Discovery of Quantitative Trait Loci Associated with Erucic Acid Content in *Brassica napus* L. Seed

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ABSTRACT

Brassica napus L. is a crucial oilseed crop and holds significant economic value for Canada. There are several seed quality types of *B. napus,* including canola with low erucic acid (used for edible purposes), as well as genotypes with high erucic acid, mainly used for industrial applications. This research was directed at increasing erucic acid levels in high erucic acid rapeseed (HEAR). The goal was to identify and characterize minor-effect quantitative trait loci (QTL) related to erucic acid content in two distinct doubled haploid (DH) populations, CBLD2 and CBER2. Both populations utilized two HEAR parents to fix the known *FAE1* genes and focus on identifying minor QTL impacting erucic acid content. The populations were phenotype in two locations over two years. Erucic acid content was evaluated using gas chromatography and the DH populations were genotyped using genotyping-by-sequencing (GBS). QTL were detected on various chromosomes, illustrating the polygenic regulation of erucic acid. Specifically, significant QTL were discovered on chromosomes A04, A07, and C08 in CBLD2, explaining up to 27 %, 21 %, and 22 % of phenotypic variation, respectively. In the CBER2 population, stable QTL on chromosomes A01, A02, and A05 accounted for 7-9 %, 7-9 %, and 11-18 % of variation, respectively. Analysis also revealed a positive correlation between erucic acid and oil content, and no significant correlation between erucic acid and other agronomic traits. This suggests erucic acid content is regulated independently from other agronomic characteristics. These insights into the genetic foundation of erucic acid content in *B. napus* are crucial for marker-assisted breeding, aiming to enhance erucic acid levels for improved agricultural and industrial uses.

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1. General Introduction

Brassica napus, known as canola or oilseed rape, ranks as the world's second-largest oilseed crop (FAO, 2023; Statista, 2024) and holds significant economic importance for Canadian agriculture (Hwang et al., 2016; Malabat et al., 2003). By the end of 2022, Canada's export of *B. napus* seed, oil, and meal reached a total of $14.4 billion CAD (Statistics Canada; COPA, 2023). Primarily cultivated for producing edible seed oil, *B. napus* also harbors erucic acid, a monounsaturated omega-9 fatty acid, drawing attention for its unique industrial applications (Leonard, 1992; Li et al., 2012; Rajcan et al., 1999). Erucic acid is utilized as a high performance lubricant, plasticizer, and raw material in the manufacturing of surfactants and emulsifiers due to its high temperature resistance, hydrophobicity and water resistance (Guan et al., 2014; Hannoufa et al., 2014; Jarvis et al., 2021; Wu et al., 2000; Wang et al., 2022). Erucic acid is a renewable oil and plays a pivotal role in the production of hundreds of industrial products (Nath et al., 2009). This vast utilization has led to the development of high erucic acid rapeseed (HEAR) cultivars, specifically engineered to elevate the erucic acid content beyond 45 % (Przybylski & Eskin, 2011; Wang et al., 2022). Given the increasing industrial need for erucic acid, exploring mechanisms to further enhance erucic acid content has become a current research topic (Wang et al., 2022).

The production of erucic acid in *B. napus* is significantly influenced by a complex interplay of genetic and environmental factors (Shi et al., 2003). Environmental

conditions such as climate, soil type, crop management like planting density, fertilization, and weather patterns interact with the plant's genetics to affect the levels of erucic acid produced (Davoudi et al., 2019; Khan et al., 2018; Sanyal & Linder, 2013; Uğur et al., 2010; Yaniv et al., 1995). At the genetic level, the synthesis of erucic acid involves interactions among various fatty acids and competition for substrates and enzymes within the plant (Mietkiewska et al., 2004). Previous research has revealed the pivotal role of the fatty acid elongase 1 (*FAE1*) gene in modulating erucic acid content (Mietkiewska et al., 2007; Nath et al., 2009; Peng et al., 2010; Shi et al., 2017; Yan et al., 2015). Fatty acid elongase 1 is found in high erucic acid rapeseed genotypes and is essential to produce erucic acid. The two functional copies of *FAE1* are located on chromosomes A08 and C03 (Jarvis et al., 2021; Liu et al., 2022; Puyaubert et al., 2005; Roscoe et al., 2001). Other related genes such as 3-Ketoacyl-Acp synthase 2 (*KAS2*), 3-Ketoacyl-Acp synthase 3 (*KAS3*), and fatty acid desaturases 2 (*FAD2*) also influence the content of erucic acid in seed. These latter genes play a minor role in the complex network of biochemical pathways that regulate erucic acid synthesis and modification in plants (Dar et al., 2017; Peng et al., 2010; Wang et al., 2013).

The demand for erucic acid underscores its economic and industrial value, driving research to not only optimize its content in *B. napus* but also to understand the genetic mechanisms underlying its production (Yan et al., 2015). Studies utilizing quantitative trait locus (QTL) analysis and genome-wide association studies (GWAS) have identified numerous loci and single nucleotide polymorphisms (SNPs) associated with variation in erucic acid content. In addition to the previously explored *FAE1* and *FAD2*, QTL affecting

erucic acid production have also been discovered on other chromosomes (Javed et al., 2016; Qiu et al., 2006; Zhao et al., 2008; Zhao et al., 2019; Zhu et al., 2019) along with SNP markers related to erucic acid content (Guan et al., 2019; Javed et al., 2016; Tang et al., 2019; Yusuf et al., 2022; Zhao et al., 2019). These findings further elucidate the genetic factors contributing to the variability in erucic acid level in *B. napus*, offering insight for targeted breeding and genetic modification efforts.

Previous research has largely concentrated on crossing canola cultivars or crosses between HEAR and canola parental genotypes, with little focus on HEAR × HEAR populations. Cuthbert & McVetty (2011) hybridization experiments with HEAR cultivar demonstrated that only one out of 45 HEAR F_1 hybrids exhibited heterosis for high erucic acid content. Given these results, it is essential to continue more in-depth studies on high erucic acid genotypes.

The objective of this research was to perform QTL analysis on two doubled haploid populations produced through HEAR x HEAR parental crosses, identifying minor QTL related to the content of erucic acid in *B. napus* seed. The discovery of these QTL regions will contribute to future breeding efforts aimed at enhancing erucic acid, thereby improving the erucic acid produced per unit of land.

2. Literature Review

2.1. Background

2.1.1. Introduction of *Brassica* Genus

One of the most widely cultivated plant families, the *Cruciferae* or *Brassicaceae*, provides a variety of nutrients and phytochemicals for human nutritional needs (Gupta, 2013). There are 3709 recognized species in the family's 338 genera (Warwick et al., 2009). The majority of genera include *Draba* (Jordon et al., 2010), *Erysimum* (Moazzeni et al., 2014), *Lepidium* (Johnson, 1986), *Cardamine* (Heenan, 2017) and *Alyssum* (Küpper et al., 2001). The *Brassicaceae* are distributed in all but a few regions of the earth, probably originating in the 'Irano-Turanian' region, and have the most significant number of genera and species (Schmidt & Bancroft, 2011). The Mediterranean region is next in terms of species abundance, with about 630 species (290 of which are endemic) in 113 genera (Schmidt & Bancroft, 2011). The *Brassicaceae* tribe of the crucifer family is where the *Brassica* genus is found (Warwick et al., 2009). Thirty-nine species in the *Brassica* genus are cultivated as a condiments, animal feed, oil crops and vegetables (Warwick et al., 2009). *Brassica* species, including *Brassica napus* L., *Brassica rapa* L., *Brassica juncea* L., *and Brassica. oleracea* L. are widely grown for economic importance (Rakow, 2004; Schmidt & Bancroft, 2011). The *Brassicaceae* family, with its extensive range of genera and species, including the economically important *Brassica* genus, provides diverse functions for humans and is globally distributed (Rakow, 2004).

Brassica rapa, grown for its oil, is thought to have emerged close to the Mediterranean Coast (Larkan et al., 2014; Schmidt & Bancroft, 2011). Around the 1970s, *B. rapa* was predominantly planted in Western Canada; however, by the 1990s, only 15 % to 20 % of those hectares of land in Canada were still home to *B. rapa*. *Brassica rapa* typically needs fewer frost-free days to complete its life cycle than other *Brassica* species and is also more tolerant to spring frosts (Downey & Röbbelen, 1989).

Brassica oleracea is extensively found across Europe, including Greece, Turkey, Italy, and other countries (Maggioni et al., 2020). As an important agronomic species, *B. oleracea* encompasses a wide range of crop types (Golicz et al., 2016; Liu et al., 2014). In Canada, *B. oleracea* is predominantly cultivated as a vegetable crop for its edible leaves. The major *B. oleracea* crops include broccoli (*B. oleracea* var. *italica*), cauliflower (*B. oleracea* var. *botrytis*), Brussels sprouts (*B. oleracea* var. *gemmifera*), regular cabbage (*B. oleracea* var *capitate*), and kale (*B. oleracea* var. *sabellica*). In 2021, these crop types yielded a combined total production of 217,936 metric tonnes, generating export revenues of 114 million dollars (Statistics Canada, 2021). Compared to other species in the *Brassicaceae* family, *B. oleracea* maintains robust growth in low temperatures and can briefly withstand environments as cold as -10 °C, in addition to exhibiting excellent storage tolerance (Song et al., 2020).

Early 20th-century cytogeneticists used interspecific crosses and meiotic analyses to clarify the chromosomal relationships between the genomes of the diploid species *B. rapa* (genome AA, 2n = 20), *B. nigra* (genome BB, 2n = 16), and *B. oleracea* (genome CC, 2n = 18), as well as their naturally occurring amphidiploids *Brassica carinata* A. (genome

AABB, 2n = 34), *B. napus* (genome AACC, 2n = 38) and *B. juncea* (genome BBCC, 2n = 36) (Morinaga, 1934; Plieske & Struss, 2001) (**Figure 2.1**).

2.1.1.1. Introduction and Development of *B. napus*

The origin of *B. napus* is unclear, and some academics assert that it came from Mediterranean Europe, while others argue that it has multiple origins (Afzal et al., 2008; Rakow, 2004). The origin of *B. napus* probably arose naturally from an interspecific cross between *B. oleracea* and *B. rapa* about 1000 years ago (Morinaga, 1934) **(Figure 2.1)**. Canola (*Brassica napus*) is among the most significant oil crops, and it is grown almost everywhere in the world (Leff et al., 2004;Friedt et al., 2018). Canola is defined as "*seed having less than 2 % erucic acid and less than 30 micromoles* of anyone or any mix*ture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy- 4-pentenyl glucosinolate per gram of air-dry, oil-free solid" (*Canola Council of Canada, 2022*).* The University of Manitoba started the *Brassica* breeding program in the early 1950s and successfully produced the first canola cultivar named Tower in 1974 (Daun et al., 2011; Canola Council of Canada, 2022). Statistics Canada showed that the amount of canola seeded in Canada increased from 8.41 million hectares in 2020 to 9.09 million in 2021 before falling to 8.66 million hectares in 2022 (Statistics Canada, 2022). Despite these fluctuations, canola remains the second-largest crop grown in the country after wheat (*Triticum aestivum* L.) (Statistics Canada, 2022). In 2022, Canada produced. 18.2 million tonne of canola, of which Manitoba produced 2.9 million tonnes (15.9 %). While the primary production regions in Saskatchewan and Alberta accounted for 52 %

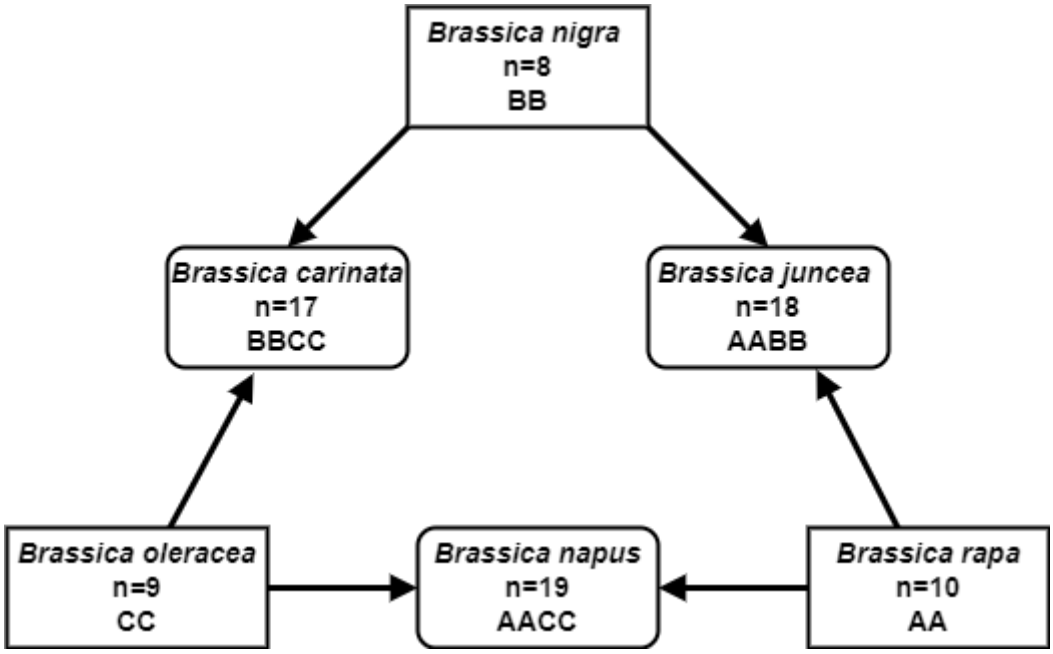


Figure 2.1 Genetic interrelationships among six cultivated *Brassica* species illustrated in the 'Triangle of U' Framework (adapted from Nagaharu, 1935; Kim et al., 2018)

and 31 % of the yield, respectively (Statistics Canada, 2022; Canola Council of Canada, 2023). This production results in a 29.9 billion Canadian dollar economic benefit (Canola Council of Canada, 2022).

2.2. Introduction of Erucic Acid and High Erucic Acid Rapeseed

Erucic acid (EA; C22:1 ω-9; C22:1 Δ13C; cis-13-docosanol acid), a long-chain monounsaturated fatty acid, is known for its diverse industrial applications (Wang et al., 2022). This acid is predominantly found in the seed of *Brassicaceae* family plants and in the liver oils of deep-sea fish like salmon, trout, and cod (Ackman, 2007). Erucic acid is synthesized during the seed formation stage, characterized by the accumulation of fatty acids of various chain lengths and saturation levels (Slabas et al., 2001). Erucic acid serves multiple applications and holds potential for new future uses (Han et al., 2001; Heath & Earle, 1995). Erucic acid is considered a versatile, clean, and environmentally friendly fatty acid, and it can be used as a renewable raw material to replace petroleum (Bao et al., 1998; Knutsen et al., 2016; Wu et al., 2000). Erucic acid, due to its hydrophobic properties and excellent lubricating capabilities, serves as a chemical intermediate across various industries. For example, through hydrogenation reactions, erucic acid can be transformed into saturated straight-chain fatty acids, which are utilized as stabilizers in the plastics, pharmaceutical, and food industries (Bährle-Rapp, 2007). When oxidized, erucic acid yields tridecanedioic acid and nonanoic acid, which are raw materials for synthetic nylon production and can also be used to create fragrances and plasticizers resistant to low temperatures and light exposure (Wang et al., 2022). By-products of erucic acid, such as

erucic acid amide, are also employed as plasticizers, anti-adhesive agents, waterproofing agents, and lubricants (Wu et al., 2007; Taylor et al., 2011; Leonard, 1992). High-erucic acid *B. napus* oil, through alkali-catalyzed transesterification, can be processed into fuel for oil extraction machines (Qi & Wang, 2009; Mcvetty & Duncan, 2015). In 2022, the global demand for erucic acid was 260 thousand tonnes, with an annual increase of 3.81% expected until it reaches 322 thousand tonnes by 2028 (TechSci Research, 2023). Beyond its applications in the polymer industry, erucic acid and its derivatives find utility across a diverse range of sectors, including but not limited to detergents, photography, cosmetics, pharmaceuticals, inks, paper, textiles, lubricants, food products and fuels (Aukema & Campbell, 2011).

High erucic acid rapeseed (HEAR) is a specialty rapeseed that is defined by its high erucic acid content, above 45 % (McVetty & Scarth, 2002; Nath et al., 2009; Taylor et al., 1994). The level of erucic acid in the seed of high erucic acid rapeseed is approximately 10-15 % higher than its levels in unmodified rapeseed genotypes (Friedt & Lühs, 1998; Nath et al., 2018; Taylor et al., 1994). The oil market for high erucic acid content is worth more than USD 100 million annually (Jadhav et al., 2005). The USA, Europe, and Japan lead the importation of high erucic acid oil (Bhardwaj & Hamama, 2000).

The first *B. rapa* HEAR cultivar was developed by Agriculture and Agri-Food Canada (AAFC) and named R-500 (Canola Council of Canada, 2023). The first *B napus* HEAR cultivar, known as the Reston, was registered by the University of Manitoba in 1982. Reston contains 40 to 48 percent erucic acid and has medium levels of glucosinolates. Since 1982, numerous HEAR cultivars have been developed. The cultivar HR696, was

tested by the Western Canadian Co-operative Canola/Rapeseed HEAR Contract Registration in 1997 and 1998, respectively. HR696 reached a mean erucic acid content of 55.2 % from dry seed extraction, averaging 26 % higher seed yield, 25 g kg^{-1} higher seed oil and 21 g kg^{-1} higher crude meal protein compared to Reston. This cultivar was eventually named MillenniUM03 summer rape (McVetty et al., 2000). Developed by the Department of Plant Sciences at the University of Manitoba, it was issued a certificate of restricted registration in November 1999 by the Canadian Food Inspection Agency (McVetty et al., 2000). Subsequently, with the most notable being the Red River and HYHEAR series developed by the University of Manitoba (McVetty et al., 2014). The Red River 1861 cultivar, which is Roundup Ready™, exhibited 52.6 % erucic acid content in its seed oil and yielded 19 % more than the previously developed HEAR cultivar MillenniUM03, demonstrating tolerance to the broad-spectrum glyphosate-based herbicide Roundup® (McVetty et al., 2012). HYHEAR 1 summer rape was the world's first hybrid Roundup Ready™ high erucic acid, low glucosinolate cultivar. HYHEAR 1 was characterized by erucic acid content of 52.2 % and low glucosinolate content in the seed meal (Duncan et al., 2017). HYHEAR 1 has a 33 % higher seed yield and produces more oil than MillenniUM03 summer rape. HYHEAR 2 Roundup Ready® summer rape (Duncan et al., 2016) and HYHEAR 3 Roundup Ready® summer rape (Duncan et al., 2017) were subsequently developed. The HYHEAR 2 hybrid cultivar has an erucic acid seed oil content of 51.6 %, while the HYHEAR 3 cultivar has an erucic acid seed oil content of 50.9 % (Duncan et al., 2016). The two cultivars also have higher seed yield than the other HEAR cultivars. Both cultivars received restricted registration certificates in 2015 and 2016

(Duncan et al., 2016; McVetty et al., 2014). The cultivar Evolve represents a significant milestone as the world's first Ogura INRA CMS hybrid summer *B. napus* cultivar. It is distinguished by its high erucic acid content (52.3%) and low glucosinolate levels in the seed meal. Utilizing the Ogura Institute National de la Recherche Agronomique (INRA), cytoplasmic male sterility (CMS) system, Evolve has shown exceptional agronomic performance in field trials, outperforming the Red River 1861 cultivar in terms of seed yield and oil content (Duncan et al., 2021; Aukema & Campbell, 2011).

High erucic acid rapeseed is of increasing interest to more and more countries to meet their industrial demand for erucic acid (Wu et al., 2008). Today, HEAR breeding focuses on developing HEAR hybrids for commercial production in Western Canada. Ultimately, increasing the erucic acid content improves the unit production per hectare (Wang et al., 2022). From an industrial point of view, the higher the erucic acid content the more economically efficient the extraction process becomes (Li et al., 2012). Reaching and exceeding 66 % erucic acid will reduce the cost of purification and make it more widely accepted for industrial processes (Li et al., 2012). The commercial value of high erucic acid oil demands further research focused on HEAR agronomic and seed quality developments (Wang et al., 2022).

Brassica napus is an ideal species to meet today's erucic acid requirements (Raboanatahiry et al., 2021). High erucic acid rapeseed cultivars have an inherent advantage as a large-scale crop adapted to a wide range of climates and cultivated on a large scale in many countries worldwide (Leff et al., 2004; Friedit et al., 2018). High erucic acid rapeseed has strong self-compatibility and a high selfing rate to produce

economically viable yield (Wang et al., 2022). High erucic acid rapeseed can be rotated with other crops, ideal for 4-year crop rotations in Canada, which is very helpful in increasing yield and reducing pests and diseases (Norton et al., 1999). Increasing HEAR yield is the most cost-effective and suitable method to meet the demand for erucic acid.

2.3. Biosynthesis and Assembly of Erucic Acid in *B. napus*

Erucic acid is synthesized from sucrose (Gooch, 2007). Sucrose is hydrolyzed into glucose and fructose, which are then metabolized through glycolysis to produce pyruvate. This pyruvate is subsequently converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) (Chandel, 2021). As the precursor of fatty acid synthesis, acetyl-CoA is first catalyzed by acetyl-CoA carboxylase (ACCase) to form malonyl-CoA (Gooch, 2007; Li-beisson et al., 2010). The fatty acid synthesis complex (FAS) then utilizes acetyl-CoA and malonyl-CoA as substrates (Bonaventure et al., 2003). During this process, the substrate undergoes numerous condensation reactions, which include reduction, dehydration, and re-reduction steps. These reactions are catalyzed by enzymes such as 3-ketoacyl-ACP synthase III (KAS III), leading to the formation of C4:0-ACP. Subsequently, 3-ketoacyl-ACP synthase I (KAS I) extends the fatty acid chain by two carbons per cycle, repeatedly generating until C16:0-ACP (Bonaventure et al., 2003; Li-beisson et al., 2010; Salas & Ohlrogge, 2002). Extension of the generated C16:0-ACP to C18:0-ACP occurs by using 3-ketoacyl-ACP synthase II (KAS II). Subsequently, C18:0-ACP is desaturated to form C18:1-ACP with another product. The fatty acid synthesis complex releases the final

saturation products into the cytoplasm as free fatty acid acyl-ACP, catalyzed by thioesterases (Bonaventure et al., 2003; Li-beisson et al., 2010; Salas & Ohlrogge, 2002). The free fatty acid is activated by long-chain acyl-CoA synthetase (LACS) to produce acyl-CoA, which is transported to the endoplasmic reticulum (ER), where the fatty acid chain is desaturated and extended (Salas & Ohlrogge, 2002; Tjellström et al., 2013) (**Figure 2.2)**.

Oleic acid (C18:1) is desaturated by fatty acid desaturase 2 (FAD2) to form linoleic acid (C18:2), and by fatty acid desaturase 3 (FAD3) to form linolenic acid (C18:3) (Okuley et al., 1994; Li et al., 2012). The fatty acid elongation enzyme complex found at the ER membrane is used to synthesize very long-chain fatty acids (VLFCA). Very long chain fatty acids range from 20 carbon chains to 26-carbon chains in length (Harwood, 1996; Harwood, 1980). Labeling Malonyl-CoA shows that Acyl-CoA produces VLCFA. Moreover, VLCFA form microsomal lipids in the presence of Acyl-CoA elongation activity, during which ATP is absorbed (ATP-dependent-elongation) (Harwood, 1980; Post-Beittenmiller, 1996; Domergue et al., 1999). During the Acyl-CoA elongation, it is successfully demonstrated that the addition of 2 carbon units to Stearoyl-CoA (18:0-CoA) resulted in the continuous production of Arachidonoyl-CoA (20:0-CoA), Behenoyl-CoA (22:0-CoA) and Lignoceroyl-CoA (24:0-CoA) (Lessire et al., 1985) **(Figure 2.2)**.

