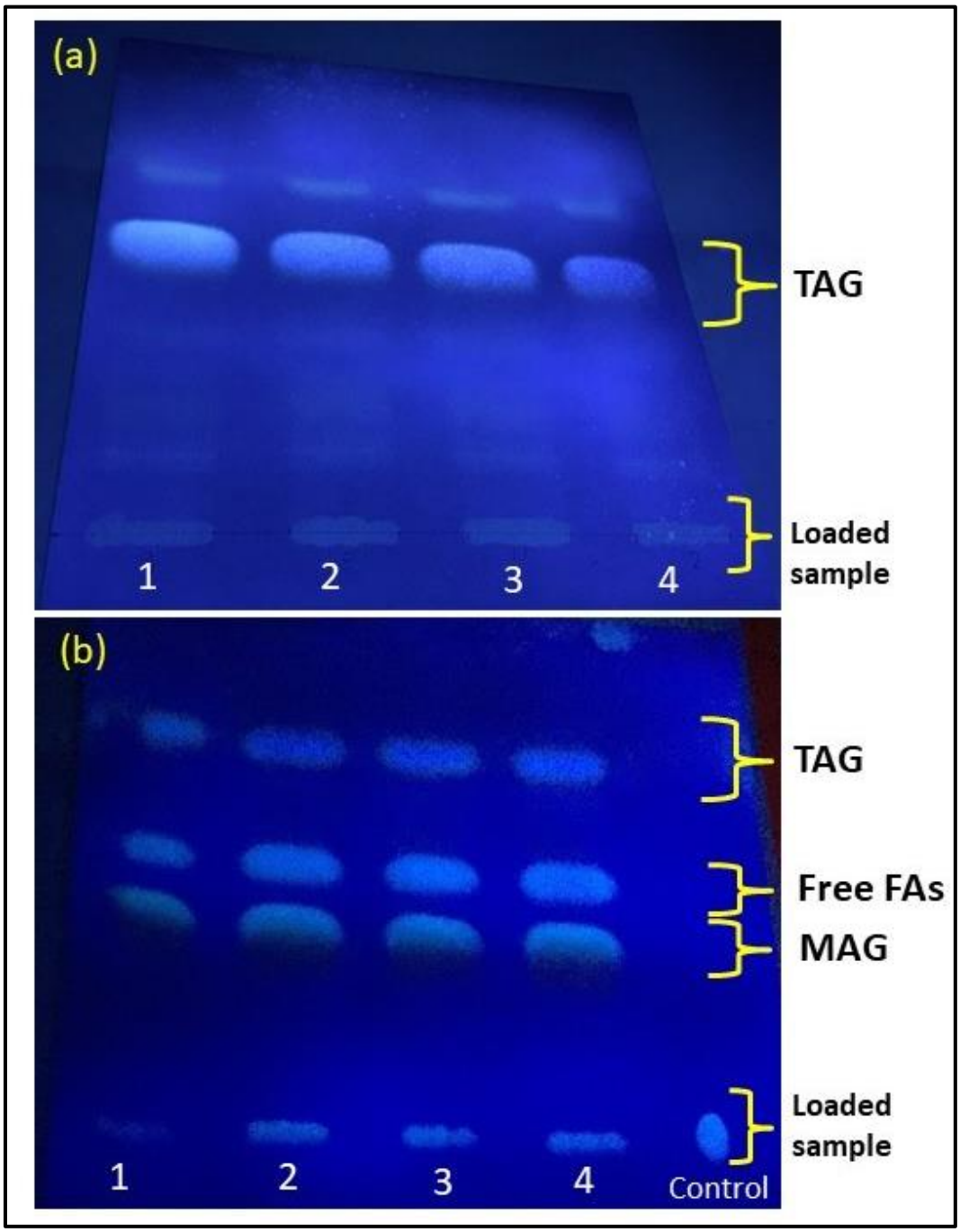

Figure 6.9 Thin layer chromatograms visualised under the UV light
(a) Separation of the TAG from the total lipids mixture (b) Separation of the sn-2 MAG, free fatty acids and undigested TAG after digesting the TAG with lipases from Rhizomucor miehei

6.4.5.3 The *sn-2* Positional Analysis

Total lipids were extracted from HELP lines (2-91-2, 2-91-3, 2-91-4 and 2-91-5), Maplus, Cabriolet and K0472 in four replicates each using the method described in Section 0. The *sn-2* positional distributions of the triacylglycerol (TAG) of these samples were analysed with the help from Peter J. Eastmond and Harrie van Erp at the Rothamsted Research, Harpenden, UK. Lipids were extracted and 10 µl of this sample was used for deriving the fatty acid methyl esters (FAMEs) using the method described in Erp, Menard and Eastmond, 2014. These were analysed in the gas chromatography and the results are shown in Table 6.7. These results were used for estimating the total lipid concentration and the total lipid content ranged from 7 to 43 µg/µl. Total lipids (1500 µg) were loaded on the thin layer chromatography (TLC) plate to separate the TAGs (Figure 6.9a) and the TAGs were extracted from the silica chromatography plate using the method described in Section 2.9.

Table 6.7 Percentages of the fatty acid compositions of the HELP lines and parents measured from the total lipids extracted

Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:1	24:1
2-91-2	2.2	0.2	0.8	26.7	1.6	3.1	0.6	2.4	0.4	55.0	0.9
2-91-3	2.1	0.0	0.6	26.8	1.2	2.7	0.6	2.1	0.4	56.8	0.9
2-91-4	2.4	0.2	0.6	25.3	1.9	3.7	0.6	2.6	0.4	56.5	0.9
2-91-5	2.2	0.0	0.6	23.9	2.0	4.2	0.6	2.2	0.5	59.6	0.9
Maplus-1	4.1	0.0	0.8	13.9	16.3	6.4	0.6	4.1	0.6	47.6	0.8
Maplus-2	4.3	0.0	0.8	10.0	16.5	8.5	0.5	4.3	0.5	50.7	1.1
Maplus-3	4.0	0.2	0.8	14.6	16.1	7.2	0.6	4.3	0.6	45.6	0.9
Maplus-4	3.9	0.0	0.7	10.9	15.9	8.7	0.5	3.9	0.5	50.6	1.0
Cab-1	4.1	0.0	1.1	75.6	8.2	9.1	0.4	4.1	0.0	0.0	0.0
Cab-2	4.1	0.0	1.0	76.1	7.6	9.0	0.4	4.1	0.0	0.0	0.0
Cab-3	4.8	0.0	1.0	72.9	9.6	10.1	0.0	4.8	0.0	0.0	0.0
Cab-4	4.6	0.0	1.0	72.7	9.6	9.7	0.4	4.6	0.3	0.0	0.0
K0472-1	3.4	0.2	1.1	87.1	1.8	3.5	0.5	3.6	0.3	0.0	0.2
K0472-2	3.6	0.0	1.0	86.7	2.0	4.8	0.0	3.6	0.0	0.0	0.0
K0472-3	3.4	0.0	1.1	86.7	2.0	4.2	0.5	3.4	0.0	0.0	0.0
K0472-4	3.7	0.0	1.5	85.8	2.3	4.6	0.0	3.7	0.0	0.0	0.0

Cab is Cabriolet

The fatty acids were analysed from these samples and the results are given in Table 6.8. The fatty acid compositions of the TAGs were found to be similar to the total lipid compositions. These TAGs were digested by using the lipase enzyme from *Rhizomucor miehei*. It digested the fatty acids from the *sn-1* and *sn-3* positions and thus, left the

fatty acid at the central *sn-2* position of the TAG. The *sn-2* monoacylglycerol (MAG) was then separated from the mixture by using TLC as shown in Figure 6.9b. Figure 6.9b also shows the separation of MAGs, undigested TAGs and free fatty acids. MAGs was extracted from the TLC plate and FAMEs were derivatized from it for analysis by the gas chromatography. The fatty acids results of the MAG at the *sn-2* position are given in Table 6.9. Mean and standard deviation of TAGs and MAGs of each of the four samples – HELP, Maplus, Cabriolet and KO472 is shown in Figure 6.10.

Table 6.8 Triacylglycerol (TAG) fatty acid compositions of the HELP lines and parents

Line	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:1
2-91-3	1.8	0.8	26.4	1.2	2.5	0.5	7.9	0.0	0.4	57.5	0.9
2-91-4	2.3	1.0	25.9	1.6	3.2	0.6	7.1	0.0	0.4	57.0	0.9
2-91-5	1.9	0.7	24.8	2.1	3.9	0.6	5.7	0.0	0.5	58.9	0.9
Maplus-1	3.2	0.9	13.4	15.4	6.1	0.6	8.7	0.9	0.6	49.4	0.9
Maplus-3	3.5	0.9	9.8	15.4	8.3	0.4	7.0	1.8	0.5	51.9	1.1
Maplus-4	3.7	1.2	13.7	14.5	6.8	0.6	9.0	1.4	0.6	47.5	0.9
Cab-1	4.0	1.6	73.6	8.0	8.5	0.4	1.8	0.0	0.3	1.5	0.2
Cab-2	3.6	1.2	75.7	7.4	8.4	0.4	2.0	0.0	0.3	0.8	0.2
Cab-3	4.1	1.1	72.8	9.3	9.3	0.4	1.8	0.0	0.3	0.6	0.3
Cab-4	4.1	1.1	73.0	9.2	9.1	0.4	1.9	0.0	0.3	0.7	0.2
K0472-1	3.3	1.5	86.6	1.9	3.4	0.4	1.9	0.0	0.3	0.4	0.2
K0472-2	3.2	1.0	86.2	1.9	4.4	0.4	2.0	0.0	0.3	0.3	0.2
K0472-3	3.0	1.2	86.6	1.8	3.9	0.5	2.3	0.0	0.4	0.3	0.0
K0472-4	2.9	1.2	85.8	2.1	4.4	0.5	2.4	0.0	0.4	0.4	0.0

Cab is Cabriolet

Table 6.9 The *sn-2* monoacylglycerol's (MAG) fatty acid compositions

Line	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:1
2-91-3	0.8	0.6	84.4	3.8	8.7	0.0	0.9	0.6	0.0	0.4	0.0
2-91-4	2.0	1.8	76.9	5.2	10.5	0.0	0.9	2.2	0.0	0.5	0.0
2-91-5	0.6	0.5	77.5	6.4	13.2	0.0	0.9	0.5	0.0	0.6	0.0
Maplus-1	0.5	0.3	32.6	47.3	18.6	0.0	0.3	0.4	0.0	0.1	0.0
Maplus-3	0.9	0.7	23.9	48.2	25.2	0.0	0.3	0.6	0.0	0.2	0.0
Maplus-4	0.9	0.8	32.3	44.6	20.3	0.0	0.3	0.7	0.0	0.2	0.0
Cab-1	0.5	0.3	68.3	14.2	16.3	0.0	0.0	0.4	0.0	0.0	0.0
Cab-2	0.4	0.3	69.5	13.3	16.0	0.0	0.1	0.4	0.0	0.0	0.0
Cab-3	0.6	0.5	64.2	17.2	17.0	0.0	0.0	0.5	0.0	0.0	0.0
Cab-4	0.9	0.7	65.5	16.8	15.2	0.0	0.0	0.9	0.0	0.0	0.0
K0472-1	1.9	1.2	86.2	3.4	6.0	0.0	0.0	1.3	0.0	0.0	0.0
K0472-2	0.7	0.5	86.5	3.6	8.1	0.0	0.1	0.5	0.0	0.0	0.0
K0472-3	0.5	0.5	87.7	3.3	7.5	0.0	0.0	0.5	0.0	0.0	0.0
K0472-4	0.6	0.5	86.6	3.7	8.1	0.0	0.0	0.4	0.0	0.0	0.0

Cab is Cabriolet

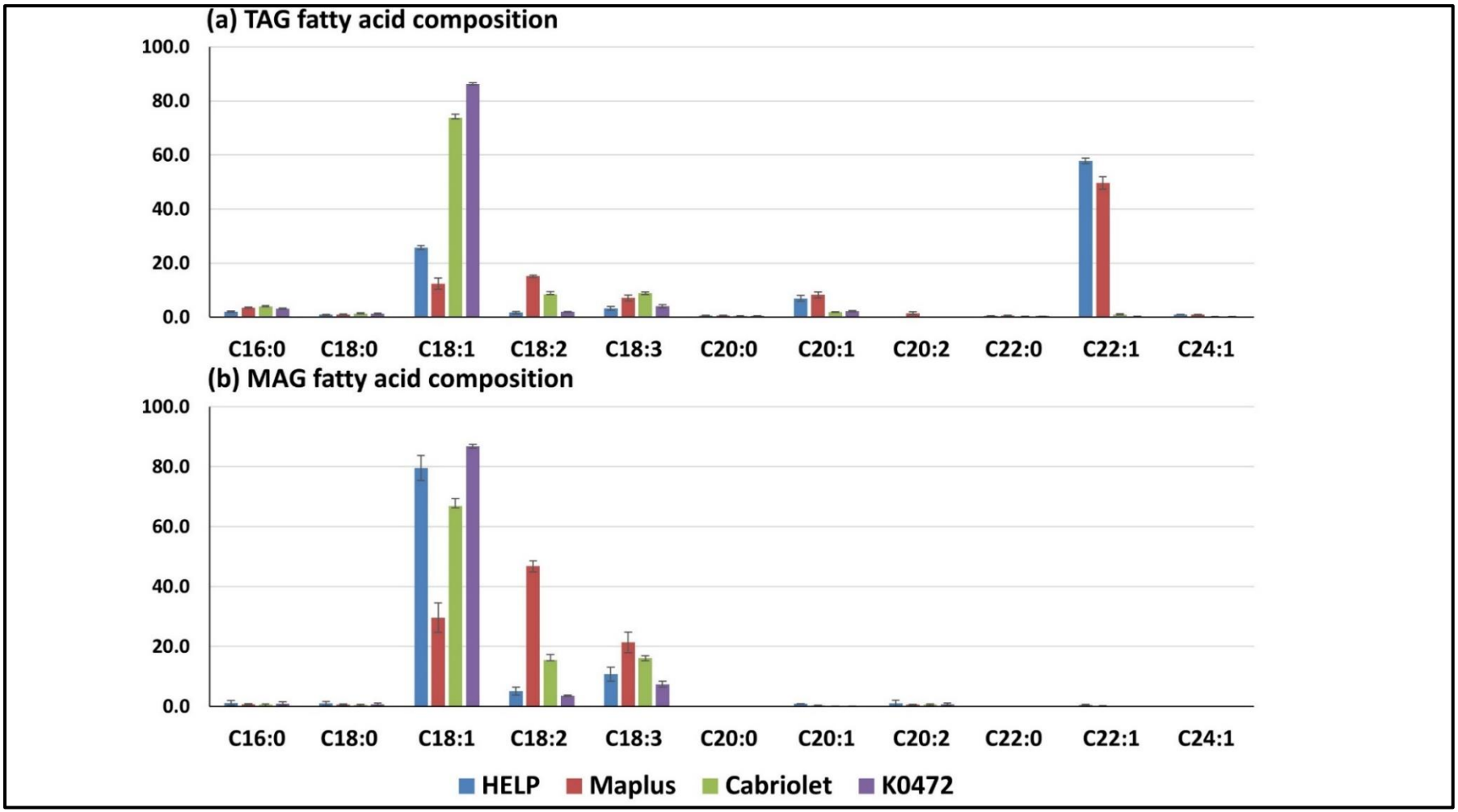

Figure 6.10 Mean fatty acid compositions of TAG and *sn-2* MAG of HELP lines, Maplus, Cabriolet and K0472

Four biological replicates were used for each sample and the graph shows the fatty acid profiles of (a) Triacylglycerol (TAG) (b) sn-2 monoacylglycerol (MAG)

Approximately 0.5% of erucic acid was found at the *sn-2* position of the HELP lines as compared to 0.2% found in the HEAR, Maplus. In total, there was ~1% increment in the very long chain fatty acids at the *sn-2* position in the HELP lines as compared to HEAR, Maplus. In the HELP lines, 80% oleic acid, 5% linoleic acid and 11% linolenic acid were present at the *sn-2* position while in Maplus, 47% linoleic acid, 30% oleic acid and 21% linolenic acid were present at this position. In K0472, the same amount of oleic acid (87%) was found in the TAG and *sn-2* MAG's fatty acids profiles. Thus, mostly oleic acid was present at the *sn-2* position, resulting in the formation of triolein (triglyceride formed with glycerol and three units of oleic acid) in K0472. In Cabriolet, 67% oleic acid, 15% linoleic acid and 16% linolenic acid was found at the *sn-2* position. *B. napus* lysophosphatidic acid acyltransferase (*Bn-LPAAT*) is responsible for the second acylation at the *sn-2* position and has a high specificity for the acyl moieties. It possesses a poor affinity for the erucoyl moiety and other acyl moieties higher than 18 Carbons in length (Kunst, Taylor and Underhill, 1992; Frentzen, 1993). Thus, mostly C18 fatty acids were present the central position of the TAG of all of these lines.

6.4.6 Comparing Maplus x Mutant Combinations

HELP lines were developed by cross-pollinating Maplus with four different mutants and thus, four different HELP lines were produced which were grouped into four separate groups. Mean values and standard deviation of polyunsaturates and very long chain fatty acids of these four groups are given in Table 6.10.

One-way ANOVA (analysis of variance) was used for comparing these four HELP groups, significant p-values of 6.32×10^{-5} and 0.00204 were found for the PUFAs and VLCFAs, respectively. Post-hoc test, Tukey was used to know the differences in the individual group combinations and the results are depicted in Table 6.11. M2444-HELP (9.1% PUFAs, 60.5% VLCFAs) was significantly different from K0472-HELP (5.4% PUFAs, 63.4% VLCFAs) in both polyunsaturates and very long chain fatty acids content. It was also significantly different from K0047-HELP (7.4% PUFAs, 63.3% VLCFAs) in VLCFAs content. There were no significant differences found in M0830-HELP (5.1% PUFAs, 61.2% VLCFAs) and K0472-HELP for either PUFAs or VLCFAs content. K0047-HELP had significantly different PUFA content than all except M2444-HELP. M2444-HELP had significantly different PUFAs than both K0472-HELP and M0830-HELP.

Overall, there doesn't seem to be as an expected trend – lower PUFAs do not give quantitatively higher VLCFAs. Thus, the trend is not consistent. The differences in these fatty acid values may be too small to identify any trend clearly. PUFA levels differ by only 3.7% between the highest and lowest HELP group. It may be a very small value to show a quantitative effect on the VLCFAs content in the different HELP groups.

Table 6.10 Mean values of PUFAs and VLCFAs values of HELP lines in four groups

S. No.	HELP group	PUFAs (Mean ± SD)	VLCFAs (Mean ± SD)
1	K0047-HELP	7.4 ± 1.0	63.3 ± 0.6
2	K0472-HELP	5.4 ± 0.9	63.4 ± 1.8
3	M0830-HELP	5.1 ± 0.5	61.2 ± 0.4
4	M2444-HELP	9.1 ± 2.3	60.5 ± 1.5

SD is the standard deviation

Table 6.11 Results of the post-hoc test: Tukey for four HELP groups

S. No.	Comparison	PUFAs (p-value)	VLCFAs (p-value)
1	K0472-HELP-K0047-HELP	0.0052695*	0.999775
2	M0830-HELP-K0047-HELP	0.0351529*	0.099459
3	M2444-HELP-K0047-HELP	0.1271516	0.006587*
4	M0830-HELP-K0472-HELP	0.9810468	0.088825
5	M2444-HELP-K0472-HELP	0.0001812*	0.005657*
6	M2444-HELP-M0830-HELP	0.0013899*	0.878159

*Significantly different

6.4.7 Summary

HELP lines were developed by cross-pollinating Maplus with four *Bna.fad2.C5* mutants, M0830 (4.6% PUFAs), K0472 (5.9% PUFAs), K0047 (6.4% PUFAs) and M2444 (6.9% PUFAs) and an allelic series of "2x *Bna.FAE1*^{Map}" in the low polyunsaturated background was established. It was followed by selections and self-pollination for four generations. HELP lines were selected in the F_2 plants (F_3 seeds) for the combinations, 'Maplus x K0472' and 'Maplus x K0047' and; in the F_3 plants (F_4 seeds) for the combinations, 'Maplus x M0830' and 'Maplus x M2444'. The fatty acid compositions were measured for the HELP lines and parents. Higher amounts of erucic acid, very long chain fatty acids and oleic acid and; lower amounts of polyunsaturated fatty acids

and saturated fatty acids were found in the HELP lines as compared to high erucic acid variety, Maplus.

HELP lines were derived from four different mutants and thus, the four groups were compared to each other. 'Maplus x M2444' HELP (9.1% PUFAs, 60.5% VLCFAs) and 'Maplus x K0472' HELP (5.4% PUFAs, 63.4% VLCFAs) showed the quantitative variations between very long chain fatty acids and polyunsaturates. All other HELP groups showed a significant difference in either VLCFAs or PUFAs or none. But no conclusions could be drawn from the present study about the influence of quantitative differences in PUFAs on the VLCFAs content of HELP line. This may be due to very small differences present in the PUFAs between different HELP groups to show a significant influence on the VLCFAs content.

HELP lines developed from the cross 'Maplus x K0472' and 'Maplus x K0047' potentially have the most suitable background for cultivation in the UK as the parent, Maplus is a winter-type variety grown in Europe. K0472 and K0047 are the mutants developed from Cabriolet which is also a winter-type rapeseed. Moreover, these had low glucosinolates levels in their seeds. HELP lines from the cross 'Maplus x K0047' had the lowest glucosinolates, specifically progoitrin in their seeds. These HELP lines could potentially serve as a valuable material for the high erucic, low polyunsaturates and low glucosinolates rapeseed. HELP lines developed from the cross 'Maplus x K0472' were multiplied at higher scale in replicated field conditions at the walled gardens, University of York, UK. The fatty acid compositions were analysed and up to 66% very long chain fatty acids were found with less than 5% polyunsaturated fatty acids. Glucosinolates levels of less than 15 µmol per gram of seeds were also observed in these HELP lines.

In addition to the fatty acids and glucosinolates content, the positional analysis was carried out to see if any trierucin was accumulated in the HELP lines. In the HELP lines, ~1% increase was found in the very long chain fatty acids accumulated at the *sn-2* position as compared to Maplus. *Bn-LPAAT* is highly specific and has poor affinity for VLCFAs (Kunst, Taylor and Underhill, 1992; Frentzen, 1993) and thus, only trace amounts of VLCFAs were found at the *sn-2* position in the samples analysed.

6.4.8 Conclusion

An allelic series of "2 x *Bna.FAE1*$^{Map"}$ in low polyunsaturated background involving *Bna.fad2.C5* alleles of various effect from the different mutant lines – M0830, K0472, K0047 and M2444 were developed in the Maplus background. The influence of quantitatively reducing the polyunsaturated fatty acids content on the very long chain fatty acids could not be observed in the present study. This may be due to small differences present in the PUFAs of the different groups to observe a significant effect on the VLCFAs.

7. Summary, Discussion and Future Directions

7.1 Summary and Outcomes

7.1.1 Two Major Genes Control the Erucic Acid Synthesis

Genetic association studies involve a correlation analysis between the trait and genetic variation in order to identify potential candidate genes controlling a specific trait. Associative transcriptomics (AT) technology was developed by Harper *et al.*, 2012 and it uses transcriptome sequencing for identifying the markers associated with the traits in polyploids such as *B. napus*. For the present study, the fatty acid compositions were analysed on 404 accessions under the RIPR project (RIPR, 2014) and the erucic acid content varied from 0 to 52%, reflecting a wide range of crop types present in the panel. AT was conducted for the erucic acid content on the *B. napus* diversity panel comprising of 383 accessions as the functional genotypes were available for only 383 accessions. The major peaks were found on the A8 and C3 chromosomes (Havlickova *et al.*, 2018) which corresponded to the known QTLs controlling the erucic acid synthesis (Harvey and Downey, 1964; Ecke and Breeding, 1995; Howell, Lydiate and Marshall, 1996; Jourdren *et al.*, 1996; Thormann *et al.*, 1996; Fourmann *et al.*, 1998; Qiu *et al.*, 2006; Smooker *et al.*, 2011). *FAE1* is not expressed in the leaves and in the AT analysis, its orthologues were present near the centres of the association peaks on A8 and C3 chromosomes. These loci are represented as *Bna.FAE1.A8* and *Bna.FAE1.C3* in the present study while these can also be represented as '*eru1* and *eru2'; 'e1 and e2'; 'BN-FAE1.1* and *BN-FAE1.2'* and; *'BnaA.FAE1.a* and *BnaC.FAE1.a'* as described in various other studies (Howell, Lydiate and Marshall, 1996; Fourmann *et al.*, 1998; Smooker *et al.*, 2011). Well-defined peaks were also detected in the regions involved in the glucosinolates controls on the chromosomes A2, A9, C2 and C9. It shows the co-selection of the two traits – erucic acid and glucosinolates (double zero rapeseed lines, canola) during the course of time in the modern rapeseed cultivars. Many minor association peaks were also observed

in the Manhattan plot for the erucic acid content and a potential candidate, Cab033920.1 (orthologue of AT2G34770.1) on the A5 chromosome having a role in the very long chain fatty acid synthesis in Arabidopsis was identified. This candidate gene was analysed with the knocked out T-DNA lines of *A. thaliana* but no change was observed in the fatty acids compositions of the mutants. Thus, the analysis of the candidate, Cab033920.1 did not provide any support for its involvement in controlling the very long chain fatty acids content. It was followed by browsing the genes related to the fatty acid elongation pathway in the minor association peaks but new loci influencing the erucic acid content were not identified in the present study. Finally, AT was conducted on two sub-sets of erucic acid content – one with high erucic acid genotypes having more than 30% erucic acid and another with low erucic acid genotypes having less than 5% erucic acid but weak associations were observed and no candidate genes related to the erucic acid biosynthesis were identified.

7.1.2 *Bna.FAD2* Alleles Influence the Fatty Acid Compositions

Bna.FAD2 family controls the desaturation step of the oleic acid (C18:1) to linoleic acid (C18:2) during the fatty acid biosynthesis and it is present in four copies (*Bna.FAD2.A1, Bna.FAD2.A5, Bna.FAD2.C1* and *Bna.FAD2.*C5) in the *B. napus* genome (Scheffler *et al.*, 1997). *Bna.FAD2.A1* is unlikely to be functional in *B. napus* due to mutations in the open reading frame. One base pair deletion (thus, frameshift mutation) in *Bna.FAD2.A5* copy in the variety, Cabriolet makes it non-functional. *Bna.FAD2.C1* copy is deleted in Cabriolet (Yang *et al.*, 2012; Wells *et al.*, 2014). Wells *et al.*, 2014 developed an EMS-mutagenized Cabriolet population, JBnaCAB_E having an allelic variation in the copy *Bna.fad.C5* leading to an increase in the oleic acid content and a decrease in the polyunsaturates levels in the mutants. Thus, we believe that this copy is partially functional and in the present study, thus, *Bna.FAD2* family is assumed to be partially functional, i.e., three copies are non-functional and one copy is partially functional (mutated). One of the mutants, K0472 (oil profile: ~85% C18:1, ~6% PUFAs, 0% C22:1) was selected from the JBnaCAB_E population and a pilot experiment was conducted to observe the influence of partially functional *Bna.FAD2* family on the very long chain fatty acids and polyunsaturates in *B. napus* by cross-pollinating K0472 to a high erucic acid cultivar, Ningyou 7 (oil profile: ~12% C18:1, ~21% PUFAs, ~50% C22:1;

functional *Bna.FAD2s* and functional *Bna.FAE1s*). The F_1 progeny was back-crossed to Cabriolet (oil profile: ~76% C18:1, ~19% PUFAs, 0% C22:1) which was the parental background for mutagenesis and the F_1B_1 progeny was self-pollinated for 6 generations accompanied by various genotypic and phenotypic selections. A unique specification of the rapeseed – high erucic acid and low polyunsaturates rapeseed (HELP) having a genotypic construct, "4 x *Bna.fad2*^{K0472} and 2 x *Bna.FAE1*^{NY7}" was developed in the present study. Winter oilseed rape (WOSR) are more suitable for cultivation in Europe (Röbbelen, 1991; McVetty *et al.*, 2016), so the flowering time of the HELP lines was assessed to select winter types. Partially functional *Bna.FAD2* family influenced the fatty acid profiles of the HELP lines ($F_1B_1S_5$ seeds) – reduced the PUFAs (C18:2 and C18:3) content to less than 7%, increased the erucic acid content up to 60%, increased the very long chain fatty acids levels (C20:1, C22:1, and C24:1) up to 65%, increased the oleic acid content up to 26% and reduced the levels of the saturated fatty acids to 4% in comparison to Ningyou 7. Thus, due to partially functional *Bna.FAD2* in the HELP lines, the lower amount of oleic acid went into desaturation pathway and thus, reducing the linoleic acid and linolenic acid (C18:3) accumulation. Thereby, overall PUFA levels were reduced in the HELP lines. In addition, more oleic acid was available as a substrate for the elongation pathway increasing the erucic acid, very long chain fatty acids and oleic acid levels. Therefore, these results supported our hypothesis – the influence of partially functional *Bna.FAD2* family on the levels of VLCFAs and PUFAs. Glucosinolates content was also measured in the HELP lines and a wide range of values was reported due to high glucosinolates present in the parent, Ningyou 7 and low glucosinolates in the other parental genotypes, K0472 and Cabriolet.

7.1.3 HELP Development in a Suitable Background

Both Maplus and Ningyou 7 are high erucic acid varieties having similar oil profiles but Maplus is a winter-type oilseed rape while Ningyou 7 is a semi-winter rapeseed. Also, Maplus is a commercial high erucic acid rapeseed variety grown in Europe. In addition, Ningyou 7 based HELP were grown in the field in the season 2017-18 and lower yields were observed as compared to the standard winter type rapeseed varieties. It may be due to Ningyou 7's background in the HELP lines. Thus, Maplus would be a more

suitable background for cultivation in Europe. So, HELP lines from the Ningyou 7 background were cross-pollinated to Maplus (as female) to produce Maplus based HELP. Similar changes in the fatty acid profiles were observed in this HELP like Ningyou 7 based HELP, showing the stability of this approach to increase VLCFAs and lower PUFA levels. The resulting HELP lines showed the effect of partially functional *Bna.FAD2* family – very long chain fatty acids content increased up to 66%, erucic acid content elevated up to 60% and polyunsaturated decreased to less than 7%. The total monounsaturated fatty acids level of up to 93% were observed in the HELP lines. To observe the differences in the *Bna.FAE1* alleles of these two high erucic acid cultivars, the fatty acid compositions of Maplus based HELP and Ningyou 7 based HELP were compared but no significant differences were found. Thus, there may be allelic differences in the *Bna.FAE1* sequences of the two high erucic acid varieties, Maplus and NY7 but it does not affect the levels of very long chain fatty acids accumulated. A range of glucosinolates content was found in these HELP lines due to a variable amount of glucosinolates present in the parental genotypes. But the low glucosinolates HELP could be easily selected as the loci controlling the erucic acid and glucosinolates are unlinked and are present on the different chromosomes.

7.1.4 Quantitative Effect of *Bna.FAD2* Alleles

Various mutants were developed in the Cabriolet background by specifically targeting one of the functional copies (*Bna.FAD2.C5*) of the *Bna.FAD2* family by Wells *et al.*, 2014. These mutants had many allelic variations in this copy, showing an effect on the oleic acid and polyunsaturates content. Four mutants – M0830, K0472, M2444 and K0047 had high oleic acid and low PUFAs (HOLP) in their seeds and were used for the present study. Their polyunsaturates (C18:2 and C18:3) content varied from 4.6 to 6.9% with a different position of the mutation in the copy *Bna.fad2.C5*. HELP lines were developed by cross-pollinating Maplus with HOLP and an allelic series of "2x *Bna.FAE1*^{Map}" in the low polyunsaturated background was developed. In the HELP lines, higher amounts of very long chain fatty acids, erucic acid and oleic acid and; lower amounts of polyunsaturated fatty acids and saturated fatty acids were observed like the previously developed HELP lines. HELP derived from the cross, 'Maplus x K0472' had the highest very long chain fatty acids content and low polyunsaturated

fatty acids level. Four types of HELP lines – 'Maplus x M0830', 'Maplus x K0472', 'Maplus x M2444' and 'Maplus x K0047', were compared to each other to observe the quantitative effect of *Bna.FAD2* alleles on the very long chain fatty acids content and polyunsaturated fatty acids content. It was not possible to conclude the influence of quantitative differences in PUFAs on the VLCFAs content of HELP lines from the present study as no consistent trend was observed as expected. It may be due to small differences present in the PUFA levels between these groups in order to show a significant change in the VLCFAs content. Both the parents used for the development of these HELP lines were winter-type, so the HELP lines from these crosses are expected to have the most suitable background for cultivation in Europe as compared to the HELP developed by using NY7. Glucosinolates content was also measured and low glucosinolates were observed in many HELP lines. Low progoitrin (one of the glucosinolates having an anti-nutritional effect) HELP lines were found in the cross 'Maplus x K0047' and these lines could serve as a valuable material for studying the genetic basis of this change. In addition, trierucin levels were analysed in the HELP lines to observe any erucic acid or eicosenoic acid accumulating at the central *sn-2* position of the TAG molecule. Approx. 1% increase was found in the very long chain fatty acids at the *sn-2* position in the HELP lines in comparison to Maplus.

7.2 Discussion

7.2.1 Overall Conclusions

Associative transcriptomics showed the already known genes controlling erucic acid content – *Bna.FAE1.A8* and *Bna.FAE1.C3* and it was not possible to underpin any new loci affecting the erucic acid biosynthesis in the present study but there may be modifiers present in the *B. napus* genome to fine-tune the erucic acid content.

Partially functional *Bna.FAD2* family in the HELP lines removed the draw of oleic acid into the desaturation pathway and hence decreased the polyunsaturated fatty acids. This led to an increment in the oleic acid levels and thus, more substrate was available for the elongation pathway, increasing the very long chain fatty acids pool catalysed by *Bna.FAE1*. Thus, these results supported our initial hypothesis of introducing

partially functional *Bna.FAD2* family in the high erucic background to increase VLCFAs and decrease PUFAs. Using the same approach of introducing partially functional *Bna.FAD2* family in the high erucic acid cultivars, HELP lines were developed from many combinations as shown in Table 7.1. Total very long chain fatty acid values of up to 66% and erucic acid levels of up to 60% were found in the HELP lines. Polyunsaturated fatty acids ranged from 4 to 7% in most of the HELP lines.

Table 7.1 Various HELP lines developed by the combination of HEAR and mutants

S. No.	Cross description of HELP lines
1	Cabriolet x (K0472 x Ningyou 7)
2	Maplus x (Cabriolet x (K0472 x Ningyou 7))
3	Maplus x M0830
4	Maplus x K0472
5	Maplus x M2444
6	Maplus x K0047

Maplus based HELP and Ningyou 7 based HELP were compared to each other and no significant differences were found in their fatty acid compositions. Thus, contradicting our hypothesis of Maplus *FAE1* alleles providing more erucic acid or VLCFAs than Ningyou 7 *FAE1* alleles. So, there may be allelic differences present in the Maplus *FAE1* and Ningyou 7 *FAE1* but they don't affect the VLCFAs levels.

Finally, quantitative differences were compared for HELP lines developed by cross-pollinating Maplus with four different mutants (M0830, K0472, M2444 and K0047) but no consistent trend was found between the different HELP groups. It could be due to minimal differences present in the PUFA levels of the four mutants to show a significant effect on the very long chain fatty acids.

The *sn-2* positional analysis of HELP lines showed that there were 1.4% very long chain fatty acids accumulated at the *sn-2* position as compared to 0.5% in Maplus. The highest levels of VLCFAs found in the HELP lines is 66% which is similar to the theoretical limit of erucic acid described in the literature due to high specificity of *Bn-LPAAT* for the fatty acids longer than 18 Carbons (Cao, Oo and Huang, 1990; Lassner, Lardizabal and Metz, 1996; Katavic *et al.*, 2001).

Irrespective of the parental combination used, HELP lines showed the same trend of high levels of erucic acid, VLCFAs, oleic acid and overall MUFAs and; low levels of

polyunsaturated fatty acids. In addition, lower levels of saturated fatty acids were observed in the HELP lines in comparison to respective high erucic acid cultivar. It shows the stability of the non-transgenic approach used in the present study. HELP lines developed from the combinations 'Maplus x K0472' and 'Maplus x K0047' are the most suitable HELP lines according to the fatty acids profiles, winter-type, Maplus background and low glucosinolates content.

Previous studies have used the transgenic approaches to increase the erucic acid content in *B. napus* (studies are reviewed in Sanyal *et al.*, 2015) and the present study has used a non-transgenic method to introduce genes of low polyunsaturates (developed by mutagenesis) in order to increase the VLCFAs and erucic acid levels.

7.2.2 Eicosenoic Acid and Erucic Acid levels

During the elongation step in the fatty acid biosynthesis, first oleic acid is converted to eicosenoic acid and then eicosenoic acid is converted to erucic acid by a four-step elongation mechanism (Lassner, Lardizabal and Metz, 1996). Both of these elongation steps are catalysed by the same enzyme in *B. napus* (Kondra and Stefansson, 1965). Coonrod *et al.*, 2008 have shown the epistasis effects of the eicosenoic acid content and thus, making it difficult to control the levels of eicosenoic acid between the generations. Sasongko and Möllers, 2005 also observed a negative correlation between the eicosenoic acid and erucic acid levels in the progeny produced by crossing high erucic acid rapeseed with high oleic acid rapeseed. Some studies suggest different alleles of *FAE1* gene have a different potential of producing erucic acid (Mahmood *et al.*, 2003). In Arabidopsis, *FAE1* prefers oleic acid as a substrate than eicosenoic acid and thus increasing the eicosenoic acid levels (Katavic *et al.*, 2001). In the present study, a strong negative correlation was found in the erucic acid and eicosenoic acid levels in the HELP lines. So, there are allelic differences in *Bna.FAE1* that may affect their specificity for the substrate or there may be the epistasis effects in the HELP lines, making the levels of the eicosenoic acid variable. For the industrial uses, the rapeseed oil is distilled to produce pure erucic acid and the fatty acids having 20 Carbons and higher remains in the distilled product (Walp and Tomlinson, 2004). So, eicosenoic acid is present in the distilled product and thus, the presence of

eicosenoic acid should not affect the use of the HELP oil. Uniform levels of the very long chain fatty acids were reported in the HELP lines in the present study.

7.2.3 Increasing VLCFAs Beyond 66%

Erucic acid (erucoyl CoA) is excluded from the *sn-2* position of the triacylglycerol (Brockerhoff, 1971) due to the low affinity of Bn-LPAAT for erucoyl-CoA as a substrate (Bernerth and Frentzen, 1990). This limits the amount of erucic acid in *B. napus* seeds to only 65 to 66% (Cao, Oo and Huang, 1990; Katavic *et al.*, 2001). In the present study, the total content of VLCFAs has been found up to 66% in the HELP lines which is the maximum theoretical level possible. Transgenic methods have been used to incorporate *LPAAT* from another species in combination with over-expressing *FAE1* and high erucic acid up to 72% have been found (Nath, Becker and Möllers, 2007; Nath, 2008). It has been a decade but no such rapeseed is available in the market for cultivation as there are many tight regulations for commercializing the varieties developed by using the transgenic methods. RNAi was used to suppress *FAD2* in order to increase erucic acid levels in *B. carinata* (Jadhav *et al.*, 2005) but the stability of this technology is hard to predict in a polyploid like *B. napus*. Thus, non-transgenic methods are always preferred for the crop improvement and commercial uses. Mutants developed by using EMS mutagenesis, a non-transgenic method (Wells *et al.*, 2014), were used to introduce the partially functional *Bna.FAD2* family in the high erucic acid varieties in the present study. The resulting HELP lines have been observed to be stable in their fatty acid compositions across generations.

Apart from transgenic methods, non-transgenic approaches could be used to break the theoretical barrier of 66%. *B. napus* elongases are unable to insert fatty acids with carbons length of more than 18 at the *sn-2* position. One approach could be a wide cross with a species having an enzyme variant and selections could be done as alien introgression. The other approach could be to screen a radiation panel (Maplus gamma radiation panel is available in the Bancroft group) for variants for erucic acid and to test whether the enzyme specificity has changed. The maximum amount of erucic acid using the transgenic methods by combination of the above transgenic approaches has been 72% (Nath, Becker and Möllers, 2007; Nath, 2008) and the non-transgenic methods may result in the same levels.

7.2.4 HELP Oil – Potential 'Green Feedstock' for the Industry

The usage of the high erucic acid oil has been increasing worldwide and rapeseed is the major source of erucic acid production (Zanetti *et al.*, 2012). The fatty acid profile of the HELP oil could be an ideal blend for the oleochemical industry due to its high content of erucic acid (or VLCFAs) and ultra-low levels of polyunsaturates (Sanyal *et al.*, 2015). The major use of high erucic acid rapeseed is in the production of erucamide (Zanetti *et al.*, 2012). Erucamide is produced by reacting purified erucic acid with ammonia at elevated temperature and pressure. The distilled product comprises mostly erucic acid and eicosenoic acid (Molnar, 1974; Walp and Tomlinson, 2004). Thus, HELP oil could be an ideal source for erucamide production due to its high very long chain fatty acids content and low PUFA composition.

The low levels of PUFAs are known to increase the thermal stability of the oil. Moreover, low PUFAs reduce the nitrous oxide emissions from the oil (Knothe and Dunn, 2003; Durrett, Benning and Ohlrogge, 2008). Oxidative stability of the Ningyou 7 HELP genotypes was measured using the Rancimat method (Läubli, Bruttel and Schalc, 1988) at the Biorenewables Development Centre (BDC) based at the University of York by Raymond Sloan. The induction period (time before the rapid deterioration of fat occurs) of HELP lines was found to be 2.7 times higher than the commercially refined rapeseed oil. Also, HELP lines were found to have the shelf-life stability for 1.6 years as compared to 0.55 year for the commercially refined rapeseed oil (the detailed results are not presented in the thesis). Thus, HELP lines have an additional attribute of high oxidative stability and long shelf-life. HELP oil had very low content of saturated fatty acids (3-4%) and it is desirable for the industrial applications (specifically for bio-diesel production) as it improves the cold-temperature flow characteristics by lowering the cloud point and pour point (Browse *et al.*, 1998; Durrett, Benning and Ohlrogge, 2008). In addition, the oleic acid levels were found to more than double in the HELP lines as compared to parental high erucic cultivars. Combining the high levels of erucic acid, eicosenoic acid and oleic acid makes the total mono-unsaturated fatty acids of up to 93% in the HELP lines. The high levels of MUFAs are desirable for the industrial applications (Durrett, Benning and Ohlrogge, 2008).

The leftover after the extraction of the oil from rapeseed is used for feeding the livestock and low levels of glucosinolates are desirable in the feedstock (Alexander *et al.*, 2008). HELP lines from the crosses, 'Maplus x K0472' and 'Maplus x K0047' had low levels of glucosinolates and thus, the high protein meal left after the extraction of the HELP oil could be used safely for the animal feed.

To summarize, HELP oil has a high potential for serving as a valuable 'green feedstock' for the industry and it could lower the processing costs of the industry as well. It would serve as a sustainable and renewable resource with faster biodegradability, low or no toxicity and fewer greenhouse emissions.

7.3 Future Directions

The main attributes for a varietal selection of oilseed rape by the breeders, companies and farmers are the oil content and seed yield in addition to other characteristics. So, these are very important parameters to measure for proposing a variety in the market. Thus, it would be a good step to measure these attributes for HELP lines. Oil content of HELP lines was measured at the Rothamsted Research, Harpenden, UK using time-domain nuclear magnetic resonance (TD-NMR) but a small quantity was used for the analysis (sufficient seeds were not available at the time of analysis). No effect on the oil content was seen in the HELP lines as compared to the commercial HEAR cultivar, Maplus.

Increasing the erucic acid and decreasing the polyunsaturated fatty acids is desirable for the industry (Sanyal *et al.*, 2015) and the HELP oil has this composition of an industrial suitable rapeseed oil. But for knowing the industrial potential of the HELP oil, it is necessary to measure the industrial characteristics of the oil such as – solubility, viscosity, lubricity, energy density, flash point (lowest temperature at which vapours ignite), cloud point (temperature at which fine crystals are formed that can block fuel filters), pour/melt point (temperature beyond the cloud point where fuel becomes gel-like and can't be poured), calorific value (amount of heat released during combustion), cetane number (measure of ignition quality relative to cetane as standard), iodine value (indicates the amount of unsaturation in fatty acids), ash percentage (total amount of minerals), sulphur percentage etc. followed by

comparisons with the commercially available plant-based oils. So, it would be an ideal step to measure the industrial parameters for proposing the HELP oil for use in the industry.

HELP lines from the cross 'Maplus x K0047' had very low levels of progoitrin (2-hydroxy but-3-enyl glucosinolates) of less than 1 µmol per gram of the seeds and the total glucosinolates were less than 12 $\mu molg^{-1}$ in these HELP lines. Parental genotypes, Maplus and K0047, had higher glucosinolates than the progeny as well. Progoitrin is a type of glucosinolate, hydrolysed by myrosinases to form goitrin which is known to be anti-nutritional as it reduces the production of thyroid hormones (Greer and Deeney, 1959; Stoewsand, 1995). The low progoitrin in the HELP lines could be due to changes in the regulatory genes or chain elongation enzymes such as AT2G25450 (Hansen *et al.*, 2008). Therefore, these HELP lines could serve as an ideal experimental material for studying the genetic basis affecting the levels of the progoitrin in *B. napus*. These lines could also be used for transfer of the low levels of progoitrin in other genotypes.

7.4 Key Findings

The following are the key findings from the present study,

- *Bna.FAD2* family influenced the erucic acid and polyunsaturates levels in *B. napus* rapeseed. When partially functional, it increased the erucic acid content (and VLCFAs pool) and decreased the polyunsaturated fatty acids.
- A unique specification of the rapeseed – high erucic acid rapeseed in the low polyunsaturated acid background (HELP) was developed in the present study using a non-transgenic method. HELP has many potential applications for the industry and is a renewable and sustainable resource. Moreover, HELP oil has high oxidative stability and long shelf life.
- The allelic differences in the *Bna.FAE1s* of the two different high erucic acid varieties (Maplus and Ningyou 7) do not affect the accumulation of erucic acid and VLCFAs. Irrespective of the high erucic acid variety used, the same effect of partially functional *Bna.FAD2* family was observed in the HELP lines.

- HELP lines with low progoitrin, developed in the present study, could serve as an ideal experimental material for studying the genetic basis of the control of progoitrin synthesis which is considered as main anti-nutritional glucosinolates in the rapeseed.

8. Appendices

I. The Fatty Acid Compositions of RIPR Panel

The fatty acid methyl esters were analysed from the seeds of 404 accessions under the RIPR panel by Vasilis Gegas at Limagrain UK Ltd (Havlickova *et al.*, 2018).