Four key enzymes: 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and trans-2,3-enoyl-CoA reductase (ECR) are used by the fatty acid elongation enzyme complex to successively add two carbon units to an expanding acyl chain (Haslam & Kunst, 2013; Huai et al., 2015). Four sequential

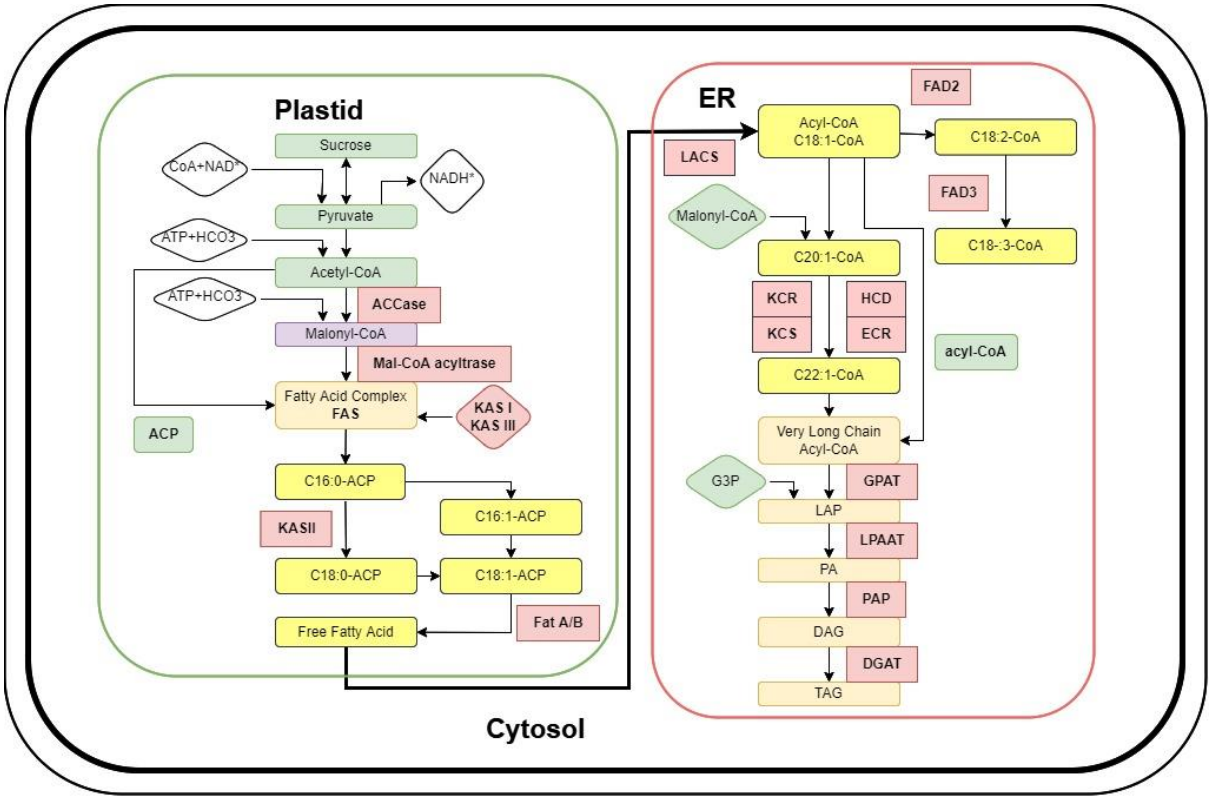


Figure 2.2 Biosynthesis process of erucic acid in *Brassica napus* (adapted from Wang et al., 2022)

ACCase, acetyl-CoA carboxylase.
KAS, 3-ketoacyl-ACP synthase.
LACS, long-chain acyl-CoA synthase.
FAD2, fatty acid desaturase 2.
FAD3, fatty acid desaturase 3.
LA, linoleic acid.
ALA, linolenic acid.
KCS, 3-ketoacyl-CoA synthase.
KCR, 3-ketoacyl-CoA reductase.
HCD, 3-hydroxyacyl-CoA dehydratase.
ECR, trans-2,3-enoyl-CoA reductase.
G3P, glycerol-3-phosphate.
GPAT, glycerol-3-phosphate acyltransferase.
LPA, lysophospholipids.
LPAAT, lysophosphatidic acid acyltransferase.
PA, phosphatidic acid; PAP, phosphatidic acid phosphorylase.
DAG, diacylglycerol.
DGAT, diacylglycerol acyltransferase.
TAG, triacylglycerol.

reactions are involved in each elongation cycle (Huai et al., 2015). A long-chain acyl-CoA and malonyl-CoA are combined by KCS, followed by KCR's reduction to 3-hydroxyacyl-CoA. Finally, HCD's dehydration of 3-hydroxyacyl-CoA is followed by KCR's reduction to elongated acyl-CoA (Cao et al., 2010; Fan et al., 2018; Harwood, 1996; Haslam & Kunst, 2013) (**Figure 2.3**). 3-ketoacyl-CoA synthase is a rate-limiting enzyme in the initial step of the fatty acid elongation reaction (Katavic et al., 2000). It is encoded by the fatty acid elongase 1 (*FAE1*) gene (Katavic et al., 2000). 3-ketoacyl-CoA is thus, a crucial regulatory target for modifying the erucic acid content via genetic engineering (Nath et al., 2009; Saini et al., 2019; Shi et al., 2015) (**Figure 2.3**).

In summary, the synthesis of erucic acid involves a complex biochemical pathway starting from sucrose, leading to the formation of various fatty acids through a series of enzymatic reactions, highlighting the potential for genetic modification to alter erucic acid content.

2.4. Prospects and Research Directions for Research on Erucic Acid

Promoting erucic acid for industrial use is a critical research goal (Jadhav et al., 2005). Traditional techniques can be used to raise erucic acid levels in *Brassicaceae* family plants (Guan et al., 2014). However, the theoretical maximum content of erucic acid in *B. napus* is 66.6 % (Grosdidier et al., 2017). Erucic acid is assembled and stored as triacylglycerols (Brough et al., 1996). Erucic acid is unable to reach the sn-2 position in triacylglycerols (Brough et al., 1996). Erucic acid can only bind to the ends of the glycerol backbone at

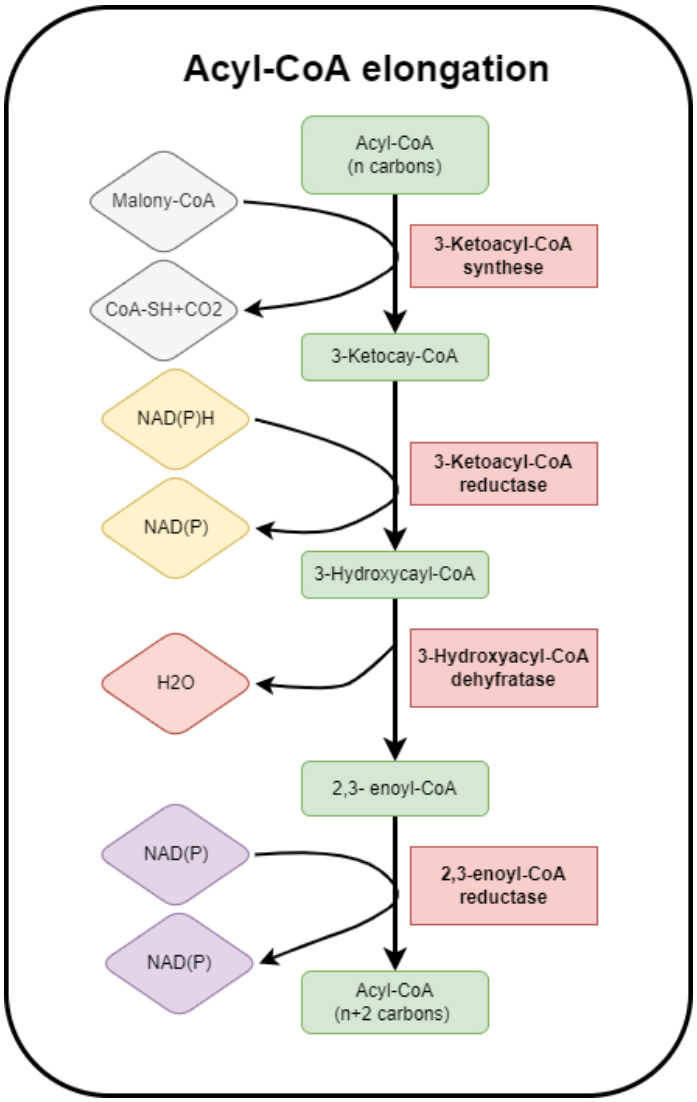


Figure 2.3 Enzymatic processes and intermediate reactions in Aryl-CoA Elongation (adapted from Wang et al., 2022).

positions sn-1 and sn-3 (Guan et al., 2014). Overcoming the inherent limitations of plants in esterifying erucic acid at the sn-2 position of the glycerol molecule presents a significant challenge (Guan et al., 2014; McVetty & Scarth, 2002). Genetic engineering can also be used to increase erucic acid content (Wang et al., 2022). Single-gene and multi-genes can be biotechnologically edited to increase the content of erucic acid (Wang et al., 2022).

Another potential area of study is research on novel high-erucic acid species and new genotypes. Erucic acid is found in many plants, but some plants are not suitable as fundamental resources for the production of industrial use of erucic acid (Jadhav et al., 2005; Knutsen et al., 2016). *Brassica carinata* L. has a relatively high erucic acid content of about 41 % of total seed oil content, but its low seed yield, long growth period, and poor nutritional quality have prevented it from becoming commercially produced on a large scale (Getinet et al., 1996; Jiang et al., 2007). *Thlaspi arvense* L. is another annual weed in the *Brassicaceae* family that can survive in extreme environments and high light intensity (Qi et al., 2018). It has an erucic acid content of 30 to 55 % (Qi et al., 2018) and has a propensity for seed pods to break after seed maturity, limiting its commercial production (Qi et al., 2018; Saghai-Maroof et al., 1984). *Crambe abyssinica* R.E.Fr., notable for its high erucic acid content (55-60%) in seeds, is widely distributed across various countries, reflecting its adaptability to diverse climatic and agricultural conditions (Carlsson, 2009). This plant is particularly appreciated for its quick reproductive cycle, high oil and protein content in seed, and potential for high yield (Qi et al., 2018; Saghai-Maroof et al., 1984). However, its global commercial utilization is limited, mainly due to

the high content of sulfur glycosides in the seed meal, which poses restrictions for its use as animal feed (Saghai-Maroof et al., 1984). The only plant found with more than 66 % erucic acid in total oil extracted from seed is *Tropaeolum majus* L., which belongs to the *Tropaeolaceae* family and has the highest erucic acid content outside of *Brassicaceae* species (Taylor et al., 2009). Although it has an erucic acid content of 75~80 %, the oil content in seed is only 6–10 %, making the collection and reproduction of this tiny seed challenging (Taylor et al., 2009).

Research on erucic acid focuses on the utilization of suitable plants for industrial production, with a growing emphasis on promoting erucic acid for industrial purposes. Traditional techniques have limitations in amplifying erucic acid content, but genetic engineering offers promising avenues for its increase through gene-editing and synthetic biology (Wang et al., 2022). Exploring novel high-erucic acid species and genotypes presents potential opportunities despite challenges related to seed yield and nutritional quality.

2.5. Erucic Acid Content in *B. napus*

2.5.1. Introduction of Gene Effect on Erucic Acid Production

Erucic acid production is influenced by the cytoplasm and is determined by the embryo's genotype (Li-beisson et al., 2010; Liu et al., 2022). Research has indicated that two pairs of major genes, specifically *FAE1*, along with multiple other genes, collaboratively determine seed erucic acid content (Alemayehu & Becker, 2001; Jourdren et al., 1996).

Their expression is also influenced by a range of modifiers or minor genes, as well as environmental conditions during growth (Wilmer et al., 1997). The implications of these genetic and environmental interactions in erucic acid synthesis will be discussed in the following sections.

2.5.2. Increasing Erucic Acid Content Through Increasing Extension Efficiency

The fatty acid elongase1 (*FAE1*) gene was found in *C. abyssinica, Tropaeolum majus* L.*, B. napus,* and *Arabidopsis thaliana* L. (Rossak et al., 2001). Cloning of the *FAE1* gene for expression in plants has demonstrated that *FAE1* can increase erucic acid content in various plants (Rossak et al., 2001; Wang et al., 2008). The expression characteristics of the *FAE1* gene is seed-specific (Roscoe et al., 2001). In HEAR cultivars of *B. napus*, two functional copies of the *FAE1* gene are located on chromosomes A08 and C03, each showing over 98 % sequence identity (Das et al., 2002). The fatty acid elongase complex is present in HEAR and cannot be detected in canola, primarily related to the lack of KCS enzyme activity (Puyaubert et al., 2005; Roscoe et al., 2001). 3-ketoacyl-CoA synthase (KCS) enzyme is the rate-limiting enzyme in the first biosynthesis step of the fatty acid extension reaction, and its level of activity results in different levels of erucic acid in different plants (Shi et al., 2015, 2017; Tian et al., 2011). Mietkiewska et al. (2007) demonstrated that transferring the *FAE1* gene from *C. abyssinica* into an *A. thaliana* mutant genotype, which was originally devoid of erucic acid, resulted in an increase of erucic acid content in *A. thaliana* seed to 12.8 % (Mietkiewska et al., 2007). Transferring

the *FAE1* gene from *T. majus* to a wild-type *A. thaliana* increased the erucic acid content from 2.1 to 9.6 % (Mietkiewska et al., 2004). At the same time, Li et al. (2012) conducted a similar experiment for *B. carinata* where they extracted the *FAE1* gene from *B. carinata* and transferred it to *C. abyssinica*, resulting in an increase in the seed content of erucic acid from 35.5 to 51.9 %. (Li et al., 2012). The *FAE1* gene extracted from various plant species can enhance erucic acid content, which indicates that the *FAE1* gene is a seed-specific expression enzyme and is involved in the process of fatty acid elongation.

In contrast to HEAR, the assessment of erucic acid-containing phenotypes in low erucic acid rapeseed (LEAR) is also contingent upon the identification of loss-of-function mutations in the *FAE1.1* and *FAE1.2* genes (Das et al., 2002; Wu et al., 2008). This situation was found that the early appearance of zero erucic acid *B. napus* cultivar called Oro. Oro was due to a sharp decrease in erucic acid content caused by a targeted mutation at base 845 in *FAE1* (Zeng & Cheng, 2014). Suppressing the expression of the *FAE1* gene through RNA interference (RNAi) has been shown to significantly decrease the erucic acid content in *B. napus*, reducing it from 40 % to below 3 % (Shi et al., 2015). The intron-spliced hairpin RNA (ihpRNA) technique can also be used for *B. napus* to inhibit erucic acid content from 40 to 0.36 % by restriction of *FAE1* gene expression (Tian et al., 2011). Utilizing RNAi and CRISPR/Cas9 technologies to inhibit the expression of *BnaFAE1* (two homologous copies of *FAE1*) and *BnaFAD2* genes (*B. napus* fatty acid desaturase 1 and 2) has been effective in reducing the erucic acid content in *B. napus*. Specifically, these methods have decreased the erucic acid levels from 42.25 % to 2.02 % in HEAR and from 0.87 % to virtually 0 % in LEAR (Jarvis et al., 2021; Liu et al., 2014; Shi et al., 2017).

Therefore, it is evident that inhibiting *FAE1* genetic expression is practical in reducing the amount of erucic acid.

The *FAE1* genes control the majority of erucic acid, but other modifier genes also play a role. When the multi-gene expression vector BnFAE (*Brassica napus* L. FAE) + LdLPAAT (*Limnanthes douglasii* R.Br. LdLPAAT) + CaFAD2-RNAi (*Crambe abyssinica* R.E.Fr. FAD2) was introduced into *C. abyssinica*, the erucic acid content in the transgenic offspring rose from the wild type with 60 % to 77 % (Li et al., 2012). These minor variations in erucic acid indicate that certain modifier genes can also have a slight influence on erucic acid levels.

2.5.3. Increasing Erucic Acid Content by Inhibiting Competing Substrates

A possible reason for restricting the erucic acid content is competition between other fatty acids during biosynthesis (Mietkiewska et al., 2004). Oleic acid (C18:1) is synthesized by lengthening the carbon chain through the action of the FAE1 enzyme. Meanwhile, the FAD2 enzyme also conducts the desaturation synthesis of polyunsaturated fatty acids (PUFAs) to produce C18:2 and C18:3 (Harwood, 1996; Harwood, 1980). The FAD2 enzyme is a crucial step in synthesizing PUFAs, so inhibition of *FAD2* gene expression is also critical to increasing erucic acid content (Li et al., 2012). Nevertheless, suppression of the *FAD2* gene increased the amount of oleic acid, which provided a sufficient substrate (oleic acid) for erucic acid synthesis and increased erucic acid content by a small amount (Jadhav et al., 2005; Qi et al., 2018). Jadhav et al. (2005) also found that with a co-suppressed *FAD2* gene, the erucic acid content in *B. carinata*

surged from an initial 12 % to 27 % by suppression of *FAD2*. Similarly, When the antisense *FAD2* gene was expressed in *B. carinata* to inhibit translation, the erucic acid content increased from 5 % to 19 % (Jadhav et al., 2005). In a control experiment using the high erucic acid content *B. napus* cultivar CY2, it was demonstrated suppression of the *FAD2* gene through RNAi led to an increase in erucic acid content by 42.3-45.6 % (Shi et al., 2017). These experiments demonstrated that limiting *FAD2* was effective in elevating erucic acid content in the seed.

2.5.4. Increasing the Erucic Acid Content by Improving Assembly Efficiency

By increasing assembly efficiency, the erucic acid content in seed can be raised (Maisonneuve et al., 2010; Maraschin et al., 2019). Optimizing diacylglycerol acyltransferase (DGAT) specificities can achieve an increase in erucic acid (Maraschin et al., 2019). In the biosynthesis of triacylglycerol, the enzyme DGAT, which acts as a rate-limiting factor, catalyzes the final step and uniquely utilizes acetyl-CoA as the acyl donor (Bates et al., 2009; Fan et al., 2018; Li-beisson et al., 2010). DGAT has two types, DGAT1 and DGAT2, and each type has four isomers. Each isomer drives and enhances the formation of a specific fatty acid (Fan et al., 2018). It was discovered that the BnDGAT2 isomer responded to 22:1-CoA differently in LEAR and HEAR seeds, being more active in HEAR genotypes and producing 6–14 times more triacylglycerol when using 22:1-CoA than in LEAR seeds (Demski et al., 2019).

2.5.5. Increasing Erucic Acid Content by Limiting or Improving Endogenous LPAAT

Reducing or limiting the content of endogenous lysophosphatidic acid acyltransferase (LPAAT) may increase the level of erucic acid in *Brassicaceae* plants (Qi et al., 2018). In *Brassicaceae*, it is not possible to use erucic acid as a ground substance to catalyze the integration of a fatty acid to the sn-2 position of triacylglycerol. However, LPAAT enzyme possesses a strong capability to transfer erucic acid to the sn-2 position of triacylglycerol (Li-beisson et al., 2010). The erucic acid content in *B. napus* seeds can be maximized by introducing the *Limnanthes douglasii* R.Br. lysophosphatidic acid acyltransferase (LdLPAAT), incorporating erucic acid on the sn-2 position (Lassner et al., 1995). Other research also demonstrated that the use of endogenous CaLPAAT2 in *C. abyssinica* to increase the ability of erucic acid to link to sn-2 (Jarvis et al., 2021; Qi et al., 2018). The results demonstrated that the erucic acid content of the CaLPAAT2-RNAi transgenic T1 seed oil was a mean of 64.5 % higher compared to the wild type, ranging from 63.1 % to 66.3 % (Jarvis et al., 2021; Qi et al., 2018).

The overexpression of *FAE1* and *LPAAT* held promise for increasing erucic acid at the sn-2 position of the glycerol backbone (Katavic et al., 2001; Nath et al., 2009). Nevertheless, empirical observations have shown that introducing both *FAE* and *LPAAT* into *B. napus* genotypes with already high levels of erucic acid resulted in an increase in erucic acid content from 54 % to 63 % (Nath et al., 2009). The limitation in erucic acid reaching beyond 66 % could be attributed to factors such as a scarcity of fatty acyl groups

available for chain elongation, competition in desaturation processes, or irreversible attachment to storage lipids (Katavic et al., 2001; Nath et al., 2009).

2.5.6. Increasing Erucic Acid Content Through Utilizing Unique Promoters

Utilizing unique promoters can aid in increasing erucic acid content (Saini et al., 2019). The level of gene transcription may also be constrained by the promoter and its collaborators, such as enhancers or silencers (Baumann et al., 2012). Thus, rather than only concentrating on boosting erucic acid content in seed, an approach could involve enhancing erucic acid levels through the use of specific promoters (Mietkiewska et al., 2004). One study showed that expressing *FAE1* in *A. thaliana* with the 35S promoter increased erucic acid levels from an initial 2.1 % to between 3.2 % and 4.0 % (Mietkiewska et al., 2004). By replacing the 35S promoter with the Napin promoter, *A. thaliana* was able to produce almost five times more erucic acid than the control group, increasing from 2.1 % to 9.6 % (Mietkiewska et al., 2004). These experiments indicate that different promoters can lead to variations in erucic acid levels.

2.5.7. Environment Affects Erucic Acid Production

Environmental factors also play a crucial role in influencing erucic acid content and its production (Shi et al., 2003). Elements such as the climate, lodging angle, planting density, and fertilizer type can impact erucic acid production (Davoudi et al., 2019; Khan et al., 2018; Sanyal & Linder, 2013; Uğur et al., 2010; Yaniv et al., 1995). For instance, the same variety of *Eruca sativa* L. produced 46.64 % erucic acid during spring sowing, which

increased to 54.79 % in autumn sowing (Uğur et al., 2010). Recent studies have shown a negative correlation between temperature and oil content in *B. napus* seeds, as well as fatty acid composition in mature seed, especially when there is manual intervention (Zhu et al., 2012). For every 5 °C temperature increase, the erucic acid content in the seed of two *B. napus* genotypes decreased by 14 % and 7 %, respectively (Zhu et al., 2012). The phenomenon of lodging has been observed to influence the erucic acid content in plants. Specifically, in the *B. napus* cultivar Huayouza 62, a change in the lodging angle from vertical to horizontal corresponded with an increase in erucic acid content from 1.13 % to 1.49 % (Khan et al., 2018). Similarly, for the same cultivar, erucic acid content rose from 1.05 % to 1.46 % when plant density decreased from 45 plants/m^2 to 15 plants/m^2 (Khan et al., 2018). The variation in erucic acid content due to changes in lodging and planting density are attributed to altered plant physiology, which affects photosynthesis and metabolism, particularly the pathways involved in fatty acid synthesis. Additionally, the availability of resources varies under different lodging and planting densities, reducing competition and enhancing overall metabolic activities, including fatty acid synthesis. While nitrogen fertilization enhances erucic acid production, different nitrogen rates and forms can influence its content (Safavi et al., 2018). An element like selenium reduces erucic acid content in seed. After spraying a sodium selenate (Na_2SeO_4) solution on the leaf surface of the *B. napus* cultivar LEAR, the erucic acid content in seeds slightly decreased from 0.32 % to 0.29 % (Davoudi et al., 2019). In summary, environmental factors, including climatic conditions, planting stand, lodging level, planting density,

temperature, and fertilizer application methods, significantly affect erucic acid production and content.

2.6. Linkage Mapping

A linkage or genetic map represents a chromosome based on recombination frequencies (Campbell & Reece, 2005). A linkage map illustrates the relative positions of genes along a chromosome (Sanders & Bowman, 2021). The foundation of such a map is the presumption that the distance between two genetic loci influences the likelihood of a genetic crossover event (Campbell & Reece, 2005). To construct a linkage map for a specific chromosome, experiments crossing two parents are employed to gather the necessary recombination frequencies (Sanders & Bowman, 2021). Gene distances on this map are expressed in map units, termed Centimorgans (cM), where one map unit corresponds to a 1 % recombination frequency (RF) ((Postma & Silverman, 2008)). The data's fit determines the order of genes on the chromosome. Genes that are either far apart or located on different chromosomes (with a recombination frequency more than 50 %, RF>50) are considered to assort independently and are unlinked. By determining recombination frequencies for numerous gene pairs, a comprehensive linkage map can be created, showcasing the order and relative distances of genes on the chromosome.

2.7. Introduction of Molecular Markers, Sequencing, and Genotyping-by-Sequencing Methodology

2.7.1. Molecular Markers

In genetics, numerous genetic traits of interest are closely linked to specific genes (Stich & Melchinger, 2010). Identifying these genotype-phenotype connections has become a primary objective in genetic research (Oraguzie & Wilcox, 2007). Many genes, in combination with environmental factors, influence quantitative traits. The relationship between these quantitative traits and genes is explored through genetic mapping. Essentially, genetic mapping establishes the connection between targeted inherited genes and potential inherited traits (Stich & Melchinger, 2010; Oraguzie & Wilcox, 2007).

One of the most significant advancements in molecular genetics has been the investigation and use of molecular markers to detect and leverage DNA polymorphisms (Mammadov et al., 2012). Molecular markers can be described as (1) alleles located in a specific DNA region that can be tracked, (2) a DNA fragment with a known genomic location, or (3) a gene that typically displays distinct phenotypic expression. These indirect selection markers are employed to label a chromosome, or locus and to identify an individual or cell that possesses it (Semagn et al., 2006).

Molecular markers are distinct DNA sequences located at determined sites within the genome and are inherited according to the principles of genetics and inheritance (Semagn et al., 2006). These markers and the marked genes are closely located on the same chromosome and remain so across generations. Markers can be present on every

chromosome, and by determining the distance between each marker and genes, a genetic linkage map can be constructed (Kucuktas et al., 2009). This map facilitates the analysis of the relationship between traits of interest and genes, promoting marker-assisted selection and enabling the introduction of desired genes.

Depending on the type of study, one can choose from various molecular techniques (Semagn et al., 2006). These markers facilitate the genotyping of populations, allowing for differentiation and subsequent phenotyping based on the traits of interest (Hall et al., 2010). There are several types of these markers, with the most common ones being restriction fragment length polymorphisms (RFLPs) (Jarcho , 2001), amplified fragment length polymorphisms (AFLPs) (Savelkoul et al., 1999), simple sequence repeats (SSRs) (Powell et al., 1996), and single-nucleotide polymorphisms (SNPs) (Stich & Melchinger, 2010).

2.7.2. Restriction Fragment Length Polymorphisms (RFLPs)

In 1974, restriction fragment length polymorphism (RFLPs) markers were first used to map the genetics of an adenovirus serotype with a temperature-sensitive mutation (Grodzicker et al., 1974). Later, these markers were used in the human genome mapping project in the 1980s and were subsequently adopted for plant genome mapping (Botstein et al., 1980; Helentjaris et al., 1986). Restriction fragment length polymorphisms leverage restriction enzymes to reveal patterns of DNA fragment size differences within individual organisms (Semagn et al., 2006). Following enzymatic cleavage, the resultant fragments are segregated based on size through electrophoresis, subsequently transferred onto a

membrane, and then hybridized using a radiolabeled probe (Botstein et al., 1980; Semagn et al., 2006).

Variations in DNA sequences at restriction sites can lead to the gain, loss, or relocation of a restriction site. As a result, when DNA is digested by restriction enzymes, different individuals, populations, and species will produce varying numbers and sizes of DNA fragments (Mohler & Schwarz, 2005; Semagn et al., 2006). Mohler and Schwarz (2005) noted the decline in the use of RFLPs in marker-assisted breeding can be attributed to the labor-intensive, multi-step nature of the protocol and the necessity for radioactive materials for effective fragment detection. Nonetheless, the advent of straightforward markers derived from sequenced RFLP probes offers a chance to preserve valuable polymorphisms identified in earlier gene mapping research (Mohler & Schwarz, 2005).

2.7.3. Amplified Fragment Length Polymorphisms (AFLPs)

Amplified fragment length polymorphisms (AFLPs) markers are a type of polymerase chain reaction (PCR)-based marker (Mullis et al., 1992). Polymerase chain reaction is a technique in molecular biology that facilitates the enzymatic amplification of DNA in minute quantities (Semagn et al., 2006). After its invention in 1983, the PCR-based marker technique became widely used in various markers, including random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), and DNA amplification fingerprinting (DAF) (Semagn et al., 2006). The AFLP marker method combines the effectiveness of RFLP and the adaptability of PCR-based techniques, achieved by

attaching primer adaptors to the DNA following restriction (Lynch & Walsh, 2001). AFLP's unique feature is its ability to screen representative DNA regions distributed randomly across the genome based on the 'genome representation' characteristic (Kundan et al., 2014). AFLP can generate markers for the DNA of any organism without the need for an initial investment in primers, probe development, or sequence analysis (Paun & Schönswetter, 2012). Due to its reliability and reproducibility, this technology was extensively used in botanical research (Mohler & Schwarz, 2005).