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000001	0.0	5.2	2.3	66.0	18.2	6.6	0.0	1.2	0.3	0.2
a-0000002	0.0	4.8	2.0	64.0	19.1	8.5	0.0	1.2	0.3	0.2
a-0000003	0.0	5.6	1.9	62.7	19.2	8.8	0.0	1.4	0.5	0.1
a-0000004	0.3	4.9	1.4	56.6	23.6	11.5	0.0	1.3	0.2	0.2
a-0000005	0.0	4.9	2.3	61.9	20.2	9.1	0.0	1.3	0.3	0.1
a-0000006	0.0	6.1	1.6	56.6	24.7	9.0	0.0	1.4	0.5	0.3
a-0000007	0.1	5.4	1.6	57.9	24.2	8.8	0.0	1.5	0.4	0.2
a-0000008	0.0	4.4	1.9	64.6	19.7	7.2	0.0	1.4	0.4	0.4
a-0000009	0.0	4.4	1.6	65.7	20.1	6.5	0.1	1.4	0.2	0.1
a-0000010	0.0	4.8	1.3	63.2	22.1	7.0	0.1	1.2	0.3	0.2
a-0000011	0.0	4.4	1.8	66.9	18.0	7.1	0.0	1.4	0.2	0.3
a-0000012	0.0	5.5	1.6	58.9	21.4	11.1	0.0	1.1	0.3	0.3
a-0000013	0.0	4.9	1.2	55.7	25.2	11.4	0.0	1.3	0.0	0.5
a-0000014	0.0	4.9	1.7	67.0	18.2	6.5	0.1	1.3	0.3	0.1
a-0000015	0.0	5.1	1.7	59.5	24.2	7.7	0.1	1.2	0.3	0.2
a-0000016	0.0	4.5	1.8	65.4	17.3	8.3	0.1	1.6	0.3	0.6
a-0000017	0.0	5.3	1.6	60.3	20.9	9.5	0.0	1.5	0.5	0.3
a-0000018	0.0	4.7	1.8	61.6	22.4	7.6	0.2	1.3	0.4	0.2
a-0000019	0.0	6.0	2.7	57.9	23.2	9.2	0.0	0.9	0.0	0.0
a-0000020	0.0	5.2	1.6	58.6	22.2	10.4	0.0	1.3	0.3	0.4
a-0000021	0.0	4.7	1.6	60.7	21.8	10.0	0.0	1.2	0.0	0.0
a-0000022	0.0	4.6	1.6	66.9	18.1	6.9	0.0	1.4	0.1	0.4
a-0000023	0.0	4.6	1.6	65.7	18.9	7.5	0.0	1.3	0.3	0.0
a-0000024	0.0	4.7	1.6	66.5	18.5	6.7	0.0	1.5	0.3	0.2
a-0000025	0.0	4.6	1.8	66.5	18.1	7.5	0.0	1.3	0.1	0.0
a-0000026	0.0	5.2	1.7	59.6	22.9	8.8	0.0	1.4	0.2	0.3
a-0000027	0.0	4.7	1.7	57.9	23.2	10.5	0.4	1.4	0.2	0.0
a-0000028	0.0	4.2	1.4	62.9	20.5	9.4	0.0	1.3	0.1	0.2
a-0000029	0.0	5.1	1.7	62.7	19.9	8.7	0.0	1.4	0.2	0.3
a-0000030	0.0	4.6	1.4	58.2	24.1	10.1	0.0	1.4	0.2	0.3
a-0000031	0.0	5.5	1.6	61.9	21.0	8.2	0.0	1.3	0.3	0.2
a-0000032	0.0	4.8	1.9	68.6	15.6	7.4	0.0	1.3	0.2	0.3
a-0000033	0.0	4.9	1.8	63.9	19.2	8.3	0.2	1.3	0.4	0.2
a-0000034	0.0	5.1	1.7	58.5	22.8	9.5	0.2	1.4	0.4	0.4
a-0000035	0.0	4.4	2.4	69.0	15.7	6.8	0.0	1.3	0.2	0.2
a-0000036	0.0	5.0	1.9	63.7	19.7	8.2	0.0	1.2	0.1	0.2
a-0000037	0.0	3.6	0.7	14.6	15.6	8.3	0.0	8.9	0.4	47.8
a-0000038	0.0	5.6	1.6	59.5	22.7	9.0	0.0	1.3	0.1	0.2

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000039	0.0	5.1	2.0	63.0	19.9	8.0	0.0	1.4	0.4	0.4
a-0000040	0.0	5.3	2.0	66.7	16.4	7.6	0.1	1.4	0.3	0.2
a-0000041	0.0	4.6	1.6	60.6	21.5	9.8	0.0	1.2	0.3	0.4
a-0000042	0.3	4.2	3.2	69.0	15.7	6.3	0.0	1.1	0.4	0.0
a-0000043	0.0	5.0	1.8	65.0	19.9	6.6	0.0	1.3	0.3	0.3
a-0000044	0.0	5.0	1.6	60.3	22.3	9.0	0.2	1.2	0.3	0.0
a-0000045	0.0	5.3	2.0	63.5	19.6	7.4	0.1	1.3	0.4	0.3
a-0000046	0.0	4.7	1.9	67.7	17.1	6.5	0.0	1.5	0.1	0.5
a-0000047	0.0	5.1	1.6	61.9	19.6	9.6	0.0	1.4	0.4	0.3
a-0000048	0.6	5.7	1.9	62.2	19.4	8.4	0.0	1.3	0.2	0.4
a-0000049	0.0	5.4	1.7	63.7	19.6	8.2	0.2	1.2	0.2	0.1
a-0000050	0.0	5.9	1.3	55.7	24.2	11.0	0.0	1.2	0.2	0.3
a-0000051	0.1	5.6	1.6	58.2	23.5	9.1	0.2	1.3	0.3	0.3
a-0000052	0.0	5.2	2.0	62.8	19.0	8.8	0.1	1.4	0.4	0.2
a-0000053	0.0	5.4	1.5	59.7	21.8	10.0	0.0	1.2	0.2	0.2
a-0000054	0.0	4.9	1.5	62.4	21.3	8.2	0.0	1.3	0.2	0.1
a-0000055	0.0	5.7	1.6	60.7	21.9	8.2	0.0	1.2	0.3	0.3
a-0000056	0.0	4.9	1.9	65.7	17.9	8.0	0.0	1.2	0.3	0.0
a-0000057	0.0	5.6	1.5	63.4	19.1	8.9	0.0	1.3	0.2	0.1
a-0000058	0.0	4.7	1.5	63.8	18.9	9.7	0.0	1.2	0.3	0.0
a-0000059	0.0	5.1	1.9	62.4	20.5	8.1	0.0	1.4	0.4	0.1
a-0000060	0.0	4.6	1.8	62.3	22.0	7.8	0.0	1.1	0.2	0.0
a-0000061	0.0	4.2	1.6	65.5	19.5	7.9	0.0	1.1	0.2	0.1
a-0000062	0.4	5.5	1.5	60.1	21.3	9.8	0.0	1.2	0.2	0.0
a-0000063	0.0	4.8	1.7	62.8	20.1	8.9	0.0	1.2	0.3	0.4
a-0000064	0.0	4.8	1.4	61.4	21.2	9.8	0.0	1.1	0.1	0.2
a-0000065	0.0	4.7	2.5	65.9	17.7	7.0	0.2	1.4	0.4	0.3
a-0000066	0.0	4.9	1.8	66.1	18.1	7.7	0.0	1.1	0.1	0.1
a-0000067	0.0	5.4	1.6	58.5	22.9	9.6	0.1	1.3	0.3	0.4
a-0000068	0.0	4.4	1.6	63.8	18.4	9.6	0.0	1.3	0.2	0.6
a-0000069	0.0	5.0	2.1	64.0	20.0	7.1	0.1	1.2	0.3	0.2
a-0000070	0.0	4.4	1.6	65.3	18.7	8.6	0.0	1.5	0.1	0.0
a-0000071	0.0	4.1	2.0	66.2	18.2	7.8	0.0	1.3	0.3	0.2
a-0000072	0.0	4.9	1.6	60.6	21.1	9.8	0.1	1.3	0.4	0.2
a-0000073	0.0	4.6	2.0	65.5	18.2	7.9	0.2	1.3	0.3	0.1
a-0000074	0.0	4.3	1.6	28.9	15.9	9.2	0.1	17.1	0.0	22.9
a-0000075	0.0	5.0	1.7	62.0	21.7	8.2	0.0	1.2	0.3	0.0
a-0000076	0.4	5.2	1.9	64.3	18.4	8.1	0.0	1.4	0.3	0.2
a-0000077	0.0	5.3	1.6	62.7	20.3	8.6	0.0	1.3	0.2	0.1
a-0000078	0.3	5.4	2.0	64.8	17.8	7.6	0.2	1.4	0.3	0.1
a-0000079	0.0	5.1	1.9	62.1	21.3	7.9	0.0	1.3	0.1	0.4
a-0000080	0.0	6.0	1.7	59.4	23.2	8.2	0.0	1.2	0.3	0.0
a-0000081	0.0	5.0	1.5	63.3	21.7	6.8	0.0	1.3	0.2	0.2
a-0000082	0.1	5.4	1.9	57.9	23.2	9.6	0.2	1.2	0.3	0.3
a-0000083	0.1	5.1	2.3	65.9	17.1	7.8	0.2	1.2	0.3	0.2
a-0000084	0.0	5.1	2.0	64.7	18.2	7.9	0.1	1.3	0.2	0.5
a-0000085	0.0	4.9	1.8	62.2	21.5	8.0	0.0	1.2	0.2	0.1
a-0000086	0.0	4.7	1.7	61.8	21.5	8.8	0.2	1.2	0.3	0.0

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000087	0.1	4.3	1.8	67.8	16.8	7.2	0.2	1.4	0.3	0.2
a-0000088	0.0	5.1	1.2	53.6	24.7	11.6	0.0	2.5	0.2	1.0
a-0000089	0.0	5.1	1.6	58.6	21.3	10.1	0.2	1.5	0.3	1.4
a-0000090	0.0	5.2	1.9	62.0	20.3	8.2	0.1	1.6	0.5	0.1
a-0000093	0.0	4.7	1.7	65.6	18.8	7.7	0.0	1.3	0.2	0.1
a-0000096	0.0	4.2	1.4	27.8	16.6	6.3	0.0	18.9	0.3	24.6
a-0000097	0.1	5.0	1.9	63.1	19.8	8.4	0.0	1.2	0.4	0.1
a-0000098	0.3	5.3	1.6	59.7	21.7	9.4	0.2	1.3	0.3	0.3
a-0000099	0.5	4.9	2.4	64.0	19.1	7.7	0.0	1.2	0.3	0.0
a-0000101	0.0	5.0	1.6	60.5	21.3	10.2	0.0	1.2	0.3	0.1
a-0000102	0.0	3.8	1.3	18.4	15.2	9.9	0.0	9.1	0.8	41.7
a-0000103	0.0	5.4	1.7	62.4	19.9	9.2	0.0	1.3	0.2	0.0
a-0000105	0.0	4.8	1.3	62.3	20.8	8.9	0.1	1.2	0.3	0.2
a-0000106	0.0	4.7	1.9	64.6	18.8	8.4	0.0	1.5	0.2	0.0
a-0000107	0.1	4.2	1.0	18.4	16.9	8.4	0.0	9.8	0.4	40.9
a-0000108	0.0	3.9	0.9	14.7	15.2	9.5	0.0	7.3	0.8	47.8
a-0000109	0.0	3.0	1.0	15.6	14.9	7.5	0.0	7.4	0.7	50.0
a-0000110	0.0	3.6	1.1	15.2	15.1	9.0	0.0	8.2	0.7	47.1
a-0000111	0.0	2.9	1.0	14.7	13.2	9.6	0.0	7.2	0.8	50.6
a-0000112	0.0	3.8	1.1	18.2	15.5	7.2	0.0	10.3	0.6	43.3
a-0000113	0.0	5.2	2.3	62.1	20.9	7.2	0.0	1.5	0.6	0.4
a-0000114	0.0	3.6	0.7	14.7	15.2	8.5	0.0	9.1	0.5	47.7
a-0000115	0.0	5.2	1.8	60.1	22.4	8.2	0.0	1.1	0.4	0.7
a-0000116	0.1	5.6	1.9	60.2	20.8	10.1	0.0	1.2	0.3	0.0
a-0000117	0.0	4.1	0.7	12.0	15.9	10.1	0.1	6.8	0.6	49.7
a-0000118	0.0	3.0	1.0	16.4	14.4	8.6	0.0	7.7	0.7	48.1
a-0000120	0.0	4.7	1.5	62.5	20.7	8.0	0.2	1.4	0.3	0.7
a-0000121	0.1	5.9	1.6	55.2	23.7	12.0	0.0	1.2	0.3	0.2
a-0000122	0.0	4.9	1.3	60.0	20.8	10.9	0.0	1.5	0.3	0.3
a-0000123	0.0	5.4	1.5	61.0	21.6	8.6	0.0	1.4	0.2	0.3
a-0000124	0.1	5.2	1.6	63.0	19.6	8.8	0.0	1.4	0.1	0.3
a-0000125	0.3	4.8	3.3	67.7	16.2	5.8	0.2	1.2	0.4	0.1
a-0000126	0.0	5.8	1.9	60.6	20.4	9.9	0.0	1.2	0.2	0.0
a-0000127	0.0	5.0	1.9	62.0	21.7	7.7	0.1	1.2	0.3	0.1
a-0000128	0.0	5.1	1.8	64.1	18.3	8.8	0.0	1.2	0.3	0.3
a-0000129	0.0	3.8	1.0	18.7	13.5	7.7	0.2	9.2	0.5	45.4
a-0000130	0.0	3.6	0.7	13.3	13.9	11.9	0.0	7.5	0.7	48.3
a-0000131	0.0	3.7	1.6	24.3	14.5	5.8	0.0	8.0	1.0	41.1
a-0000133	0.0	3.2	1.0	17.7	14.1	7.1	0.0	9.2	0.5	47.3
a-0000135	0.0	5.4	1.6	58.5	22.3	10.1	0.0	1.3	0.3	0.5
a-0000136	0.0	5.3	1.4	59.1	23.5	9.0	0.0	1.2	0.2	0.4
a-0000137	0.0	4.4	2.1	66.4	18.4	7.2	0.0	1.2	0.3	0.0
a-0000138	0.0	4.1	1.2	17.6	14.9	7.9	0.0	9.7	0.6	44.1
a-0000139	0.0	3.6	0.8	13.7	13.9	10.4	0.0	5.6	0.8	51.3
a-0000140	0.0	4.9	1.8	63.0	20.8	7.9	0.2	1.2	0.1	0.1
a-0000141	0.0	4.5	1.9	64.2	18.1	8.3	0.0	1.5	0.2	1.3
a-0000142	0.0	5.1	1.8	60.4	21.5	9.7	0.0	1.2	0.2	0.2
a-0000143	0.0	3.7	1.1	17.9	16.0	7.7	0.0	8.7	0.7	44.3

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000144	0.0	3.6	1.1	16.1	14.1	8.1	0.2	8.6	0.6	47.6
a-0000145	0.0	3.7	1.1	17.9	15.0	10.4	0.0	10.3	0.6	41.0
a-0000146	0.0	4.9	1.6	62.5	19.7	10.0	0.0	1.1	0.2	0.2
a-0000147	0.0	5.0	1.6	60.8	21.1	9.9	0.2	1.2	0.3	0.1
a-0000148	0.0	5.2	1.6	64.6	18.0	8.9	0.0	1.3	0.3	0.1
a-0000149	0.0	3.8	1.2	16.7	13.5	9.1	0.2	9.2	0.7	45.7
a-0000151	0.0	4.3	1.8	65.0	19.5	7.6	0.1	1.3	0.3	0.0
a-0000152	0.0	4.4	2.0	60.2	24.1	7.9	0.0	1.1	0.1	0.1
a-0000153	0.0	4.2	2.3	63.3	21.4	7.3	0.0	1.3	0.2	0.0
a-0000154	0.0	5.2	1.6	61.2	20.7	9.5	0.0	1.3	0.2	0.1
a-0000155	0.0	5.6	1.6	58.9	22.3	10.2	0.0	1.2	0.1	0.1
a-0000156	0.0	5.3	1.6	59.2	20.8	11.6	0.0	1.3	0.2	0.0
a-0000157	0.0	4.8	1.6	64.8	18.9	8.6	0.0	1.2	0.2	0.0
a-0000158	0.0	3.9	1.2	19.4	12.8	7.6	0.2	10.8	0.7	43.4
a-0000159	0.0	3.7	1.0	15.8	14.5	8.7	0.0	9.4	0.6	46.4
a-0000160	0.0	3.9	1.0	14.2	16.3	8.8	0.0	7.6	0.6	47.6
a-0000161	0.0	4.2	1.3	22.5	18.3	7.9	0.0	15.4	0.4	30.2
a-0000162	0.0	3.3	1.3	14.4	14.9	9.3	0.0	7.0	1.0	48.8
a-0000163	0.0	4.1	1.2	20.6	15.2	7.9	0.0	9.6	0.5	40.8
a-0000164	0.0	3.7	0.8	14.6	14.1	9.2	0.1	7.8	0.6	48.9
a-0000165	0.0	3.8	1.0	14.5	13.9	9.0	0.0	7.8	0.7	49.5
a-0000166	0.0	3.4	1.1	18.2	11.8	6.8	0.0	9.5	0.6	48.4
a-0000167	0.0	2.9	1.0	17.7	12.8	8.2	0.2	8.0	0.6	48.6
a-0000168	0.0	3.8	1.1	16.1	15.0	9.2	0.0	8.2	0.6	46.1
a-0000169	0.0	3.4	1.2	22.2	15.2	7.7	0.0	11.2	0.6	38.5
a-0000170	0.0	3.8	0.9	15.6	13.7	9.7	0.2	7.8	0.7	47.6
a-0000171	0.0	3.9	1.0	15.8	15.8	10.5	0.1	12.1	0.5	40.1
a-0000172	0.0	3.7	0.9	16.8	12.8	8.5	0.3	9.0	0.6	47.4
a-0000173	0.0	3.7	1.0	15.0	15.3	9.2	0.0	6.7	0.8	48.4
a-0000174	0.0	3.4	0.8	13.9	14.6	8.4	0.1	7.2	0.6	50.8
a-0000175	0.0	4.8	1.7	61.4	21.4	9.4	0.2	1.2	0.1	0.0
a-0000176	0.0	3.6	1.0	19.3	14.1	8.2	0.0	8.8	0.6	44.4
a-0000177	0.0	3.2	1.1	17.6	15.0	7.5	0.0	7.8	0.7	47.2
a-0000178	0.0	4.0	1.0	16.4	15.7	7.2	0.0	8.6	0.8	46.4
a-0000179	0.0	3.7	0.8	10.3	15.2	12.7	0.0	5.4	1.0	50.9
a-0000180	0.0	3.6	1.0	14.4	13.7	8.8	0.0	7.0	0.7	50.8
a-0000181	0.0	5.3	1.4	48.7	31.6	11.0	0.0	1.4	0.4	0.2
a-0000182	0.1	5.1	2.3	65.6	18.0	7.7	0.0	1.0	0.3	0.0
a-0000183	0.0	3.6	0.8	14.0	12.7	9.8	0.0	6.3	0.7	52.2
a-0000184	0.0	3.8	2.5	40.3	16.1	5.1	0.3	15.6	0.1	16.2
a-0000185	0.0	3.7	0.9	22.2	17.1	8.2	0.1	8.9	0.5	38.4
a-0000186	0.0	3.8	1.6	19.1	14.5	8.7	0.2	9.7	0.8	41.7
a-0000187	0.0	4.6	1.5	20.7	16.8	9.1	0.2	14.0	0.3	32.9
a-0000188	0.0	4.1	1.1	17.8	16.4	8.7	0.0	9.0	0.4	42.4
a-0000189	0.0	3.5	1.2	23.6	13.6	7.8	0.0	9.3	0.9	40.2
a-0000190	0.0	3.9	1.0	19.2	15.6	10.3	0.0	6.7	0.8	42.6
a-0000191	0.0	5.1	2.0	63.1	19.0	9.0	0.0	1.3	0.3	0.3
a-0000193	0.0	3.5	0.8	12.3	17.9	9.8	0.0	6.6	0.6	48.3

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000194	0.0	3.7	0.8	13.6	16.1	9.3	0.0	8.2	0.6	47.9
a-0000195	0.1	5.2	1.7	62.3	21.7	7.2	0.1	1.2	0.3	0.2
a-0000197	0.0	4.2	1.0	19.2	14.1	8.4	0.0	11.0	0.6	41.6
a-0000198	0.0	3.3	1.2	21.9	13.9	7.0	0.2	10.8	0.6	41.2
a-0000199	0.0	3.3	0.8	18.5	16.0	8.6	0.2	8.5	0.7	43.2
a-0000200	0.0	3.4	0.9	15.3	15.3	7.1	0.0	6.6	0.6	50.9
a-0000201	0.0	3.7	1.2	16.0	15.3	7.4	0.0	7.1	0.9	48.7
a-0000202	0.0	4.9	1.2	22.0	17.1	9.1	0.0	13.3	0.5	31.9
a-0000203	0.0	3.5	1.0	19.2	17.1	8.4	0.2	7.4	0.7	42.8
a-0000204	0.0	4.3	1.1	19.2	16.0	6.8	0.0	8.9	0.6	43.0
a-0000206	0.0	3.6	1.0	16.1	15.9	8.0	0.0	8.3	0.4	46.7
a-0000207	0.1	5.3	1.7	62.1	19.5	9.4	0.2	1.4	0.3	0.0
a-0000208	0.1	4.2	1.0	15.6	15.7	11.2	0.0	8.4	0.7	43.3
a-0000209	0.0	3.5	1.7	20.3	14.1	6.8	0.0	9.6	1.1	43.0
a-0000210	0.0	3.2	1.8	26.9	11.5	6.1	0.0	10.8	1.0	38.9
a-0000211	0.0	3.2	1.3	23.6	14.4	8.3	0.0	11.4	0.6	37.1
a-0000212	0.0	3.5	0.8	12.9	14.5	9.1	0.0	5.6	1.1	52.6
a-0000213	0.0	4.2	0.9	11.2	14.2	12.0	0.3	6.0	0.8	50.5
a-0000214	0.0	3.8	1.2	12.9	17.1	11.1	0.2	8.6	0.6	44.5
a-0000216	0.0	5.1	1.5	60.8	23.2	7.8	0.0	1.4	0.2	0.1
a-0000217	0.0	4.1	1.9	64.2	20.1	8.1	0.0	1.3	0.3	0.0
a-0000218	0.1	5.1	1.8	59.0	23.8	8.5	0.0	1.2	0.2	0.4
a-0000219	0.1	4.5	1.8	66.8	18.7	6.6	0.0	1.3	0.3	0.2
a-0000221	0.0	3.8	2.0	65.9	20.8	5.9	0.0	1.5	0.3	0.0
a-0000222	0.0	3.6	1.6	26.5	13.8	6.8	0.0	9.1	1.1	37.8
a-0000223	0.0	3.3	1.4	18.8	15.2	9.4	0.3	7.8	0.8	43.1
a-0000224	0.0	3.3	1.5	19.6	17.2	7.1	0.0	9.1	0.6	41.5
a-0000225	0.0	3.5	0.8	12.9	15.8	12.0	0.0	7.7	0.6	46.7
a-0000226	0.0	3.6	1.0	14.2	14.6	13.4	0.0	8.3	0.7	44.3
a-0000227	0.0	4.5	1.8	59.6	22.2	10.4	0.0	1.2	0.0	0.4
a-0000228	0.0	4.7	1.8	54.5	25.8	11.4	0.0	1.3	0.1	0.3
a-0000229	0.0	4.5	1.3	58.4	19.3	14.4	0.1	1.5	0.2	0.3
a-0000232	0.0	4.5	2.1	68.9	15.0	8.3	0.0	1.1	0.0	0.1
a-0000233	0.0	3.4	0.8	14.1	13.7	11.4	0.1	7.6	0.5	48.3
a-0000234	0.0	2.8	1.2	19.8	14.5	10.3	0.0	9.5	0.7	41.5
a-0000235	0.0	4.0	2.2	71.6	13.5	6.4	0.2	1.4	0.3	0.4
a-0000236	0.0	4.9	2.4	63.6	21.9	5.1	0.0	1.6	0.4	0.2
a-0000237	0.0	4.3	2.0	67.7	17.8	6.4	0.0	1.5	0.4	0.1
a-0000238	0.0	2.5	0.9	17.7	15.1	10.7	0.0	6.5	0.7	46.0
a-0000241	0.0	5.1	1.3	53.0	27.2	11.2	0.0	1.4	0.2	0.5
a-0000242	0.0	4.8	1.6	60.5	21.3	9.6	0.1	1.5	0.3	0.2
a-0000247	0.0	3.0	2.4	23.6	12.9	6.2	0.0	10.7	1.1	40.4
a-0000248	0.0	4.3	1.6	61.5	20.3	10.5	0.0	1.4	0.3	0.1
a-0000249	0.0	4.9	1.8	59.6	23.8	8.5	0.0	1.2	0.3	0.0
a-0000250	0.0	4.8	2.2	52.4	25.3	13.3	0.1	1.2	0.1	0.4
a-0000251	0.0	3.3	1.4	26.8	13.8	6.2	0.0	12.3	0.5	36.0
a-0000253	0.1	5.2	2.1	56.2	24.7	9.5	0.0	1.3	0.4	0.4
a-0000254	0.1	6.0	1.7	52.4	28.0	10.4	0.0	1.3	0.3	0.1

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000255	0.0	4.4	1.9	66.3	17.3	7.9	0.0	1.5	0.5	0.1
a-0000256	0.3	4.8	1.6	59.2	20.8	12.1	0.0	1.2	0.3	0.0
a-0000257	0.0	4.5	2.0	63.8	21.0	7.3	0.1	1.2	0.0	0.1
a-0000258	0.0	4.0	1.0	12.7	15.3	11.9	0.0	6.8	0.9	47.6
a-0000259	0.0	4.4	1.7	63.1	21.2	8.0	0.0	1.5	0.3	0.0
a-0000260	0.0	4.1	2.2	69.8	16.4	5.7	0.0	1.3	0.4	0.2
a-0000261	0.0	4.7	1.9	59.8	23.2	8.9	0.0	1.1	0.3	0.1
a-0000262	0.0	4.8	1.3	15.7	20.6	7.4	0.3	8.1	0.9	40.9
a-0000264	0.0	4.4	1.7	65.7	17.5	8.8	0.0	1.3	0.1	0.4
a-0000265	0.0	4.6	1.3	19.3	17.7	11.2	0.0	11.2	0.4	34.4
a-0000266	0.0	3.8	1.1	13.8	16.6	9.7	0.0	8.6	0.8	45.6
a-0000267	0.0	3.7	1.8	26.8	15.2	6.5	0.2	12.4	0.6	32.9
a-0000268	0.0	3.1	1.2	24.7	13.9	7.3	0.0	10.1	0.6	39.1
a-0000269	0.0	4.1	1.9	37.8	16.9	8.4	0.2	13.4	0.0	17.3
a-0000270	0.0	3.1	1.6	29.7	12.0	6.0	0.0	19.1	0.3	28.2
a-0000271	0.1	3.5	1.4	20.4	14.5	7.6	0.3	12.7	0.6	39.0
a-0000272	0.0	4.7	1.8	63.4	20.0	8.3	0.1	1.3	0.4	0.0
a-0000273	0.0	3.8	1.4	16.1	16.5	8.8	0.0	8.1	0.8	44.4
a-0000274	0.0	5.0	1.9	63.0	18.4	10.0	0.0	1.4	0.3	0.3
a-0000275	0.0	4.4	2.2	64.3	21.1	5.9	0.2	1.3	0.4	0.2
a-0000276	0.0	3.8	1.8	66.9	18.1	7.1	0.1	1.6	0.5	0.1
a-0000277	0.0	4.7	2.6	68.1	16.5	6.6	0.0	1.2	0.4	0.0
a-0000278	0.0	3.0	1.9	23.8	14.0	7.8	0.0	10.5	1.0	38.1
a-0000279	0.0	4.1	1.2	21.9	17.1	11.6	0.2	10.2	0.4	33.5
a-0000280	0.0	3.9	1.3	26.9	14.0	7.9	0.0	17.1	0.3	28.7
a-0000281	0.0	5.0	1.7	56.8	25.1	9.1	0.2	1.5	0.5	0.3
a-0000282	0.0	4.1	1.8	64.5	19.0	8.9	0.0	1.3	0.3	0.0
a-0000283	0.1	4.9	2.3	63.3	18.9	9.2	0.0	1.2	0.2	0.2
a-0000284	0.0	4.6	1.9	62.4	21.6	7.6	0.2	1.4	0.2	0.2
a-0000285	0.0	4.8	1.9	62.0	21.9	7.6	0.1	1.2	0.3	0.0
a-0000286	0.0	4.0	1.2	16.0	16.7	10.6	0.0	8.5	0.6	42.7
a-0000287	0.0	4.5	1.7	60.0	22.5	10.3	0.0	1.2	0.1	0.0
a-0000288	0.0	3.6	1.7	23.7	16.2	8.3	0.2	10.8	0.6	35.1
a-0000289	0.0	4.5	1.4	58.1	21.9	11.6	0.0	1.4	0.2	0.9
a-0000290	0.0	4.6	2.0	60.7	22.2	8.3	0.2	1.5	0.5	0.2
a-0000291	0.0	5.7	2.0	56.4	24.8	8.8	0.2	1.4	0.3	0.4
a-0000292	0.0	6.1	1.6	55.4	23.4	12.3	0.0	1.2	0.0	0.2
a-0000293	0.0	4.8	1.3	56.6	24.7	10.4	0.6	1.3	0.3	0.0
a-0000294	0.0	4.5	1.6	61.7	20.8	9.8	0.0	1.5	0.2	0.0
a-0000295	0.0	3.7	1.6	65.1	19.9	7.9	0.2	1.3	0.2	0.1
a-0000296	0.0	4.4	2.0	63.4	21.1	6.7	0.2	1.4	0.2	0.6
a-0000297	0.2	4.7	1.5	61.5	20.3	9.6	0.2	1.5	0.2	0.3
a-0000298	0.0	4.1	1.9	61.8	21.4	8.8	0.1	1.4	0.2	0.4
a-0000299	0.0	4.4	2.1	64.8	18.7	8.3	0.0	1.4	0.2	0.4
a-0000300	0.4	5.0	2.1	60.6	21.6	8.6	0.0	1.3	0.4	0.2
a-0000301	0.0	4.9	1.6	59.4	23.0	9.3	0.0	1.4	0.2	0.3
a-0000302	0.0	4.4	1.6	61.2	22.3	8.5	0.2	1.5	0.3	0.0
a-0000303	0.0	4.9	1.8	58.0	20.5	13.1	0.0	1.4	0.4	0.1

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Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000305	0.0	3.7	1.6	63.0	21.2	8.3	0.2	1.5	0.3	0.4
a-0000306	0.0	4.4	2.1	68.7	17.1	5.9	0.0	1.4	0.4	0.1
a-0000307	0.0	4.8	2.1	63.4	20.4	8.1	0.0	1.1	0.3	0.0
a-0000308	0.0	4.5	1.7	62.3	19.7	9.9	0.2	1.2	0.2	0.4
a-0000309	0.0	3.5	1.4	23.4	12.1	7.7	0.0	9.9	0.8	41.3
a-0000310	0.0	3.1	1.0	16.8	14.8	11.1	0.0	7.2	0.8	45.3
a-0000311	0.0	4.0	1.5	60.0	22.8	9.8	0.0	1.4	0.3	0.2
a-0000312	0.0	4.3	1.6	60.2	21.9	9.9	0.0	1.4	0.1	0.5
a-0000313	0.0	4.3	2.3	64.9	18.8	7.9	0.1	1.3	0.3	0.0
a-0000314	0.0	4.4	1.9	63.4	19.4	9.0	0.0	1.3	0.3	0.2
a-0000315	0.0	3.5	1.3	22.8	16.5	9.2	0.0	10.0	0.7	36.2
a-0000316	0.0	5.6	2.6	57.6	22.3	9.0	0.2	1.5	0.5	0.7
a-0000317	0.0	3.8	1.7	21.5	13.3	8.0	0.0	6.6	1.0	44.1
a-0000318	0.0	5.0	1.9	59.0	23.4	9.1	0.0	1.3	0.2	0.2
a-0000319	0.0	4.5	1.9	59.4	23.4	9.0	0.2	1.2	0.4	0.0
a-0000321	0.0	3.8	1.3	24.1	15.6	8.9	0.2	11.2	0.4	34.5
a-0000322	0.0	4.0	1.6	66.6	19.0	7.7	0.0	1.2	0.1	0.0
a-0000323	0.0	4.4	1.9	61.8	20.2	9.8	0.0	1.5	0.2	0.2
a-0000326	0.2	5.0	2.7	65.4	16.9	7.6	0.5	1.3	0.5	0.0
a-0000327	0.0	3.6	1.7	34.4	12.7	8.2	0.0	15.8	0.2	23.5
a-0000328	0.0	3.4	1.2	29.0	17.5	9.2	0.0	16.6	0.1	22.9
a-0000329	0.0	3.8	1.8	65.0	20.3	7.6	0.0	1.4	0.2	0.0
a-0000330	0.0	4.7	1.7	58.8	23.5	9.0	0.0	1.5	0.3	0.8
a-0000332	0.0	4.7	2.1	64.2	19.3	7.8	0.0	1.5	0.4	0.2
a-0000333	0.0	4.6	1.7	64.5	19.3	8.2	0.2	1.1	0.3	0.0
a-0000334	0.0	3.8	1.5	29.7	15.2	8.9	0.0	13.3	0.4	27.1
a-0000335	0.0	4.1	2.2	65.7	19.6	6.3	0.2	1.3	0.3	0.2
a-0000336	0.0	3.8	1.7	32.3	13.8	8.7	0.0	14.4	0.3	25.0
a-0000337	0.0	4.7	2.7	61.2	22.3	7.5	0.0	1.2	0.3	0.1
a-0000338	0.0	3.2	1.3	17.3	15.9	9.9	0.0	9.1	0.7	42.7
a-0000339	0.0	4.7	2.2	66.2	18.3	6.6	0.2	1.2	0.4	0.3
a-0000340	0.0	4.4	1.9	65.8	20.0	6.6	0.2	1.2	0.1	0.0
a-0000342	0.0	3.3	1.2	16.8	14.5	10.2	0.2	7.1	1.0	45.7
a-0000343	0.0	3.7	1.2	18.5	16.2	10.6	0.0	10.2	0.5	39.1
a-0000345	0.0	3.3	1.3	18.8	14.4	9.1	0.0	9.8	0.7	42.7
a-0000346	0.0	4.6	2.1	46.3	21.5	7.0	0.2	8.7	0.2	9.6
a-0000347	0.1	5.1	2.7	60.1	23.2	7.1	0.0	1.2	0.3	0.1
a-0000348	0.0	5.6	2.1	57.3	23.9	9.8	0.0	1.2	0.0	0.2
a-0000349	0.0	4.5	2.1	64.2	19.0	8.3	0.2	1.3	0.3	0.2
a-0000350	0.0	4.8	2.1	63.4	18.8	9.2	0.0	1.5	0.2	0.1
a-0000351	0.0	5.2	1.8	58.1	24.1	9.1	0.0	1.3	0.1	0.3
a-0000352	0.0	5.2	2.3	62.1	21.5	7.3	0.2	1.2	0.2	0.0
a-0000353	0.0	4.1	1.1	17.9	15.5	7.7	0.0	9.4	0.6	43.9
a-0000354	0.0	3.6	1.2	21.3	17.0	9.7	0.2	10.1	0.5	36.4
a-0000355	0.0	4.5	2.7	69.9	15.4	5.7	0.0	1.4	0.4	0.1
a-0000356	0.0	4.6	2.1	64.8	18.9	7.0	0.2	1.6	0.4	0.4
a-0000357	0.0	5.1	2.5	62.1	21.4	7.9	0.0	1.0	0.1	0.0
a-0000359	0.0	4.2	1.9	64.8	20.9	6.0	0.1	1.2	0.2	0.6

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Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000360	0.0	3.4	2.4	66.3	18.0	7.9	0.0	1.6	0.2	0.1
a-0000361	0.0	3.8	1.9	25.9	17.9	11.5	0.0	14.9	0.1	24.0
a-0000362	0.0	3.7	2.1	54.5	12.4	5.6	0.0	14.3	0.1	7.2
a-0000365	0.0	3.9	1.5	21.9	16.7	8.8	0.1	12.6	0.3	34.1
a-0000366	0.0	4.9	1.9	56.1	23.5	11.4	0.0	1.7	0.3	0.2
a-0000367	0.0	3.6	1.4	22.3	16.5	7.3	0.2	11.1	0.5	37.1
a-0000368	0.0	3.6	1.8	24.9	14.9	8.0	0.0	12.7	0.4	33.6
a-0000369	0.0	5.3	1.9	60.9	22.2	8.3	0.0	1.2	0.2	0.0
a-0000370	0.0	4.3	2.1	63.7	19.4	7.8	0.3	1.4	0.4	0.8
a-0000371	0.0	4.2	2.1	48.1	17.3	9.6	0.2	11.5	0.1	6.9
a-0000372	0.0	4.6	2.0	66.4	16.4	8.5	0.1	1.2	0.2	0.5
a-0000373	0.0	4.6	2.1	63.5	20.3	7.4	0.3	1.3	0.3	0.3
a-0000374	0.0	3.2	0.9	14.5	14.5	10.9	0.0	8.3	0.8	46.9
a-0000377	0.0	4.2	3.0	61.6	19.9	9.3	0.0	1.6	0.4	0.0
a-0000378	0.1	4.5	2.4	65.1	19.2	7.1	0.0	1.2	0.3	0.1
a-0000379	0.1	3.5	1.5	21.8	14.2	9.5	0.2	11.2	0.6	37.3
a-0000380	0.0	4.8	1.8	62.1	21.7	8.1	0.0	1.3	0.3	0.0
a-0000381	0.0	4.8	1.7	58.2	23.0	10.5	0.0	1.2	0.4	0.2
a-0000382	0.0	5.6	1.9	53.1	27.4	10.1	0.0	1.0	0.8	0.0
a-0000383	0.0	5.3	1.8	56.1	25.7	9.6	0.0	1.2	0.1	0.3
a-0000384	0.0	2.6	0.9	18.5	14.3	10.3	0.1	8.2	0.6	44.5
a-0000385	0.4	4.0	1.4	20.6	17.7	8.4	0.2	11.0	0.5	35.9
a-0000386	0.0	3.5	1.1	19.7	13.2	8.9	0.0	9.8	0.6	43.3
a-0000387	0.0	3.2	1.1	20.1	15.7	9.8	0.0	10.3	0.5	39.3
a-0000391	0.0	4.5	1.9	68.2	16.7	6.9	0.0	1.4	0.3	0.1
a-0000393	0.0	4.9	1.7	60.9	21.5	9.5	0.0	1.2	0.2	0.2
a-0000394	0.0	5.0	1.6	65.4	19.0	7.6	0.0	1.1	0.3	0.0
a-0000395	0.0	3.8	1.4	23.0	16.4	11.3	0.2	8.8	0.6	34.6
a-0000396	0.1	3.2	1.0	20.2	10.5	7.7	0.3	8.1	0.7	48.2
a-0000397	0.0	5.2	1.6	62.6	18.8	9.4	0.0	1.7	0.4	0.3
a-0000398	0.0	5.7	1.5	58.2	21.2	11.3	0.0	1.4	0.3	0.4
a-0000399	0.0	4.0	1.4	26.1	15.6	10.8	0.0	10.2	0.4	31.6
a-0000401	0.0	4.3	1.8	33.8	15.7	9.0	0.0	13.5	0.2	21.8
a-0000403	0.0	3.9	1.3	20.2	18.3	13.2	0.2	8.8	0.4	33.7
a-0000405	0.0	5.6	1.5	59.5	20.0	11.6	0.0	1.6	0.3	0.0
a-0000406	0.0	4.0	1.4	19.3	17.8	8.6	0.2	9.9	0.8	38.0
a-0000409	0.0	4.7	1.9	26.4	17.5	12.2	0.0	10.6	0.2	26.7
a-0000411	0.0	4.5	1.8	25.9	18.9	10.2	0.5	11.8	0.2	26.6
a-0000414	0.0	4.0	1.9	29.9	18.3	9.3	0.0	10.1	0.5	26.0
a-0000415	0.0	4.2	1.2	20.2	15.5	9.5	0.3	8.9	0.7	39.5
a-0000418	0.0	3.5	1.6	31.0	15.8	9.9	0.2	11.4	0.2	26.4
a-0000419	0.0	4.4	2.0	33.1	18.1	8.6	0.0	11.0	0.1	22.9
a-0000420	0.0	4.4	1.6	27.3	19.7	9.5	0.3	11.6	0.0	25.5
a-0000421	0.0	3.7	1.4	28.1	18.5	9.4	0.0	10.9	0.4	27.8
a-0000422	0.0	4.1	1.1	27.0	21.3	10.3	0.0	9.8	0.0	26.3
a-0000423	0.0	4.3	2.1	53.4	18.4	5.6	0.0	9.9	0.0	6.3
a-0000424	0.0	3.6	1.5	20.9	13.6	10.9	0.2	8.8	0.8	39.8
a-0000426	0.0	3.6	1.6	24.0	13.2	8.3	0.6	10.9	0.7	37.4

Appendix I Continued

Accession ID	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
a-0000430	0.0	4.1	1.5	28.9	15.5	10.0	0.2	11.4	0.4	28.1
a-0000431	0.0	3.8	1.3	21.2	16.7	10.6	0.0	7.6	0.9	38.1
a-0000433	0.0	4.7	2.3	32.3	19.6	8.6	0.4	9.2	0.7	22.1
a-0000439	0.0	5.0	1.9	27.3	18.6	11.4	0.0	9.6	0.9	25.2
a-0000440	0.0	3.9	1.5	26.7	16.6	8.3	0.0	9.8	0.7	32.7
a-0000441	0.0	4.0	1.9	30.5	14.2	7.2	0.0	11.2	0.5	30.6
a-0000442	0.0	3.8	1.7	23.2	17.2	8.5	0.0	8.7	0.8	36.3
a-0000497	0.0	5.5	1.9	59.8	22.8	8.4	0.0	1.3	0.2	0.0
a-0000498	0.0	4.0	1.5	72.9	10.0	9.6	0.0	1.6	0.2	0.2
a-0000499	0.0	4.9	1.8	63.1	19.2	8.9	0.1	1.5	0.3	0.2
a-0000500	0.0	4.8	1.7	63.1	19.6	8.4	0.1	1.8	0.3	0.1
a-0000501	0.0	3.8	0.9	10.8	16.5	12.7	0.0	4.9	1.4	49.0
a-0000502	0.0	4.8	2.0	65.1	18.9	7.5	0.2	1.2	0.3	0.1
a-0000503	0.0	5.2	2.0	62.3	20.6	8.2	0.2	1.3	0.4	0.1
a-0000505	0.0	5.2	1.6	64.0	19.3	8.5	0.0	1.3	0.2	0.0
a-0000506	0.0	4.9	1.6	63.1	20.1	8.7	0.0	1.3	0.2	0.2
a-0000508	0.0	3.3	0.9	10.1	15.2	13.2	0.0	4.9	0.9	51.4
a-0000509	0.0	3.3	1.0	17.0	14.2	7.3	0.0	8.3	0.7	48.3
a-0000510	0.1	5.6	1.7	60.8	19.3	10.1	0.2	1.8	0.3	0.2
a-0000511	0.0	6.3	2.4	54.9	24.2	6.8	0.0	2.2	0.4	2.6
a-0000512	0.0	5.5	1.5	60.2	21.8	9.0	0.0	1.4	0.4	0.3
a-0000514	0.0	4.8	2.7	61.5	21.2	8.0	0.0	1.2	0.0	0.5
a-0000515	0.1	4.9	1.8	63.8	20.5	7.0	0.2	1.3	0.4	0.2
a-0000516	0.0	4.9	1.3	62.0	21.0	9.2	0.0	1.4	0.1	0.1
a-0000517	0.0	4.4	2.2	63.9	18.9	9.1	0.0	1.2	0.1	0.4
a-0000518	0.0	4.4	2.0	61.1	21.2	8.8	0.2	1.4	0.5	0.5
a-0000519	0.0	4.3	1.7	68.2	17.0	7.2	0.0	1.2	0.2	0.2
a-0000520	0.0	4.3	1.5	66.0	18.6	8.3	0.0	1.2	0.3	0.0
a-0000521	0.0	5.2	1.7	61.5	20.8	9.2	0.1	1.2	0.3	0.2
a-0000522	0.0	4.9	1.7	61.5	20.9	9.5	0.0	1.2	0.3	0.0

II. New Primer Pairs for Regions Flanking *Bna.FAE1*

Fourteen primer pairs were designed based on the SNPs flanking the *Bna.FAE1.A8* and *Bna.FAE1.C3 r*egions in the GWAS data that may act as modifier loci for controlling the erucic acid biosynthesis.

CDS model	Primer name	Sequence	Length	Tm (°C)	Length (bp)
Cab035976.1	EA_A8.1F	CCTGAACTACATGGCTGCCT	20	59.8	980
	EA_A8.1R	TGGCAAGTAGAATCACAAGCA	21	57.9	
Cab035974.1	EA_A8.2F	CCTCTGAGGGGTTGAAACAC	20	58.1	832
	EA_A8.2R	CCTGTCTACCCTCACACCAA	20	58.6	
Cab035991.2	EA_A8.3F	GCAGAGAAGAAAGCCCAAGG	20	58.8	1189
	EA_A8.3R	TGAGCTGTCCATCCCAAACT	20	58.9	
Cab035992.1	EA_A8.4F	TGTACCGGAAAATGGACCAGA	21	59.0	516
	EA_A8.4R	ATGATCTGCGCTCTTTCGTG	20	58.7	
Cab036061.1	EA_A8.5F	CGACGAGATGCAGTTGAAGG	20	59.0	1793
	EA_A8.5R	CCTTCCTGGTCATGGTAGCA	20	59.1	
Cab033414.2	EA_A8.6F	GGAGACGCTTCCAACGGTAA	20	60.0	748
	EA_A8.6R	ACCTCAACAACATCAGGCCT	20	59.2	
Cab035852.1	EA_A8.7F	ACAGTGCCGGAGAATCTGAA	20	59.0	374
	EA_A8.7R	GTCGCAACAGCTAGCTTCTC	20	59.0	
Cab035874.1	EA_A8.8F	CGTGATGGTTCTAATCGGCG	20	59.1	818
	EA_A8.8R	CCATGAACGGCTACTCACGT	20	60.1	
Cab035955.1	EA_A8.9F	GCTCTCTTTCTTCTCCGACCA	21	59.4	771
	EA_A8.9R	TTGTCTGCTTGCGGTGTAAC	20	59.1	
Cab040805.1	EA_A8.10F	GCGGAGACAAGAAAGAGAAGAAG	23	59.3	1083
	EA_A8.10R	ACTCCTTTGGGCGGCTTAAT	20	59.7	
Bo3g168710.1	EA_C3.11F	GCTGCCAATCTTGATTGCCT	20	59.2	2093
	EA_C3.11R	TGGCAAGTAGAATCACAACCA	21	57.5	
	EA_C3.11S	ACCAAAGTCCCGCATGTTTT	20	58.6	1241
Bo3g162450.1	EA_C3.13F	GTCCCACACGTTAACAGCG	19	59.1	1889
	EA_C3.13R	TATACCACGCTTTCCCTGCA	20	59.1	
Bo3g164280.1	EA_C3.15F	CATGCGTTCGTCTCCAACTC	20	59.3	856
	EA_C3.15R	TACCATGAACGGCTGCTCA	19	59.0	
Bo3g168860.1	EA_C3.17F	CATGCGTCTTGTGGGATGTT	20	58.8	1209
	EA_C3.17R	GCCCTCCAAACGCCCATA	18	58.2	

III. Base Pair Change Information for the CDS Models

Base pair changes in the sequence of the CDS models used for designing the primers flanking the *Bna.FAE1* region.