2.7.4. Simple Sequence Repeats (SSRs)

Simple sequence repeats (SSRs), also known as microsatellites, are a type of PCR-based marker (Litt & Luty, 1989). SSRs target simple repetitive DNA sequences that are 2-6 base pairs long or even longer (Jonah et al., 2011; Semagn et al., 2006). Microsatellites are commonly characterized by dinucleotide repeats, exemplified as $(CA)^n$, where 'n' represents the repeat count, typically varying between 10 and 100 (Semagn et al., 2006). The development of primers for SSRs involves cloning arbitrary DNA segments from the species of interest (Semagn et al., 2006). Owing to the uniform distribution of SSR markers across the eukaryotic genome, these markers are co-dominant, highly polymorphic, and stable, making them ideal for constructing genetic maps (Hearne et al., 1992). However, a significant challenge in developing SSR technology is the inaccuracy of genomic libraries and the difficulty in managing the vast number of SSR clones that emerge during sequencing (Hearne et al., 1992; Semagn et al., 2006).

2.7.5. Single Nucleotide Polymorphisms (SNPs)

Variation in an organism's sequence can be found between individuals, populations, and subspecies. Single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) have been observed at intervals of 170 bp and 540 bp in gene sequences, respectively (Drenkard et al., 2000; Garg et al., 1999). Due to the abundance and distribution of these polymorphisms across the genome, SNP markers have become a valuable genetic tool for constructing genetic maps (Nasu et al., 2002). A SNP marker represents a single base change in the DNA sequence, making allele identification by size differences on a gel challenging compared to previous methods (Ghosh et al., 2002). The utility of SNPs relies on efficient sequence information identification (Ghosh et al., 2002; Mohler & Schwarz, 2005). As a practical second-generation genetic marker, SNPs are widely diverse throughout the *B. napus* genome (Hayward et al., 2012; Mohler & Schwarz, 2005). SNPs can differentiate between homozygous and heterozygous loci and support automated and high-throughput genotyping. These advantages make SNP markers particularly appealing for applications like marker-assisted selection (MAS) (Hayward et al., 2012; Mohler & Schwarz, 2005; Semagn et al., 2006).

2.7.6. Development of Molecular Markers in *B napus* and Erucic Acid-Specific Markers

In the realm of *B. napus* molecular marker development, a chronological advancement from traditional techniques to high-density SNP arrays and erucic acid-specific markers is evident. The initial phase employed restriction enzyme-based systems, such as RFLP,

AFLP, and SSR, for QTL mapping and genetic linkage construction, albeit with limitations in accuracy and resolution (Liu et al., 2013). Subsequently, with the maturation of sequencing technologies and high-density SNP genotyping arrays, the advent of new high-throughput sequencing methods marked an advancement in molecular marker technology, predominantly genome-specific, enhancing both the scope and accuracy (Clarke et al., 2016). In the molecular marker development for *B. napus*, particularly for erucic acid content, a chronological progression is evident. Early studies established that erucic acid content is controlled by two major genes with additive effects, mapped in rapeseed using QTL methods (Downey, 1964; Ecke et al., 1995; Jourdren et al., 1996; Thormann et al., 1996). Subsequent research utilized primers designed from *A. thaliana* and *B. napus FAE1* sequences, confirming that a two-base deletion in the *B. napus* C-homeolog led to the low erucic acid phenotype (Fourmann, 1998). Further advancement came with the segregation of 99 DH lines into high, medium, and low erucic acid content groups, identifying two potential QTL controlling erucic acid concentration using RFLP markers. This was complemented by identifying four and three SNPs in *FAE1.1* and *FAE1.2*, respectively, differentiating low and high erucic acid genotypes in *Brassica* (Gupta et al., 2004)

In a study by Nath et al. (2009), transgenic approaches that involved cloning and amplifying *FAE1.1* and *FAE1.2* in F_4 progeny of a cross between wild type *B. napus* and HEAR, followed by binary plasmid construction and agrobacterium-mediated transformation generated transgenic plants. Polymerase chain reaction analysis in F_2 plants indicated the potential non-functionality of ectopic expression of the *FAE1.1* gene

(Nath et al., 2009). Wang et al. (2010) found three SNPs/indels in *FAE1* gene in 30 commercial LEAR cultivars using EcoTILLING, strongly correlating with seed erucic acid content. This study also mapped phylogenetic divergence of *FAE1* in the A and C genomes post-speciation from *Arabidopsis* (Wang et al., 2010). Cao et al. (2010) utilized SSR markers in QTL studies, uncovering genetic drag between erucic acid content and oil content, advocating backcrossing and marker-assisted selection to overcome this breeding challenge (Cao et al., 2010).

Wu et al. (2015) isolated the *FAE1* gene in 30 LEAR cultivars, comparing it with the *FAE1* sequence of the HEAR cultivar Zhongyou 821. Specific primers developed through restriction enzyme and high-resolution melting (HRM) curve analysis identified mutations including a two-base deletion (e_{C2}), a point mutation (e_{A1}), a four-base deletion (e_{C4}), and a single base transversion four-base deletion (e_{A*}), further substantiating the role of the e_{C2} mutation in *FAE1.2* in the low erucic acid phenotype (Wu et al., 2015). Yan et al. (2015) designed unique cloning for promoter sequences of *FAE1*, identifying 26 SNPs causing 13 amino acid changes in *B. rapa* (Yan et al., 2015). Clarke et al. (2016) introduced a high-density SNP genotyping array containing 52,157 markers for tetraploid *B. napus*, also applicable to its erucic acid analysis for diploid ancestors *B. oleracea* and *B. rapa* (Clarke et al., 2016). Lastly, Gill et al. (2021) employed kompetitive allele-specific PCR (KASPar) genotyping assay in *B. juncea* for low erucic acid varieties, cloning and sequencing *FAE1.1* and *FAE1.2* genes in high and low erucic acid genotypes of *B. juncea, B. rapa*, and *B. nigra*. This study revealed amino acid changes due to SNPs in *FAE1.1* and confirmed the role of *FAE1* gene mutations in reducing erucic acid content

through decreased KCS enzyme activity (Gill et al., 2021).

2.8. The *B. napus* Reference Genome

With the advancement and establishment of whole genome sequencing technology, with the advancement and establishment of whole genome sequencing technology, there has been a surge in basic agricultural research (Kersey, 2019). Before attempting to edit any known or potentially functional elements within a genome, a well-sequenced genome is essential (Kersey, 2019). This sequencing technique not only traces the evolutionary history of plants but also establishes connections between traits and polymorphisms (Kersey, 2019). Research in whole-genome sequencing is pivotal for purposes such as disease resistance, identifying new species, and creating genetic markers for marker-assisted selection (Kersey, 2019).

Due to the intricate technical process and cost constraints of the Sanger sequencing method, genome sequencing initially focused on organisms with smaller genomes. The first plant species to be sequenced was *A. thaliana* in 2001, followed by rice (*Oryza sativa* L.) in 2005 (Goff et al., 2014). Later, the advent of massively parallel next-generation sequencing (NGS) machines dramatically increased sequencing efficiency. These machines have since been employed to sequence thousands of organisms (Kersey, 2019). In 2014, a collaborative effort among scientists led to the short-read sequencing of the French *B. napus* winter cultivar Darmor-bzh genome. Scientists assembled the Darmor-bzh genome using long-read-length sequencing data combined with optical and genetic mapping in 2020 (Rousseau-Gueutin et al., 2021). The genome size, determined to be 862

Mbp, was verified by comparing Oxford Nanopore Technologies (ONT) sequencing with contiguity 50N and 90N tests (Rousseau-Gueutin et al., 2021). Rousseau-Gueutin et al. (2021) detailed the extensive mapping of genetic markers, stating, "*A total of 39,495 markers deriving from the Illumina 8K, 20K, and 60K arrays were genetically mapped on the integrated map, totaling 2777.7 cM. The sequence contexts of all single-nucleotide polymorphism markers that were genetically mapped were blasted against our B. napus Darmor-bzh assembly to validate the quality of our assembly and to help order and orient the scaffolds. Of these 39,495 genetically mapped markers, 36,391 were physically anchored on the final Darmor-bzh assembly. The genetic and physical positions were discordant for only 618 markers (0.02 %) due to an inaccurate position on the genetic map (variation of a few centimorgans in almost all cases)*" (Rousseau-Gueutin et al., 2021, p. 5).

Several other *B. napus* species have also been sequenced, including Westar (Song et al., 2020), Express617 (Lee et al., 2020), Tapidor3 (Song et al., 2020), Shengli3 (Song et al., 2020), and Zhongsuang11 (Song et al., 2020). Beyond *B. napus*, other species and sub-genomes have also been successfully sequenced. For instance, *B. rapa* (genotype Z1) was sequenced using ONT technology in 2018 (Belser et al., 2018), while *B. nigra* (genotype NI100) was sequenced with the same technology in 2020 (Perumal et al., 2020). Additionally, *B. oleracea* (genotype HDEM) was sequenced by ONT in 2018 (Guo et al., 2021). The sequencing of an increasing number of species is paving the way for enhanced scientific research in the future.

The first plant pan-genome was published in 2014 by Li et al., who analyzed seven accessions of soybean (*Glycine max* L.). Their research demonstrated that these seven

varieties represented about 87 % of the genetic diversity in soybeans. They discovered that approximately 80 % of the pan-genome was present across all seven germplasms, representing the species' core genome (Li et al., 2014). Subsequently, Song et al (2020) conducted a pan-genome comparative analysis of eight *B. napus* accessions. The study entailed the de novo assembly and annotation of eight distinct *B. napus* genomes, utilizing an integration of PacBio sequencing, Illumina paired-end short read sequencing, and Hi-C technologies. The findings revealed considerable intraspecific variation among these eight *B. napus* genomes. Nonetheless, a significant proportion, exceeding 76 % of the genes, were identified in syntenic regions, while less than 9.4 % of the genes with large-effect mutations were detected in these reference genomes (Song et al., 2020). Consequently, these eight genomes were categorized into semi-winter oilseed rapeseed (ZS11, Zheyou7, Gangan, and Shengli), spring-type oilseed rapeseed (Westar and No2127), and winter-type oilseed rapeseed (Darmor-bzh and Tapidor). By integrating these eight genomes, a high-quality pan-genome of *B. napus*, based on the Darmor-bzh, was reconstructed. This genome is approximately 1.8 Gb in size, comprising 105,672 gene clusters, of which about 56 % are core gene clusters, approximately 42 % are dispensable gene clusters, and around 2 % are specific gene clusters (Song et al., 2020).

2.9. The *Brassica* 60K Illumina® Infinium™ SNP Chips

The *Brassica napus* 60K Illumina® Infinium™ SNP chip has been widely adopted by the international rapeseed community (Mason et al., 2017). The widespread adoption of this

method is attributed to its capability for swift data collection and straightforward analysis, which are particularly advantageous for high-throughput genotyping, especially when paired with the availability of new reference genome sequences (Mason et al., 2017). The *Brassica* 60K SNP chip provides a platform for genetic analysis in the *Brassica* genome, introducing high-density SNP markers for genotyping in *B. napus* (Mason et al., 2017). This chip utilizes Illumina® Bead-Chip technology (Mason et al., 2017). The Bead-Chip works by amplifying the entire genome and then conducting an allele-specific hybridization based on the selected SNPs (Hayward et al., 2012). These hybridization reactions occur on the Bead-Chip, and specific alleles are detected through fluorescence using the Bead-Chip array reader (Hayward et al., 2012). The Infinium *Brassica* array comprises 58,464 SNPs, leading to its "60K" designation (Sharpe, 2012; Mason et al., 2017). Out of these, 89 % are deemed functional and are part of the working set arrays (Mason et al., 2017).

The *Brassica* 60K SNP chip array boasts unparalleled advantages (Mason et al., 2017). Its widespread adoption can be attributed to its cost-effectiveness, user-friendliness, ease of sample preparation, uncomplicated data analysis, and consistent reproducibility, which are key characteristics of this approach (Mason et al., 2017). This SNP chip array has found extensive use in research concerning *B. napus*, particularly in genome-wide association mapping studies (GWAS) and in verifying the accuracy of whole genome sequencing (Wei et al., 2016; Rousseau-Gueutin et al., 2021).

2.10. Genotyping-By-Sequencing (GBS)

Genotyping-by-sequencing is a method for detecting SNPs using Illumina® next-generation sequencing technology (Elshire et al., 2011). The standard protocol uses methylation restriction enzymes to reduce genome complexity (He et al., 2014). After enzymatic digestion, PCR is performed to increase the fragment pool to create GBS libraries. Then, the GBS libraries are sequenced by Illumina® next generation sequencing technologies (NGS), usually resulting in 100-150 bp reads. The next step is to go through the aligned reference genome and find the SNPs. SNPs need to be checked for coverage and duplication by technical means after finding them (Elshire et al., 2011; He et al., 2014). Since millions of sequences exist in the GBS library and 10,000 to 100,000 SNPs are generated after sequencing, a large amount of data needs to be rigorously screened to expect the most accurate SNPs (Bradbury et al., 2007; Elshire et al., 2011; Glaubitz et al., 2014).

Early detection of maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) using the GBS method by segmenting the genome with up to 96 restriction enzymes resulted in 25,185 SNPs mapped in maize and 24,186 SNPs mapped in barley (Elshire et al., 2011). One of the varieties of doubled haploid barley species (OWB003) was 99 % mapped by GBS (Elshire et al., 2011). Recent experiments have also demonstrated that GBS can be applied to soybean (*Glycine max* L.) and *B. napus* (Lees et al., 2016; Sonah et al., 2013).

Genotyping-by-sequencing streamlines the process of gene sequencing, facilitates comparison with reference genomes, and offers comprehensive coverage of the genome

(Chen et al., 2013; Elshire et al., 2011). Genotyping-by-sequencing is adept at discovering a significant number of SNP markers in large genomes. It also mitigates alignment issues in genetically diverse species by minimizing the repetitive regions of the genome (Lees et al., 2016). Given its ability to yield a rich set of SNPs, GBS is well-suited for GWAS and QTL analysis, and phylogenetic assort (Sonah et al., 2013). The GBS method boasts a relatively simple library preparation process with reduced genome representation (Lees et al., 2016). There are no stringent requirements for DNA quality, and initial DNA concentrations ranging from 100-200 ng are acceptable (Lees et al., 2016). Another significant advantage of GBS is that it does not always necessitate a reference genome for sequence mapping the markers (Elshire et al., 2011). These markers can also function as dominant markers, enabling alternative genetic analyses in the genomes of any species, even without prior knowledge of its genetic structure (Elshire et al., 2011).

Genotyping-by-sequencing is a cost-effective method, especially for populations with complex genomes or limited reference genome resources (Elshire et al., 2011). Given its numerous advantages, this technology holds the potential for rapid and affordable sequencing in future populations (Lees et al., 2016). Genotyping-by-sequencing can be extensively employed for studies on genetic diversity and heterozygosity (Lees et al., 2016).

2.11. Analysis of Quantity Trait Loci for Erucic Acid Content in *B. napus*

Often, multiple genes are involved in the regulation of a single trait, which is also subject

to the influence of environmental variables (Ackerly et al., 2000; Leips & Mackay, 2002), such traits are termed quantitative traits (MacKay et al., 2009). Many vital agricultural traits, such as crop productivity, seed quality, tolerance to environmental stresses, and certain types of disease resistance, fall under this category (Amar et al., 2008; Rajpal et al., 2016). A statistical analysis technique can be used to identify quantitative trait locus. Quantitative trait loci are loci that either influence or are associated with a specific trait (Asins et al., 2010). To understand the relationship between phenotypic and genotypic data, QTL analysis is employed to study the genetic variation among individuals within a population (Kearsey & Farquhar, 1998).

2.11.1. Introduction of Quantitative Trait Loci Analysis

Quantitative trait loci analysis hinges on the two parents of the population that produce the QTL. The two parents should be genetically distinct concerning the trait of interest, and it is essential to differentiate between the genetic markers of the two parental genotypes (Byrne, 2005). The chosen markers for this purpose are typically the best-suited genotyping molecular markers, including SNP, SSR, RFLPs, and transposable element positions (Casa et al., 2000; Vignal et al., 2002; Gupta & Rustgi, 2004; Henry, 2006). For QTL analysis, the F_1, derived from crosses between distinct parental genotypes, are utilized to produce the DH population. The DH lines should have substantial phenotypic variation and can be used for genetic mapping (Huang et al., 2011).The phenotypic trait of interest will often segregate with molecular markers located in the regions associated with the phenotype (Huang et al., 2011). Conversely, molecular

markers outside the associated region will not have a significant relation to the trait (Byrne, 2005). QTL analysis determines whether phenotypic differences primarily arise from a few loci with substantial influence or many loci with minimal effects, and it assesses the extent of influence of each locus (Grisel, 2000).

The conventional methodology for conducting a QTL mapping experiment involves the following steps: (1) Choose two parental genotypes with a significant difference in the allele influencing variation in a trait; (2) cross the parents to produce a mapping population; (3) analyze the phenotypic mapping population and assign the traits of interest; (4) generate molecular data for a sufficient portion of the population using evenly spaced polymorphic markers to construct a genetic map; and (5) implement a statistical procedure to identify molecular markers associated with the trait of interest (Rajpal et al., 2016).

Numerous factors impact the success of a QTL analyses. Substantial sample size is essential for QTL analysis. A limited sample size might overlook QTL with minor effects and could result in overestimating the effect size of the detected QTL (Grisel, 2000; Mackay, 2001). Various interactions can also influence QTL, including genotype-by-sex (G x S), genotype-by-environment(G x E), and epistatic interactions between QTL (Forbes et al., 2004; Lauter & Doebley, 2002; Leips & Mackay, 2002; Nuzhdin et al., 2005; Wilson et al., 2006).

2.11.2. Quantity Trait Loci for Traits in *B. napus*

Quantitative trait loci have been primarily documented in *B. napus* traits such as seed oil,

erucic acid content, glucosinolate contents, disease resistance, and yield (Wang et al., 2013; Qiu et al., 2006). An analysis of 403 SSR markers across 348 doubled haploid genotypes identified 63 QTL associated with seed oil content, with 2.6-17.9 % of phenotypic variation in the population (Wang et al., 2013). Furthermore, the research also pinpointed 24 consensus QTL explaining 3.6-11.7 % of the variation in oil content across 11 chromosomes in *B. napus* (Wang et al., 2013). In a subsequent study, the TNDH population was derived from a cross between Ningyou7 and Tapidor, which have erucic acid contents of 56.2 % and 3.9 %, respectively. This study identified four major QTL that controlled 45.0 % and 30.0 % of erucic acid content on chromosomes A08 and C03, respectively, using multiple marker types (Qiu et al., 2006).

2.11.3. Consensus of Erucic Acid Quantitative Trait Loci

Earlier research determined that the synthesis of erucic acid was controlled by two genes with additive effects (Thomasson, 1956; Stefansson & Hogen., 1964). A subsequent study identified these two erucic acid genes and three QTL affecting seed oil content using RFLPs on 135 genotypes from the segregating doubled haploid population of *B. napus* (Ecke et al., 1995). The primary QTL affecting erucic acid content were previously identified on Linkage Groups (LG) 7 and LG 15 from a cross between Victor and Tapidor cultivars (Burns et al., 2003). After crossing parents with a 95 % difference in phenotype, it was found that the QTL on LG 7 could correspond to the *eru1* locus on chromosome A08 and the QTL on LG 15 might be equivalent to the *eru2* locus on Chromosome C03 (Lincoln et al., 1993; Burns et al., 2003). Potential QTL for erucic acid were located on

chromosomes A01, A08, and C03 in the paralogous/homoeologous regions of the *B. napus* genome (Parkin et al., 2003). All these QTL align with the long arm of the *A. thaliana* chromosome C4 (Parkin et al., 2003; Isobel et al., 2005). The identified region aligns with the *Brassica* species' *FAE1* homolog, suggesting its potential role as a key gene in the regulation of erucic acid synthesis. The *FAE* gene probably exists in the corresponding regions of the *B. napus* genome in proximity to each of these three QTL (Lassner et al., 1996; Roscoe et al., 2001).

Advancing from the initial understanding of erucic acid synthesis, subsequent research has sharpened the focus on specific QTL, enhancing the comprehension of their roles in *Brassica* species. A high-stringency genetic linkage map with 277 loci was developed from the TNDH population based on 188 doubled haploid genotypes (Qiu et al., 2006). After analysis using MapQTL software and two years of phenotypic data, it was identified that the major QTL controlling erucic acid were on chromosomes A08 and C03 (Qiu et al., 2006). In the high erucic acid parent, the average expression levels of phenotypic variation on chromosomes A08 and C03 were 45.1 % and 30.4 % erucic acid, respectively (Qiu et al., 2006). Besides the major QTL for elevated erucic acid, Qiu et al. (2006) also identified minor erucic acid QTL on chromosomes C01 and C02, affecting 5.8 % and 6.6 % of erucic acid content, respectively. However, QTL on chromosomes A06 and A10 were detected in only one of four experimental environments, with the logarithm of odds (LOD) values around 2.3-2.7 (Qiu et al., 2006). Another study, which selected 200 individual genotypes by hybridizing Tapidor and Victor in backcross experiments using the RFLP method, also identified the two major QTL for erucic acid on chromosomes A08

and C03, named *eru1* and *eru2*, respectively (Burns et al., 2003). The *eru1* locus was positioned at the proximal end of chromosome A08 with a 95 % confidence interval of approximately 12 cM. In comparison, the *eru2* locus was mapped to the distal end of chromosome C03 with a 95 % confidence interval of about 26 cM (Burns et al., 2003). Collectively, the *eru1* and *eru2* loci contribute to 64 % of the overall variance in erucic acid levels observed within the backcross 1 population. However, there are still several QTL in unidentified regions that have yet to be accounted for (Burns et al., 2003).

Subsequent research identified the locus primarily responsible for the *B. napus* biosynthesis of erucic acid in seed as *BnA8.FAE1* on chromosome A08 and *BnC3.FAE1* on chromosome C03. These findings were further validated in subsequent studies (Cao et al., 2010; Wang et al., 2008; Wu et al., 2008). A more detailed study using 202 doubled haploid TNDH populations, tested across 11 environments, revealed more precise QTL for erucic acid content (Cao et al., 2010). In addition to confirming the previously identified QTL *qEA.A08.1*, another QTL with a smaller effect, termed *qEA.A08.2*, was discovered on the same chromosome, located 7 cM away from *qEA.A08.1* (Cao et al., 2010). The overlap of both *qEA08.1* and the newly discovered *qEA.A08.2* with the oil content QTL *qOC.A08.3* and *qOC.A08.5* indicates a potential relationship between erucic acid levels and oil content (Cao et al., 2010). In some experiments, QTL *qEA.A08.2* was not detected, which might be attributed to potential environmental factors (Cao et al., 2010). The accumulated data suggests that chromosome A08 may contain QTL other than *BnA8.FAE1* and that erucic acid content is linked to oil content.

A new mapping population of 156 doubled haploid genotypes was developed by selecting two spring canola varieties: Polo 9 and Topas (Javed et al., 2016). After two years of field trials, it was determined that positions at 4.01 cM and 17.70 cM on chromosome 10 were associated with QTL for oil content, erucic acid, and linoleic acid contents (Javed et al., 2016). The most significant arachidic acid content (a saturated fatty acid with 20 carbons) QTL was identified as *qC20:0-C3d* (located at 154.55 cM, the minor QTL were also found on chromosomes A01, A10, C01, C03, C05, C08, and C09. This QTL had the highest LOD value and accounted for 26.51 % of the phenotypic variation in arachidic acid content (Javed et al., 2016). The enzyme responsible for elongating from C18 to C20 is the same as the one that elongates C20 to C22; both are utilized by FAE enzyme (Post-Beittenmiller, 1996; Zeng & Cheng, 2014). This similarity in elongation processes could lead to the colocalization of QTL, suggesting that the QTL affecting erucic acid might also be present at the qC20:0-C3d locus (Javed et al., 2016).

Research on erucic acid synthesis in *B. napus* has evolved from identifying two major additive genes to mapping specific QTL on chromosomes A08 and C03, with further studies revealing additional minor QTL and suggesting a link between erucic acid, other fatty acids and oil content, as well as potential environmental influences on these traits.

2.12. Objectives

The current research aims to identify chromosomal regions associated with the phenotypic expression of erucic acid in *B. napus* seed. Determining the loci that control

erucic acid traits and analyzing phenotypes across different environments will offer valuable insight for the development of seed with higher erucic acid content and the breeding of new HEAR cultivars. The objective of this study was to delve deeper into the understanding of minor QTL associated with elevated levels of erucic acid in *B. napus*. The hypothesis is that one or more minor QTL in the *B. napus* genome significantly influences the erucic acid content in the seed. Two HEAR doubled haploid mapping populations, CBLD2 and CBER2, comprising a total of 373 individuals selected for variation in seed erucic acid content, were subjected to QTL mapping analysis using GBS. The research aimed to uncover the distribution of minor QTL, that influence erucic acid levels in populations where both parents had high erucic acid content.

3. Discovery and Mapping of Quantitative Trait Loci Contributing to Erucic Acid Content in CBLD2 Population Seed

3.1. Abstract

Brassica napus L., commonly known as canola or rapeseed, is an economically significant crop due to its high oil content and quality. Erucic acid is a major component of *B. napus* oil and plays a crucial role in determining its nutritional and industrial properties. Erucic acid is used in a wide range of industrial applications, often as a highly effective industrial lubricant, and is in high demand as a raw material in modern manufacturing. The objective of this research was to identify and analyze the minor-effect quantitative trait loci (QTL) responsible for high levels of erucic acid in *B. napus.*

A doubled haploid (DH) population was generated from parental genotypes with contrasting erucic acid content. The DH population (CBLD2) consisted of 183 individuals. The population was compared in a randomized complete block design in two locations in 2020 and 2021. Fatty acid analyses were conducted using gas chromatography. DNA samples were extracted from the leaf tissue of each genotype, and genotyping was performed using modified genotyping-by-sequencing (GBS). Genetic maps were produced from the detected SNPs and InDels. Inclusive composite interval QTL mapping (ICIM) was employed to detect significant QTL with high statistical confidence. Multiple QTL associated with erucic acid content were detected. These QTL were distributed across chromosomes A04, A07, A10, C01, C02, C04, and C08. The most influential QTL

were located on chromosomes A04, A07 and C08. Specifically, the QTL *qEA_CBLD2_A04* on chromosome A04, positioned between 23.0 and 23.4 cM, accounted for 3.5 - 12.5 % of the phenotypic variation (R^2). On chromosome A07, the QTL *qEA_CBLD2_A07 was* located between 7.1 and 8.2 cM and explains 3.5 - 9.0 % of the phenotypic variation. Lastly, the QTL *qEA_CBLD2_C08* on chromosome C08, spanning 1.5 to 9.8 cM, contributes to 3.11 - 9.8 % of the phenotypic variation. These results indicate the polygenic nature of erucic acid regulation. The identified QTL provides valuable insight into the genetic basis of the erucic acid high quantity trait in *B. napus*. These findings can facilitate marker-assisted selection programs aimed at developing *B. napus* genotypes with increased erucic acid content.