CDS	SNPs	Maplus	Ningyou 7	Cabriolet
		Base pair change		
Cab035976.1	Cab035976.1:1025:G	G	G	S
	Cab035976.1:1042:C	C	C	M
	Cab035976.1:1092:G	C	C	S
Cab035974.1	Cab035974.1:195:G	T	T	K
	Cab035974.1:204:T	C	C	Y
	Cab035974.1:294:A	C	C	M
Cab035991.2	Cab035991.2:1887:T	T	T	Y
	Cab035991.2:1956:G	G	G	R
	Cab035991.2:2085:G	G	G	R
	Cab035991.2:2508:T	T	T	Y
Cab035992.1	Cab035992.1:3001:G	G	G	R
Cab036061.1	Cab036061.1:804:A	T	T	A
Cab033414.2	Cab033414.2:244:C	S	G	S
	Cab033414.2:156:T	Y	C	C
	Cab033414.2:402:A	R	A	R
Cab035852.1	Cab035852.1:1830:A	R	G	R
Cab035874.1	Cab035874.1:189:C	T	Y	Y
Cab035955.1	Cab035955.1:48:T	C	Y	Y
Cab040805.1	Cab040805.1:141:G	G	A	R
Bo3g168710.1	Bo3g168710.1:1092:A	R	R	A
	Bo3g168710.1:834:C	C	C	M
	Bo3g168710.1:1020:T	Y	Y	T
	Bo3g168710.1:762:G	G	G	R
Bo3g162450.1	Bo3g162450.1:1590:C	Y	C	Y
	Bo3g162450.1:453:T	Y	T	Y
	Bo3g162450.1:168:T	K	T	K
	Bo3g162450.1:78:C	Y	C	Y
	Bo3g162450.1:414:A	R	A	R
	Bo3g162450.1:1548:A	R	A	R
Bo3g164280.1	Bo3g164280.1:21:G	R	A	R
	Bo3g164280.1:225:T	K	G	K
	Bo3g164280.1:141:C	Y	T	C
	Bo3g164280.1:477:A	R	G	R
Bo3g168860.1	Bo3g168860.1:726:A	A	A	R
	Bo3g168860.1:987:G	R	R	G
	Bo3g168860.1:975:T	Y	Y	T

IV. The Fatty Acids Results of $F_1B_1S_2$ seeds of the cross, 'Cabriolet x (K0472 x Ningyou 7)'

Single seed fatty acid analysis (Section 2.7.1) data (percentages) of 17 lines of $F_1B_1S_2$ seeds and controls having ~10 biological replicates each.

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Ningyou 7	2	2_02	2.84	0.21	0.84	11.42	13.49	11.11	0.71	5.55	0.54	0.70	51.19	0.28	1.12
Ningyou 7	2	2_03	2.90	0.17	0.75	11.34	13.37	9.73	0.70	4.49	0.40	0.86	53.83	0.35	1.12
Ningyou 7	2	2_04	3.30	0.15	0.91	8.61	16.46	11.70	0.69	3.85	0.59	0.97	50.98	0.41	1.39
Ningyou 7	2	2_05	3.03	0.14	0.66	9.42	15.26	13.64	0.55	4.14	0.52	0.69	50.41	0.28	1.27
Ningyou 7	2	2_06	3.16	0.15	0.68	8.94	15.62	12.10	0.57	4.03	0.56	0.75	51.75	0.34	1.34
Ningyou 7	2	2_07	3.29	0.18	0.99	14.19	15.10	10.64	0.74	7.38	0.60	0.65	44.78	0.31	1.14
Ningyou 7	2	2_08	3.42	0.25	0.68	9.57	16.14	13.82	0.61	4.12	0.55	0.77	48.72	0.27	1.09
Ningyou 7	2	2_09	3.00	0.12	0.70	10.81	14.96	13.31	0.57	4.84	0.52	0.71	49.02	0.26	1.17
Ningyou 7	2	2_10	3.21	0.24	0.73	10.89	15.41	12.49	0.68	4.07	0.45	0.84	49.45	0.34	1.20
Cab x (K0472 x Ningyou 7) A3-42	1	1_01	2.61	0.26	0.78	33.97	1.53	2.91	0.58	16.48	0.11	0.28	39.85	0.07	0.58
Cab x (K0472 x Ningyou 7) A3-42	1	1_02	2.69	0.21	0.83	33.41	1.50	2.77	0.65	12.89	0.09	0.33	44.02	0.06	0.56
Cab x (K0472 x Ningyou 7) A3-42	1	1_03	2.59	0.31	0.69	33.62	1.92	3.68	0.52	13.99	0.13	0.29	41.52	0.07	0.68
Cab x (K0472 x Ningyou 7) A3-42	1	1_04	2.31	0.32	0.46	27.08	2.15	4.76	0.48	4.91	0.04	0.36	56.21	0.10	0.82
Cab x (K0472 x Ningyou 7) A3-42	1	1_05	2.45	0.32	0.67	30.73	1.58	3.55	0.55	8.35	0.03	0.45	50.35	0.13	0.83
Cab x (K0472 x Ningyou 7) A3-42	1	1_06	2.64	0.33	0.74	32.79	1.85	4.07	0.59	12.21	0.11	0.35	43.64	0.08	0.60
Cab x (K0472 x Ningyou 7) A3-42	1	1_08	2.44	0.21	0.97	34.40	1.60	3.05	0.68	16.17	0.14	0.31	39.51	0.07	0.45
Cab x (K0472 x Ningyou 7) A3-42	1	1_09	2.62	0.26	0.79	32.56	1.81	3.46	0.66	13.41	0.07	0.39	43.29	0.08	0.60
Cab x (K0472 x Ningyou 7) A3-92	3	3_01	2.39	0.25	0.77	33.83	1.56	3.53	0.55	13.47	0.08	0.31	42.37	0.10	0.80
Cab x (K0472 x Ningyou 7) A3-92	3	3_02	2.79	0.29	1.07	41.44	1.29	2.85	0.63	19.23	0.08	0.30	29.21	0.10	0.71
Cab x (K0472 x Ningyou 7) A3-92	3	3_03	2.68	0.27	1.07	43.83	1.10	2.42	0.61	19.67	0.08	0.29	27.06	0.14	0.78
Cab x (K0472 x Ningyou 7) A3-92	3	3_04	2.36	0.15	0.98	34.87	1.23	2.65	0.68	14.44	0.04	0.36	41.36	0.08	0.77
Cab x (K0472 x Ningyou 7) A3-92	3	3_05	2.68	0.22	1.11	43.16	1.31	3.03	0.51	18.77	0.08	0.24	28.10	0.08	0.72

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) A3-92	3	3_06	2.84	0.30	1.02	43.17	1.23	3.10	0.55	19.93	0.10	0.27	26.79	0.06	0.65
Cab x (K0472 x Ningyou 7) A3-92	3	3_07	2.31	0.20	0.78	31.76	1.30	2.61	0.67	10.14	0.04	0.41	48.86	0.13	0.78
Cab x (K0472 x Ningyou 7) A3-92	3	3_08	2.82	0.29	1.08	44.12	1.26	3.11	0.58	19.63	0.11	0.27	25.91	0.10	0.72
Cab x (K0472 x Ningyou 7) A3-92	3	3_09	2.52	0.24	0.90	35.64	1.44	2.93	0.60	14.13	0.06	0.30	40.37	0.10	0.77
Cab x (K0472 x Ningyou 7) A3-92	3	3_10	2.95	0.28	1.17	57.15	1.13	2.59	0.44	19.85	0.10	0.22	13.51	0.11	0.51
Cab x (K0472 x Ningyou 7) A8-24	4	4_01	2.93	0.32	1.38	50.00	1.55	3.72	0.57	22.11	0.09	0.23	16.33	0.11	0.67
Cab x (K0472 x Ningyou 7) A8-24	4	4_02	2.53	0.25	1.08	39.83	1.19	2.57	0.72	17.20	0.07	0.34	33.12	0.14	0.96
Cab x (K0472 x Ningyou 7) A8-24	4	4_03	2.94	0.42	2.00	53.28	1.25	2.35	0.93	19.18	0.06	0.31	16.37	0.20	0.71
Cab x (K0472 x Ningyou 7) A8-24	4	4_04	3.70	0.25	2.00	86.26	1.96	3.33	0.61	1.21	0.05	0.26	0.00	0.25	0.12
Cab x (K0472 x Ningyou 7) A8-24	4	4_05	3.46	0.24	1.88	87.13	1.66	3.55	0.50	1.10	0.05	0.21	0.00	0.16	0.06
Cab x (K0472 x Ningyou 7) A8-24	4	4_06	3.65	0.23	1.88	87.62	1.41	3.02	0.49	1.08	0.03	0.23	0.00	0.22	0.14
Cab x (K0472 x Ningyou 7) A8-24	4	4_07	2.65	0.20	1.34	50.68	1.56	3.50	0.61	20.13	0.10	0.24	18.02	0.12	0.85
Cab x (K0472 x Ningyou 7) A8-24	4	4_08	2.66	0.22	1.48	53.28	1.21	2.92	0.60	22.20	0.10	0.17	14.51	0.09	0.55
Cab x (K0472 x Ningyou 7) A8-24	4	4_09	3.58	0.25	2.00	85.53	2.16	4.22	0.57	1.11	0.00	0.22	0.00	0.23	0.13
Cab x (K0472 x Ningyou 7) A8-24	4	4_10	2.72	0.17	1.42	47.41	2.08	4.33	0.70	17.34	0.07	0.35	22.06	0.18	1.18
K0472	5	5_01	3.50	0.28	1.24	87.99	1.52	3.12	0.47	1.32	0.05	0.28	0.00	0.15	0.10
K0472	5	5_02	3.59	0.30	1.12	88.30	1.57	2.78	0.46	1.33	0.05	0.25	0.02	0.14	0.10
K0472	5	5_03	4.20	0.41	1.11	86.47	1.93	3.45	0.45	1.34	0.00	0.29	0.02	0.21	0.12
K0472	5	5_04	3.63	0.24	1.16	88.46	1.53	2.69	0.46	1.32	0.04	0.25	0.00	0.14	0.08
K0472	5	5_05	3.68	0.28	1.22	87.55	1.62	3.22	0.47	1.34	0.04	0.30	0.00	0.16	0.11
K0472	5	5_06	3.61	0.27	1.12	88.62	1.51	2.69	0.43	1.23	0.04	0.23	0.04	0.14	0.08
K0472	5	5_08	3.59	0.25	1.16	87.71	1.63	3.10	0.46	1.43	0.07	0.29	0.00	0.17	0.13
K0472	5	5_09	3.60	0.23	1.18	88.31	1.57	2.79	0.44	1.33	0.01	0.27	0.01	0.15	0.10
K0472	5	5_10	3.73	0.24	1.32	87.71	1.62	2.85	0.53	1.35	0.02	0.31	0.00	0.17	0.12
Cab x (K0472 x Ningyou 7) A9-68	6	6_01	2.10	0.16	0.73	29.55	1.52	2.96	0.59	9.47	0.08	0.35	51.89	0.08	0.52

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) A9-68	6	6_02	2.57	0.18	0.75	23.41	6.76	6.56	0.59	8.51	0.23	0.41	49.44	0.08	0.52
Cab x (K0472 x Ningyou 7) A9-68	6	6_03	2.58	0.21	0.67	23.20	4.53	7.39	0.64	5.96	0.13	0.47	53.54	0.10	0.58
Cab x (K0472 x Ningyou 7) A9-68	6	6_04	2.54	0.18	0.80	23.25	6.11	6.28	0.60	9.52	0.28	0.39	49.51	0.07	0.48
Cab x (K0472 x Ningyou 7) A9-68	6	6_05	2.51	0.19	0.65	23.55	4.54	6.24	0.57	6.63	0.12	0.44	53.89	0.10	0.58
Cab x (K0472 x Ningyou 7) A9-68	6	6_06	3.02	0.28	0.74	23.41	5.79	7.15	0.62	6.22	0.16	0.55	51.31	0.13	0.64
Cab x (K0472 x Ningyou 7) A9-68	6	6_07	2.50	0.22	0.58	22.65	4.37	6.70	0.58	6.11	0.14	0.46	55.01	0.10	0.60
Cab x (K0472 x Ningyou 7) A9-68	6	6_08	2.67	0.21	0.72	22.87	6.20	6.66	0.61	10.76	0.23	0.37	48.07	0.08	0.56
Cab x (K0472 x Ningyou 7) A9-68	6	6_09	2.40	0.23	0.73	31.08	1.55	3.19	0.62	9.43	0.10	0.38	49.72	0.08	0.50
Cab x (K0472 x Ningyou 7) A9-68	6	6_10	2.66	0.17	0.66	24.42	4.39	6.80	0.55	7.98	0.14	0.37	51.28	0.08	0.49
Cab x (K0472 x Ningyou 7) A20-10	7	7_01	3.03	0.28	1.09	37.54	1.72	4.28	0.61	12.30	0.07	0.32	37.84	0.08	0.84
Cab x (K0472 x Ningyou 7) A20-10	7	7_02	2.85	0.26	1.52	39.52	1.57	3.48	0.82	14.26	0.10	0.37	34.39	0.13	0.72
Cab x (K0472 x Ningyou 7) A20-10	7	7_03	2.37	0.20	1.41	37.06	1.30	3.38	0.83	13.27	0.07	0.46	38.72	0.14	0.80
Cab x (K0472 x Ningyou 7) A20-10	7	7_04	2.94	0.22	1.28	39.69	1.40	3.80	0.67	14.35	0.08	0.30	34.34	0.14	0.80
Cab x (K0472 x Ningyou 7) A20-10	7	7_05	2.95	0.31	1.38	37.45	1.47	3.49	0.72	13.93	0.09	0.35	36.83	0.16	0.86
Cab x (K0472 x Ningyou 7) A20-10	7	7_06	2.81	0.27	1.26	37.17	1.85	4.42	0.63	12.27	0.09	0.38	37.85	0.10	0.89
Cab x (K0472 x Ningyou 7) A20-10	7	7_07	2.95	0.17	1.92	51.29	1.28	3.42	0.79	17.50	0.09	0.29	19.29	0.20	0.82
Cab x (K0472 x Ningyou 7) A20-10	7	7_08	2.88	0.27	1.49	47.71	1.31	3.69	0.67	19.00	0.05	0.28	21.78	0.12	0.73
Cab x (K0472 x Ningyou 7) A20-10	7	7_10	2.77	0.22	1.32	49.25	1.16	3.62	0.57	20.08	0.07	0.28	20.05	0.11	0.51
Cab x (K0472 x Ningyou 7) A20-22	8	8_01	3.99	0.71	1.42	40.50	1.97	5.65	0.75	20.65	0.08	0.34	22.78	0.18	0.96
Cab x (K0472 x Ningyou 7) A20-22	8	8_02	3.10	0.30	1.53	48.57	1.58	3.84	0.76	19.82	0.13	0.35	19.14	0.13	0.75
Cab x (K0472 x Ningyou 7) A20-22	8	8_03	3.61	0.26	1.38	85.65	1.69	4.01	0.59	1.78	0.07	0.47	0.04	0.24	0.21
Cab x (K0472 x Ningyou 7) A20-22	8	8_04	3.35	0.37	1.49	86.20	1.50	4.10	0.60	1.58	0.08	0.40	0.00	0.17	0.15
Cab x (K0472 x Ningyou 7) A20-22	8	8_05	3.18	0.32	1.18	46.69	1.32	4.26	0.68	21.91	0.12	0.29	19.22	0.10	0.73
Cab x (K0472 x Ningyou 7) A20-22	8	8_06	2.85	0.30	1.19	39.93	1.48	3.87	0.75	18.20	0.10	0.38	30.07	0.14	0.75
Cab x (K0472 x Ningyou 7) A20-22	8	8_07	3.19	0.33	1.51	50.13	1.35	3.43	0.67	21.42	0.11	0.30	16.66	0.16	0.75

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) A20-22	8	8_08	3.18	0.42	1.65	49.48	1.36	3.40	0.81	20.14	0.07	0.34	18.18	0.18	0.79
Cab x (K0472 x Ningyou 7) A20-22	8	8_09	3.67	0.33	1.57	85.19	2.04	3.99	0.62	1.76	0.00	0.44	0.03	0.21	0.14
Cab x (K0472 x Ningyou 7) A20-22	8	8_10	2.66	0.31	1.44	53.35	0.98	2.98	0.68	23.36	0.06	0.34	13.33	0.11	0.41
Cab x (K0472 x Ningyou 7) A20-24	9	9_01	2.66	0.20	1.04	27.24	3.71	4.77	0.92	6.04	0.07	0.75	51.27	0.28	1.04
Cab x (K0472 x Ningyou 7) A20-24	9	9_02	2.62	0.19	1.00	30.03	3.28	5.40	0.82	10.71	0.11	0.48	44.23	0.20	0.92
Cab x (K0472 x Ningyou 7) A20-24	9	9_03	2.51	0.27	1.06	33.45	1.11	2.96	0.89	10.01	0.03	0.56	45.81	0.21	1.12
Cab x (K0472 x Ningyou 7) A20-24	9	9_04	2.91	0.24	0.91	28.41	3.97	5.46	0.80	7.94	0.12	0.52	47.58	0.19	0.93
Cab x (K0472 x Ningyou 7) A20-24	9	9_05	2.38	0.20	0.74	26.61	4.09	5.62	0.66	5.70	0.09	0.59	51.97	0.22	1.14
Cab x (K0472 x Ningyou 7) A20-24	9	9_06	3.01	0.36	1.68	33.74	4.00	4.33	1.28	10.69	0.12	0.77	38.60	0.43	1.01
Cab x (K0472 x Ningyou 7) A20-24	9	9_07	2.93	0.28	1.03	28.73	4.17	5.81	0.92	8.92	0.15	0.61	45.08	0.33	1.04
Cab x (K0472 x Ningyou 7) A20-24	9	9_08	2.73	0.26	0.84	28.71	3.95	5.40	0.69	10.24	0.11	0.44	45.47	0.16	1.01
Cab x (K0472 x Ningyou 7) A20-24	9	9_09	2.91	0.23	1.28	27.77	6.11	6.71	0.94	11.15	0.27	0.66	40.67	0.32	0.98
Cab x (K0472 x Ningyou 7) A20-24	9	9_10	2.89	0.31	0.86	23.94	5.10	6.19	0.85	3.38	0.11	0.74	54.05	0.34	1.22
Cab x (K0472 x Ningyou 7) A20-35	10	10_01	2.65	0.27	1.22	50.95	1.53	3.30	0.60	21.51	0.12	0.28	17.02	0.10	0.45
Cab x (K0472 x Ningyou 7) A20-35	10	10_02	2.51	0.31	0.88	32.66	1.59	3.56	0.58	11.77	0.08	0.32	45.08	0.10	0.56
Cab x (K0472 x Ningyou 7) A20-35	10	10_03	2.37	0.17	1.37	39.87	1.37	3.11	0.81	17.62	0.15	0.38	32.09	0.10	0.59
Cab x (K0472 x Ningyou 7) A20-35	10	10_04	2.42	0.29	0.94	33.10	1.44	3.41	0.71	9.95	0.06	0.45	46.42	0.11	0.69
Cab x (K0472 x Ningyou 7) A20-35	10	10_05	2.27	0.23	1.21	32.44	1.42	3.17	0.91	9.51	0.05	0.62	47.26	0.17	0.75
Cab x (K0472 x Ningyou 7) A20-35	10	10_06	2.39	0.22	1.19	38.53	1.29	3.12	0.72	17.44	0.07	0.36	33.99	0.10	0.58
Cab x (K0472 x Ningyou 7) A20-35	10	10_07	2.35	0.17	1.60	40.76	1.26	2.75	1.01	17.47	0.10	0.45	31.41	0.14	0.55
Cab x (K0472 x Ningyou 7) A20-35	10	10_08	2.37	0.27	1.06	32.18	1.66	3.63	0.84	8.84	0.05	0.55	47.68	0.14	0.72
Cab x (K0472 x Ningyou 7) A20-35	10	10_09	3.23	0.20	1.29	87.34	1.36	3.24	0.58	1.93	0.05	0.39	0.13	0.15	0.12
Cab x (K0472 x Ningyou 7) A20-48	11	11_01	3.77	0.64	1.55	43.68	1.70	4.52	0.88	20.97	0.08	0.40	20.95	0.19	0.66
Cab x (K0472 x Ningyou 7) A20-48	11	11_02	3.11	0.65	1.85	35.60	1.34	3.06	1.42	8.02	0.07	1.10	42.01	0.61	1.16
Cab x (K0472 x Ningyou 7) A20-48	11	11_03	3.27	0.42	1.83	48.84	1.06	3.76	1.06	18.12	0.10	0.50	19.76	0.45	0.84

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) A20-48	11	11_04	3.39	0.27	1.75	47.71	1.26	3.53	0.90	21.23	0.08	0.36	18.63	0.18	0.71
Cab x (K0472 x Ningyou 7) A20-48	11	11_05	2.80	0.28	0.83	31.05	1.59	4.50	0.67	8.38	0.04	0.52	48.36	0.14	0.83
Cab x (K0472 x Ningyou 7) A20-48	11	11_06	3.02	0.35	1.38	36.92	1.05	3.44	0.88	16.89	0.05	0.42	34.83	0.14	0.64
Cab x (K0472 x Ningyou 7) A20-48	11	11_07	2.84	0.45	1.02	31.59	1.07	3.32	0.82	5.03	0.00	0.66	51.73	0.27	1.20
Cab x (K0472 x Ningyou 7) A20-48	11	11_08	2.94	0.38	1.27	31.15	1.98	4.07	0.96	8.20	0.07	0.65	47.33	0.17	0.83
Cab x (K0472 x Ningyou 7) A20-48	11	11_09	3.20	0.43	2.00	37.38	1.10	3.43	1.39	11.15	0.05	0.83	37.52	0.57	0.94
Cab x (K0472 x Ningyou 7) A20-48	11	11_10	2.98	0.39	0.93	35.01	1.41	4.09	0.70	14.99	0.10	0.40	37.93	0.16	0.91
Cab x (K0472 x Ningyou 7) A20-54	12	12_01	3.01	0.30	0.83	25.06	4.06	6.59	0.77	6.34	0.09	0.64	51.34	0.17	0.80
Cab x (K0472 x Ningyou 7) A20-54	12	12_02	2.16	0.24	0.70	29.55	1.27	2.85	0.70	6.27	0.04	0.50	54.91	0.11	0.71
Cab x (K0472 x Ningyou 7) A20-54	12	12_03	2.85	0.24	0.67	18.94	7.13	8.88	0.76	4.25	0.14	0.71	54.63	0.15	0.65
Cab x (K0472 x Ningyou 7) A20-54	12	12_04	2.54	0.19	0.64	23.14	3.68	6.90	0.70	4.79	0.15	0.66	55.67	0.16	0.79
Cab x (K0472 x Ningyou 7) A20-54	12	12_05	2.69	0.32	0.61	20.76	4.50	8.78	0.68	2.59	0.05	0.77	56.87	0.24	1.15
Cab x (K0472 x Ningyou 7) A20-54	12	12_06	2.77	0.37	0.65	19.82	4.93	9.24	0.64	3.28	0.06	0.81	56.34	0.19	0.91
Cab x (K0472 x Ningyou 7) A20-54	12	12_07	2.90	0.74	0.72	28.94	1.92	3.87	0.75	4.16	0.00	0.79	54.03	0.19	0.99
Cab x (K0472 x Ningyou 7) A20-54	12	12_08	3.01	0.25	0.94	26.39	3.67	6.06	0.76	9.73	0.11	0.42	47.95	0.11	0.61
Cab x (K0472 x Ningyou 7) A20-54	12	12_09	2.56	0.27	0.71	29.45	1.41	3.12	0.72	6.93	0.04	0.50	53.58	0.10	0.60
Cab x (K0472 x Ningyou 7) A20-54	12	12_10	2.83	0.31	0.64	23.85	3.91	7.04	0.68	4.90	0.08	0.56	54.16	0.14	0.91
Cab x (K0472 x Ningyou 7) A20-73	13	13_01	3.61	0.39	0.85	19.14	7.98	8.99	0.73	3.94	0.13	1.04	51.68	0.36	1.15
Cab x (K0472 x Ningyou 7) A20-73	13	13_02	2.48	0.29	0.82	31.48	0.92	3.21	0.76	7.90	0.03	0.57	50.07	0.22	1.26
Cab x (K0472 x Ningyou 7) A20-73	13	13_03	3.39	0.40	1.03	24.89	4.46	6.54	1.11	5.04	0.07	1.01	50.39	0.45	1.22
Cab x (K0472 x Ningyou 7) A20-73	13	13_04	2.82	0.29	0.70	22.45	4.52	7.88	0.70	3.81	0.07	0.85	54.44	0.29	1.17
Cab x (K0472 x Ningyou 7) A20-73	13	13_05	3.04	0.27	0.80	19.09	7.40	8.39	0.76	4.89	0.18	0.87	52.75	0.37	1.20
Cab x (K0472 x Ningyou 7) A20-73	13	13_06	3.06	0.28	1.10	25.67	4.14	7.70	0.93	8.21	0.11	0.73	46.54	0.36	1.19
Cab x (K0472 x Ningyou 7) A20-73	13	13_07	2.81	0.25	0.96	29.06	3.15	8.67	0.78	14.28	0.10	0.44	38.24	0.19	1.08
Cab x (K0472 x Ningyou 7) A20-73	13	13_08	2.45	0.37	0.70	29.14	1.19	3.61	0.72	4.81	0.03	0.67	54.90	0.21	1.21

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) A20-73	13	13_09	2.61	0.26	0.78	29.50	1.29	4.04	0.72	4.16	0.00	0.73	54.36	0.24	1.31
Cab x (K0472 x Ningyou 7) A20-73	13	13_10	3.12	0.34	0.75	22.89	4.70	9.59	0.69	6.13	0.15	0.64	49.36	0.29	1.34
Cab x (K0472 x Ningyou 7) A20-94	14	14_01	2.43	0.22	0.83	29.18	1.61	3.67	0.73	5.83	0.03	0.64	53.76	0.17	0.91
Cab x (K0472 x Ningyou 7) A20-94	14	14_02	2.73	0.29	1.74	53.35	0.95	2.34	0.79	22.89	0.07	0.34	13.89	0.16	0.47
Cab x (K0472 x Ningyou 7) A20-94	14	14_03	3.25	0.31	1.68	52.12	1.10	3.13	0.66	21.17	0.11	0.27	15.52	0.14	0.55
Cab x (K0472 x Ningyou 7) A20-94	14	14_04	2.71	0.34	1.20	40.06	1.48	3.32	0.61	15.64	0.08	0.29	33.58	0.12	0.59
Cab x (K0472 x Ningyou 7) A20-94	14	14_05	2.96	0.25	1.29	43.64	1.46	3.22	0.75	20.22	0.08	0.32	24.99	0.12	0.68
Cab x (K0472 x Ningyou 7) A20-94	14	14_06	2.79	0.30	1.09	39.13	1.53	3.72	0.65	16.96	0.07	0.30	32.61	0.11	0.73
Cab x (K0472 x Ningyou 7) A20-94	14	14_07	2.84	0.23	1.08	38.98	1.48	3.60	0.60	19.33	0.10	0.26	30.81	0.12	0.58
Cab x (K0472 x Ningyou 7) A20-94	14	14_08	2.95	0.41	0.69	28.70	2.02	5.53	0.56	6.14	0.06	0.46	51.24	0.13	1.11
Cab x (K0472 x Ningyou 7) A20-94	14	14_09	2.93	0.31	1.21	49.70	1.59	3.73	0.57	22.12	0.11	0.25	16.75	0.11	0.61
Cab x (K0472 x Ningyou 7) A20-94	14	14_10	2.59	0.19	1.34	39.79	1.65	3.41	0.81	17.26	0.15	0.39	31.55	0.15	0.71
Cab x (K0472 x Ningyou 7) C2-16	15	15_01	2.63	0.33	1.08	35.38	1.36	3.33	0.68	16.55	0.08	0.32	37.47	0.09	0.71
Cab x (K0472 x Ningyou 7) C2-16	15	15_02	3.23	0.47	1.36	53.47	1.64	3.69	0.49	20.44	0.18	0.18	14.17	0.13	0.56
Cab x (K0472 x Ningyou 7) C2-16	15	15_03	2.64	0.34	0.90	32.88	1.55	3.71	0.66	12.97	0.05	0.35	43.13	0.10	0.72
Cab x (K0472 x Ningyou 7) C2-16	15	15_04	3.07	0.32	1.03	52.14	1.48	3.49	0.44	23.49	0.07	0.19	13.74	0.09	0.46
Cab x (K0472 x Ningyou 7) C2-16	15	15_05	2.70	0.34	0.91	36.28	1.54	3.34	0.55	14.48	0.07	0.30	38.70	0.07	0.72
Cab x (K0472 x Ningyou 7) C2-16	15	15_06	3.16	0.42	1.13	47.03	1.52	3.97	0.52	24.69	0.16	0.24	16.47	0.09	0.60
Cab x (K0472 x Ningyou 7) C2-16	15	15_07	3.77	0.36	1.23	87.75	1.56	3.05	0.45	1.23	0.04	0.26	0.00	0.19	0.10
Cab x (K0472 x Ningyou 7) C2-16	15	15_08	3.74	0.41	1.29	86.84	1.74	3.32	0.49	1.39	0.08	0.32	0.00	0.23	0.15
Cab x (K0472 x Ningyou 7) C2-16	15	15_09	2.44	0.26	0.72	28.98	1.46	3.33	0.67	7.06	0.08	0.48	53.66	0.10	0.75
Cab x (K0472 x Ningyou 7) C2-16	15	15_10	2.87	0.40	1.01	35.89	1.46	2.94	0.59	14.51	0.06	0.30	39.22	0.08	0.65
Cab x (K0472 x Ningyou 7) C2-20	16	16_01	2.81	0.25	0.86	26.70	3.15	5.39	0.73	7.80	0.07	0.45	51.02	0.11	0.66
Cab x (K0472 x Ningyou 7) C2-20	16	16_02	2.67	0.31	0.82	24.41	3.65	6.00	0.76	4.55	0.07	0.61	55.30	0.14	0.73
Cab x (K0472 x Ningyou 7) C2-20	16	16_03	2.60	0.29	0.86	24.77	3.58	5.82	0.72	5.46	0.07	0.58	54.36	0.15	0.75

Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) C2-20	16	16_04	2.62	0.25	0.93	24.60	3.61	5.80	0.77	5.48	0.09	0.57	54.35	0.16	0.78
Cab x (K0472 x Ningyou 7) C2-20	16	16_05	2.74	0.24	0.94	25.18	3.57	5.43	0.79	6.36	0.07	0.55	53.30	0.13	0.68
Cab x (K0472 x Ningyou 7) C2-20	16	16_06	2.34	0.24	0.71	28.63	1.30	3.01	0.73	4.49	0.00	0.56	56.96	0.14	0.88
Cab x (K0472 x Ningyou 7) C2-20	16	16_07	2.76	0.32	1.00	25.51	3.56	5.69	0.79	8.77	0.10	0.50	50.19	0.11	0.69
Cab x (K0472 x Ningyou 7) C2-20	16	16_08	2.64	0.31	0.98	25.50	3.83	5.04	0.82	5.08	0.08	0.63	54.22	0.13	0.73
Cab x (K0472 x Ningyou 7) C2-20	16	16_09	2.39	0.28	0.73	29.50	1.37	2.93	0.66	6.42	0.02	0.52	54.23	0.13	0.81
Cab x (K0472 x Ningyou 7) C2-20	16	16_10	2.70	0.27	0.84	23.56	3.59	6.55	0.74	6.30	0.09	0.58	53.90	0.12	0.75
Cab x (K0472 x Ningyou 7) C2-54	17	17_01	2.74	0.26	0.75	28.09	3.60	5.78	0.58	12.44	0.10	0.36	44.40	0.11	0.79
Cab x (K0472 x Ningyou 7) C2-54	17	17_02	2.90	0.30	1.00	28.61	2.92	5.31	0.70	15.80	0.10	0.33	41.28	0.09	0.68
Cab x (K0472 x Ningyou 7) C2-54	17	17_03	2.75	0.22	1.05	37.76	1.46	3.20	0.62	21.78	0.08	0.30	29.92	0.11	0.75
Cab x (K0472 x Ningyou 7) C2-54	17	17_04	3.19	0.32	0.96	30.32	3.86	5.50	0.65	14.15	0.11	0.34	39.66	0.12	0.82
Cab x (K0472 x Ningyou 7) C2-54	17	17_05	3.23	0.29	0.86	27.30	5.81	6.51	0.60	12.94	0.20	0.31	41.10	0.10	0.75
Cab x (K0472 x Ningyou 7) C2-54	17	17_06	2.96	0.33	0.78	26.43	3.91	5.30	0.60	9.03	0.11	0.35	49.27	0.11	0.82
Cab x (K0472 x Ningyou 7) C2-54	17	17_07	2.56	0.32	0.91	35.24	1.66	4.13	0.61	18.44	0.09	0.30	34.74	0.10	0.90
Cab x (K0472 x Ningyou 7) C2-54	17	17_08	2.88	0.30	0.97	31.05	3.50	4.80	0.64	15.01	0.12	0.30	39.54	0.10	0.78
Cab x (K0472 x Ningyou 7) C2-54	17	17_09	2.78	0.30	0.79	25.76	3.49	5.53	0.66	7.58	0.10	0.43	51.78	0.14	0.67
Cab x (K0472 x Ningyou 7) C2-54	17	17_10	2.74	0.31	0.74	27.07	3.43	5.05	0.59	9.75	0.11	0.37	49.01	0.09	0.74
Cab x (K0472 x Ningyou 7) C2-54	19	19_01	2.42	0.31	0.82	31.88	1.67	3.19	0.57	10.93	0.06	0.30	47.04	0.07	0.76
Cab x (K0472 x Ningyou 7) C2-78	19	19_02	3.54	0.43	0.94	35.96	1.89	3.65	0.59	21.83	0.11	0.25	30.00	0.08	0.71
Cab x (K0472 x Ningyou 7) C2-78	19	19_03	2.70	0.32	0.91	39.87	1.41	2.98	0.53	20.80	0.08	0.25	29.33	0.09	0.72
Cab x (K0472 x Ningyou 7) C2-78	19	19_04	3.05	0.30	1.00	44.24	1.29	2.50	0.53	23.21	0.09	0.19	23.07	0.08	0.46
Cab x (K0472 x Ningyou 7) C2-78	19	19_05	2.55	0.33	0.81	32.19	1.43	3.13	0.64	12.28	0.07	0.32	45.52	0.07	0.67
Cab x (K0472 x Ningyou 7) C2-78	19	19_06	2.44	0.31	0.86	31.53	1.14	2.60	0.65	10.96	0.06	0.36	48.39	0.07	0.63
Cab x (K0472 x Ningyou 7) C2-78	19	19_07	2.58	0.25	0.95	33.44	1.17	2.31	0.61	13.48	0.02	0.32	44.24	0.05	0.56
Cab x (K0472 x Ningyou 7) C2-78	19	19_08	2.77	0.34	0.86	37.74	1.55	3.77	0.52	19.93	0.10	0.24	31.37	0.11	0.70

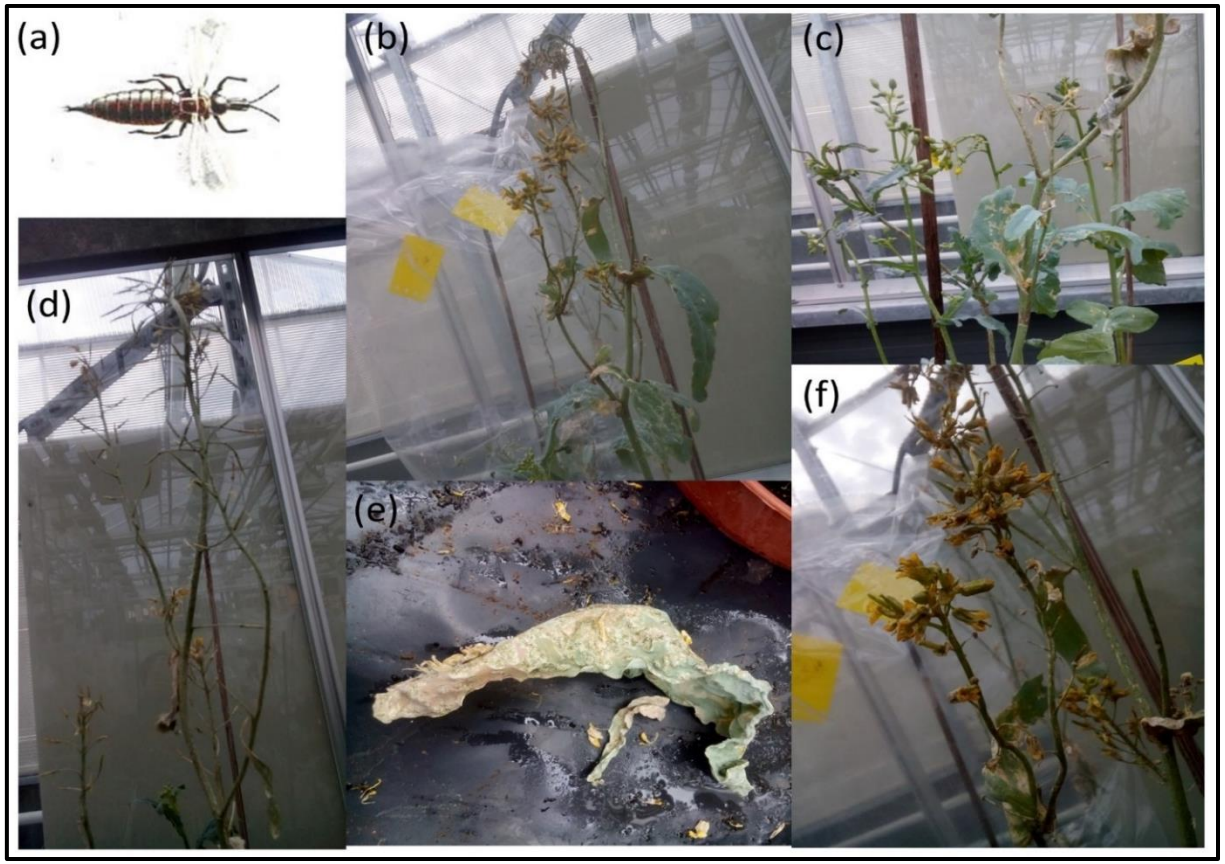
Appendix IV Continued

Cross Description ($F_1B_1S_2$ seeds)	Code	Rep	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:02	22:0	22:1	24:0	24:1
Cab x (K0472 x Ningyou 7) C2-78	19	19_09	2.42	0.26	0.81	33.64	1.44	3.16	0.57	15.22	0.09	0.28	41.27	0.07	0.78
Cab x (K0472 x Ningyou 7) C2-78	19	19_10	2.68	0.38	0.77	30.81	1.62	2.97	0.58	7.35	0.06	0.38	51.53	0.12	0.75
Cab x (K0472 x Ningyou 7) C2-88	20	20_01	3.28	0.61	2.45	40.70	1.92	3.35	1.56	17.61	0.08	0.75	26.58	0.40	0.71
Cab x (K0472 x Ningyou 7) C2-88	20	20_02	2.77	0.36	0.94	34.19	1.58	3.13	0.65	14.10	0.10	0.29	41.17	0.09	0.63
Cab x (K0472 x Ningyou 7) C2-88	20	20_03	2.78	0.38	0.84	32.33	1.60	3.49	0.61	12.75	0.09	0.34	43.96	0.09	0.74
Cab x (K0472 x Ningyou 7) C2-88	20	20_04	2.84	0.33	0.84	33.68	1.58	2.91	0.60	14.68	0.10	0.27	41.41	0.09	0.67
Cab x (K0472 x Ningyou 7) C2-88	20	20_05	2.53	0.36	0.72	29.91	1.61	3.23	0.67	6.81	0.04	0.49	52.79	0.09	0.75
Cab x (K0472 x Ningyou 7) C2-88	20	20_07	2.44	0.23	0.81	32.65	1.52	2.94	0.61	13.91	0.08	0.33	43.67	0.10	0.69
Cab x (K0472 x Ningyou 7) C2-88	20	20_08	2.43	0.27	1.18	32.88	1.32	2.59	0.78	16.20	0.07	0.32	41.24	0.08	0.64
Cab x (K0472 x Ningyou 7) C2-88	20	20_09	2.57	0.32	0.82	33.56	1.67	3.29	0.57	14.17	0.09	0.31	41.87	0.07	0.69
Cab x (K0472 x Ningyou 7) C2-88	20	20_10	2.74	0.31	0.96	33.62	1.53	2.78	0.64	15.63	0.06	0.32	40.74	0.06	0.62

Cab is Cabriolet and Code is the identity used for the FAMEs analysis.

V. Thrips Damage to the Rapeseed Plants in the Glasshouse

The following figure shows a thrip (a), and whole plant, flower and leaf damage by the thrips in the glasshouse (b to f)



VI. The Fatty Acids Analysis of the HELP Lines ($F_1B_1S_3$ seeds)

The fatty acids were analysed on 14 HELP lines along with the controls NY7 and Cabriolet by the single seed method using 11 replicates each. The HELP lines were derived from the cross 'Cabriolet x (K0472 x Ningyou 7)'.

Code	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
1_1	E12-2	2.9	0.3	0.6	31.7	1.8	4.4	0.6	4.6	0.4	52.0	0.8
1_2	E12-2	2.8	0.3	0.6	31.2	1.7	4.3	0.6	4.1	0.5	53.1	0.8
1_3	E12-2	2.8	0.3	0.8	34.5	1.9	3.6	0.6	8.7	0.4	45.4	0.9
1_4	E12-2	3.0	0.4	0.6	30.5	2.0	5.3	0.5	3.6	0.4	52.9	0.8
1_5	E12-2	2.7	0.3	0.5	32.1	2.1	6.1	0.5	5.6	0.3	49.0	0.8
1_6	E12-2	2.7	0.4	0.6	32.4	1.9	5.8	0.5	4.1	0.4	50.2	0.9
1_8	E12-2	3.5	0.4	0.8	36.2	2.1	5.3	0.6	7.8	0.4	42.2	0.7
1_10	E12-2	2.7	0.4	0.6	32.0	1.8	4.8	0.6	5.2	0.4	50.9	0.7
1_11	E12-2	3.2	1.0	0.7	28.6	3.3	8.5	0.4	1.3	0.7	51.0	1.4
2_1	E12-26	2.8	0.3	0.7	35.3	2.0	5.2	0.5	6.7	0.4	45.3	0.8
2_2	E12-26	2.9	0.3	0.7	35.0	1.8	3.8	0.5	5.8	0.5	47.7	0.9
2_3	E12-26	2.8	0.2	0.6	32.9	1.9	4.8	0.5	3.6	0.5	51.2	0.9
2_5	E12-26	2.7	0.2	0.7	33.8	2.2	4.3	0.6	5.6	0.5	48.6	0.8
2_6	E12-26	2.6	0.3	0.7	33.6	2.0	4.8	0.6	4.7	0.4	49.6	0.7
2_7	E12-26	3.0	0.3	0.8	35.1	2.4	4.4	0.6	7.1	0.4	45.2	0.8
2_8	E12-26	2.7	0.2	0.7	33.6	1.9	4.3	0.6	5.4	0.5	49.3	0.7
3_1	E12-33	3.2	0.5	0.6	34.4	2.0	5.2	0.5	6.2	0.3	46.3	0.8
3_2	E12-33	2.9	0.4	0.7	34.6	2.2	6.3	0.5	6.2	0.3	45.0	0.9
3_3	E12-33	2.4	0.3	0.5	32.0	1.8	5.5	0.5	3.2	0.4	52.4	1.0
3_4	E12-33	2.8	0.4	0.7	33.1	1.7	5.4	0.6	6.0	0.4	48.1	0.9
3_5	E12-33	2.5	0.2	0.4	31.0	2.0	5.8	0.4	3.4	0.4	52.8	0.9
3_6	E12-33	2.8	0.2	0.5	31.9	1.6	5.1	0.6	3.6	0.5	52.2	1.0
3_7	E12-33	2.9	0.3	0.4	30.0	1.8	6.7	0.4	2.1	0.5	53.8	1.1
3_8	E12-33	2.9	0.3	0.5	31.3	1.9	5.8	0.5	3.2	0.0	52.7	0.9
3_9	E12-33	2.8	0.5	0.5	33.0	1.9	5.0	0.5	3.7	0.0	51.2	0.9
3_10	E12-33	2.7	0.3	0.5	33.8	1.7	4.6	0.4	5.9	0.3	48.8	0.9
3_11	E12-33	2.7	0.3	0.5	31.2	1.7	5.0	0.6	3.9	0.4	52.8	0.8
4_1	E12-34	2.9	0.4	0.6	32.7	1.6	4.1	0.5	4.9	0.4	51.0	0.7
4_2	E12-34	2.9	0.4	0.5	31.7	1.9	5.4	0.5	3.6	0.4	51.9	0.9
4_3	E12-34	3.1	0.3	0.6	35.6	1.7	4.1	0.6	7.4	0.4	45.5	0.7
4_4	E12-34	2.8	0.3	0.6	33.7	1.6	4.1	0.5	7.0	0.3	48.4	0.7
4_5	E12-34	3.3	0.4	0.6	35.2	2.0	4.7	0.6	6.2	0.4	45.8	0.8
4_6	E12-34	3.1	0.3	0.7	36.4	1.6	3.3	0.6	8.6	0.0	44.8	0.7
4_7	E12-34	3.3	0.4	0.7	35.1	1.9	4.2	0.6	7.1	0.4	45.7	0.7
4_8	E12-34	3.3	0.5	0.5	33.1	2.1	5.2	0.6	3.7	0.4	49.8	0.9