3.2. Introduction

Brassica napus L. is a major global oilseed crop (FAO, 2022). High erucic acid *B. napus* cultivars are particularly valuable for industrial applications, such as in the production of plastics, lubricants, and erucic acid derivatives (Friedt & Lühs, 1998; McVetty & Duncan, 2015). The demand for high erucic acid oil is substantial. For example, the United States imported approximately 18 million kg to meet industrial needs in 2000 (Aukema & Campbell, 2011; Przybylski & Eskin, 2011). In 2005, Puyaubert et al. (2005) projected that by 2010, the global demand for erucic acid would reach approximately 46.29 million kg annually. Thus, the significant growth in *B. napus* production and the escalating global demand for high erucic acid oil not only highlight the economic importance of *B. napus*

but also underscore its evolving role in meeting both food and industrial needs worldwide.

Erucic acid production is influenced by environmental conditions, genetics and their interactions (Shi et al., 2003). Agronomic factors such as planting density and fertilization also contribute to variation in erucic acid content (Davoudi et al., 2019; Khan et al., 2018; Sanyal & Linder, 2013; Uğur et al., 2010; Yaniv et al., 1995). The synthesis of erucic acid also involves interactions with other fatty acids and enzymes, with competition for substrates being a key factor (Mietkiewska et al., 2004). Hence, the synthesis and modulation of erucic acid content in *B. napus* are influenced by a combination of genetic factors, which dictate the potential for enzyme production, and environmental conditions that, together with post-translational modifications, impact enzymatic activity, each contributing to the dynamics of the biosynthetic pathway.

Historically, research on *B. napus* has focused on developing low erucic acid cultivars, such as canola, which has significantly lower erucic acid content compared to traditional rapeseed cultivars (Ecke et al., 1995; Vera et al., 2007). The *FAE1* gene, isolated from various plants, plays a crucial role in modulating erucic acid content (Amar et al., 2008; Guan et al., 2019; Javed et al., 2016; Qiu et al., 2006; Thambugala et al., 2020; Wang et al., 2008). Special high erucic acid-low glucosinolate rapeseed (HEAR) cultivars, with greater than 45 % erucic acid content are becoming increasingly important as renewable feedstocks (Aukema & Campbell, 2011; Heath & Earle, 1995; McVetty & Duncan, 2015). In HEAR cultivars, two functional copies of *FAE1* are located on chromosomes A08 and C03 (Puyaubert et al., 2005; Roscoe et al., 2001). The absence of *FAE1* in canola suggests

its link to reduced 3-ketoacyl-CoA synthase (KCS) enzyme activity and caused the low erucic acid content in seed (Puyaubert et al., 2005; Roscoe et al., 2001). The need for high erucic acid *B. napus* cultivars reflects the evolving demands of agriculture products and the importance of genetic research in crop development.

Quantitative trait loci (QTL) analysis is a powerful tool for identifying genomic regions contributing to erucic acid variation in *B. napus*. Li et al. (2016) identified 24 QTL across 11 chromosomes, explaining 4.1-28.4 % of the phenotypic variation in erucic acid content by using the *Brassica* 60K SNP Illumina Infinium ™ SNP array (Li et al., 2016). In addition to major QTL on chromosomes A08 and C03, minor QTL influencing erucic acid production have been discovered on other chromosomes (Qiu et al., 2006; Zhao et al., 2008). QTL analysis and genome-wide association studies (GWAS) have further identified multiple SNP markers related to erucic acid content (Jones et al., 1995; Qu et al., 2017; Salas & Ohlrogge, 2002). Hence, advancements in QTL analysis for erucic acid content in *B. napus* not only deepens our understanding of its genetic control, but also paves the way for more precise and efficient breeding advancements for this trait.

Given the increasing industrial demand for erucic acid, enhancing its content in *B. napus* seed is essential for large-scale production (Wang et al., 2022). This research aims to identify additional minor QTL that impacts erucic acid content in seed, contributing to the development of high erucic acid *B. napus* cultivars.

3.3. Materials and Methods

3.3.1. Plant Material

CBLD2 was developed by crossing the female parent (FP) 268-2 ZSDH6550/LLHR10282 with the male parent (MP) CB131037-6-8. Female parent (268-2 ZSDH6550/LLHR10282) was obtained through single plant selection from a cross between the 268-2 ZSDH6550 doubled haploid line and LLHR10282 (an F_4 generation cross between 06C421 and LLHR499-1-49). Male parent (CB131037-6-8) was developed by backcrossing the Castor summer rape cultivar (McVetty et al., 1997) with the VT 500 cultivar. The FP 268-2 ZSDH6550/LLHR10282 contains a mean of 53.7 % erucic acid in the total seed extracted oil, while the MP CB131037-6-8 contains 50.3 %. Pollen from the F_1 generation underwent microspore culture, producing doubled haploid progeny (Weber et al., 2005; Collard et al., 2005).

3.3.2. Field Evaluation

The doubled haploid CBLD2 population, consisting of 183 genotypes, was planted in 2020 and 2021 in two environments each year (a total of 4 environments). In 2020, it was grown at two field research stations: the University of Manitoba Point Field Research Station in Winnipeg, MB, Block 8 (49.811352, -97.123632), and the Ian N. Morrison Research Farm in Carman, MB, Block 4E (49.499807, -98.029022). In 2021, CBLD2 was planted at the Ian N. Morrison Research Farm, Block 6E (49.492538, -98.032599) and the

Portage La Prairie Research Farm in Portage, MB (50.0636349, -98.3997980). Adverse environmental conditions and wildlife interference led to the destruction of most plants in the 2020 CBLD2 population at the University of Manitoba Point Field Research Laboratory. Thus, data from this location was excluded from the analysis.

The soil in Carman is characterized as 'Loamy Lacustrine' (Angad & Entz, 2002) and taxonomically classified as fine-loamy, mixed, superactive, thermic Udic Argiustolls (USDA, 2022). The soil at Portage was classified as 'Clay Lacustrine' (Government of Canada, 2017) and taxonomically identified as very fine, smectitic, mesic Vertic Endoaquolls (USDA, 2022).

Each field experimental design was a randomized complete block design (RCBD) with three replicates. Each replicate utilized 0.5 g of seed, planted in 3 m rows with a 0.4 m row spacing. The spring *B. napus* HEAR cultivar MilleniUM03 (Erucic acid content is 55.2 %) (McVetty et al., 2000) was planted every twentieth entry as a check. The reason for choosing MilleniUM03 as the check is its well-documented stable high erucic acid cultivar, which provides a reliable benchmark for evaluating other agronomic traits under various field conditions, ensuring consistency in our analyses. Guard rows bordered all exterior edges of the experiment. Agronomic traits, including days-to-flower, plant height, lodging score and days-to-maturity were recorded for each plot. These agronomic traits are important to breeders and canola growers, and they may influence or correlate with erucic acid synthesis. Studying these traits alongside with erucic acid content helps breeders understand the integrated physiological and genetic factors that impact crop performance and oil quality under different environmental conditions. Days-

to-flower, also known as flowering time, was noted as the total number of days from sowing until half of the plants in a specific row began to flower (Liu et al., 2022; Song et al., 2021). Plant height was measured from the ground to the topmost pods during the pod-filling stage, by selecting random plants within each row (Li et al., 2016). Lodging scores, assessed at the maturity stage, were determined based on the angle between the vertical growth habit and the ground, using a visual scale ranging from 1 (completely upright, minimal lodging) to 5 (entirely flat on the ground, severe lodging) (Udall et al., 2004). Finally, days-to-maturity, or maturity time, was calculated as the number of days from sowing until approximately 60-70% of the seed in the main raceme changed color from green to dark brown or black (Vera et al., 2007; Canola Council of Canada, 2001).

For the 2020 experiment at Carman, seedbed preparation was conducted in the fall of 2019. Soil was cultivated twice by disc harrow. Seed was treated with a mixture of Helix® (Pioneer, Winnipeg, MB, Canada), Lumiderm™ (Corteva, Indianapolis, IN, US), and Butoe™ (Bayer CropScience, Leverkusen, Germany) at a concentration of 2.24 mL/100 g to prevent seed-borne diseases and insect damage. Seed were planted on May 21, with a soil temperature of 12 °C. On May 22, 2020, a weed control mixture of 0.3 L/ha Conqure® II Group 14 (pyraflufen-ethyl) (Nufarm, Winnipeg, MB, Canada), and Pardner® Group 6 (bromoxynil combo) (Bayer CropScience, Leverkusen, Germany) plus Roundup WeatherMAX 9® (Bayer CropScience, Leverkusen, Germany) in 1.7 L/ha was applied as a pre-emergence burn-off complex for weed suppression. Flea beetles (*Phyllotreta Cruciferae* Goeze.) were controlled with 1.5 L/ha of Matador® 120 EC Group 3A (Lambda-cyhalothrin) (Syngenta Canada, Guelph, ON, Canada) on May 29, 2020, at the 1-2 leaf

stage, with a subsequent application on June 12, August 11, and August 20, 2020. On June 4, 2020, 493.2 kg/ha of 33-12-0-5 (N-P-K-S) fertilizer was surface applied. On June 19, a mixture of Merge surfactant (1.0 %), 0.5 L/ha of Lontrel™ 360 (Dow AgroSciences, Midland, MI, USA), 0.7 L/ha of Poast® Ultra herbicide (BASF, Ludwigshafen, Germany), and 0.03 kg/ha Muster® (DuPont Canada, Mississauga, ON, Canada) was applied to control broadleaf and grassy weeds at the 2-3 leaf stage. Harvesting, utilizing a Wintersteiger small plot combine (Nursery Master Classic Wintersteiger, Salt Lake City, UT, US) was conducted on August 25, 2020. Seed was stored in a drying room until their moisture content was below 6 %, then manually cleaned using a spiral seed cleaner (Can-Seed Equipment Ltd, Saskatoon, SK, Canada). A 30 g sample per replication of the total yield was analyzed for seed quality.

The field methodology in Carman and Portage in 2021 were similar to those conducted in Carman in 2020. In Carman 2021, Edge® granular herbicide was applied at a rate of 27.9 kg/ha, and 33-12-0-5 (N-P-K-S) fertilizer at 504.2 kg/ha were applied prior to cultivation. In Portage, Edge® granular herbicide was applied at 27.9 kg/ha along with 33-12-0-5 (N-P-K-S) fertilizer at 504.4 kg/ha during cultivation. One week prior to planting in 2021, the seedbed was prepared using a vibra-shank cultivator followed by circular harrows. Treated seed were sown in Carman on May 11, 2021, and in Portage on May 14, 2021. At the time of sowing, the soil temperature at both locations was approximately 12 °C. For flea beetle control, Matador® 120 EC was applied at 0.2 L/ha on June 3 and June 15, 2021, in Carman and on June 1 and 8, 2021, in Portage. For weed management, a mixture of Lontrel™ 360 (0.5 L/ha) Poast® Ultra (0.7 L/ha) and Muster®

(0.03 kg/ha) was applied at the 2-3 leaf stage on June 14, 2021, in both locations. Due to the hot and dry weather in 2021, no insecticide was applied both in Carman and Portage. Manual weeding, targeting green foxtail (*Setaria viridis* L.), Canada thistle (*Cirsium arvense* L.), and other volunteer plants, was conducted twice per season in both Carman and Portage. The first weeding in Carman occurred on June 2 and July 15, 2021, while in Portage, it was carried out on June 26 and 29, 2021. The Carman experiment was harvested on September 6, 2021, and the Portage field on August 26, 2021. Both fields were harvested using a Wintersteiger small-plot combine in a straight-cut manner.

3.3.3. Seed Quality Analysis

The erucic acid content in the seed of each genotype was assessed using gas chromatography (GC) analysis, while total seed oil and protein content were tested through near-infrared spectroscopy (NIR) at the University of Manitoba's Seed Quality Lab. The University of Manitoba's Seed Quality Lab is annually certified by the Canadian Grain Commission.

Gas chromatography separates and analyzes vaporizable mixtures at varying temperatures. This process enables the identification and quantification of individual substances within the mixture (Hougen & Bodo, 1973; Liu, 1994). The analysis was performed using a Scion Instruments GC-430 (Scion Instruments, Austin, TX, US) equipped with a CP-Wax 52 CB capillary column and a flame ionization detector (Kim et al., 2007). The column featured a 0.025-micron polyethylene glycol phase coating on fused silica, measuring 15 m x 0.32 mm in diameter (Varian, Walnut Creek, CF, US). Gas

chromatography involved setting the column oven from 190 °C to 240 °C and the detector at 280 °C, utilizing ultra-high purity (UHP) helium carrier gas at a flow rate of 2.0 mL/min. A split vent ratio of 100:1 and a septum purge flow of 4.0 mL/min were established. Seed (300 mg) was finely crushed and placed in a 13 x 100 mm test tube, mixed with 5 mL of heptane, and left overnight for oil extraction. The subsequent supernatant was transferred to a new tube, combined with 1 mL of 0.5 N sodium methoxide reagent (10.8g sodium methylate powder in 500 mL anhydrous methanol), and shaken for 4 h on an Eberbach™ shaker (Eberbach Corporation, Belleville, MI, US) to transesterify triglycerides into fatty acid methyl esters. After adding 100 µL of 0.3 % acetic acid solution and gentle stirring, the mixture was left to stand at 4 °C for 1-2 hours. Then, 1.5 mL of the clarified solution was transferred to a 2 mL autosampler vial. The reference standard used was Nu-Check standard, code GLC # 421 (Nu-Chek Prep, Elysian, MN, US), with one standard example employed per every 20 samples to ensure accurate and precise fatty acid methyl ester sample preparation. Peak areas and erucic acid content was manually identified using the Varian Star Workstation software system version 6.0 (Varian, Walnut Creek, CA, US). Results are expressed as percentages, encompassing the content of 15 fatty acids ranging from C14 to C24.

Total oil and protein content in the seed was determined using a Foss NIR System (Model 6500) (Foss NIRSystems Inc, Laurel, MD, US). The process adhered to the protocols established by the American Oil Chemist's Society for evaluating seed protein, oil, and moisture content (Daun, 1994; Javed et al., 2016; Kim et al., 2007). In this procedure, 6 g seed samples were prepared and placed in standard ring cups for scanning

by the NIR system. The system gathered spectral data by measuring the reflectance of the seed across a wavelength range of 400 to 2500 nm. The 400 to 2500 nm wavelength range was chosen for the NIR system because it encompasses both the visible and near-infrared spectra, allowing for comprehensive analysis of the seed chemical composition and physical attributes. The WinISI II software (Version 1.04a) was employed to process and analyze the reflectance data statistically. The findings were reported as percentages of seed protein and oil in relation to the total weight of the whole dry seed (7-9 % moisture). All seeds were tested within 120 days post-harvest. One genotype was omitted from the phenotypic analysis due to abnormal growth or seed mix-up.

3.3.4. Statistical Analysis

Statistical analysis was conducted using SAS® version 9.4 (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was performed using PROC GLIMMIX to determine the statistical significance of the effects of genotype and environment on the multiple agronomic traits and erucic acid content in the total dry seed extracted oil. Genotype and environment were considered fixed effects, while replication nested within each environment, and the interaction between genotype and environment were treated as random effects. Additionally, this project explored the relationships among these agronomic traits by calculating Pearson correlation coefficients, providing insight into how these traits are interrelated within the CBLD2 population. The homogeneity of variance was assessed through SAS® PROC GLM to determine whether the data could be combined.

For data analysis, the best linear unbiased estimator (BLUEs) was utilized, conducted by the multi-environment trial analysis with R for Windows (META-R) software, version 6.0 (Biometrics and Statistics Unit, Genetic Resource Program, Batán, México & Plant Breeding, Genetics, and Biotechnology Program, Michigan State University, US). The BLUEs were used to plot unbiased linear estimator with the least variance (Alvarado et al., 2020; Maud & Shin, 1986). META-R facilitated the analysis of BLUEs, setting individual analyses and combining data across environments. When determining the BLUEs, genotypes and the covariate were treated as fixed effects, while all other factors were regarded as random effects (Alvarado et al., 2020). In field experiments, the primary advantage of using BLUEs was its ability to provide precise and unbiased estimates of fixed effects, enabling researchers to accurately isolate and understand the impact of specific controlled variables on phenotypic traits.

3.3.5. DNA Extraction and Prepare GBS Illumina Library.

3.3.5.1. DNA Extraction

DNA was extracted from leaves using a cetyltrimethylammonium bromide (CTAB) extraction (Tamari et al., 2013). Leaf samples (1-2 leaves per samples) were collected from plant at growth stage 3 (stem elongation or "bolting" stage) (Canola Council of Canada, 2017c). Approximately 0.5 g of fresh leaf tissue was placed in 1.5 mL microcentrifuge tubes and frozen in liquid nitrogen for 60 s. The leaf tissue was then ground to a fine powder using a plastic pestle. Subsequently, 500 µL of 2X CTAB buffer (2 % CTAB, 20 mM ethylenediaminetetraacetic acid, 100 mM tris-hydroxymethyl-

aminomethane, and 1.4 M sodium chloride, pH 7.5) was added to the ground leaf tissue and gently mixed. The samples were incubated at 65 °C for 60 min, followed by the addition of 500 µL of chloroform (isoamyl: alcohol 24:1). The samples were capped, shaken for 10 min, and centrifuged at 12,000 rpm for 10 min to separate phases. The supernatant was transferred to a new 1.5 mL tube, mixed gently with isopropanol (2-propanol) at 0.5 times the volume, and then centrifuged at 8000 rpm for 30 s to precipitate the DNA. The resultant DNA precipitate was washed twice with 800 µL of 70 % ethanol, centrifuged at 6300 rpm for 2 min, and air-dried overnight. The sediment was redissolved in 400 µL of distilled water.

For each genotype sample, the DNA solution was transferred to a 96-well plate. DNA samples were purified using Omega Mag-Bind@ TotalPure magnetic beads NGS (Opentrons Labworks, Norcross, GA, US). After bringing 0.4 times the volume of magnetic bead solution to room temperature then shaking, the magnetic bead solution was added to each well of the 96-well plate containing DNA samples, and the mixture was left for 15 min. The 96-well plate containing the DNA mixture was placed on the NEBNext® magnetic rack (New England Biolabs, Ipswich, MA, US) for over 30 s until the solution was clarified. The solution was discarded via pipetting, and the beads were washed once with 60 µL of 80 % ethanol and then air-dried overnight. The sample was redissolved in 50 µL of distilled water and stored at -20 °C.

3.3.5.2. PCR and GBS illumina® Library Prepared

Unlike standard GBS libraries protocol, which necessitates DNA cleavage by a restriction

enzyme (Elshire et al., 2011), the PCR-based modification protocol omits this enzyme cleavage step, replacing it with DNA fragment amplification via PCR. This method, based on the protocol published by Elshire et al. (2011), was modified by Dr. Genyi Li's lab at the University of Manitoba. Instead of using endonuclease restriction enzymes to digest genomic DNA and reduce genome complexity as in the published method, a two-round step PCR was employed across all genotypes in this project to simplify the *Brassica* genome.

In the first PCR round, a primer was prepared, comprising four short-length P5-end primers and six short-length P7-end primers. The newly formulated PCR Master Mix consisted of 8.6 µL double-distilled water (DDH2O), 1 µL 10X PCR buffer (500 mM KCl, 100 mM Tris, 1 % Triton, 1.5 mM $MgCl_2$, pH 9.3), 0.15 µL dNTPs, 0.15 µL forward primer, 0.15 µL reverse primer, and 0.15 µL taq polymerase. The PCR Master Mix solution was divided into 10 µL volumes in each well and placed into a 384-well plate. DNA samples were transferred to the 384-well plate using a stainless steel 96-needles replicator. The replicator was gently oscillated to ensure thorough mixing of DNA with the PCR Master Mix solution. After securing the plate cover, the PCR procedure was executed in the Eppendorf 950010061 MasterCycler® ep 384 Thermal Cycler (Eppendorf Canada, Mississauga, ON, Canada). PCR parameters were set as follows: an initial cycle at 94 °C for 3 min, cycle 2 at 94 °C for 50 s, cycle 3 at 35 °C for 50 s, and cycle 4 at 72 °C for 45 s. The entire PCR process was repeated 25 times **(Table 3.1)**.

The second round of PCR followed the first round, utilizing one P5-end primer and 186 P7-end primers. A new 384-well plate was employed, with preparation mirroring

Table 3.1 Steps of the first and second rounds of PCR for preparing GBS library of CBLD2 samples.

	Program	Time	Temperature	Cycle
PCR #1	Cycle 1: Initial Denaturation	180 s	94 °C	X25
	Cycle 2: Denature	50 s	94 °C	
	Cycle 3: Anneal primer	50 s	35 °C	
	Cycle 4: extend DNA	45 s	72 °C	
PCR #2	Cycle 1: Initial Denaturation	180 s	94 °C	X25
	Cycle 2: Denature	50 s	94 °C	
	Cycle 3: Anneal primer	50 s	50 °C	
	Cycle 4: Extend DNA	40 s	72 °C	

that of the first-round PCR Master Mix, albeit with altered forward and reverse primers. The stainless steel 192-needles replicator was immersed into the first-round PCR products, and DNA was transferred to the second-round PCR Master Mix solution. An aluminum foil PCR plate seal was applied before proceeding with the PCR. The PCR process parameters were largely consistent with the first round, with the exception that the temperature in cycle 3 was adjusted from 35 °C to 50 °C for 50 s, and the time in cycle 4 was reduced from 45 s to 40 s **(Table 3.1)**.

After the second round of PCR, all PCR products were pooled into 1.5 mL microcentrifuge tubes. The PCR products were checked using a 1 % agarose gel through electrophoresis to identify the PCR product. The PCR product was again purified by 1.2-time ratio Omega Mag-Bind@ magnetic bead adsorption.

3.3.5.3. DNA Quality

The NanoDrop™ 2000 spectrophotometer and its associated software (Thermo Fisher Scientific, Waltham, MA, US) were utilized for DNA quantity analysis. Initially, 2 µL of distilled water was loaded onto the pedestal as a blank. Subsequently, 2 µL of each DNA sample was applied using a micropipette. The quality of all samples was deemed acceptable based on absorbance measurements at 230 nanometers (nm), 260 nm, and 280 nm. Each genotype sample was measured three times, and average A 260/230, and A 260/280 ratios were calculated, with acceptable ratios being 1.8 - 2.0 and above 2.0, respectively. The 260/230 ratios indicated that the qualified samples were free from carbohydrate, phenol, chloroform, and the 260/280 ratios indicated the protein

contaminants (Thermo Scientific, 2000). Upon confirming the DNA's quality and purity, it was sent for off-campus pair-end second-generation sequencing.

3.3.5.4. Genotyping by Sequencing Bioinformatics.

Hiseq Nova 6000 sequencing machines performed the Illumina® sequencing. Sequencing analysis (single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) calling) was performed by Genoseq company (Genoseq, Wuhan, Hubei, RPC). The raw reads were filtered to obtain clean reads. Adapter sequences were removed from the reads using "Cutadapt" software (version 1.13) (Martin, 2018). Low-quality bases were removed from the reads using "Trimmomatic" software (version 0.36), with a sliding window of 4 bp to calculate the average quality, and all subsequent bases were removed if they were below 15 (Bolger et al., 2014). The "Dash" command was used to determine the length of the reads that needed to be greater than 50bp (Chhangawala et al., 2015). The burrow-wheeler aligner (BWA) software compares the differences between short sequences with low variance and the reference genome (Li & Durbin, 2010; Li & Homer, 2010). The "BWA-MEM" algorithm of BWA software (version 0.7.15-r1140) was selected to compare the sequencing data with the reference genome sequence (ZS11-v20200127) (http://cbi.hzau.edu.cn/cgi-bin/rape/download_ext). The comparison sequence was generated and converted to the BAM format. Reads in the BAM file was sorted using 'SortSam' from the Picard toolkit (version 1.91). 'SortSam' was used to count the coverage of bases on the reference genome, with the main criteria being genome coverage and coverage depth (Jiang et al., 2019; Sims et al., 2014).

Genome analysis toolkit (GATK) software was used for variant calling of high-throughput sequencing data (McKenna et al., 2010). For locating SNPs as well as InDels in DNA and RNAseq data, GATK was considered an industry standard (McKenna et al., 2010). Using GATK (version 3.7.0) software with the “HaplotypeCaller” module, the BAM files obtained from the comparison with the reference genes were processed to generate GVCF files. The “GenotypeGVCF” module was used to perform the SNPs and InDels detection. The GATK output variation information was saved in VCF format, containing all variants between the sample and the reference gene. Finally, the SNPs and InDels data were filtered separately by “hard-filtering germline short variants." The filtered files included multiple annotations to select specific thresholds. Variables that met the threshold were marked as "PASS," and those that did not meet the threshold were marked as "FAIL." Only variants marked as "PASS" were selected for the output file. For hard filtering, the output file was further manually filtered with the following conditions: 1) Markers that had missing data greater than 50 % were excluded; and 2) the relative heterozygosity of the offspring over 25 % was excluded.