Appendix VI Continued

Code	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
4_9	E12-34	2.9	0.5	0.6	34.3	1.9	3.9	0.5	5.4	0.4	49.0	0.8
4_10	E12-34	3.2	0.4	0.6	33.5	2.1	4.5	0.6	5.2	0.0	49.1	0.8
4_11	E12-34	3.4	0.4	0.7	35.4	1.9	4.2	0.6	8.1	0.3	44.3	0.7
5_1	E12-38	2.8	0.3	0.8	36.1	2.1	5.1	0.6	9.8	0.3	41.6	0.7
5_2	E12-38	2.7	0.3	0.7	36.2	1.9	4.9	0.6	8.2	0.3	43.8	0.6
5_3	E12-38	2.8	0.3	0.7	34.1	2.0	4.4	0.6	6.7	0.4	47.3	0.7
5_4	E12-38	2.9	0.4	0.7	35.6	2.4	5.7	0.6	6.4	0.0	44.7	0.6
5_5	E12-38	2.7	0.2	0.6	35.5	2.4	5.5	0.6	6.1	0.4	45.2	0.8
5_6	E12-38	2.7	0.2	0.7	33.8	2.2	6.2	0.5	6.8	0.4	46.0	0.6
5_7	E12-38	3.0	0.3	0.7	34.7	1.9	4.5	0.5	7.9	0.4	45.4	0.8
5_8	E12-38	2.6	0.3	0.6	33.9	2.1	6.1	0.5	5.1	0.4	47.5	0.8
5_9	E12-38	2.9	0.3	0.8	36.6	1.9	3.4	0.6	8.8	0.4	43.5	0.7
5_10	E12-38	2.7	0.3	0.6	32.8	2.0	5.3	0.5	4.2	0.0	50.8	0.9
5_11	E12-38	2.8	0.3	0.7	36.3	1.9	5.1	0.6	7.2	0.3	44.0	0.6
6_1	E16-6	3.2	0.4	0.8	39.0	1.7	4.0	0.5	12.3	0.3	37.1	0.7
6_2	E16-6	2.9	0.4	0.6	31.8	2.2	5.8	0.5	3.8	0.4	50.7	0.8
6_3	E16-6	3.1	0.5	0.6	33.2	2.3	5.9	0.5	4.4	0.4	48.3	0.8
6_4	E16-6	2.9	0.6	0.6	33.2	2.4	6.1	0.5	4.3	0.3	48.4	0.7
6_5	E16-6	3.1	0.6	0.6	33.9	2.4	6.2	0.5	3.6	0.4	47.9	0.8
6_6	E16-6	3.0	0.5	0.5	33.0	2.3	7.4	0.4	2.9	0.5	48.5	0.9
6_7	E16-6	2.8	0.4	0.8	33.4	1.8	4.4	0.6	7.5	0.3	47.5	0.6
6_8	E16-6	3.1	0.5	0.7	33.9	2.6	5.4	0.5	6.0	0.4	46.1	0.7
6_9	E16-6	3.2	0.5	0.7	32.7	2.3	5.7	0.6	3.6	0.4	49.6	0.8
6_10	E16-6	3.0	0.5	0.7	35.0	2.3	6.2	0.5	6.9	0.3	43.9	0.8
7_1	E16-8	2.8	0.4	0.7	35.2	1.7	3.8	0.5	10.1	0.3	44.0	0.6
7_2	E16-8	2.9	0.4	0.7	33.5	1.9	4.9	0.5	7.0	0.0	47.6	0.7
7_3	E16-8	3.0	0.4	0.7	35.2	1.9	4.6	0.5	9.5	0.3	43.2	0.6
7_4	E16-8	2.9	0.4	0.6	32.2	1.9	4.6	0.5	6.2	0.3	49.6	0.7
7_5	E16-8	3.2	0.5	0.6	34.0	2.2	5.1	0.5	6.2	0.4	46.4	0.7
7_6	E16-8	2.9	0.5	0.8	34.0	1.7	4.7	0.6	8.6	0.3	45.3	0.6
7_7	E16-8	3.1	0.5	0.7	34.3	2.0	4.6	0.5	7.6	0.3	45.9	0.6
7_8	E16-8	2.8	0.5	0.8	35.4	2.1	4.1	0.5	9.1	0.3	43.8	0.6
7_9	E16-8	3.1	0.5	0.8	36.5	2.2	5.1	0.5	9.1	0.3	41.2	0.7
7_10	E16-8	2.8	0.4	0.7	32.9	1.6	4.2	0.6	6.7	0.3	49.3	0.5
7_11	E16-8	3.0	0.5	0.7	33.4	1.6	4.1	0.6	8.4	0.3	46.9	0.5
8_1	E16-10	3.1	0.5	0.7	32.8	2.1	4.9	0.6	5.7	0.4	48.5	0.7
8_2	E16-10	2.9	0.4	0.7	32.4	1.9	4.3	0.6	4.7	0.4	51.0	0.6
8_3	E16-10	3.2	0.4	0.7	33.6	1.7	4.3	0.5	6.5	0.3	48.2	0.6
8_4	E16-10	2.9	0.4	0.7	33.0	1.9	4.5	0.5	6.8	0.3	48.2	0.7
8_5	E16-10	2.7	0.3	0.8	32.0	1.5	3.8	0.6	6.9	0.3	50.4	0.5
8_6	E16-10	3.0	0.4	0.6	32.8	2.0	4.7	0.5	4.4	0.4	50.4	0.8
8_7	E16-10	3.0	0.4	0.7	32.6	2.0	5.0	0.6	5.4	0.4	49.2	0.7
8_8	E16-10	2.9	0.4	0.6	32.1	1.7	4.4	0.6	5.1	0.4	51.2	0.6

Appendix VI Continued

Code	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
8_9	E16-10	2.8	0.3	0.8	32.7	1.6	3.9	0.6	7.4	0.0	49.3	0.6
8_10	E16-10	3.1	0.3	0.7	33.7	1.7	4.1	0.5	8.0	0.3	46.9	0.6
8_11	E16-10	3.1	0.3	0.8	33.8	1.7	4.0	0.6	7.3	0.3	47.6	0.5
9_1	E16-11	3.2	0.4	0.8	36.7	1.7	3.4	0.5	11.3	0.3	41.1	0.7
9_2	E16-11	3.0	0.3	0.7	34.2	1.6	3.9	0.5	9.6	0.3	45.3	0.6
9_3	E16-11	2.6	0.2	0.8	36.7	1.7	3.8	0.6	11.4	0.3	41.2	0.7
9_4	E16-11	2.9	0.4	0.7	34.3	1.9	4.8	0.5	7.5	0.3	45.9	0.8
9_5	E16-11	2.8	0.3	0.8	36.7	1.6	3.8	0.5	11.0	0.3	41.5	0.6
9_6	E16-11	3.5	0.5	0.7	34.4	2.0	5.5	0.5	7.6	0.3	44.3	0.6
9_7	E16-11	2.9	0.4	0.7	35.1	2.2	4.9	0.5	7.7	0.3	44.5	0.8
9_8	E16-11	3.2	0.6	0.6	34.4	2.1	5.0	0.5	5.0	0.3	47.8	0.7
9_9	E16-11	2.8	0.3	0.8	35.7	1.9	4.2	0.5	10.0	0.3	43.0	0.6
9_10	E16-11	2.5	0.3	0.6	31.9	2.2	6.2	0.6	4.7	0.4	49.8	1.0
9_11	E16-11	3.2	0.4	0.8	34.7	2.0	4.2	0.6	8.8	0.3	44.3	0.6
10_1	E16-20	3.0	0.5	0.6	32.5	2.1	5.7	0.5	3.8	0.5	50.0	0.7
10_2	E16-20	3.0	0.4	0.6	31.4	1.8	5.3	0.6	3.9	0.5	51.8	0.7
10_3	E16-20	2.9	0.4	0.5	31.1	1.9	5.3	0.6	2.9	0.5	53.1	0.7
10_4	E16-20	2.8	0.4	0.7	31.9	1.9	4.7	0.6	5.2	0.4	50.7	0.7
10_5	E16-20	3.0	0.5	0.6	31.9	2.3	5.7	0.5	4.5	0.4	49.8	0.8
10_6	E16-20	3.1	0.6	0.6	31.0	2.3	6.7	0.5	2.5	0.6	51.2	1.0
10_7	E16-20	3.0	0.6	0.6	31.1	2.2	5.9	0.6	2.9	0.5	51.8	0.9
10_8	E16-20	3.1	0.6	0.6	31.5	2.6	6.7	0.5	3.2	0.5	49.8	0.8
10_9	E16-20	3.0	0.6	0.7	31.7	2.1	6.1	0.6	4.3	0.5	49.8	0.7
10_11	E16-20	2.9	0.5	0.7	33.0	1.9	4.8	0.5	6.1	0.3	48.6	0.7
11_1	E16-28	3.2	0.5	1.0	34.5	1.9	4.5	0.7	9.7	0.3	43.1	0.6
11_2	E16-28	3.2	0.4	0.8	34.4	2.0	4.5	0.6	8.2	0.3	45.0	0.6
11_3	E16-28	3.2	0.3	0.8	33.7	1.7	4.1	0.6	8.1	0.3	46.7	0.6
11_4	E16-28	2.9	0.5	0.7	33.9	2.3	5.5	0.5	6.0	0.4	46.6	0.8
11_5	E16-28	3.1	0.5	0.8	33.8	2.1	5.3	0.6	6.5	0.3	46.4	0.6
11_6	E16-28	3.2	0.5	0.8	36.3	2.2	5.0	0.5	7.8	0.3	42.5	0.8
11_7	E16-28	3.1	0.4	0.8	34.1	1.9	4.7	0.6	8.0	0.3	45.6	0.6
11_8	E16-28	3.1	0.4	0.9	34.8	2.0	4.3	0.5	9.7	0.3	43.4	0.6
11_9	E16-28	3.0	0.4	0.8	38.1	1.7	3.9	0.5	11.5	0.2	39.2	0.6
11_10	E16-28	3.0	0.4	0.7	33.7	1.9	5.9	0.6	7.3	0.4	45.4	0.8
11_11	E16-28	3.2	0.5	0.8	35.5	2.2	5.5	0.5	7.5	0.3	43.4	0.7
12_1	E16-31	2.8	0.3	0.6	32.7	2.1	4.9	0.5	5.6	0.4	49.5	0.6
12_2	E16-31	2.8	0.3	0.5	31.2	2.1	5.5	0.6	3.3	0.0	52.9	0.8
12_3	E16-31	2.8	0.4	0.5	31.3	2.2	5.9	0.5	4.1	0.4	51.2	0.8
12_4	E16-31	2.7	0.6	0.5	30.4	2.0	6.2	0.5	3.5	0.4	52.4	0.9
12_5	E16-31	2.9	0.4	0.7	32.8	2.0	4.3	0.6	7.6	0.3	47.8	0.6
12_6	E16-31	2.9	0.4	0.5	31.0	2.2	5.8	0.5	3.4	0.0	52.4	0.8
12_7	E16-31	2.8	0.5	0.6	31.9	2.1	5.1	0.5	3.9	0.4	51.5	0.8
12_8	E16-31	2.8	0.5	0.6	31.8	1.8	5.5	0.6	5.0	0.4	50.3	0.7

Appendix VI Continued

Code	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
12_9	E16-31	3.0	0.5	0.7	32.4	1.8	4.7	0.5	6.6	0.4	48.9	0.6
12_10	E16-31	2.9	0.5	0.6	31.3	2.1	6.1	0.5	4.3	0.4	50.6	0.9
12_11	E16-31	2.8	0.4	0.7	32.5	1.9	4.8	0.6	6.6	0.4	48.8	0.7
13_1	E16-38	2.8	0.4	0.7	31.5	1.8	4.5	0.6	5.6	0.0	51.6	0.6
13_2	E16-38	2.8	0.4	0.7	32.2	1.7	4.3	0.5	6.6	0.0	50.2	0.6
13_3	E16-38	2.5	0.3	0.8	32.7	1.7	4.8	0.6	5.3	0.0	50.5	0.8
13_4	E16-38	2.8	0.4	0.7	31.1	1.9	4.7	0.7	4.4	0.4	52.4	0.7
13_5	E16-38	3.0	0.4	0.7	31.5	1.8	4.6	0.6	5.7	0.4	50.7	0.7
13_6	E16-38	2.8	0.5	0.6	31.0	2.5	4.9	0.7	2.6	0.6	53.0	0.8
13_7	E16-38	3.1	0.3	0.8	32.9	2.0	4.9	0.6	7.0	0.4	47.6	0.6
13_8	E16-38	3.1	0.5	0.5	32.1	2.3	5.0	0.4	6.5	0.2	48.7	0.6
13_9	E16-38	2.7	0.4	0.6	31.1	2.0	5.6	0.6	3.7	0.4	52.0	0.7
13_10	E16-38	2.9	0.5	0.7	31.6	2.1	5.8	0.6	4.4	0.4	50.4	0.7
13_11	E16-38	3.0	0.4	0.8	32.2	1.9	5.2	0.6	5.6	0.4	49.3	0.6
14_1	E16-39	2.9	0.4	0.7	34.1	2.0	4.6	0.5	8.3	0.3	45.6	0.7
14_2	E16-39	3.0	0.4	0.7	31.7	2.0	4.7	0.6	5.0	0.4	50.7	0.7
14_3	E16-39	2.8	0.4	0.8	31.6	1.8	5.1	0.7	5.4	0.4	50.3	0.7
14_4	E16-39	3.0	0.4	0.7	32.9	1.8	4.4	0.5	7.9	0.0	47.8	0.7
14_5	E16-39	2.7	0.4	0.5	30.2	2.0	5.3	0.5	3.3	0.5	53.6	0.8
14_6	E16-39	2.9	0.5	0.7	31.6	1.8	4.8	0.6	4.9	0.4	51.1	0.6
14_7	E16-39	2.8	0.4	0.8	33.1	1.9	4.9	0.6	7.2	0.0	47.5	0.7
14_8	E16-39	2.9	0.5	0.7	31.7	1.9	5.9	0.6	3.7	0.5	50.7	0.8
14_9	E16-39	2.8	0.3	0.7	31.7	1.7	4.7	0.6	5.7	0.4	50.8	0.6
14_10	E16-39	2.9	0.4	0.6	31.2	1.9	5.4	0.6	4.0	0.4	52.0	0.7
14_11	E16-39	2.7	0.4	0.6	30.3	2.0	5.6	0.5	3.9	0.4	52.5	0.8
15_1	NY7	4.4	0.4	0.7	14.0	17.4	11.6	0.6	4.0	0.6	45.3	0.9
15_2	NY7	4.1	0.2	0.9	15.2	18.8	6.6	0.7	4.4	0.8	47.1	1.1
15_3	NY7	5.5	0.3	0.7	9.6	21.8	11.8	0.5	2.9	0.8	45.0	1.1
15_4	NY7	4.0	0.3	0.7	16.3	20.1	10.9	0.5	7.2	0.5	38.6	1.0
15_5	NY7	3.3	0.2	0.8	13.1	15.6	12.3	0.6	4.0	0.8	48.5	1.0
15_6	NY7	3.8	0.3	0.5	9.0	19.6	18.6	0.4	2.2	0.8	43.6	1.2
15_7	NY7	3.8	0.2	0.7	16.1	16.1	9.8	0.5	3.7	0.6	47.5	1.0
15_8	NY7	3.4	0.4	0.8	16.7	17.3	9.8	0.5	4.4	0.6	45.2	1.1
15_9	NY7	3.5	0.2	0.8	17.3	15.7	9.9	0.5	4.0	0.7	46.5	0.9
15_10	NY7	3.9	0.3	1.0	29.2	14.3	9.7	0.5	13.3	0.2	26.5	0.8
15_11	NY7	3.4	0.2	0.6	13.6	16.2	12.9	0.4	3.2	0.0	48.4	1.1
16_1	Cab	5.3	0.4	0.8	68.3	11.0	12.0	0.4	1.4	0.3	0.0	0.2
16_2	Cab	4.7	0.3	0.8	72.0	10.0	10.0	0.4	1.4	0.3	0.0	0.1
16_3	Cab	4.5	0.3	0.7	72.0	9.3	11.1	0.3	1.4	0.2	0.0	0.1
16_4	Cab	4.8	0.3	0.8	70.8	10.5	10.6	0.4	1.4	0.2	0.0	0.1
16_5	Cab	4.6	0.3	0.8	72.6	9.3	10.4	0.4	1.5	0.0	0.0	0.1
16_6	Cab	5.2	0.4	0.8	65.8	12.4	13.2	0.4	1.5	0.2	0.0	0.2
16_7	Cab	5.0	0.4	0.8	68.7	11.1	11.9	0.4	1.4	0.3	0.0	0.1

Appendix VI Continued

Code	Line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:1
16_8	Cab	4.4	0.3	0.8	78.0	7.9	6.4	0.4	1.4	0.2	0.0	0.1
16_9	Cab	5.1	0.3	0.8	69.2	10.7	11.6	0.4	1.4	0.3	0.0	0.2
16_10	Cab	4.9	0.4	0.8	72.4	11.4	8.1	0.4	1.3	0.2	0.0	0.1
16_11	Cab	4.9	0.3	0.8	71.9	11.0	8.9	0.4	1.4	0.3	0.0	0.1

NY7 is Ningyou 7 and Cab is Cabriolet.

VII. Comparison of the Two Fatty Acid Measurements Methods

Comparison of the bulk and single seed methods of the fatty acid analysis on the HELP lines ($F_1B_1S_3$ seeds)

	18:1		PUFAs		20:1		22:1		VLCFAs	
Genotype	**Bulk***	**Single⁺**	**Bulk**	**Single**	**Bulk**	**Single**	**Bulk**	**Single**	**Bulk**	**Single**
E12-2	26.2	32.1	5.0	7.4	4.6	5.0	57.6	49.6	62.2	57.1
E12-26	28.2	34.2	4.7	6.5	5.9	5.5	54.6	48.1	60.4	54.7
E12-33	27.5	32.4	4.3	7.3	6.1	4.3	55.7	50.5	61.8	57.9
E12-34	26.4	34.2	4.3	6.2	5.3	6.1	57.2	47.8	62.5	53.9
E12-38	27.6	35.1	4.2	7.2	7.4	7.0	54.6	45.4	62.0	52.6
E16-6	26.4	33.9	4.5	7.9	6.0	5.5	56.1	46.8	62.1	54.8
E16-8	28.2	34.2	4.2	6.4	8.1	8.1	52.6	45.7	60.7	52.2
E16-10	27.4	32.9	4.1	6.1	7.3	6.2	54.2	49.2	61.4	55.3
E16-11	29.6	35.0	4.2	6.4	8.9	8.6	50.7	44.4	59.6	50.8
E16-20	25.4	31.7	4.9	7.8	4.3	3.9	58.1	50.7	62.4	58.5
E16-28	27.8	34.8	4.6	6.8	7.0	8.2	53.8	44.3	60.8	51.1
E16-31	26.2	31.7	4.6	7.4	5.4	4.9	57.0	50.6	62.3	57.9
E16-38	25.8	31.8	4.7	6.9	5.2	5.2	56.8	50.6	62.0	57.5
E16-39	27.1	31.8	4.4	6.9	7.0	5.4	54.7	50.2	61.7	57.2
Ningyou 7	13.2	15.5	21.8	28.8	4.9	4.8	50.8	43.8	55.7	72.6
Cabriolet	73.3	71.1	18.1	20.8	1.6	1.4	0.0	0.0	1.7	20.8

*Bulk –Bulk seeds FAMEs analysis

⁺Single –Single seed FAMEs analysis (average values)

VIII. The Fatty Acid Analysis of the Cross, 'Maplus x HELP' (F_4 Seeds)

HELP lines used for crossing with Maplus were derived from the cross, 'Cabriolet x (K0472 x Ningyou 7)'. The fatty acid compositions were analysed on 96 HELP lines, 10 biological replicates of Maplus, 2 biological replicates of NY7, 2 biological replicates Cabriolet and 2 biological replicates of K0472. Three technical replicates were used for each sample and the following Table shows the mean value of these replicates. The coding of the line is discussed in detail in the Appendix IX.

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
1	5-10-1	0.0	2.2	0.2	0.8	27.8	1.7	3.6	0.7	11.0	0.0	0.4	51.0	0.0	0.7	5.2	62.6	4.1	90.7
2	5-10-2	0.0	2.2	0.3	1.1	29.2	1.2	2.9	0.9	13.5	0.0	0.4	47.6	0.1	0.6	4.1	61.7	4.7	91.2
3	5-10-3	0.0	2.2	0.2	0.9	27.3	1.6	3.4	0.8	10.6	0.0	0.5	51.6	0.2	0.7	5.0	62.9	4.6	90.4
4	5-10-4	0.0	2.3	0.2	1.2	29.4	1.2	3.1	1.0	14.4	0.0	0.5	46.2	0.0	0.5	4.4	61.1	5.0	90.7
5	5-10-5	0.0	2.9	0.4	1.0	29.2	2.3	4.1	0.8	11.2	0.0	0.4	46.9	0.1	0.7	6.4	58.8	5.2	88.4
6	5-10-6	0.0	2.2	0.2	0.6	25.4	1.7	4.4	0.6	8.2	0.0	0.4	55.3	0.0	0.9	6.1	64.4	3.8	90.0
7	5-10-7	0.0	2.4	0.2	0.7	26.4	2.1	4.4	0.7	9.9	0.0	0.4	51.7	0.1	1.1	6.5	62.6	4.3	89.2
8	5-10-8	0.0	2.3	0.2	1.2	28.0	1.6	3.7	0.9	12.6	0.0	0.4	48.2	0.1	0.8	5.3	61.5	4.9	89.8
9	5-10-9	0.1	2.5	0.3	0.9	28.5	1.4	3.1	0.7	12.8	0.0	0.4	48.1	0.0	1.2	4.5	62.2	4.6	91.0
10	5-10-10	0.1	2.3	0.2	0.9	27.5	1.4	3.4	0.8	10.6	0.0	0.4	51.0	0.0	1.4	4.8	63.0	4.5	90.7
11	6-15-1	0.0	1.9	0.2	0.6	25.3	1.6	4.0	0.6	6.1	0.0	0.6	58.1	0.2	0.9	5.5	65.1	3.9	90.6
12	6-15-2	0.0	2.0	0.2	0.5	22.0	2.4	5.4	0.6	4.6	0.0	0.7	60.6	0.1	0.9	7.8	66.1	3.9	88.3
13	6-15-3	0.0	2.1	0.3	0.7	26.3	1.7	3.5	0.7	8.9	0.0	0.5	54.5	0.1	0.8	5.2	64.1	4.1	90.7
14	6-15-4	0.0	2.1	0.2	0.8	26.5	1.7	3.8	0.7	8.0	0.0	0.5	54.6	0.2	0.8	5.5	63.5	4.2	90.2
15	6-15-5	0.0	2.1	0.2	0.7	27.9	1.3	3.0	0.6	9.0	0.0	0.4	53.9	0.1	0.7	4.3	63.7	3.8	91.8
16	6-15-6	0.0	2.2	0.3	0.6	24.8	2.2	4.3	0.6	6.8	0.0	0.6	56.6	0.1	0.8	6.5	64.3	4.1	89.4
17	6-15-7	0.0	2.0	0.2	0.6	26.6	1.7	4.0	0.6	8.0	0.0	0.4	54.9	0.1	0.8	5.7	63.7	3.8	90.5

Appendix VIII Continued

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
18	6-15-8	0.0	1.9	0.2	0.5	26.8	1.2	2.8	0.5	7.1	0.0	0.4	57.7	0.1	0.9	4.0	65.6	3.4	92.6
19	6-15-9	0.0	2.1	0.2	0.5	25.1	1.7	4.3	0.5	6.1	0.0	0.5	57.8	0.1	0.9	6.1	64.9	3.8	90.2
20	6-15-10	0.0	1.9	0.3	0.4	24.5	2.2	4.5	0.5	4.9	0.0	0.2	59.6	0.1	0.9	6.7	65.4	3.1	90.1
21	6-30-1	0.0	2.4	0.0	0.9	31.9	1.3	2.9	0.4	11.0	0.0	0.4	48.4	0.0	0.2	4.3	59.7	4.2	91.6
22	6-30-2	0.0	2.4	0.2	0.8	28.2	1.6	3.7	0.7	10.2	0.0	0.3	51.0	0.0	0.8	5.4	62.0	4.2	90.4
23	6-30-3	0.0	2.4	0.2	0.7	26.7	2.2	4.7	0.6	9.5	0.0	0.4	51.7	0.0	0.8	6.9	62.0	4.2	89.0
24	6-30-4	0.1	2.3	0.1	0.7	29.1	1.3	2.7	0.6	10.3	0.0	0.3	51.2	0.0	1.2	4.0	62.7	4.0	91.9
25	6-30-5	0.0	2.3	0.2	0.7	25.2	2.0	4.7	0.6	8.4	0.0	0.4	54.7	0.0	0.7	6.7	63.8	4.1	89.2
26	6-30-6	0.0	2.6	0.4	0.7	25.4	2.4	5.0	0.5	8.7	0.1	0.3	53.1	0.1	0.7	7.5	62.5	4.2	88.3
27	6-30-7	0.0	2.5	0.3	0.8	28.0	1.5	3.3	0.8	9.4	0.0	0.5	52.3	0.0	0.7	4.8	62.4	4.5	90.7
28	6-30-8	0.0	2.5	0.4	0.9	30.3	1.5	3.6	0.7	11.8	0.0	0.4	47.2	0.0	0.9	5.1	59.8	4.5	90.4
29	6-30-9	0.0	2.1	0.2	0.7	26.9	1.4	3.4	0.5	8.5	0.0	0.4	55.0	0.0	0.9	4.7	64.4	3.7	91.5
30	6-30-10	0.0	2.8	0.4	0.8	28.4	1.4	3.2	0.7	10.7	0.0	0.4	50.4	0.1	0.7	4.6	61.8	4.9	90.6
31	6-47-1	0.0	2.1	0.2	1.2	32.4	1.0	2.1	0.9	13.3	0.0	0.4	45.6	0.0	0.6	3.2	59.6	4.7	92.2
32	6-47-2	0.0	2.1	0.2	0.7	29.1	1.3	2.7	0.6	8.3	0.0	0.4	54.0	0.0	0.7	4.0	63.1	3.7	92.3
33	6-47-3	0.0	2.2	0.2	0.8	28.2	1.2	2.8	0.7	9.2	0.0	0.3	53.6	0.0	0.7	4.0	63.4	4.0	91.9
34	6-47-4	0.0	2.2	0.3	0.7	27.3	1.3	3.2	0.7	6.5	0.0	0.5	56.4	0.2	0.8	4.5	63.7	4.3	91.2
35	6-47-5	0.0	2.1	0.3	0.7	28.3	1.1	2.8	0.7	7.6	0.0	0.5	55.2	0.0	0.8	3.9	63.6	3.9	92.1
36	6-47-6	0.0	2.1	0.2	0.8	29.3	1.1	2.8	0.7	9.6	0.0	0.4	52.3	0.1	0.7	3.9	62.6	4.0	92.1
37	6-47-7	0.0	1.8	0.2	0.7	26.8	1.1	2.8	0.7	8.3	0.0	0.4	56.3	0.0	0.8	3.9	65.4	3.7	92.3
38	6-47-8	0.0	1.9	0.2	0.7	27.8	1.1	2.8	0.7	8.3	0.0	0.4	55.3	0.0	0.8	4.0	64.3	3.7	92.3
39	6-47-9	0.0	2.0	0.3	1.2	29.6	1.1	2.4	0.9	10.4	0.0	0.5	50.7	0.2	0.8	3.4	61.9	4.8	91.8
40	6-47-10	0.0	2.0	0.2	0.8	28.3	1.1	2.4	0.7	8.7	0.0	0.4	54.6	0.1	0.7	3.5	64.1	3.9	92.6
41	6-5-1	0.0	2.0	0.2	0.7	29.3	1.1	2.3	0.6	11.9	0.0	0.3	51.0	0.0	0.6	3.4	63.4	3.7	92.9
42	6-5-2	0.0	2.0	0.2	0.7	28.6	1.2	2.4	0.6	11.4	0.0	0.4	52.0	0.0	0.6	3.5	64.0	3.7	92.8
43	6-5-3	0.0	2.0	0.2	0.6	27.7	1.2	2.7	0.5	9.3	0.0	0.4	54.8	0.0	0.6	3.9	64.7	3.4	92.7
44	6-5-4	0.0	2.1	0.2	0.7	28.0	1.3	2.3	0.6	10.6	0.0	0.4	53.3	0.0	0.6	3.6	64.5	3.8	92.7
45	6-5-5	0.0	2.0	0.2	0.8	29.0	1.2	2.3	0.6	11.9	0.0	0.3	51.0	0.0	0.6	3.5	63.5	3.8	92.7