3.3.5.5. Linkage Mapping Analysis

Since CBLD2 was a doubled haploid population, it followed Mendel's law of segregation, exhibiting a 1:1 segregation ratio. MapDisto software (version 1.7.7) was utilized to calculate and analyze the 1:1 segregation ratio (McCartney et al., 2016). The probability segregation ratio and segregation chi-square of loci were calculated using "segregation x2s", selecting markers that closely matched a 1:1 segregation ratio and culling

monomorphic markers from the parental type. The "Binning of Redundant Markers" function and its default settings in IciMapping software (version 4.1.0.0) were employed to group co-segregated markers into bins (Meng et al., 2015). One highly recommended SNP marker from each bin (with the least missing data) was selected and imported into MapDisto to generate a linkage map (Lorieux, 2012; McCartney et al., 2016; Meng et al., 2015). The "AutoMap" function in MapDisto software was used to identify linkage groups by gradually relaxing default parameters (McCartney et al., 2016). Map distances from recombination fractions applied the Kosambi mapping function (Kosambi, 1943), utilizing a strict minimum logarithm of the odds (LOD) and maximum recombination frequency thresholds. The LOD score was set at 3.0 and recombination frequency thresholds at 0.35 cM (ensuring the maximum distance between two markers did not exceed 35 cM) (Thambugala et al., 2020). The "Color Genotypes" function in MapDisto software was employed to check for double recombinants and identify genotyping errors, culling detected rare markers with substantial bad or missing data and converting single-occurrence double crossovers to missing data (McCartney et al., 2016; Thambugala et al., 2020). To enhance the linkage map's accuracy, the physical position of the markers in the reference genome (ZS11-v20200127) was verified against the position on the linkage map (McCartney et al., 2016; Thambugala et al., 2020).

3.3.5.6. Quantitative Trait Loci Mapping Analysis

QTL analysis was conducted using Qgene software (version 4.4.0), adhering to protocols detailed by Chitwood et al. (2016), Mcclean (2022), Wang et al. (2012), and modified

protocols based on personal communication with Dr. Harmeet Chawla (Chitwood et al., 2016; Mcclean, 2022; Wang et al., 2012; Gabur et al., 2020). The software's inclusive composite interval mapping (ICIM), analysis functions were utilized to plot QTL peaks (Jones et al., 2002; Li et al., 2008; Li et al.,2015; Javed et al., 2016). Following QTL mapping with a "scanned interval" of 0.1 cM, the default forward "cofactor selection function" was analyzed to mitigate the effects of background genetic variation or population structure, enhancing the accuracy and power of QTL detection by reducing potential bias or confounding effects from these background factors (Gabur et al., 2020; Joehanes & Nelson, 2008; Li et al., 2007).

Upon completion, the system provided the output of the percentage of phenotypic variation explained (R^2 value), LOD value, and additive effect value from selected analysis functions. Visual plots and data were exported from Qgene software. QTL additive effects were reported when the LOD value exceeded 3.0 in one or more environments with significant value or in one or more environments plus the combined dataset (Gupta et al., 2015; Khowaja et al., 2009; Thambugala et al., 2020). The QTL name '*qEA_CBLD2*', followed by the specific chromosome and a distinguishing number, was used to identify the erucic acid QTL in the CBLD2 population. This naming convention helped to differentiate QTL found on the same chromosome as well as those located in different chromosomes and locations. After QTL detection, the one LOD drop-off method was employed to estimate the QTL confidence interval (Visscher & Goddard, 2004). Finally, MapChart (version 2.32) software was used to produce charts of genetic linkage maps and QTL data for enhanced understanding (Voorrips, 2002).

3.4. Result

3.4.1. Phenotypic results

In the 2020 Carman experiment, the CBLD2 population exhibited seed erucic acid content ranging from 48.5 % to 55.8 %, with a mean value of 52.7 %. In 2021 at Carman, the seed erucic acid content ranged between 46.6 % and 57.2 %, with a mean of 51.9 %. In Portage 2021, the seed erucic acid content ranged from 46.4 % to 58.0 %, with a mean of 52.9 % **(Table 3.2)**.

Overall, the parent FP 268-2 exhibited a 3.4 % higher erucic acid content compared to MP 131037-6-8. The difference in erucic acid content between the two parents ranged from approximately 2–4 %. It is noteworthy that some genotypes demonstrated transgressive segregation, exhibiting extreme phenotypic characteristics that surpassed those of their parents. Forty-four genotypes showed a mean erucic acid content that exceeded that of FP 268-2, while 17 genotypes had a mean erucic acid content lower than that of MP 131037-6-8 (**Figure 3.1**).

The analysis of *B. napus* genotypes across different environments revealed significant variation in key agronomic traits (**Table 3.3**). This variation was particularly pronounced in traits like lodging in PT2021 and oil in both CM2021 and PT2021. Days-to-flower had a mean of 48.7 days, within a range of 45.4 to 53 days. Plant height had a mean of 88.7 cm, with in a range of 77.2 to 102.8 cm. Lodging had a mean of 2.2, with a range of 1.3 to 4. Days-to-maturity had a mean of 92.7 days with a range of 90 to 97.8 days. Oil content

Table 3.2 Erucic acid content of the 182 doubled haploid genotypes of the CBLD2 population and two parents from Carman 2020, Carman 2021, and Portage 2021 experiments.

Environment	MP CB131037-6-8	FP 268-2	Mid-Parent Point	DH		
				Min	Mean	Max
CM 2020[1]	51.0	54.4	52.7	48.5	52.8	55.8
CM 2021[2]	48.9	53.7	51.3	46.7	51.9	57.2
PT 2021[3]	50.8	52.9	51.9	46.4	52.9	58.0
Combined[4]	50.3	53.7	52.1	48.0	52.6	56.7

[1]**CM2020:** 2020, Carman, Manitoba, Canada
[2]**CM2021:** 2021, Carman, Manitoba, Canada
[3]**PT2021:** 2021, Portage La Prairie, Manitoba, Canada
[4]**Combined:** Combined data form three environments

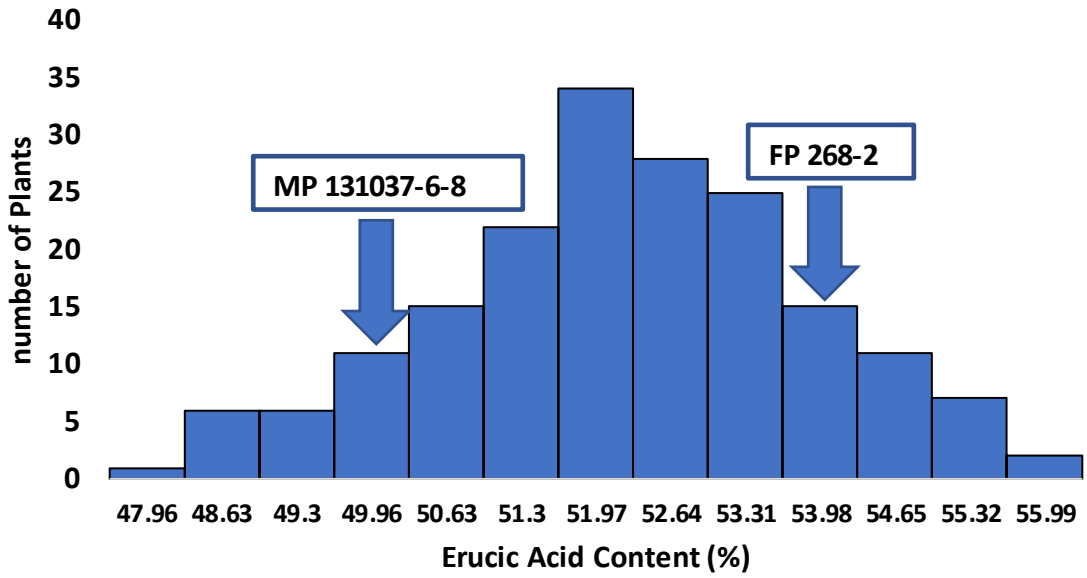


Figure 3.1 Histogram representing the erucic acid content in seed of 182 genotypes and two parental types in a CBLD2 doubled haploid mapping population: combined analysis of three environmental field experiments Carman 2020, Carman 2021, and Portage 2021 based on best linear unbiased estimator (BLUEs) data.

Table 3.3 Descriptive statistics for key agronomic traits including days-to-flower, plant height, lodging, days-to-maturity, oil, and protein content in the CBLD2 population across Carman 2020, Carman 2021, and Portage 2021 environments.

Simple Statistics					
Variable	**N[1]**	**Mean**	**Std Dev[2]**	**Minimum**	**Maximum**
meanFLR[3]	185	48.7	1.7	45.4	53
meanPH[4]	185	88.7	4.8	77.2	102.8
meanLODG[5]	185	2.2	0.5	1.3	4
meanMAT[6]	185	92.7	1.6	90	97.8
meanOIL[7]	185	43.6	2.2	37	48.3
meanPRO[8]	185	30	1.5	26.7	33.9

[1]**N:** Number of observations
[2]**Std Dev:** Standard deviations
[3]**FLR:** Days-to-flower - Days (Time from planting to flowering)
[4]**PH:** Plant height - Centimeters (Height of the plant)
[5]**LODG:** Lodge Value - Scale (1-5, Depending on the scoring system used for lodging resistance)
[6]**MAT:** Days-to-maturity - Days (Time from planting to maturity)
[7]**OIL:** Oil content in seed- Percentage (%) (Percentage of oil content in the seed)
[8]**PRO:** Protein content in seed - Percentage (%) (Percentage of protein content in the seed)

exhibited moderate variation, with a mean of 43.6 % and a range from 37 % to 48.3 %, while protein content had a mean of 30 % with a range of 26.7 % to 33.9 % (**Table 3.3**).

Analysis of variance (ANOVA) demonstrated a significant difference for genotype in all three environments (**Table 3.4**). These results indicated a substantial genetic influence on erucic acid accumulation in the seed. The environmental F-value was 0.6 (P<0.0001), indicating no significant differences in the combined environment. The consistency across environments underscored the inherent genetic control over erucic acid levels in the studied population. However, the interaction between genotype and environment (G*E) was significant, with an F-value of 1.7***, suggesting that genotype interaction with environmental significantly impacted erucic acid content **(Table 3.4)**.

3.4.2. Correlation Analysis of Agronomic Traits

In addition to the individual trait analyses, this research also explored the relationships between various agronomic traits within the CBLD2 population. To this end, the calculated Pearson correlation coefficients assess the strength and direction of associations between these traits. revealed notable correlations within the CBLD2 population's genetic makeup. A strong negative correlation between oil and protein content (-0.91***) and a positive correlation between days-to-flower and plant height (0.62***) were observed. Additionally, a correlation between lodging and protein content (0.20*) was identified. Erucic acid content displayed a moderate positive correlation with oil content (0.19***) and a weak negative correlation with days-to maturity (-0.09*), while showing no significant correlations with most other agronomic traits **(Table 3.5)**.

Table 3.4 Analysis of variance for days-to-flower, plant height, lodging, days-to-maturity, oil content, protein content and erucic acid content in *Brassica napus* genotypes, environment and genotype-by-environment for CBLD2 population in Carman 2020, Carman 2021, and Portage 2021.

Variation (Genotype)	EA[4]	FLR[5]	PH[6]	LODG[7]	MAT[8]	OIL[9]	PRO[10]
CM2020[1]	2.7***	7.4***	2.2***	3.4***	2.2***	3.5***	3.1***
CM2021[2]	1.8***	4.0***	2.3***	1.8***	2.2***	8.5***	4.5***
PT2021[3]	3.5***	6.1***	3.8***	43.5***	4.7***	14.2***	16.2***
Environment (combined)	0.6	2967***	84.2***	3.8	733.6** *	40.3***	50.2***
Genotype (combined)	3.7***	11.9***	5.6***	21.8***	4.3***	11.9***	7.9***
Genotype* Environment (combined)	1.7***	1.9***	1.5***	16.6***	1.5***	4.9***	3.6***

***P<0.001; **P<0.01; *P<0.05

Numbers represent the F-Values

[1]**CM2020:** 2020, Carman, Manitoba, Canada

[2]**CM2021:** 2021, Carman, Manitoba, Canada

[3]**PT2021:** 2021, Portage La Prairie, Manitoba, Canada

[4]**EA:** Erucic acid content in seed - Percentage (%) (Percentage of erucic acid in total oil content in the seed)

[5]**FLR:** Days-to-flower - Days (Time from planting to flowering)

[6]**PH:** Plant height - Centimeters (Height of the plant)

[7]**LODG:** Lodge value - Scale (1-5, depending on the scoring system used for lodging resistance)

[8]**MAT**: Days-to-maturity - Days (Time from planting to maturity)

[9]**OIL:** Oil content in seed - Percentage (%) (Percentage of oil content in the seed)

[10]**PRO:** Protein content in seed - Percentage (%) (Percentage of protein content in the seed)

Table 3.5 Pearson correlation coefficients among agronomic traits in the CBLD2 population: mean across Carman 2020, Carman 2021, and Portage 2021.

Pearson Correlation Coefficients							
	Mean FLR	**Mean PH**	**Mean LODG**	**Mean MAT**	**Mean OIL**	**Mean PRO**	**Mean EA**
Mean FLR[1]	1						
Mean PH[2]	0.62***	1					
Mean LODG[3]	-0.46***	-0.31***	1				
Mean MAT[4]	0.53***	0.46***	-0.02	1			
Mean OIL[5]	0.03	0.02	-0.23 ***	-0.54 ***	1		
Mean PRO[6]	0.05	0.03	0.20*	0.57 ***	-0.91***	1	
Mean EA[7]	-0.05	-0.09	0.1	-0.09*	0.19**	0.24	1

***P<0.001; **P<0.01; *P<0.05

digital number: Represent the strength and direction of the relationship between two variables

[1]**Mean FLR:** Mean days-to-flower - Days (Time from planting to flowering)

[2]**Mean PH:** Mean plant height - Centimeters (Height of the plant)

[3]**Mean LODG:** Mean lodging - Scale (1-5, depending on the scoring system used for lodging resistance)

[4]**Mean MAT:** Mean days-to-maturity - Days (Time from planting to maturity)

[5]**Mean OIL:** Mean oil content – Percentage (%) (Percentage of oil content in the seed)

[6]**Mean PRO:** Mean protein content – Percentage (%) (Percentage of protein content in the seed)

[7]**Mean EA:** Mean erucic acid content - Percentage (%) (Percentage of erucic acid in total oil content in the seed)

3.4.3. Marker Selection and Distribution Analysis

A total of 5,772 markers were obtained for CBLD2, of which 4,883 were SNPs and 889 were InDels. For the SNPs, 2,811 were conversion types A/G and C/T, while 2,072 were inversion types A/C, A/T, C/G and G/T, with a conversion inversion ratio (Ts/Tv) of 1.36. Table 3.6 presents the distribution and density of SNPs and InDels markers across the chromosomes in the CBLD2 population. The data showed considerable variation in the number and density of these genetic markers across different chromosomes. In terms of InDels, A10 and A07 exhibited the highest densities. The total genomic landscape showed a mean SNP density of 5.08 and InDel density of 0.93 per Mbp across the whole genome, indicating a heterogeneous distribution of these markers in the CBLD2 population **(Table 3.6)**.

3.4.4. Linkage Map

The genotypic data for the CBLD2 population was analyzed to determine the structure of the genetic map, with linkage groups generated using MapDisto (version 1.7.7). Smaller and physically misplaced linkage groups were removed, resulting in 20 linkage groups that covered 15 chromosomes. The high-throughput linkage maps were developed using the GBS protocol, selecting a total of 3822 markers and yielding 507 linkage bins. The number of linkage bins ranged from 3 on chromosome A06 to 64 on chromosome C01, averaging 26 linkage bins per linkage group and fixing the total map distance at 1312.5 cM. In some instances, one chromosome was represented by two linkage groups (**Table 3.7**).

Table 3.6 Distribution and density of SNP and Indel markers across chromosomes in the CBLD2 population.

Chr[1]	Length[2]	No. SNP[3]	SNP density[4]	No. InDels[5]	InDel density[6]
A01	38,004,428	116	3.05	25	0.66
A02	35,943,954	93	2.59	31	0.86
A03	44,868,710	384	8.56	87	1.94
A04	25,679,024	75	2.92	20	0.78
A05	45,991,561	330	7.18	36	0.78
A06	48,704,706	192	3.94	62	1.27
A07	32,302,721	288	8.92	58	1.8
A08	28,329,074	40	1.41	26	0.92
A09	65,862,748	94	1.43	39	0.59
A10	26,592,803	311	11.69	58	2.18
C01	57,880,920	543	9.38	69	1.19
C02	65,293,782	690	10.57	74	1.13
C03	79,061,710	367	4.64	58	0.73
C04	71,179,181	359	5.04	73	1.03
C05	59,550,008	387	6.5	61	1.02
C06	52,512,057	62	1.18	22	0.42
C07	60,986,212	250	4.1	29	0.48
C08	53,660,391	196	3.65	34	0.63
C09	68,416,614	106	1.55	27	0.39
Whole	960,820,60	4,883	5.08	889	0.93

[1]**Chr:** Chromosome number
[2]**Length:** Chromosome length (bp)
[3]**No. SNPs:** Numbers of SNP
[4]**SNP density:** Every 1Mbp has a number of single-nucleotide polymorphisms.
[5]**No. InDels:** Number of insertions and deletions
[6]**InDel density:** Every 1Mbp has a number of insertions and deletions

Table 3.7 Genetic map characteristics of the CBLD2 population: chromosome name, map length, bin number, mean and maximum bin intervals per chromosome, developed in MapDisto (version 1.7.7).

Chr[1]	Length (cM)[2]	No. bins[3]	Bin interval (cM)[4]	Max interval (cM)[5]
A02	4.92	4	1.23	2.20
A03.1	49.104	14	3.51	11.10
A03.2	91.179	33	2.76	25.50
A04	23.58	11	2.14	4.90
A05	78.95	37	2.13	16.40
A06.1	2.186	3	0.73	1.60
A06.2	37.92	22	1.72	8.80
A07	39.527	28	1.41	5.00
A10.1	79.139	29	2.73	11.40
A10.2	31.868	13	2.45	17.60
C01	130.142	64	2.03	22.00
C02	148.016	64	2.31	17.00
C03.1	70.249	26	2.70	26.90
C03.2	70.809	19	3.73	25.70
C04.1	87.392	32	2.73	15.80
C04.2	50.137	27	1.86	19.50
C05	107.446	27	3.98	24.80
C06	72.66	8	9.08	15.20
C07	104.97	27	3.89	15.60
C08	32.35	19	1.70	8.30

[1]**Chr:** Linkage group number (chromosome)
[2]**Length:** Linkage group total length
[3]**No. bins:** Total bins in each linkage group
[4]**Bin interval:** Mean distance between adjacent bins
[5]**Max interval:** Maximum distance between adjacent markers

3.4.5. Quantitative Trait Loci Analysis Result

Seven QTL for erucic acid content were found in CBLD2 DH population. The QTL named *qEA_CBLD2_A04, qEA_CBLD2_A07, qEA_CBLD2_A10, qEA_CBLD2_C01, qEA_CBLD2_C02, qEA_CBLD2_C04,* and *qEA_CBLD2_C08*, were detected on chromosomes A04, A07, A10, C01, C02, C04, and C08, respectively. Two QTL, *qEA_CBLD2_A04* and *qEA_CBLD2_A07* were consistently identified across all environments (CM2020, CM2021, PT2021, and the combined environments). The LOD scores ranged from 3.45 to 12.52 for *qEA_CBLD2_A04* and 3.44 to 8.98 for *qEA_CBLD2_A07*. *qEA_CBLD2_C01, qEA_CBLD2_C02, qEA_CBLD2_C04*, and *qEA_CBLD2_C08* also showed presence in multiple environments, indicating their stable influence on erucic acid content across different environments. The LOD scores and phenotypic variation explained values (R^2) suggest a moderate to high influence of these QTL on the trait, with *qEA_CBLD2_A04* in CM2021 showing a particularly high LOD score of 12.52 and explaining 27 % of the phenotypic variation (**Table 3.8 and Figure 3.2)**.

3.5. Discussion

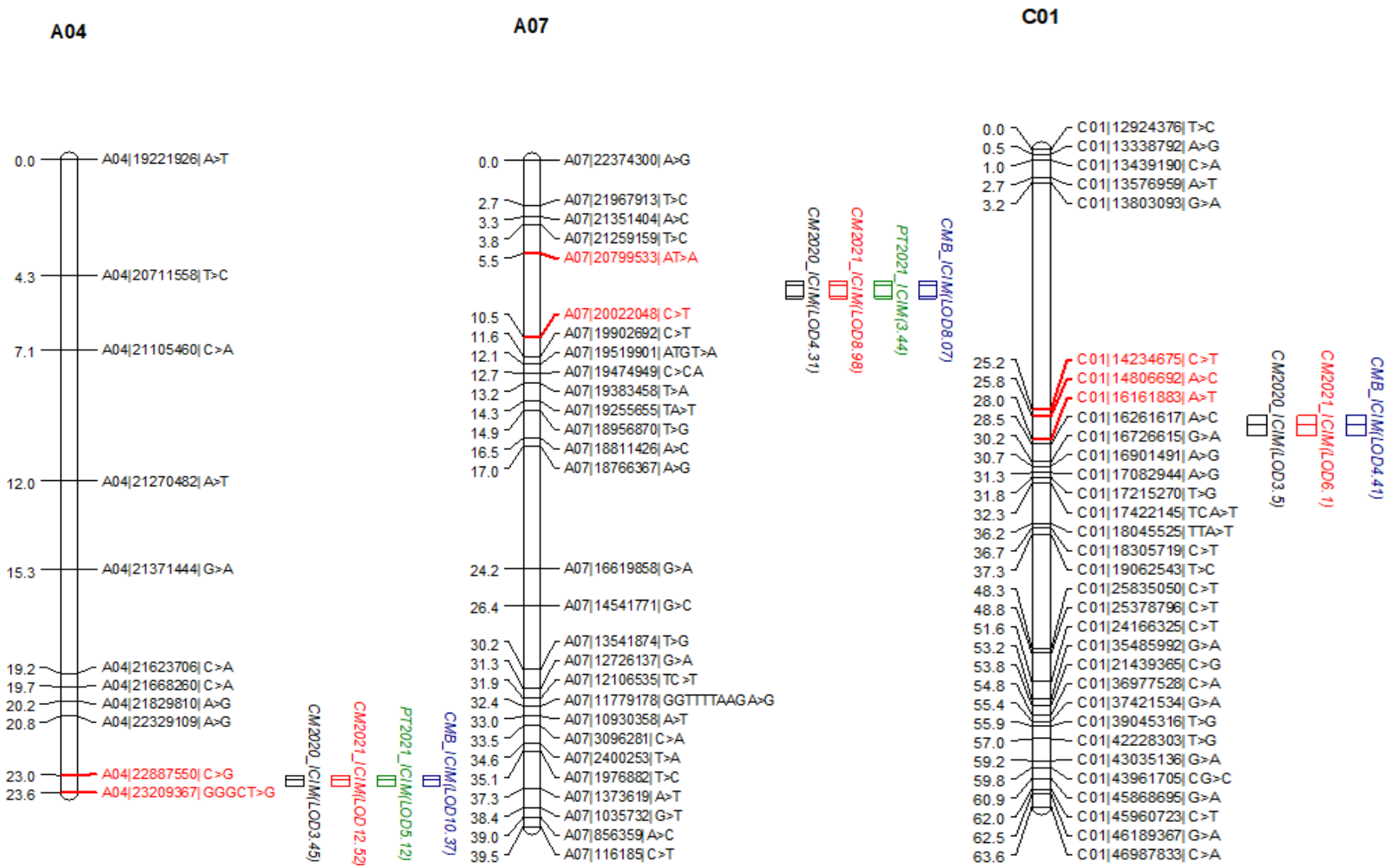
This research on B. napus focused on erucic acid, a key component in hundreds of industrial products. The research involved phenotyping and genotyping the CBLD2 DH population to create a high-density linkage map with 3822 markers spanning 1312.5 cM. Significant QTL for erucic acid content were identified on chromosomes A04, A07, C01, C02, C04, and C08. The research identified genetic loci affecting erucic acid content in

Table 3.8 Additive-effect QTL detected for erucic acid content in total oil extracted from a CBLD2 doubled haploid population dry seed using phenotypic data from three environments by using inclusive composite interval mapping and best linear unbiased estimator across Carman 2020, Carman 2021, and Portage 2021.

			ICIM[1]				Physical distance (bp)	
	Env.[2]	**Chr.[3]**	**Pos.[4]**	**Add.[5]**	**LOD[6]**	**R^2[7]**	**Left Flank**	**Right Flank**
qEA_CBLD2_A04	CM2020	A04	23.0-23.4	0.27	3.45	0.08	22,887,550	22,975,350
	CM2021	A04	23.0-23.4	0.83	12.52	0.27		
	PT2021	A04	23.0-23.4	0.41	5.12	0.12		
	CMB	A04	23.0-23.4	0.52	10.37	0.23		
qEA_CBLD2_A07	CM2020	A07	7.1-8.2	0.32	4.31	0.1	11,779,178	12,726,137
	CM2021	A07	7.1-8.2	0.71	8.98	0.21		
	PT2021	A07	7.1-8.2	0.35	3.44	0.08		
	CMB	A07	7.1-8.2	0.47	8.07	0.19		
qEA_CBLD2_A10	CM2020	A10	74.1-77.6	-0.26	2.92	0.07	12,924,376	25,489,607
qEA_CBLD2_C01	CM2020	C01	25.8-27.8	0.28	3.5	0.09	14,806,692	16,161,883
	CM2021	C01	25.8-28.5	0.58	6.1	0.14		
	CMB	C01	25.8-28.5	0.33	4.41	0.11		

qEA_CBLD2_C02	CM2020	C02	87.4-106.3	0.41	4.79	0.11	30,248,068	30,920,244
	CM2021	C02	88.7-105.6	0.53	3.71	0.09		
	CMB	C02	87.4-101.5	0.4	5.35	0.13		
qEA_CBLD2_C04	CM2020	C04	49.9-56.2	0.37	6.02	0.14	65,944,806	68,383,602
	CM2021	C04	50.1-62.7	0.57	5.23	0.13		
	CMB	C04	49.9-58.7	0.39	5.85	0.14		
qEA_CBLD2_C08	CM2020	C08	6.4-9.3	0.49	9.8	0.22	46,385,896	47,159,778
	CM2021	C08	2.5-7.8	0.43	3.11	0.08		
	CMB	C08	1.5-9.3	0.35	4.94	0.12		

[1]**ICIM**: Inclusive composite interval mapping
[2]**Env**: Environment
[3]**Chr**: Chromosome
[4]**Pos**: Position on the linkage group in centimorgans
[5]**LOD**: Logarithm of odds, $-\log_{10}$(p-value) the peak of LOD score
[6]**R^2**: Phenotypic variation explained (r^2; %)
[7]**Add**: Additive effect of allele substitution. A positive number indicated that the female parent allele increased the respective quantitative trait and vice-versa
CM2020: 2020, Carman, Manitoba, Canada
CM2021: 2021, Carman, Manitoba, Canada
PT2021: 2021, Portage La Prairie, Manitoba, Canada
CMB: Combined data set
Physical distance (bp): The marker starts position and ends position in physical map distance united by base pair


A04
0.0 A04|19221926|A>T
4.3 A04|20711558|T>C
7.1 A04|21105460|C>A
12.0 A04|21270482|A>T
15.3 A04|21371444|G>A
19.2 A04|21623706|C>A
19.7 A04|21668260|C>A
20.2 A04|21829810|A>G
20.8 A04|22329109|A>G
23.0 A04|22887550|C>G
23.6 A04|23209367|GGGCT>G
CM2020_ICIM(LOD3.45)
CM2021_ICIM(LOD12.52)
PT2021_ICIM(LOD5.12)
CMB_ICIM(LOD10.37)
A07
0.0 A07|22374300|A>G
2.7 A07|21967913|T>C
3.3 A07|21351404|A>C
3.8 A07|21259159|T>C
5.5 A07|20799533|AT>A
10.5 A07|20022048|C>T
11.6 A07|19902692|C>T
12.1 A07|19519901|ATGT>A
12.7 A07|19474949|C>CA
13.2 A07|19383458|T>A
14.3 A07|19255655|TA>T
14.9 A07|18956870|T>G
16.5 A07|18811426|A>C
17.0 A07|18766367|A>G
24.2 A07|16619858|G>A
26.4 A07|14541771|G>C
30.2 A07|13541874|T>G
31.3 A07|12726137|G>A
31.9 A07|12106535|TC>T
32.4 A07|11779178|GGTTTTAAG A>G
33.0 A07|10930358|A>T
33.5 A07|3096281|C>A
34.6 A07|2400253|T>A
35.1 A07|1976882|T>C
37.3 A07|1373619|A>T
38.4 A07|1035732|G>T
39.0 A07|856359|A>C
39.5 A07|116185|C>T
CM2020_ICIM(LOD4.31)
CM2021_ICIM(LOD8.98)
PT2021_ICIM(3.44)
CMB_ICIM(LOD8.07)
C01
0.0 C01|12924376|T>C
0.5 C01|13338792|A>G
1.0 C01|13439190|C>A
2.7 C01|13576959|A>T
3.2 C01|13803093|G>A
25.2 C01|14234675|C>T
25.8 C01|14806692|A>C
28.0 C01|16161883|A>T
28.5 C01|16261617|A>C
30.2 C01|16726615|G>A
30.7 C01|16901491|A>G
31.3 C01|17082944|A>G
31.8 C01|17215270|T>G
32.3 C01|17422145|TCA>T
36.2 C01|18045525|TTA>T
36.7 C01|18305719|C>T
37.3 C01|19062543|T>C
48.3 C01|25835050|C>T
48.8 C01|25378796|C>T
51.6 C01|24166325|C>T
53.2 C01|35485992|G>A
53.8 C01|21439365|C>G
54.8 C01|36977528|C>A
55.4 C01|37421534|G>A
55.9 C01|39045316|T>G
57.0 C01|42228303|T>G
59.2 C01|43035136|G>A
59.8 C01|43961705|CG>C
60.9 C01|45868695|G>A
62.0 C01|45960723|C>T
62.5 C01|46189367|G>A
63.6 C01|46987833|C>A
CM2020_ICIM(LOD3.5)
CM2021_ICIM(LOD6.1)
CMB_ICIM(LOD4.41)

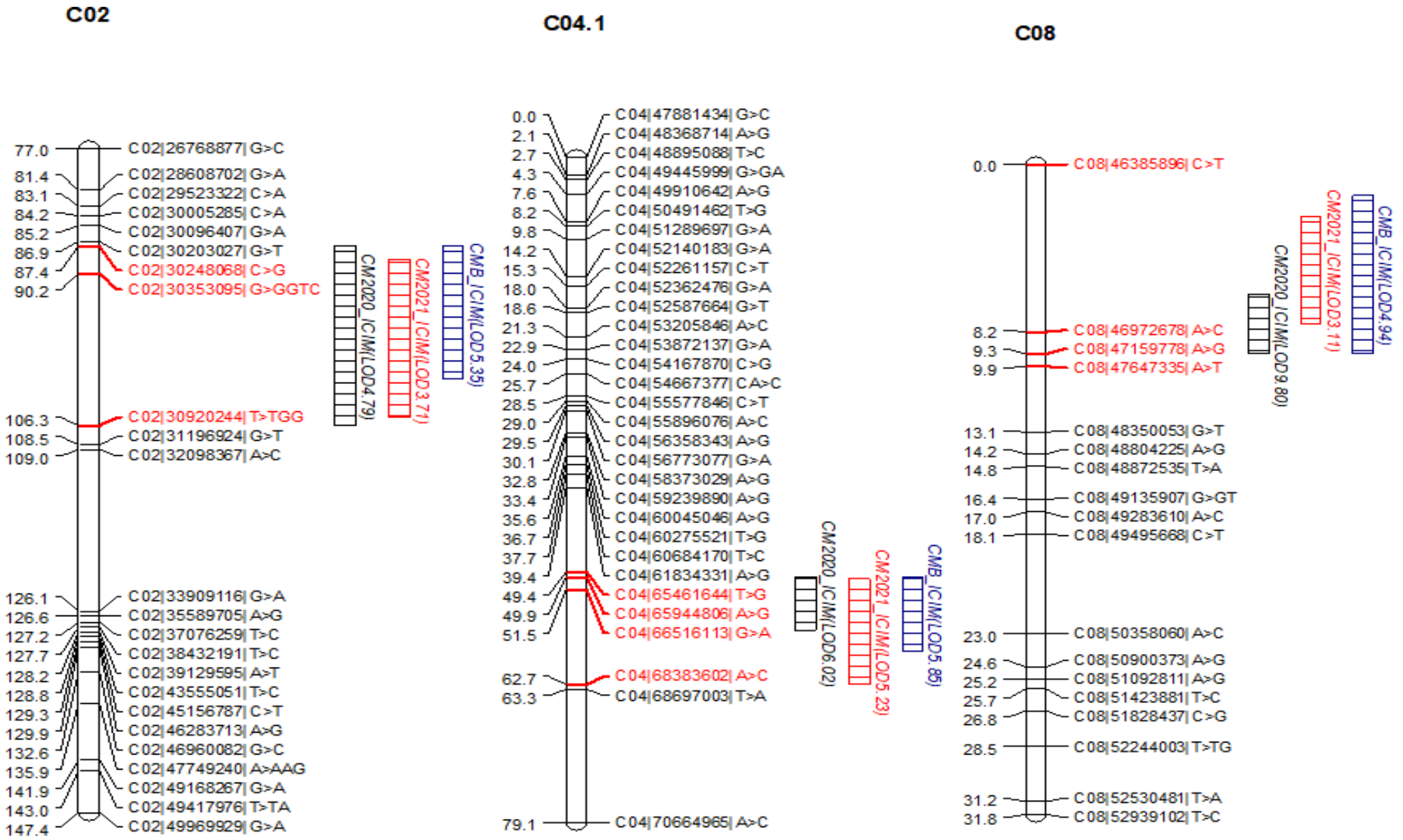

C02
77.0 C02|26768877|G>C
81.4 C02|28608702|G>A
83.1 C02|29523322|C>A
84.2 C02|30005285|C>A
85.2 C02|30096407|G>A
86.9 C02|30203027|G>T
87.4 C02|30248068|C>G
90.2 C02|30353095|G>GGTC
106.3 C02|30920244|T>TGG
108.5 C02|31196924|G>T
109.0 C02|32098367|A>C
126.1 C02|33909116|G>A
126.6 C02|35589705|A>G
127.2 C02|37076259|T>C
127.7 C02|38432191|T>C
128.2 C02|39129595|A>T
128.8 C02|43555051|T>C
129.3 C02|45156787|C>T
129.9 C02|46283713|A>G
132.6 C02|46960082|G>C
135.9 C02|47749240|A>AAG
141.9 C02|49168267|G>A
143.0 C02|49417976|T>TA
147.4 C02|49969929|G>A
CM2020_ICIM(LOD4.79)
CM2021_ICIM(LOD3.71)
CMB_ICIM(LOD5.35)
C04.1
0.0 C04|47881434|G>C
2.1 C04|48368714|A>G
2.7 C04|48895088|T>C
4.3 C04|49445999|G>GA
7.6 C04|49910642|A>G
8.2 C04|50491462|T>G
9.8 C04|51289697|G>A
14.2 C04|52140183|G>A
15.3 C04|52261157|C>T
18.0 C04|52362476|G>A
18.6 C04|52587664|G>T
21.3 C04|53205846|A>C
22.9 C04|53872137|G>A
24.0 C04|54167870|C>G
25.7 C04|54667377|CA>C
28.5 C04|55577846|C>T
29.0 C04|55896076|A>C
29.5 C04|56358343|A>G
30.1 C04|56773077|G>A
32.8 C04|58373029|A>G
33.4 C04|59239890|A>G
35.6 C04|60045046|A>G
36.7 C04|60275521|T>G
37.7 C04|60684170|T>C
39.4 C04|61834331|A>G
49.4 C04|65461644|T>G
49.9 C04|65944806|A>G
51.5 C04|66516113|G>A
62.7 C04|68383602|A>C
63.3 C04|68697003|T>A
79.1 C04|70664965|A>C
CM2020_ICIM(LOD6.02)
CM2021_ICIM(LOD5.23)
CMB_ICIM(LOD5.85)
C08
0.0 C08|46385896|C>T
8.2 C08|46972678|A>C
9.3 C08|47159778|A>G
9.9 C08|47647335|A>T
13.1 C08|48350053|G>T
14.2 C08|48804225|A>G
14.8 C08|48872535|T>A
16.4 C08|49135907|G>GT
17.0 C08|49283610|A>C
18.1 C08|49495668|C>T
23.0 C08|50358060|A>C
24.6 C08|50900373|A>G
25.2 C08|51092811|A>G
25.7 C08|51423881|T>C
26.8 C08|51828437|C>G
28.5 C08|52244003|T>TG
31.2 C08|52530481|T>A
31.8 C08|52939102|T>C
CM2020_ICIM(LOD9.80)
CM2021_ICIM(LOD3.11)
CMB_ICIM(LOD4.94)

Figure 3.2 Conditional quantitative trait loci (QTL) mapping for erucic acid content was conducted across linkage groups A04, A07, C01, C02, C04.1, and C08 in the CBLD2 population. This figure illustrates QTL for erucic acid content identified in Carman in the years 2020 and 2021 (CM2020, CM2021) and in Portage in 2021 (PT2021). Vertical bars denoted the linkage groups, with both genetic distances and physical positions alongside locus names and log of odds (LOD) values annotated adjacent to the bars. Regions with erucic acid QTL identified through conditional analysis were highlighted in red, with the LOD values providing a measure of the strength of the QTL signal.

B. napus, offering valuable markers for breeding programs aimed at optimizing the crop for high-quality erucic acid cultivar production.

These research findings aligned with the historical understanding that the erucic acid content in *B. napus* seed oil is determined by the embryo genotype (Harvey & Downey, 1964). Kondra & Stefansson (1965) and Jonsson (2009) suggested erucic acid is controlled by two major genes with multiple alleles functioning additively. This research differed from previous research, in that the population was developed from two parents that were both high in erucic acid content. Thus, this population has fixed the *FAE1* genes on chromosomes A08 and C03. This approach allowed us to identify minor QTL in genomic regions that are specifically associated with additive erucic acid content, independent of the well-characterized *FAE1* genes (Tang et al., 2021; Zhao et al., 2019). Previous *B. napus* erucic acid QTL research focused on experiments using parental genotypes with distinctly different erucic acid levels, typically involving HEAR × canola genotypes. Ecke et al. (1995) utilized genotype Mansholt's Hamburger Raps and Samourai as parent plants, focusing on high and low erucic acid cultivars though specific percentages were not detailed (Ecke et al., 1995). Burns et al. (2003) studied a DH population derived from Tapidor (low erucic acid) and Victor (high erucic acid) (Burns et al., 2003). Qiu et al. (2006) reported a significant contrast in erucic acid content between Tapidor (3.9 %) and Ninggyou7 (56.2 %) (Qiu et al., 2006). Cao et al. (2010) observed notable differences in the parental erucic acid levels of the TNDH population, with 4.2±0.9 % and 47.6±5.9 %, respectively. Subsequent studies, such as those by Zou et al. (2016), Zhao et al. (2019), and Guan et al. (2018), also focused on varying integrated

traits, analyzing genotypes with less than 3 % erucic acid in LEAR × LEAR crosses. However, Zhao et al. (2008) marked a shift by hybridizing Sollux (40.7 % erucic acid) and Gaoyou (36.4 % erucic acid), both genotypes with moderate levels of erucic acid. This study, involving 282 DH lines, identified minor QTL for erucic acid on several chromosomes (A01, A03, A09, C08, and C09), explaining 50 % of the phenotypic variance. Utilizing two HEAR genotypes as parents in the current study marked a distinct and novel approach, differing significantly from previous research that typically involved lower erucic acid content genotypes.

This research indicated a lack of strong correlation between erucic acid content and most other traits, suggesting that erucic acid content in *B. napus* was possibly independent of these agronomic characteristics. However, a notable exception was the slightly positive correlation observed between erucic acid and oil content. This interdependence implies that any modification in erucic acid levels could directly influence oil content, an aspect critical for breeding programs (Burns et al., 2003). Despite this, selectively modifying erucic acid levels without significantly impacting other key agronomic traits could be highly advantageous, allowing for the targeted improvement of specific seed characteristics while preserving overall agronomic quality (Soorni et al., 2022).

In this study, seven QTL related to erucic acid were identified in the CBLD2 DH population, with six exhibiting genetic associations across multiple environments (consensus QTL). The main QTL identified had LOD scores ranging from 3.11 to 12.52 and explained phenotypic variances ranging from 8 % to 27 %. The significance of the

identified major QTL may contribute to a deeper understanding of the genetic control of erucic acid and other fatty acids (Fan et al., 2018; McVetty & Scarth, 2002).

Previous research aligns with the identification of several additive effects of QTL found in the current research. The QTL *qEA_CBLD2_A04* was located within the physical interval of a QTL linked to eicosanoid acid (C20:1) as identified by Zhao et al. (2019), and overlaps with oil content QTL reported by Yusuf et al (2022).

In our study, the QTL *qEA_CBLD2_A07* was identified within the range of 11.8 to 12.7 Mbp. This finding closely aligns with the results reported by Guan et al. (2019), who identified oil content (OC) and linolenic acid (C18:3) QTL in a similar genomic region, specifically between 11.4 to 12.6 Mbp. This region also includes genes such as *BnaA07g12710D* (OC, Domain of unknown function (DUF702)), *BnaA07g14120D* (C18:3, Replication factor A, C-terminal), and *BnaA07g23550D* (OC, receptor like protein 11), as reported by Guan et al. (2019) and Tang et al. (2019). Additionally, *qEA_CBLD2_A07* was proximal to an erucic acid QTL physical interval, housing the candidate gene *BnaA07g05270D* (CLE for CLAVATA3/ESR-related), as noted in studies by Bilgrami et al. (2022), Guan et al. (2019), and Tang et al. (2019). In related research, Xu et al. (2015) identified the gene *BnaA07g21920D* (transferase activity, transferring acyl groups), which is physically located near the findings of this study at 17.06 Mbp. This gene is known to produce the enzyme *LPAAT4*, which plays a crucial role in enhancing erucic acid levels.

The *qEA_CBLD2_A10* QTL was found at the physical position at between 13 to 25.8 Mbp, which aligns with the seed oil content QTL on A10 at 16.9 Mbp, as found by Wang

et al., (2021), and also corresponds with the erucic acid QTL *Bn-A10-p16190708* at 15.4 Mbp (Qu et al., 2017), as well as other QTL that affected important fatty acid composition, such as *BnaA10g20970D* (AMP-dependent synthetase/ligase) at 14.5 Mbp and *BnaA10g21150D* (FAD binding) at 14.6 Mbp, that impact the C18:3 QTL on chromosome A10 (Zhu et al., 2019).

The QTL *qEA_CBLD2_C01*, identified at 13.5 Mbp in the current study, coincided with the erucic acid QTL range of 14.8 to 16.2 Mbp reported by Zou et al. (2016). In their research, Zou et al. (2016) also identified the erucic acid candidate gene *BnaC01g19560D* (ABC transporter) within this region. This QTL range also aligns with QTL for C18:3 and C18:1 (Bilgrami et al., 2022).

The QTL *qEA_CBLD2_C02*, identified in this study, was located on chromosome C02 of *B. napus*, spanning the 87.4-106.3 cM region, accounting for 9~13 % erucic acid phenotypic variation. This QTL aligns with earlier literature, where a QTL for oil content was mapped to the distal end of the linkage group on chromosome C02 (Qiu et al., 2006). The position of the erucic acid QTL (*qEA_CBLD2_C02*), identified in this study spans 30.2 to 30.9 Mbp on chromosome C02, and was previous found in GWAS studies focusing on multiple fatty acids, including C18:1, C18:2, and C18:3, as well as oil content (Qu et al., 2017). This physical location also aligns with the 30.0 Mbp position of a C18:3 fatty acid QTL identified by Zhu et al. (2019). However, it is important to note that this specific erucic acid QTL has not been previously reported.

This current research also identified the *qEA_CBLD2_C04* QTL on chromosome C04. Which accounts for 13~14 % of the phenotypic variation in erucic acid. This QTL was

previously unreported, possibly due to its low LOD score and minor contribution to phenotypic variation. This suggests *qEA_CBLD2_C04* might be a minor QTL with a subtle effect on erucic acid content, potentially more significant under specific conditions. Further investigation is needed to understand its role in the genetic control of erucic acid content in *B. napus.*

In our study, the QTL *qEA_CBLD2_C08* associated with erucic acid was identified within the range of 46.3 to 47.1 Mbp, accounting for 8 % to 22 % of the phenotypic variation. This finding is near the SNP related to erucic acid at 48.4 Mbp, as reported by Zhu et al. (2019), lending support to the significance of our discovery. This QTL location not only aligns with the oil content QTL identified by Zhu et al., (2019) but was also close to QTL for C16:0 and C16:1 fatty acids located at the 50.5 Mbp position on chromosome C08 (Yusuf et al., 2022). This overlap and close physical proximity suggested a complex interplay of genetic factors influencing fatty acid composition in this region of the *B. napus* genome.

The discovery of erucic acid QTL in *B. napus* is instrumental for breeding programs focused on augmenting erucic acid levels for industrial uses. The QTL identified in this work facilitates future marker-assisted selection, enabling breeders to efficiently select plants with high erucic acid traits early in the breeding cycle, significantly reducing time and resources. Additionally, a deeper genetic understanding is achieved, allowing scientists to precisely edit or regulate genes responsible for erucic acid synthesis, leading to genotypes with optimized fatty acid profiles. Furthermore, this knowledge of erucic acid QTL can be strategically leveraged in hybrid vigor optimization. By selecting parent

plants with complementary QTL that enhance erucic acid content, breeders can create hybrids that not only exhibit significantly increased erucic acid levels but also demonstrate improved overall agronomic performance, such as higher yield and greater stress resistance. This precise genetic targeting enables the development of hybrids that maximize the benefits of heterosis, ensuring both trait enhancement and overall plant health and productivity, thereby meeting HEAR industrial demand.

3.6. Conclusions

The goal of this research was to gain a deeper understanding of minor genes that influence erucic acid content in *B. napus* beyond the known major erucic acid genes. To date, published genetic mapping studies specifically on erucic acid content in *B. napus* are limited. While past literature has focused on how to reduce erucic acid content, there is a need for more research on erucic acid in high erucic acid *B. napus*. In this study, using a HEAR × HEAR DH population, seven QTL were identified, with QTL on chromosomes A04, A07, C02, C04, and C08 exhibiting expression across multiple environments. Future research should explore these chromosomes to better understand the control of erucic acid content. To further refine our understanding of these QTL, fine-mapping techniques should be employed to narrow down the genomic regions of interest, providing more precise locations of the genes involved. Additionally, the development and study of near-isogenic lines for these QTL regions could facilitate a detailed functional analysis, helping to determine the specific genetic contributions to erucic acid content. This focused

approach will enable breeders to more effectively utilize these loci in breeding programs, ultimately leading to the development of high erucic acid *B. napus* cultivars with enhanced agronomic traits. Many genetic regions identified in this study are considered novel and warrant further investigation. A genetic understanding of erucic acid production, coupled with the development of molecular markers ultimately usable for marker assistance selection, should improve selection for higher erucic acid content in *B. napus*. Using this information to implement genomics-based breeding strategies for erucic acid should generate new genetic material in *B. napus*, aiding the efficiency of commercial erucic acid production.

4. Discovery and Mapping of Quantitative Trait Loci Contributing to Erucic Acid Content in CBER2 Population Seed

4.1. Abstract

Brassica napus L. is a crop of considerable economic importance, largely due to its abundant oil yield and superior oil quality. Erucic acid is a long-chain monounsaturated fatty acid which is constituent of *B. napus* seed oil, dictating the end-use applications in the food industry and various industrial sectors. Notably, erucic acid is used for industrial purposes, serving as a lubricant and a valued raw material in contemporary production processes. Specifically, erucic acid is a key ingredient in the manufacture of biodegradable lubricants, nylon-13, and pharmaceuticals, where it is valued for its ability to provide high-temperature stability and low volatility. Additionally, it is used in the production of plasticizers and surfactants, which are crucial in creating flexible plastics and enhancing the cleaning power of detergents, respectively. This study aimed to uncover and characterize the minor quantitative trait loci (QTL) that contribute to elevated erucic acid levels in *B. napus,* thereby providing a foundation for enhancing its content through targeted plant breeding and genetic advancement strategies.

In the conducted research, a doubled haploid (DH) population CBER2, consisting of 190 genotypic individuals and two parental genotypes (Reston 40.6 % erucic acid content and ZSDH7970 57.9 % erucic acid content) was utilized. From 2021 to 2022, erucic acid and other agronomic traits were evaluated across three locations using a randomized

complete block design (RCBD). DNA extraction from leaf samples was performed, followed by modified genotyping-by-sequencing (GBS). A genetic map was then constructed based on detected SNPs and InDels. Using inclusive composite interval mapping (ICIM), multiple QTL associated with seed erucic acid content were identified, distributed across chromosomes A01, A02, A05, C06, and C09. Stable QTL (consensus QTL) were located on chromosomes A01, and A05 in various environments. Specifically, the QTL *qEA_CBER2_A01* on chromosome A01 was situated between 48.4 and 55.6 cM, explaining 7-9 % of the phenotypic variance (R^2). On chromosome A02, QTL *qEA_CBER2_A02* was identified between 19.4 to 27.1 cM, accounting for 7-9 % of the phenotypic variance. Finally, QTL *qEA_CBER2_A05* on chromosome A05, ranging from 6.1 to 9.9 cM, was responsible for 11-18 % of the phenotypic variance. The findings from this study underscore the complex genetic architecture underlying erucic acid synthesis in *B. napus,* providing crucial insight for the development of cultivars with optimized erucic acid content.

4.2. Introduction

Brassica napus L. is a major global oilseed crop and is Canada's second-largest crop after wheat (Leff et al., 2004; Ramchiary & Kole, 2017; Statistics Canada, 2022). In 2022, the *B. napus* industry impacted the Canadian economy by $29.9 billion, supporting over 200,000 jobs and 140,000 families (Canola Council of Canada, 2023). Breeding efforts have focused on enhancing *B. napus* seed quality, yield, oil content, and fatty acid

composition (McVetty & Scarth 2002; Roscoe et al., 2001; Fard et al., 2018).

Erucic acid is a long-chain fatty acid, occurs naturally in the plant seed of *Brassicaceae* and *Tropaeolaceae* families (Wang et al., 2022). It is mainly extracted from *B. napus* seed oil and fish liver oil (Lundebye et al., 2017). Due to environmental concerns and growing demand, relying on aquaculture for erucic acid is unsustainable, making extraction from seed more economical and viable (Ackman. 2007; Knutsen et al., 2016). Wild-type *B. napus* (rapeseed), historically cultivated for its high erucic acid content, is ideal for industrial uses, such as in lubricants and plastics (McVetty & Duncan, 2015; Wu et al., 2008). However, the recognition of erucic acid's adverse health effects in the 1970s led to the development of low-erucic acid canola cultivars (Malabat et al., 2003).

Erucic acid, which is formed by extending oleic acid, is produced in rapeseed oil through the combined actions of two crucial loci: fatty acid elongase 1.1 (*FAE1.1*) and fatty acid elongase 1.2 (*FAE1.2*) (Cao et al., 2010). The *FAE1* locus is responsible for encoding 3-ketoacyl-CoA synthase (KCS), a crucial enzyme in the elongation process of very long-chain fatty acids, as detailed in studies by Li et al. (2013, 2014) and Wang et al. (2022). Furthermore, *FAE1* is believed to encode ketoacyl-CoA thioesterase, which plays a role, although varying across different plant species, in elongating long-chain fatty acids (Gao et al., 2009). In certain plants, *FAE1* is also involved in extending medium-chain fatty acids, including C16, C18, C20 (Li et al., 2014). In *B. napus*, the erucic acid content is predominantly regulated by the combined activities of *FAE1.1* and *FAE1.2*. However, the synthesis of erucic acid is not limited to these two genes alone. Wang et al. (2013) proposed that 3-Ketoacyl-Acp synthase 2 (KAS2) and 3-Ketoacyl-Acp synthase 3 (KAS3)

might also be involved in this process, with dysfunctional *KAS2* and *KAS3* potentially altering erucic acid levels (Wang et al., 2013). Furthermore, research indicates that silencing both *FAE1* and fatty acid desaturases 2 (FAD2) genes can markedly reduce erucic acid content while still retaining a minor amount of it, and simultaneously increase the oleic acid content in seed oil to over 75 % (Dar et al., 2017; Peng et al., 2010). This intricate genetic network underscores the complexity of fatty acid biosynthesis in *B. napus* and its implications for agricultural and industrial applications.