Appendix VIII Continued

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
46	6-5-6	0.0	2.2	0.2	0.7	29.1	1.2	2.3	0.6	10.3	0.0	0.3	52.3	0.0	0.7	3.5	63.3	3.9	92.6
47	6-5-7	0.0	2.0	0.2	0.7	29.2	1.2	2.7	0.5	11.6	0.0	0.4	51.0	0.0	0.6	3.8	63.2	3.6	92.6
48	6-5-8	0.0	2.2	0.2	0.8	29.0	1.3	2.4	0.7	11.1	0.0	0.4	51.3	0.0	0.7	3.6	63.0	4.1	92.3
49	6-5-9	0.0	2.0	0.3	0.7	28.5	1.2	2.5	0.6	10.9	0.0	0.4	52.2	0.0	0.6	3.7	63.8	3.7	92.5
50	6-5-10	0.0	2.2	0.3	0.8	30.8	1.2	2.2	0.5	13.7	0.0	0.3	47.5	0.0	0.6	3.4	61.8	3.8	92.9
51	7-13-1	0.1	2.5	0.2	0.9	27.4	1.5	3.8	0.7	11.0	0.0	0.4	50.6	0.0	0.9	5.3	62.5	4.6	90.1
52	7-13-2	0.0	2.7	0.4	0.9	30.6	1.4	3.2	0.7	13.5	0.0	0.3	45.5	0.1	0.7	4.6	59.7	4.7	90.7
53	7-13-3	0.0	2.5	0.3	0.6	23.4	2.5	5.9	0.6	6.8	0.0	0.4	56.1	0.0	0.8	8.4	63.7	4.1	87.5
54	7-13-6	0.0	2.6	0.2	1.0	30.0	1.6	3.3	0.7	14.2	0.0	0.3	45.3	0.0	0.6	4.9	60.2	4.7	90.4
55	7-13-7	0.0	3.3	0.5	0.8	27.9	2.1	3.6	0.8	8.7	0.2	0.4	50.8	0.1	0.9	5.9	60.4	5.4	88.7
56	7-13-8	0.0	3.0	0.4	1.0	29.2	2.1	4.0	0.7	11.7	0.0	0.4	46.7	0.0	0.8	6.1	59.1	5.2	88.7
57	7-16-2	0.2	2.3	0.3	0.8	28.3	1.7	3.1	0.7	10.1	0.0	0.4	50.5	0.0	1.6	4.8	62.2	4.4	90.8
58	7-16-3	0.2	2.6	0.3	0.6	24.5	2.6	5.1	0.6	8.0	0.0	0.4	53.1	0.2	1.9	7.6	63.0	4.6	87.8
59	7-16-4	0.0	2.6	0.3	0.8	26.8	1.9	4.1	0.7	9.8	0.0	0.4	51.4	0.1	1.1	6.0	62.3	4.6	89.4
60	7-16-5	0.2	2.3	0.2	0.6	26.0	1.5	4.6	0.7	7.0	0.0	0.5	55.5	0.1	0.9	6.1	63.4	4.3	89.6
61	7-16-6	0.0	2.6	0.3	0.9	30.1	1.6	2.7	0.7	11.0	0.0	0.4	48.9	0.0	0.8	4.3	60.7	4.7	91.0
62	7-16-8	0.0	2.2	0.3	0.5	24.4	2.0	5.0	0.6	5.6	0.0	0.5	57.9	0.1	1.0	6.9	64.5	3.8	89.2
63	7-16-9	0.0	2.3	0.2	0.7	27.6	1.5	3.4	0.6	9.4	0.0	0.4	53.1	0.0	0.8	4.9	63.2	4.1	91.1
64	7-16-10	0.0	2.3	0.3	0.7	26.9	1.4	3.8	0.6	8.5	0.0	0.3	54.3	0.1	0.9	5.1	63.7	4.0	90.9
65	7-54-1	0.0	2.1	0.2	0.9	28.4	1.2	2.6	0.7	11.7	0.0	0.4	51.0	0.1	0.8	3.8	63.4	4.1	92.1
66	7-54-2	0.0	2.1	0.3	0.7	27.1	1.5	3.4	0.6	8.2	0.0	0.5	54.6	0.1	1.0	4.9	63.7	4.0	91.1
67	7-54-3	0.0	2.5	0.3	0.9	29.1	1.3	2.7	0.7	12.0	0.0	0.4	49.2	0.1	0.7	4.0	62.0	4.6	91.4
68	7-54-4	0.0	2.3	0.3	0.9	27.9	1.5	3.0	0.7	9.5	0.0	0.3	52.7	0.0	0.8	4.5	63.0	4.2	91.3
69	7-54-5	0.0	2.4	0.2	0.9	30.0	1.4	2.5	0.7	13.7	0.0	0.3	47.2	0.0	0.7	3.9	61.6	4.3	91.8
70	7-54-6	0.0	2.3	0.3	0.7	27.0	1.4	3.3	0.6	9.1	0.0	0.4	53.9	0.0	0.8	4.7	63.9	4.1	91.2
71	7-54-7	0.0	2.4	0.2	1.1	31.0	1.6	3.1	0.8	11.7	0.0	0.4	46.7	0.0	0.9	4.7	59.3	4.7	90.5
72	7-54-8	0.0	2.2	0.2	0.8	27.9	1.5	3.0	0.7	9.5	0.0	0.4	52.9	0.1	0.8	4.5	63.3	4.1	91.4
73	7-54-9	0.0	2.7	0.3	0.8	28.1	1.5	3.0	0.7	9.4	0.0	0.4	52.1	0.1	0.8	4.5	62.3	4.7	90.8

Appendix VIII Continued

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
74	7-54-10	0.0	3.1	0.5	1.0	29.3	1.9	3.2	0.8	9.7	0.0	0.5	49.2	0.0	0.9	5.1	59.7	5.4	89.6
75	8-20-3	0.0	2.6	0.4	0.8	30.4	1.5	3.2	0.6	11.6	0.0	0.3	47.6	0.1	0.8	4.8	60.0	4.5	90.8
76	8-20-5	0.0	2.7	0.3	0.9	28.2	2.2	4.2	0.7	13.2	0.1	0.3	46.6	0.0	0.7	6.4	60.5	4.7	88.9
77	8-20-6	0.0	2.5	0.2	0.9	31.2	2.0	3.5	0.6	13.2	0.0	0.3	44.9	0.0	0.7	5.5	58.8	4.3	90.2
78	8-20-7	0.0	2.5	0.3	0.7	29.0	1.6	3.3	0.6	11.0	0.0	0.3	49.9	0.0	0.8	4.9	61.7	4.0	91.0
79	8-20-8	0.0	2.3	0.3	0.8	27.5	1.6	3.9	0.6	10.0	0.0	0.3	51.7	0.0	1.0	5.5	62.7	4.0	90.5
80	8-32-1	0.0	2.5	0.3	0.9	24.6	2.5	4.9	0.8	7.8	0.0	0.5	54.4	0.0	0.8	7.4	63.0	4.7	88.0
81	8-32-2	0.0	2.4	0.3	0.9	25.4	2.3	4.7	0.8	9.8	0.0	0.4	52.1	0.1	0.7	7.1	62.7	4.5	88.4
82	8-32-3	0.0	2.5	0.2	0.8	24.7	2.3	4.3	0.8	6.5	0.0	0.5	56.6	0.1	0.8	6.5	63.9	4.6	88.8
83	8-32-4	0.0	2.2	0.3	0.9	26.1	1.9	4.2	0.7	11.7	0.0	0.3	51.1	0.1	0.6	6.2	63.4	4.2	89.7
84	8-32-5	0.0	2.3	0.3	0.7	23.7	2.5	4.7	0.7	6.3	0.0	0.5	57.3	0.1	1.1	7.2	64.6	4.3	88.6
85	8-32-6	0.0	2.3	0.2	0.9	27.3	1.6	3.4	0.7	10.9	0.0	0.4	51.6	0.0	0.7	5.0	63.2	4.2	90.8
86	8-32-8	0.0	2.8	0.4	0.9	25.9	2.8	4.6	0.8	7.5	0.0	0.6	52.5	0.2	1.0	7.4	61.0	5.3	87.3
87	8-32-9	0.0	2.6	0.3	1.0	25.7	2.3	5.0	0.8	7.9	0.0	0.5	52.9	0.1	1.0	7.3	61.7	5.1	87.6
88	8-32-10	0.0	2.3	0.2	1.1	27.9	1.6	4.1	0.8	13.5	0.0	0.3	47.5	0.0	0.6	5.7	61.6	4.5	89.7
89	8-85-1	0.0	2.3	0.3	0.9	28.5	1.1	3.3	0.7	11.8	0.0	0.3	50.1	0.0	0.8	4.4	62.7	4.2	91.4
90	8-85-3	0.0	2.5	0.5	0.5	25.1	1.9	5.1	0.5	6.5	0.0	0.4	56.1	0.0	1.0	7.0	63.6	3.8	89.2
91	8-85-4	0.0	2.4	0.4	0.5	25.5	1.8	4.8	0.5	7.2	0.0	0.4	55.7	0.0	0.9	6.5	63.8	3.8	89.7
92	8-85-5	0.0	2.4	0.3	0.6	27.7	1.4	3.6	0.6	9.3	0.0	0.4	52.9	0.0	0.9	5.0	63.0	4.0	91.0
93	8-85-6	0.0	3.3	0.5	1.1	29.4	1.6	3.6	0.9	9.6	0.0	0.6	48.4	0.1	0.8	5.2	58.9	6.0	88.8
94	8-85-8	0.0	2.3	0.4	0.5	25.3	1.9	5.0	0.6	7.1	0.0	0.3	55.3	0.1	1.1	7.0	63.5	3.8	89.2
95	8-85-9	0.4	2.7	0.4	0.8	29.2	1.6	5.6	0.7	10.2	0.0	0.4	47.3	0.1	0.7	7.2	58.2	5.0	87.8
96	8-85-10	0.0	2.4	0.3	0.7	28.5	1.3	3.6	0.6	10.5	0.0	0.3	50.9	0.0	0.7	4.9	62.2	4.1	91.0
97	Maplus-1	0.0	4.2	0.3	0.9	11.5	13.7	9.9	0.7	10.9	0.6	0.5	46.4	0.0	0.4	24.2	57.7	6.3	69.5
98	Maplus-2	0.0	4.3	0.3	0.9	11.1	14.2	10.1	0.7	10.5	0.6	0.5	46.3	0.1	0.5	24.9	57.3	6.5	68.7
99	Maplus-3	0.0	4.9	0.4	0.7	10.2	17.3	9.9	0.5	7.2	0.7	0.5	46.8	0.1	0.9	27.8	54.9	6.7	65.4
100	Maplus-4	0.2	4.1	0.2	0.8	14.7	16.2	9.0	0.7	9.0	0.5	0.5	42.7	0.2	1.0	25.7	52.8	6.6	67.6
101	Maplus-5	0.0	3.9	0.2	0.8	11.1	16.3	9.0	0.6	8.4	0.6	0.6	47.5	0.2	0.9	25.8	56.9	6.0	68.2

Appendix VIII Continued

S. No.	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0	24:1	PUFA	VLCFA	SAFA	MUFA
102	Maplus-6	0.0	3.5	0.2	0.7	11.9	15.6	8.3	0.6	8.1	0.5	0.6	49.1	0.2	0.8	24.4	57.9	5.6	70.0
103	Maplus-7	0.0	3.8	0.2	0.7	11.0	16.6	9.1	0.5	7.1	0.5	0.6	48.9	0.1	0.9	26.2	56.9	5.6	68.1
104	Maplus-9	0.0	4.2	0.2	1.0	15.3	16.2	7.3	0.7	9.5	0.5	0.6	43.5	0.2	0.8	24.0	53.8	6.7	69.3
105	Maplus-10	0.0	5.1	0.3	0.8	13.1	17.9	7.4	0.7	8.6	0.6	0.6	43.8	0.2	0.8	25.9	53.1	7.5	66.6
106	NY7-1	0.0	3.1	0.2	0.8	12.8	13.4	10.2	0.6	7.3	0.4	0.7	49.1	0.3	0.9	24.1	57.4	5.5	70.4
107	NY7-2	0.0	3.3	0.3	0.8	13.1	14.0	10.2	0.6	7.2	0.4	0.7	48.1	0.2	1.1	24.5	56.4	5.7	69.8
108	Cabriolet-1	0.0	4.5	0.3	1.1	72.5	8.6	10.3	0.7	1.5	0.0	0.5	0.0	0.0	0.0	18.8	1.5	6.9	74.3
109	Cabriolet-2	0.0	4.3	0.3	1.0	74.0	8.0	9.8	0.6	1.6	0.0	0.3	0.1	0.0	0.0	17.8	1.7	6.2	76.1
110	K0472-1	0.0	3.7	0.4	1.2	84.0	2.0	4.6	0.7	2.0	0.0	0.6	0.2	0.5	0.0	6.6	2.2	6.7	86.7
111	K0472-2	0.0	3.4	0.3	1.2	83.4	2.3	5.1	0.8	2.1	0.0	0.7	0.1	0.5	0.0	7.4	2.2	6.6	86.0

PUFA=18:2+18:3+20:2; VLCFA=20:1+22:1+24:1; SAFA=14:0+16:0+18:0+20:0+22:0+24:0; MUFA = 16:1+18:1+20:1+22:1+24:1

IX. Coding Description of the HELP and other Lines

The line code of the F_4 progeny of the HELP lines is made up of 3 digits separated by hyphens, i.e., 'F_2-F_3-F_4'. To replace the lengthy cross names, one or two digit code was used as described in the Table below. The first digit describes the F_2 progeny and it ranges from 1 to 13. The second letter corresponds to the F_3 progeny and third letter corresponds to the F_4 progeny. For example, line 5-10-1 is the 1st plant (F_4) of the 10th line (F_3) of the cross 'Maplus x P19-4A' (F_2); line 2-91-2 is the 2nd plant (F_4) of the 91st line (F_3) of the cross 'Maplus x K0472-4A' (F_2); line 4-87-3 is the 3rd plant (F_4) of the 87th line (F_3) of the cross 'Maplus x K0047-3E' (F_2) and so on. Letters or digits after the mutants such as M0830-1D means 'D' pod (pods were named 'A to Z' from each plant) of the 1st replicate (4 replicates were used for each mutant) of the mutant M0830. P19-4A means 'A' pod of the 4th plant of the line P19 (Chapter 4). For the F_5 progeny, new number can be added at end separated by a hyphen.

Code (describes F_2 progeny)	**Original Cross**
1	Maplus x M0830-1D
2	Maplus x K0472-4A
3	Maplus x M2444-1C
4	Maplus x K0047-3E
5	Maplus x P19-4A
6	Maplus x P19-12B
7	Maplus x P20-2C
8	Maplus x P20-6D
10*	Maplus x M0830-3E
11*	Maplus x K0472-4E
12*	Maplus x M2444-2A
13*	Maplus x K0047-1E

*These were grown later than the previous batch for a summer student. So, a different number was given.

9. Glossary

9.1 Abbreviations

4HG	4-Hydroxyglucobrassicin
4MG	4-methoxyglucobrassicin
ACCase	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ACS	Acyl-CoA synthetase
A_g	Peak area for the desulfoglucosinolate
A_s	Peak area for the internal standard
AT	Associative transcriptomics
ANOVA	Analysis of variance
BDC	Biorenewables Development Centre
BHT	Butylated hydroxy toluene
Bn-LPAAT	*Brassica napus*- lysophosphatidic acid acyltransferase
C16	Fatty acid with a chain length of 16 carbons
C18	Fatty acid with a chain length of 18 carbons
$CaCl_2$	Calcium chloride
CDS	cDNA model
C_L	Carbon length
CMLM	Compressed mixed linear model
CoA	Co-enzyme A
CPT	CDP choline: DAG choline phosphotransferase;
CTAB	Cetyl trimethyl ammonium bromide
D	Number of double bonds (if any)
DAG	Diacylglycerol
DAGAT, DGAT	1,2-diacylglycerol acyltransferase
DEFRA	Department for environment, food and rural affairs
DH	Doubled haploid
DNA	Deoxyribo nucleic acid
EA	Erucic acid
EFSA	European food safety authority
EMS	Ethyl methane sulphonate
ER	Endoplasmic reticulum
EU	European union
FAD	Fatty acid desaturases
FAE	Fatty acid elongases
FAMEs	Fatty acid methyl esters
FAO	Food and agriculture organization of the Unites Nations
FAS	Fatty acid synthase
FAT	Fatty ACP thioesterases
FID	Flame ionization detector
G3P	Glycerol-3-phosphate

GAL	Glucoalyssin
GAPIT	Genome association and prediction integrated tool
GBN	Glucobrassicanapin
GBS	Glucobrassicin
GC	Gas chromatography
GC-FID	Gas chromatography- flame ionization detector
gDNA	Genomic DNA
GEM	Gene expression marker
GIB	Glucoiberin
GLS	Glucosinolates
GLCAN	Gamma (ɣ)-linolenic acid canola
GM	Genetically modified
GNA	Gluconapin
GNL	Gluconapoleiferin
GPAT	Glycerol-3-phosphate acyl transferase
GST	Gluconasturtin
GWAS	Genome wide association study
H_2	Hydrogen
HEAR	High erucic acid rapeseed
HELP	High erucic acid rapeseed in low polyunsaturated fatty acid background (or high erucic and low polyunsaturates)
HERO	High erucic rapeseed oil
HOAR	High oleic acid rapeseed
HOCAN	High oleic canola oil
HOLL	High oleic acid and low linolenic acid rapeseed
HOLP	High oleic acid and low polyunsaturated fatty acid rapeseed
HPLC	High performance liquid chromatography
IPA	Isopropyl alcohol
ISTD	Internal standard
KAS	Keto-acyl synthase
KCS	3-ketoacyl-CoA synthase or β-ketoacyl-CoA synthase
K_g	Response factor of the desulfoglucosinolate relative to internal standard
L.	Linnaeus
LB	Left border of T-DNA
LD	Linkage disequilibrium
Ld-LPAAT	*Limanthes douglasii*- lysophosphatidic acid acyltransferase
LEAR	Low erucic acid rapeseed
LLCAN	Low linolenic canola oil
LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
LTCAN	Lauric acid canola
MAG	Mono acyl glycerol
Map	Maplus
MAS	Marker assisted selection
MCA	Malonyl-CoA
Mr	Molecular weight
mRNA	Messenger RNA

m	Mass
mt	Mutant
MUFA(s)	Mono unsaturated fatty acid(s)
N	Amount of internal standard (in micromoles)
N_2	Nitrogen gas
NaBr	Sodium bromide
NASC	Nottingham Arabidopsis stock centre
Neo	Neoglucobrassicin
NIRS	Near-infrared spectroscopy
NY7	Ningyou 7
ORF	Open reading frame
OSR	Oilseed rape
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PCA	Principal component analysis
PDAT	phospholipid: DAG acyltransferase
PCR	Polymerase chain reaction
PDCT	PC: DAG choline phosphotransferase
PLC	Phospholipase C
PLD	Phospholipase D
PRO	Progoitrin
PUFA(s)	Poly unsaturated fatty acid(s)
PSIKO	Population structure inference using kernel-PCA and optimisation
PTFE	Polytetrafluoroethylene
QTL	Quantitative trait loci
RB	Right border of t-DNA
RED	Renewable Energy Directive
RIPR	Renewable industrial products from rapeseed
RNA	Ribo nucleic acid
RNAi	RNA interference
RPKM	Reads per kb per million aligned reads
S. No.	Serial number
SAFA(s)	Saturated fatty acid(s)
sn	Stereospecific numbering
SNP	Single nucleotide polymorphism
TAG	Triacylglycerol
TAIR	The Arabidopsis information resource
TD-NMR	Time domain nuclear magnetic resonance
T-DNA	Transfer-DNA
TILLING	Targeting induced local lesions in genomes
TLC	Thin layer chromatography
TNDH	Tapidor Ningyou 7 doubled haploid population
UK	United kingdom
UV	Ultra Violet
Var.	Variety
VLCFA(s)	Very long chain fatty acid(s)
WGT	Whole genome triplication
WOSR	Winter oilseed rape

Wt	Wild type
X	Position of the carbon with a double bond

9.2 Greek Symbols

α	Alpha
β	Beta
ɤ	Gamma
ω	Omega
µ	Mu (micro)

9.3 Measurement Units

%	Percentage
°C	Degree Celsius (temperature)
1x	1 time
2x	2 times
bp	Base pairs
cm	Centimetres
g	Gram
ha	Hectare
Hz	Hertz
kb	Kilo bases
m	Metre
M	Molar (Molarity units)
mg	Milligrams
ml	Millilitres
mm	Millimetres
mM	Millimolar
N	Normal (Normality units)
ng	Nano grams
nmol	Nano mols
pH	Logarithmic scale used to measure acidity/basicity
psi	Pounds per square inch
rcl	Relative centrifugal force
rpm	Rotations per minute
µg	Micrograms
µl	Micro litres
µM	Micro moles
µmol	Micro mole
w/v	Weight by volume
v/v	Volume by volume

9.4 DNA Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

9.5 Common Names of the Fatty Acids

C9:0	Pelargonic acid
C12:0	Lauric acid
C13:0	Brassylic acid
C14:0	Myristic acid
C15:0	Penatdecanoic acid
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	α-Linolenic acid and ɣ-Linolenic acid
C20:0	Arachidic acid
C20:1	Eicosenoic acid or Gondoic acid
C20:2	Eicosadienoic acid
C22:0	Behenic acid
C22:1	Erucic acid
C22:2	Docosadienoic acid
C24:0	Lignoceric acid
C24:1	Nervonic acid

9.6 IUPAC Ambiguity codes for DNA Nucleotides

M	A or C
R	A or G
W	A or T
S	C or G
Y	C or T
K	G or T
V	A or C or G
H	A or C or T
D	A or G or T
B	C or G or T
N	G or A or T or C

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