Early studies on QTL for erucic acid content in *B. napus* have led to the identification of genes impacting its production. The TNDH population, after a two-year field experiments produced from high and low erucic acid parents, revealed that chromosomes A08 and C03 contain the major QTL accounting for 45.1 % and 30.4 % of erucic acid variation, respectively (Qiu et al., 2006). Minor QTL on chromosomes A01 and A02 were also identified, contributing to 5.8 % and 6.6 % of the variation (Qiu et al., 2006). The influence of the environment on these QTL has been well-documented, with significant QTL-environment interactions (Cao et al., 2001). Additional QTL for erucic acid were sporadically found on chromosomes A10 and A06, varying with environmental conditions (Cao et al., 2021). Subsequent research confirmed that *B. napus* linkage groups A01, A08, and C03 include the *FAE1* loci, encoding the enzyme β-ketoacyl-CoA synthase (Qiu et al., 2006).

A comprehensive association mapping analysis involving 435 individual experiments identified 16 quantitative trait nucleotides (QTNs) associated with erucic acid content across various chromosomes in *B. napus* (Guan et al., 2019). Notably, two QTNs on

chromosomes A08 and C03 emerged as primary genetic regions influencing erucic acid content in *B. napus* seed. Additional significant QTNs were identified on chromosomes A09 and C08, varying across different individuals and environmental conditions. This analysis also pinpointed candidate genes, including *BnaA08g1130D* and *BnaC03g65980D*, as potential paralogs of *KCS18*, and *BnaA08g1140D* and *BnaC03g66040D*, related to *KCS17* (Guan et al., 2019).

Moreover, association mapping in winter *B. napus* cultivars revealed QTL for erucic acid content, which did not segregate in the analyzed plant material (Honsdorf et al., 2010). Zhao et al. (2008) reported non-localized QTL for oleic and linolenic acids in crosses between two high erucic acid genotypes, suggesting that the variation in QTL numbers for each trait might reflect the underlying genetic structure (Zhao et al., 2008). While some QTL are associated with both oil and erucic acid content variations, the direct relationship between these traits is yet to be confirmed (Singh et al., 2023; Zhao et al., 2008). However, it is evident that each QTL for erucic acid content could potentially influence or be influenced by multiple fatty acid traits (Wang et al., 2013; Xiao et al., 2019). Ongoing research continues to focus on clarifying the complex network of QTL affecting erucic acid levels in *B. napus.*

On the basis of the escalating industrial need for erucic acid, boosting its concentration within *B. napus* seed emerges as a goal in rapeseed breeding (Wang et al., 2022). The objective of this study focuses on uncovering minor QTL influencing the erucic acid content in seed, thereby facilitating the creation of *B. napus* cultivars with elevated levels of erucic acid.

4.3. Materials and Methods

4.3.1. Plant Material

The CBER2 population was derived from crossing the female parent (FP) Reston and male parent (MP) ZSDH7970. Reston is cultivar developed and registered by the University of Manitoba in 1982 with the registration number 2190, and emerged from selective breeding and hybridization among Tower, Regent and Altex cultivars (McVetty et al., 2000; So & Duncan, 2021). On the other hand, ZSDH7970 is a male doubled haploid genotype, selected from the Nnx25 population for its highest erucic acid content. In terms of erucic acid levels in seed oil, Reston exhibited a mean erucic acid content of 40.6 %, while ZSDH7970 had a content of 57.9 %. Pollen was collected from the F_1 for microspore culture to generate doubled haploids (Rajcan et al., 2011; Weber et al., 2005). In this study, DH population samples were generated and subsequently propagated in the greenhouse.

4.3.2. Field Evaluation

4.3.2.1. Field Experiments

The doubled haploid CBER2 population comprised of 190 genotypes, was cultivated in the field across four environments in 2021 and 2022. In both years, CBER2 was planted at two experimental sites: the Ian N. Morrison Research Farm, Block 6E (49.492538, -98.032599) and the Portage La Prairie Research Farm in Portage, MB (50.0636349, -98.3997980). Each experiment was conducted in a randomized complete block design (RCBD), adhering to the protocols outlined in Section 2.3.2 of Chapter 2. The soil

composition in Carman has been described as 'Loamy Lacustrine' by Angad & Entz (2002), with its taxonomy falling under the category of fine-loamy, mixed, superactive, thermic Udic Argiustolls according to the USDA (2022). Meanwhile, the soil found in Portage is referred to as 'Clay Lacustrine' by the Government of Canada (2017), with a taxonomic classification as very fine, smectitic, mesic Vertic Endoaquolls (USDA, 2022).

The methodology in the 2021 field experiments was previously described in section 2.3.2.. In 2022 Carman, the seedbed was prepared by a double disk in fall 2021, Edge® granular herbicide (Gowan Canada, Winnipeg, MB, Canada) and 504.4 kg/ha of 33-12-0-5 (N-P-K-S) fertilizer was applied during cultivation. Seed was treated with a Helix® (Pioneer, Winnipeg, MB, Canada), Lumiderm™ (Corteva, Indianapolis, IN, US), and Buteo™ (Bayer CropScience, Leverkusen, Germany) mixture (2.24 mL/100 g). Land preparation prior to sowing involved a single pass with a vibra-shank cultivator equipped with a circular harrow one week before seeding. Seeding occurred on May 23, 2022 (soil temperature about 13 ^{o}C), with 3 m rows at 0.4 m spacing apart. Flea beetles (*Phyllotreta Cruciferae* Goeze.) were not chemically controlled in Carman 2022. On June 21 (at the 2-3 leaf stage), a tank mix of Merge surfactant (1.0 %), 0.5 L/ha of Lontrel™ 360 (Dow AgroSciences, Midland, MI, USA), 0.7 L/ha of Poast® Ultra herbicide (BASF, Ludwigshafen, Germany), and 0.03 kg/ha of Muster® (DuPont Canada, Mississauga, ON, Canada) was applied for weed management. Proline fungicide was sprayed on July 22, 2022, with rate of 0.4 L/ha, to combat with sclerotinia stem rot (*Sclerotinia sclerotiorum* Lib. de Bary). Manual weeding of Green Foxtail (*Setaria viridis* L.) and other volunteer plants was performed three times during growth season. On September 8, 2022, a tank mixes

desiccant treatment consisting of Heat® LQ (BASF, Mississauga, ON, Canada), Roundup WeatherMAX 9® (Bayer, Calgary, Alberta, Canada) and merge® adjuvant (BASF, Mississauga, ON, Canada) in 1.7 L/ha was applied. Straight cut harvesting was conducted on September 20, 2022, using a Wintersteiger small-plot combine (Nursery Master Classic Wintersteiger, Salt Lake City, UT, US).

The field methodologies employed in Portage during 2021 mirrored those described in section 2.3.2 Field Evaluation of the pervious chapter, adhering to the same procedures as those applied to the CBLD2 population in Portage. Portage's spring 2022 seedbed was prepared with two passes of a disc harrow and treated with Edge® granular herbicide with 504.4 kg/ha of 33-12-0-5 (N-P-K-S) fertilizer. Seed was treated with a Helix®, Lumiderm™, and Buteo™ mixture at 2.24 ml/100 g and planted on May 26, 2022, in 3 m rows spaced 0.4 m apart at a soil temperature of approximately 14 °C. Flea beetles were controlled with Matador® 120 EC (Lambda-cyhalothrin) (Syngenta Canada, Guelph, Ontario, Canada) at 0.1 L/ha on June 7, 2022 (cotyledon to 2nd leaf stage) and June 11, 2022 (1 to 4 leaf stage). Broadleaf and grassy weeds were managed with a tank mix of 1 % of Merge surfactant, 0.5 L/ha Lontrel™ 360, 0.7 L/ha of Poast® Ultra and 0.03 kg/ha Muster® applied on June 23, 2022 (2-4 leaf stage). Proline fungicide at 0.4 L/ha was sprayed on July 12, 2022, to control sclerotinia stem rot. Hand weeding was conducted twice during the growth season, Additionally, inter-row cultivation was done on June 23, 2022. A combination of Heat® LQ herbicide, Roundup WeatherMAX 9® and merge® adjuvant desiccant cocktail in 1.7 L/ha was applied as a desiccant treatment on

September 1, 2022. Harvest occurred on September 10, 2022, by straight cut using a Wintersteiger small-plot combine.

4.3.2.2. Greenhouse Experiments

Greenhouse experiments were conducted during the 2022 spring within the University of Manitoba's greenhouse complex (49.8064146007, -97.1352683805). This study was designed under controlled environmental conditions with a RCBD protocol. Each genotype was replicated three times, with each replication consisting of three plants.

The greenhouse experiment commenced on January 13, 2022, with seed sown 2.0 cm deep in 4 x 3 well cell packs (13 cm x 13 cm x 5 cm) filled with Sunshine Metro Mix potting soil (Sungro Horticulture, Vassar, MB, Canada). Seedlings were grown in a growth chamber at a constant 20 °C, with a 16-hour light/8-hour dark cycle for 14-18 days, receiving 20-20-20 (N-P-K) fertilizer (concentration of 7 g/3.78 L) at 10 days after planting (DAP) to encourage root establishment. At the 2-3 leaf stage, on February 1, 2022, each seedling was potted individually into larger plastic growers pots (14.5 cm x 15 cm) and subsequently moved to argus environmental control greenhouse (Argus-controls, Winnipeg, MB, Canada) with average temperatures of 22 °C daytime and 21 °C nighttime, humidity ranging from 23 % to 61 %, and a light cycle from 6 a.m. to 10 p.m. Light intensity, measured only from sunlight, averaged 160 μmol/m^2/s. The automatic irrigation system, initiated at 30 DAPs, was scheduled for three times daily, with durations adjusted according to growth stage. Post-flowering, irrigation was gradually reduced and ceased upon plant maturity. Fertilization occurred twice: post-transplant

(20 DAP) and during flowering (50 DAP), using Plant-Prod® water-soluble fertilizer (10-52-10) (Royal Brinkman Canada, Guelph, ON, Canada) at 15 g/3.78 L concentration. Verimark® insecticide (FCM, Mississauga, ON, Canada) was applied at a rate of 5 mL per 250 plants at 20 DAPs, for aphid and thrip control, Orthenex® 97 % SG systemic insecticide (Arysta LifeScience North American, Cary, NC, US) was used from six leaf to bolting, followed by a soil application of Beleaf® 50SG Insecticide (FCM, Mississauga, ON, Canada) at the flowering stage. Sulfur fumigation was employed at night to manage powdery mildew (*Blumeria graminis* f. sp. hordei). All treatments adhered to the manufacturers' label instructions. Harvest occurred on May 26 to June 4, 2022, when 60 % of seed color transitioned from green to brown, following the Canola Council of Canada's guidelines (Canola Council of Canada, 2012). Seed were first harvested from three plants of the same genotype dried at 40 °C for one day to reduce moisture content. After drying, a 0.2 g sample of seed was taken from each replication. These samples were then combined into a single batch for subsequent analysis of erucic acid content.

4.3.3. Seed Quality Analysis

This experiment assessed the erucic acid content in seed using gas chromatography (GC). These analyses were conducted at the University of Manitoba's Seed Quality Lab, which adheres to standards certified by the Canadian Grain Commission. For a detailed description of the methodologies, please refer to Chapter 2, Section 2.3.3.

4.3.4. Statistical Analysis

The statistical significance of genotype and environmental effects on agronomic traits and erucic acid content was determined using SAS® version 9.4, employing ANOVA with PROC GLIMMIX. This was supplemented by analysis of Pearson correlation coefficients to explore trait interrelations within the population. The best linear unbiased estimator (BLUEs) was calculated using META-R software to provide precise estimates of fixed effects across multiple environments. For a detailed description of the methodologies, please refer to Chapter 2, Section 2.3.4.

4.3.5. DNA Extraction and GBS Illumina Library Preparation

DNA was extracted from plant leaves using a CTAB method, followed by the preparation of a GBS Illumina library without the need for restriction enzyme digestion which simplified the *Brassica* genome's complexity. This PCR-based approach facilitated the amplification of DNA fragments for subsequent analysis. For a detailed description of the methodologies, please refer to Chapter 2, Section 2.3.5.

4.3.6. Advanced Genetic Analyses

Further genetic analyses included genotyping by sequencing (GBS), quality checks of DNA samples, and bioinformatics processing to identify SNPs and InDels. Linkage mapping and QTL mapping analyses were conducted to explore genetic markers and traits associated with erucic acid content. These processes utilized software and methodologies, such as MapDisto for linkage mapping and Qgene for QTL analysis, ensuring a comprehensive

genetic evaluation of the CBER2 population. For detailed descriptions of these methods, please refer to Chapter 2, Sections 2.3.5.1 to 2.3.5.6.

4.4. Results

4.4.1. Phenotypic Results

In the 2021 Carman environment, erucic acid levels varied from 40 % to 61.2 % of the total oil extracted from seed, with a mean of 53.6 % **(Table 4.1)**. In Portage, the erucic acid range was 40.4 % to 59.5 %, with a mean of 52.4 %. For 2022, Carman seed had a range of 42.2 % to 58.4 % erucic acid, with a mean of 52.5 %, while 2022 Portage seed ranged from 40.4 % to 59 %, with a mean of 53.6 %. Greenhouse seed in 2022 showed a range of 39.2 % to 59.5 %, with a mean of 54.8 % **(table 4.1).**

Field experiments conducted in Carman and Portage revealed a 16.2 % mean erucic acid content difference between the parents. In 2021 at Carman, the FP Reston had an erucic acid content of 40.8 %, while MP CB_ZSDH7970 exhibited a higher content of 60.5 %. That same year in Portage, FP Reston presented an erucic acid percentage of 40.4 % in contrast to the MP CB_ZSDH7970 (55.3 %). In 2022, the experiments in these locations showed the FP Reston with an erucic acid content of 39.9 % in Carman and 40.6 % in Portage, whereas the MP CB_ZSDH7970 had an erucic acid content of 54.6 % in Carman and 56.2 % in Portage. Most DH genotypes exhibited erucic acid levels that were intermediate to those of the parents, with minimal transgressive segregation observed **(Figure 4.1)**. Given the unexpectedly low erucic acid content recorded for the MP

Table 4.1 Summary of percentage of erucic acid content in 190 doubled haploid genotypes of the CBER2 population and two parents from Carman 2021, Carman 2022, Portage 2021, Portage 2022, and Greenhouse 2022.

Environment	FP Reston	MP CB_ZSDH7970	Mid-Parent Point	DH		
				Min	Mean	Max
CM2021[1]	40.8	60.5	50.7	40.0	53.6	61.2
PT2021[2]	40.4	55.3	47.8	40.4	52.4	59.5
CM 2022[3]	39.9	54.6	47.3	42.2	52.5	58.4
PT 2022[4]	40.6	56.2	48.4	40.4	53.6	59.0
GH 2022[5]	41.2	-	-	39.2	54.8	59.5

[1]**CM2021: 2021:** Carman, Manitoba, Canada
[2]**CM2022: 2022:** Carman, Manitoba, Canada
[3]**PT2021: 2021:** Portage La Prairie, Manitoba, Canada
[4]**PT2022: 2022:** Portage La Prairie, Manitoba, Canada
[5]**GH2022: 2022:** University of Manitoba, Greenhouse Research Complex[6]-: Data for the male parent in the Greenhouse 2022 environment is not available

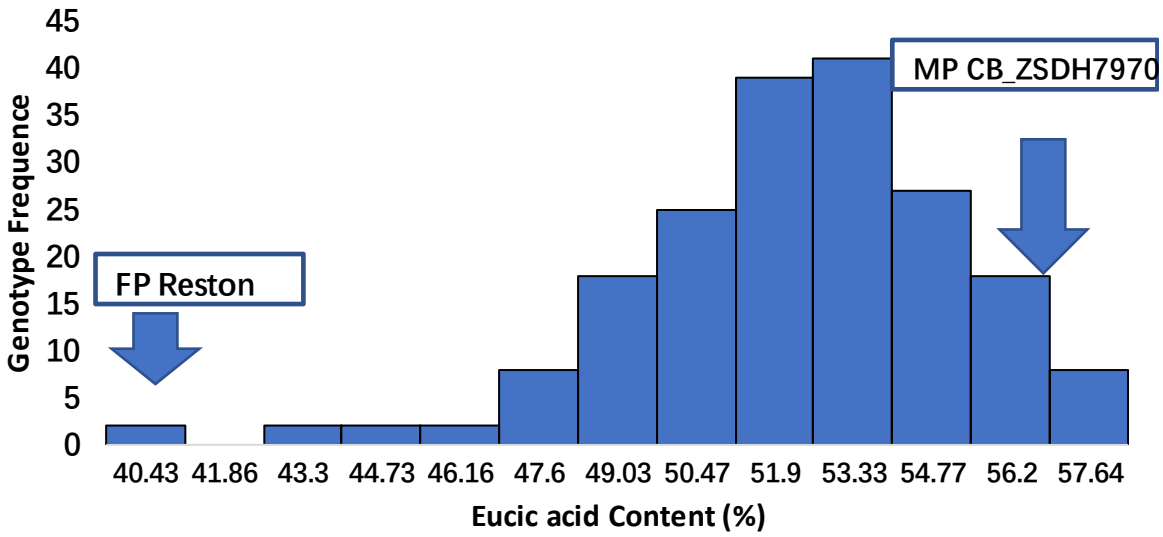


Figure 4.1 Histogram distribution of erucic acid content in seed of 190 genotypes and the two parents for the CBER2 doubled haploid mapping population: combined analysis of four environmental field experiments Carman 2021, Portage 2021, Carman 2022, and Portage 2022 based on best linear unbiased estimator (BLUEs) data.

CB_ZSDH7970 in the 2022 greenhouse data, it is suspected that there was an error in the identification of this parent genotype. Typically, this parent is known for higher erucic acid levels, therefore, the observed inconsistency strongly suggests a misidentification. To ensure the integrity of our study, results pertaining to this questionable data set have been excluded from the analysis (**Figure 4.2)**.

Across all four field environments, days-to-flower exhibited a mean of 49.4 days, with a range from 44.6 to 58.5 days **(Table 4.2)**. Plant height displayed a mean of 92.6 cm, extending from 73.3 to 116 cm. Lodging had a mean score of 3.5, with values spanning from 2.3 to 4.2. Days-to-maturity showed a mean of 94.6 days, with a duration from 91.5 to 97.6 days. Oil content in the seed had a mean of 38.4 %, ranging from 31.9 % to 44.7 %. Protein content in the seed had a mean of 31.7 %, with a range from 27.6 % to 35.5 %.

In the ANOVA analysis, the F-values and P-value indicate that the erucic acid content exhibits substantial variation across different environments and years (Carman 2021 2.2***, Carman 2022 2.9***, Portage 2021 5.5***, Portage 2022 12.8*** and Greenhouse 2022 21.78***). This pattern underscores the significant genotype-dependent variation in erucic acid content. However, the combined effect of environmental factors on erucic acid content were not significant (1.4), implying that environmental influences may exert a relatively minor impact on erucic acid content in comparison to genetic factors. This observation also suggests a certain level of stability or uniformity in the erucic acid content, with minimal variability under diverse environmental conditions and across years. Both the genotype effect (9.5***) and the genotype-by-environment (1.3**) interaction significant **(Table 4.3)**.

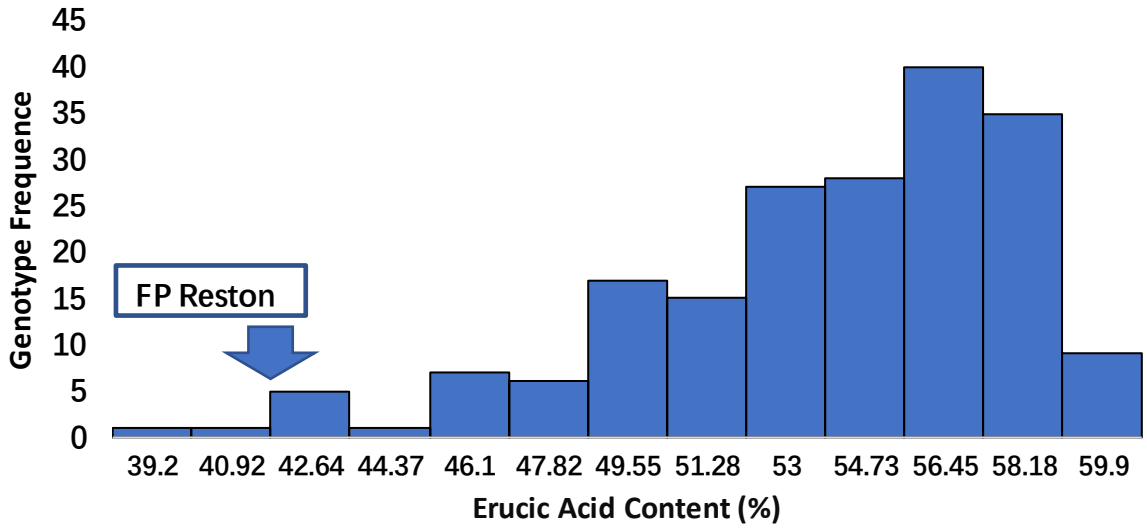


Figure 4.2 Histogram distribution of erucic acid content in seed of 190 genotypes in a CBER2 doubled haploid mapping population: analysis of Greenhouse 2022 based on best linear unbiased estimator (BLUEs) data.

Table 4.2 Descriptive statistics for key agronomic traits including days-to-flower, plant height, lodging, days-to-maturity, oil, and protein content in the CBER2 population in Carman 2021, Carman 2022, Portage 2021, and Portage 2022.

Variable	N[1]	Mean	Std Dev[2]	Minimum	Maximum
Mean FLR[3]	193	49.4	3.1	44.6	58.5
Mean PH[4]	193	92.6	8.2	73.3	116
Mean LODG[5]	193	3.5	0.3	2.3	4.2
Mean MAT[6]	193	94.6	1.6	91.5	97.6
Mean OIL[7]	193	38.4	2.4	31.9	44.7
Mean PRO[8]	193	31.7	1.5	27.6	35.5

[1]**N:** Number of observations
[2]**Std Dev:** Standard deviations
[3]**FLR:** Days-to-flower - Days (Time from planting to flowering)
[4]**PH:** Plant height - Centimeters (Height of the plant)
[5]**LODG:** Lodge value - Scale (1-5, depending on the scoring system used for lodging resistance)
[6]**MAT:** Days-to-maturity - Days (Time from planting to maturity)
[7]**OIL:** Oil content in seed- Percentage (%) (Percentage of oil content in the seed)
[8]**PRO:** Protein Content in Seed Percentage (%) (Percentage of protein content in the seed)

Table 4.3 Analysis of variance for days-to-flower, plant height, lodging, days-to-maturity, oil content, protein content and erucic acid content in *Brassica napus* genotypes, environment and genotype-by-environment for CBER2 population in Carman 2021, Carman 2022, Portage 2021, Portage 2022, and Greenhouse 2022.

Variation (Genotype)	EA[7]	FLR[8]	PH[9]	LODG[10]	MAT[11]	OIL[12]	PRO[13]
CM2021[1]	2.2***	2.5***	3.1***	2.1***	2.0***	3.7***	2.8***
CM2022[2]	2.9***	6.2***	2.8***	2.0***	1.6***	7.0***	3.6***
PT2021[3]	5.5***	8.7***	3.7***	3.1***	3.9***	16.5***	13.1***
PT2022[4]	12.8***	8.6***	5.2***	2.8***	3.3***	13.5***	10.4***
GH2022[5]	21.78***	-	-	-	-	-	-
Environment (Field Results Combined)[6]	1.4	440.0***	29.7***	25.3**	709.0***	169.4***	371.1***
Genotype (field Result combined)	9.5***	28.2***	9.7***	2.9***	4.6***	21.7***	15.3***
Genotype* Environment (Field Result combined)	1.3**	2.0***	1.3***	2.1***	1.5***	2.1***	2.0***

***P<0.001; **P<0.01; *P<0.05

Numbers represent the F-Values

[1]**CM2021:** 2021, Carman, Manitoba, Canada

[2]**CM2022:** 2022, Carman, Manitoba, Canada

[3]**PT2021:** 2021, Portage La Prairie, Manitoba, Canada

[4]**PT2022:** 2022, Portage La Prairie, Manitoba, Canada

[5]**GH2022:** 2022, University of Manitoba, Manitoba, Canada

[6] **(Field Results Combined):** Combined with CM2021, CM2022, PT2021 and PT2022

[7]**EA:** Erucic acid content in seed - Percentage (%) (Percentage of erucic acid in total oil content in the seed)

[8]**FLR:** Days-to-flower - Days (Time from planting to flowering)

[9]**PH:** Plant height - Centimeters (Height of the plant)

[10]**LODG:** Lodge value - Scale (1-5, depending on the scoring system used for lodging resistance)

[11]**MAT**: Days-to-maturity - Days (Time from planting to maturity)

[12]**OIL:** Oil content in seed - Percentage (%) (Percentage of oil content in the seed)

[13]**PRO:** Protein content in seed - Percentage (%) (Percentage of protein content in the seed)

4.4.2. Correlation Analysis of Agronomic Traits

A strong positive correlation (0.6***) between days-to-flower (FLR) and plant height (PH) and a significant negative correlation (-0.3***) between FLR and lodging (LODG) are observed. Moreover, a robust positive correlation (0.8***) between FLR and days-to-maturity (MAT) is noted. A weak negative correlation was observed between FLR and oil content (OIL), indicated by a correlation coefficient of -0.2**. Additionally, oil content (OIL) and protein content (PRO) exhibited a strong negative correlation, with a coefficient of -0.8***. A positive correlation was noted between MAT and PRO, demonstrated by a correlation coefficient of 0.4***.

Erucic acid (EA) generally shows no significant correlations with FLR, PH, LODG, and MAT, suggesting its content is largely independent of these traits in *B. napus*. However, a weak positive correlation (0.12**) between EA and OIL implies a slight linkage between oil content and erucic acid levels (**Table 4.4**).

4.4.3. Marker Selection and Distribution Analysis

In the CBER2 population, 3,169 variable markers were identified, including 2,798 SNPs with 1,625 transition (A/G and C/T) and 1,173 transversion types (A/C, A/T, C/G, and G/T), yielding a Ts/Tv ratio of 1.39, and 371 Indels. Eight DH lines were excluded due to genetic testing anomalies and suboptimal sequencing. Analysis showed SNP density per 1Mb ranged from 0.7 to 6.5 on chromosomes A09, C09, and A03, and Indel density ranged from 0.1 to 0.8 on A05 and A10, highlighting diverse genomic architecture (**Table 4.5**).

Table 4.4 Pearson correlation coefficients among agronomic traits in the CBER2 population: mean across Carman 2021, Carman 2022, Portage 2021, Portage 2022, and Greenhouse 2022.

Pearson Correlation Coefficients							
	Mean FLR	**Mean PH**	**Mean LODG**	**Mean MAT**	**Mean OIL**	**Mean PRO**	**Mean EA**
Mean FLR[1]	1						
Mean PH[2]	0.64***	1					
Mean LODG[3]	-0.33***	-0.2***	1				
Mean MAT[4]	0.75***	0.58***	-0.24***	1			
Mean OIL[5]	-0.22**	0.008	-0.14	-0.39***	1		
Mean PRO[6]	0.15*	0.07	0.08	0.36***	-0.77***	1	
Mean EA[7]	-0.42	-0.2	0.15	-0.15*	0.12**	0.12	1

***P<0.001; **P<0.01; *P<0.05

Digital number: Represent the strength and direction of the relationship between two variables

[1]**Mean FLR:** Mean days-to-flower - Days (Time from planting to flowering)

[2]**Mean PH:** Mean plant height - Centimeters (Height of the plant)

[3]**Mean LODG:** Mean lodging - Scale (1-5, depending on the scoring system used for lodging resistance)

[4]**Mean MAT:** Mean days-to-maturity - Days (Time from planting to maturity)

[5]**Mean OIL:** Mean oil content - Percentage (%) (Percentage of oil content in the seed)

[6]**Mean PRO:** Mean protein content - Percentage (%) (Percentage of protein content in the seed)

[7]**Mean EA:** Mean erucic acid content - Percentage (%) (Percentage of erucic acid in total oil content in the seed)

Table 4.5 Distribution and density of SNP and Indel markers across chromosomes in the CBRE2 population.

Chr[1]	Length[2]	No. SNP[3]	SNP density[4]	No. InDels[5]	InDel density[6]
A01	38,004,428	156	4.10	13	0.34
A02	35,943,954	139	3.87	17	0.47
A03	44,868,710	290	6.46	31	0.69
A04	25,679,024	98	3.82	13	0.51
A05	45,991,561	59	1.28	5	0.11
A06	48,704,706	105	2.16	15	0.31
A07	32,302,721	90	2.79	9	0.28
A08	28,329,074	161	5.68	12	0.42
A09	65,862,748	42	0.64	13	0.20
A10	26,592,803	110	4.14	22	0.83
C01	57,880,920	200	3.46	24	0.41
C02	65,293,782	310	4.75	36	0.55
C03	79,061,710	164	2.07	17	0.22
C04	71,179,181	264	3.71	41	0.58
C05	59,550,008	150	2.52	22	0.37
C06	52,512,057	98	1.87	14	0.27
C07	60,986,212	169	2.77	27	0.44
C08	53,660,391	149	2.78	31	0.58
C09	68,416,614	44	0.64	9	0.13
Whole	960,820,60	2,798	2.91	371	0.39

[1]**Chr:** Chromosome number
[2]**Length:** Chromosome length (bp)
[3]**No. SNPs:** Numbers of SNP
[4]**SNP density:** Every 1Mb has a number of single nucleotide polymorphisms
[5]**No. InDels:** Number of insertions and deletions
[6]**InDel density:** Every 1Mb has a number of insertions and deletions

4.4.4. Linkage Map

MapDisto (version 1.7.7) identified 22 linkage groups in the CBER2 population, corresponding to 19 chromosomes. A total of 2,966 markers were selected, distributed across 566 linkage bins, ranging from 2 on chromosome A04.1 to 62 on chromosome A03, averaging 27 bins per group. The cumulative map distance was 1,336.9 cM. Marker coverage was uneven, with some chromosomes exhibiting poor representation. This often resulted in multiple smaller linkage groups on a single chromosome, such as A04.1, A04.2, A10.1, A10.2, C03.1 and C03.2, due to gaps in coverage (**Table 4.6**).

4.4.5. Quantitative Trait Loci Analysis Result

Five QTL for erucic acid content were found in the CBER2 DH population. These QTL were designated as *qEA_CBER2_A01, qEA_CBER2_A02, qEA_CBER2_A05, qEA_CBER2_C06,* and *qEA_CBER2_C09*, were located on chromosomes A01, A02, A05, C06, and C09, respectively.

Significant findings from the analysis revealed consistent identification of erucic acid QTL *qEA_CBER2_A01* in the PT2021, with LOD scores to 3.7 9 % of phenotypic variation. The *qEA_CBER2_A02* was detected in the GH2022 and CM2022 environments. Notably, *qEA_CBER2_A05* exhibited significant effects in multiple environments (CM2021, GH2022, PT2021, and CMB), with an especially high LOD score of 7.49 in the PT2021 environment, accounting for 18 % of the phenotypic variation (**Table 4.7** & **Figure 4.3**). Environment also played a significant role in the erucic acid QTL results, and the interaction between environmental and genetic factors is evident (Cao et al., 2001; Xing et al., 2002).

Table 4.6 Genetic map characteristics of the CBER2 population: chromosome, map lengths, bin counts, mean and maximum bin intervals per chromosome developed in MapDisto.

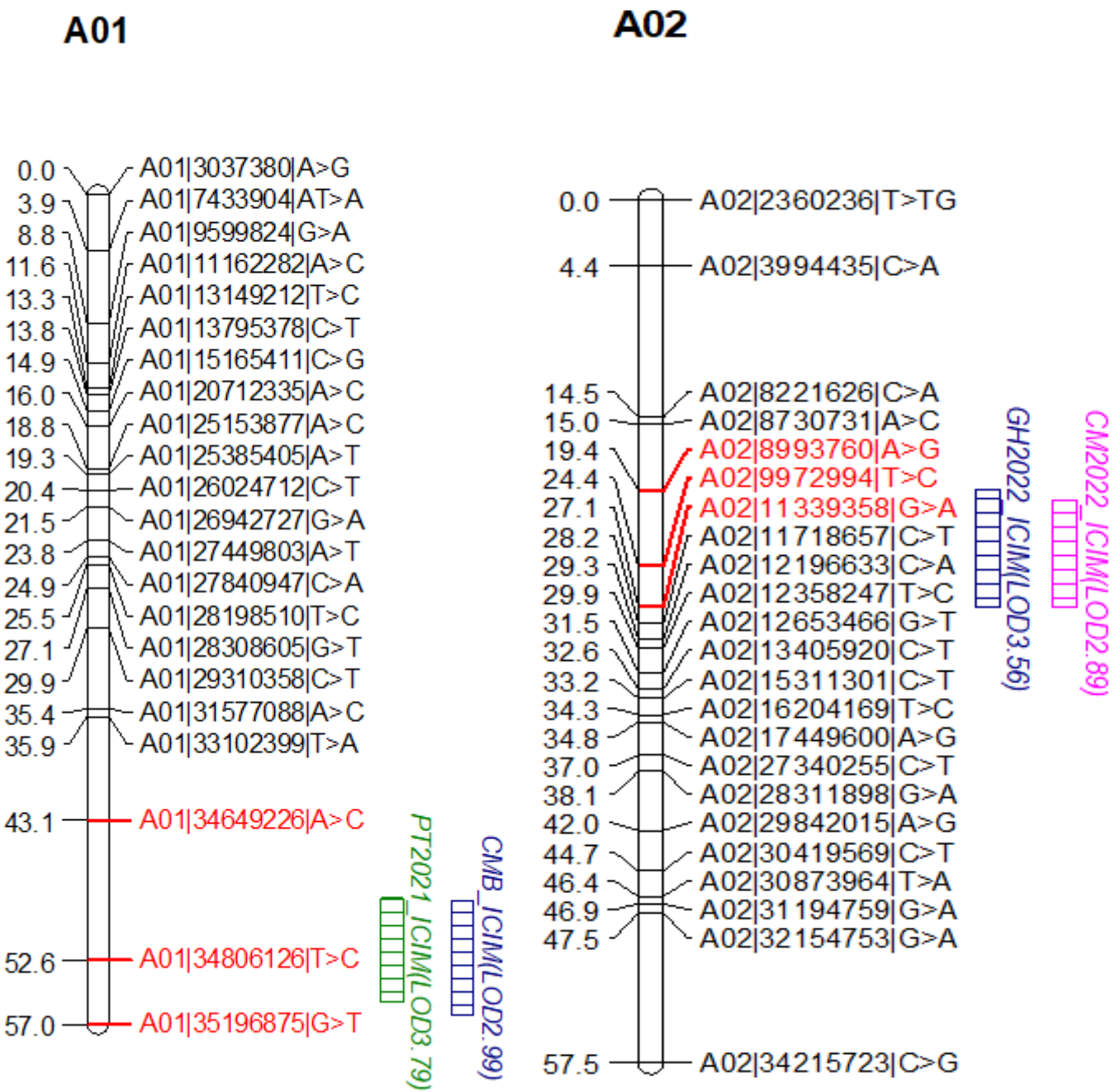
Chr[1]	Length (cM)[2]	No. bins[3]	Bin interval (cM)[4]	Max interval (cM)[5]
A01	56.968	22	2.59	9.45
A02	57.487	23	2.50	10.02
A03	104.911	62	1.69	12.92
A04.1	11.774	2	5.89	11.77
A04.2	72.373	20	3.62	19.75
A05	40.304	11	3.66	6.63
A06	75.009	20	3.75	18.37
A07	60.959	17	3.59	27.83
A08	74.163	34	2.18	8.36
A09	72.280	8	9.03	25.67
A10.1	15.956	9	1.77	4.41
A10.2	3.304	4	0.83	2.20
C01	64.242	26	2.47	8.88
C02	99.629	57	1.75	14.10
C03.1	78.906	28	2.82	7.19
C03.2	9.353	6	1.56	4.41
C04	98.780	62	1.59	6.07
C05	50.153	35	1.43	3.85
C06	62.716	20	3.14	14.69
C07	82.730	45	1.84	6.63
C08	98.914	44	2.25	12.33
C09	46.054	11	4.19	14.18

[1]**Chr:** Linkage group number (chromosome)
[2]**Length:** Linkage group total length
[3]**No. bins:** Total bins in each linkage group
[4]**Bin interval:** Mean distance between adjacent bins
[5]**Max interval:** Maximum distance between adjacent markers

Table 4.7 Additive-effect QTL detected for erucic acid content in total oil extracted from a CBER2 doubled haploid population using phenotypic data from five environments by using inclusive composite interval mapping (ICIM) and best linear unbiased estimator (BLUEs) across Carman 2021, Carman 2022, Portage 2021, Portage 2022, and Greenhouse 2022.

	ICIM[1]						Physical distance (bp)	
	Env.[2]	**Chr.[3]**	**Pos.[4]**	**Add.[5]**	**LOD[6]**	**R^2[7]**	**Left Flank**	**Right Flank**
qEA_CBER2_A01	PT2021	A01	48.4-55.6	1.01	3.79	0.09	34,649,226	34,806,126
	CMB	A01	48.5-56.4	0.76	2.98	0.07		
qEA_CBER2_A02	GH2022	A02	19.4-27.1	-1.12	3.56	0.09	8,993,760	11,339,358
	CM2022	A02	20.1-27.1	-0.64	2.89	0.07		
qEA_CBER2_A05	CM2021	A05	6.1-9.9	1.07	4.80	0.11	33,434,528	36,723,260
	GH2022	A05	6.1-9.9	1.42	5.50	0.13		
	PT2021	A05	6.1-9.9	1.40	7.49	0.18		
	CMB	A05	6.1-9.9	0.91	4.55	0.12		
qEA_CBER2_C06	PT2021	C06	39.1-53.7	0.94	3.13	0.08	13,771,207	28,439,656
qEA_CBER2_C09	GH2022	C09	15.3-23.9	1.13	3.01	0.07	53,204,230	60,986,760

[1]**ICIM**: Inclusive composite interval mapping
[2]**Env**: Environment
[3]**Chr**: Chromosome
[4]**Pos**: Position on the linkage group in centimorgans
[5]**LOD**: Logarithm of odds, -$\log_{10}$(p-value) the peak of LOD score
[6]**R^2**: Phenotypic variation explained (r^2; %)
[7]**Add**: Additive effect of allele substitution. A positive number indicated that the female parent allele increased the respective quantitative trait and vice-versa
CM2021: 2021, Carman, Manitoba, Canada
CM2022: 2022, Carman, Manitoba, Canada
PT2021: 2021, Portage La Prairie, Manitoba, Canada
PT2022: 2022, Portage La Prairie, Manitoba, Canada
CMB: Combined environment data set
Physical distance (bp): The marker starts position and ends position in physical map distance united by base pairs


A01
A02
0.0 A01|3037380|A>G
3.9 A01|7433904|AT>A
8.8 A01|9599824|G>A
11.6 A01|11162282|A>C
13.3 A01|13149212|T>C
13.8 A01|13795378|C>T
14.9 A01|15165411|C>G
16.0 A01|20712335|A>C
18.8 A01|25153877|A>C
19.3 A01|25385405|A>T
20.4 A01|26024712|C>T
21.5 A01|26942727|G>A
23.8 A01|27449803|A>T
24.9 A01|27840947|C>A
25.5 A01|28198510|T>C
27.1 A01|28308605|G>T
29.9 A01|29310358|C>T
35.4 A01|31577088|A>C
35.9 A01|33102399|T>A
43.1 A01|34649226|A>C
52.6 A01|34806126|T>C
57.0 A01|35196875|G>T
PT2021_ICIM(LOD3.79)
CMB_ICIM(LOD2.99)
0.0 A02|2360236|T>TG
4.4 A02|3994435|C>A
14.5 A02|8221626|C>A
15.0 A02|8730731|A>C
19.4 A02|8993760|A>G
24.4 A02|9972994|T>C
27.1 A02|11339358|G>A
28.2 A02|11718657|C>T
29.3 A02|12196633|C>A
29.9 A02|12358247|T>C
31.5 A02|12653466|G>T
32.6 A02|13405920|C>T
33.2 A02|15311301|C>T
34.3 A02|16204169|T>C
34.8 A02|17449600|A>G
37.0 A02|27340255|C>T
38.1 A02|28311898|G>A
42.0 A02|29842015|A>G
44.7 A02|30419569|C>T
46.4 A02|30873964|T>A
46.9 A02|31194759|G>A
47.5 A02|32154753|G>A
57.5 A02|34215723|C>G
GH2022_ICIM(LOD3.56)
CM2022_ICIM(LOD2.89)

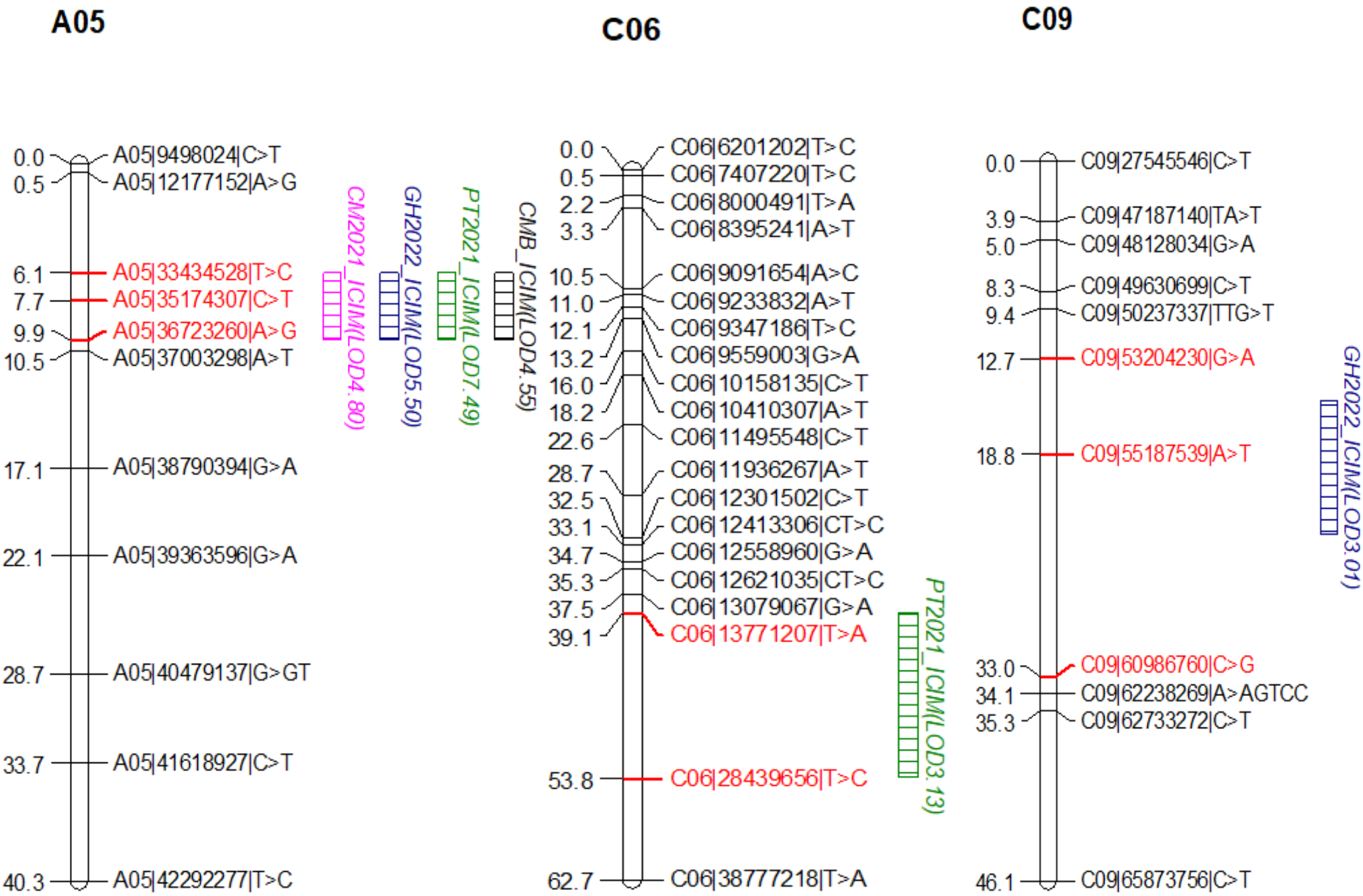

A05
0.0 A05|9498024|C>T
0.5 A05|12177152|A>G
6.1 A05|33434528|T>C
7.7 A05|35174307|C>T
9.9 A05|36723260|A>G
10.5 A05|37003298|A>T
17.1 A05|38790394|G>A
22.1 A05|39363596|G>A
28.7 A05|40479137|G>GT
33.7 A05|41618927|C>T
40.3 A05|42292277|T>C
CM2021_ICIM(LOD4.80)
GH2022_ICIM(LOD5.50)
PT2021_ICIM(LOD7.49)
CMB_ICIM(LOD4.55)
C06
0.0 C06|6201202|T>C
0.5 C06|7407220|T>C
2.2 C06|8000491|T>A
3.3 C06|8395241|A>T
10.5 C06|9091654|A>C
11.0 C06|9233832|A>T
12.1 C06|9347186|T>C
13.2 C06|9559003|G>A
16.0 C06|10158135|C>T
18.2 C06|10410307|A>T
22.6 C06|11495548|C>T
28.7 C06|11936267|A>T
32.5 C06|12301502|C>T
33.1 C06|12413306|CT>C
34.7 C06|12558960|G>A
35.3 C06|12621035|CT>C
37.5 C06|13079067|G>A
39.1 C06|13771207|T>A
53.8 C06|28439656|T>C
62.7 C06|38777218|T>A
PT2021_ICIM(LOD3.13)
C09
0.0 C09|27545546|C>T
3.9 C09|47187140|TA>T
5.0 C09|48128034|G>A
8.3 C09|49630699|C>T
9.4 C09|50237337|TTG>T
12.7 C09|53204230|G>A
18.8 C09|55187539|A>T
33.0 C09|60986760|C>G
34.1 C09|62238269|A>AGTCC
35.3 C09|62733272|C>T
46.1 C09|65873756|C>T
GH2022_ICIM(LOD3.01)

Figure 4.3 Conditional quantitative trait loci (QTL) mapping for erucic acid content was conducted across linkage groups A01, A02, A05, C06 and C09 in the CBER2 population. This figure illustrates QTL for erucic acid content identified in Carman in 2021 and 2022 (CM2021, CM2022) and in Portage in 2021 and 2022 (PT2021, PT2022). Vertical bars denoted the linkage groups, with both genetic distances and physical positions alongside locus names and Log of Odds (LOD) values annotated adjacent to the bars. Regions with QTL identified through conditional analysis were highlighted in red, with the LOD values providing a measure of the strength of the QTL signal.

4.5. Discussion

This research focused on the genetic control of erucic acid in a population developed from two HEAR parental genotypes. A linkage map featuring 2,966 markers over 1,336.9 cM was developed, identifying QTL on chromosomes A01, A02, A05, C06, and C09, with LOD scores ranging from 2.8 to 7.5 and explaining 7 % to 18 % of phenotypic variance. The study revealed erucic acid genetic stability across environments, providing insight for future breeding methods aimed at producing high erucic acid genotypes. Additionally, a bi-parental QTL analysis with two high erucic acid parental genotypes targeted minor QTL, avoiding the redundancy of well-known *FAE1* genes on A08 and C03 (Cao et al., 2010; Saini et al., 2019; Wu et al., 2008; Zhu et al., 2019). This strategy revealed the polygenic nature of erucic acid synthesis and the complex genetic factors influencing its content.

Previous QTL research associated with erucic acid content (Cao et al., 2010; Ecke et al., 1995; Jourdren et al., 1996; Qiu et al., 2006; Xu et al., 2015; Zou et al., 2016) and related association mapping studies (Jourdren et al., 1996; Li et al., 2014; Tang et al., 2019; Zhao et al., 2019) have identified multiple erucic acid QTL in *B. napus*. Major erucic acid QTL located on chromosomes A08 and C03 were found to represent the *FAE1* genes, predominantly controlling over 70 % of the variation in *B. napus* (Burns et al., 2003; Qiu et al., 2006). A comparative analysis revealed that the sequence similarity between the two functional copies: *FAE1.1* (*BnFAE.A08*) and *FAE1.2* (*BnFAE.C03*), in HEAR exceeded 98 % (Das et al., 2002; Yan et al., 2015). The presence of SNPs in *FAE1*, when comparing

low and high erucic acid genotypes, reaffirmed the significance of *FAE1* in determining erucic acid content (Roscoe et al., 2001; Wang et al., 2022). Research has demonstrated that the functional loss of *FAE1* in rapeseed using in the method like gene transfer (Li et al., 2012; Mietkiewska et al., 2004, 2007; Yang et al., 2012; Nath et al., 2009), RNA interference (Peng et al., 2010; Shi et al., 2015; Tian et al., 2011), intron-spliced hairpin RNA (ihpRNA) (Tian et al., 2011), and CRISPR/Cas9-mediated genome editing leads to change in erucic acid content (Jarvis et al., 2021; Yunhao Liu et al., 2022). Significant success has been realized in obtaining low erucic acid rapeseed through the manipulation of *FAE1.*

In high erucic acid rapeseed, most studies have largely focused on the role of *FAE1*, with less discussion on the impact of other alleles. Exploring potential minor QTL remains a promising avenue for future research. Therefore, exploring minor QTL represented a critical pathway for broadening our understanding of the genetic complexity underlying this trait. Along these lines, Qiu et al. (2006) discovered a QTL for erucic acid on chromosome A01 through the construction of a genetic linkage map of the TNDH population. This QTL was located at positions 53-77 cM on the chromosome's short arm distal end, contributing 5.8 % to the phenotypic variation in erucic acid content. This region closely aligned with the QTL *qEA_CBER2_A01* identified in our study at 48.4-55.6 cM. Parkin et al. (2003) identified QTL related to erucic acid content on chromosomes A01, A08, and C03. These QTL aligned with the long arm of chromosome 4 in *Arabidopsis thaliana*. This arrangement, aligning with the *A. thaliana FAE1* homolog candidate gene

for erucic acid biosynthesis in *B. napus*, indicates the likelihood of upstream homologous genes in the genomic region on chromosome A01 (Parkin et al., 2005). Subsequent studies, such as Javed et al. (2016), also inferred the presence of a QTL for erucic acid on A01 through the analysis of QTL for multiple unsaturated fatty acids like oleic acid (C18:1). Yusuf et al. (2022) identified multiple fatty acid QTL (16:0; 18:1; 18:2) and oil content QTL at the physical location of 31-34 Mbp on chromosome A01. Yusuf et al. (2022) found the QTL physical location was in proximity or coincides with the QTL *qEA_CBER2_A01* found in the current study at 34.6-34.8 Mbp. Within the QTL intervals, Yusuf et al. (2022) also identified potential candidate genes including fatty acid desaturase 2 (*FAD2*) homolog *BnaA01G0369500ZS*, lysophosphatidic acid acyltransferase 5 (*LPAAT5*) homolog *BnaA01G0323800ZS*, and glycerol-3-phosphate acyltransferase 5 (*GPAT5*) homolog *BnaA01G0373800ZS* (Yusuf et al., 2022). Although direct physical locations for erucic acid QTL overlapping with those identified in this study were not reported in the literature, Teh & Möllers (2016) highlighted the mapping of *LPAAT* and *FAD2* enzymes near our discovered QTL regions on chromosome A01. These enzymes play a crucial role in enhancing the assembly of triacylglycerol (TAG), reducing the saturation levels of 18:2 and 18:3 fatty acids and thus, increasing C20:1 and erucic acid content in the seed oil as noted by Möllers & Schierholt (2002) and Zhao et al. (2008).

A study of Qiu et al. (2006) reported the occurrence of erucic acid QTL on the *B. napus* chromosome A02, positioned at 84-116 cM, accounting for 6.6 % of the variation. This observation was further substantiated in subsequent studies (Javed et al., 2016; Zhao et

al., 2022). Later, Raboanatahiry et al. (2017) identified multiple QTL for oil and various fatty acids across different cultivars on chromosome A02, with three of them impacting oil content QTL (*qOC-A2-4-KN* 6.5-19.73 Mbp; *qOC-A2-3-TN* 8.1-10.3 Mbp; *qOC-A2-1-Z5* 8.9-18.7 Mbp). These previously reported QTL exhibited physical proximity to the QTL *qEA_CBER2_A02* (QTL location in 8.9-11.3 Mbp) identified in our study. Raboanatahiry et al. (2017) also discovered several candidate genes potentially involved in fatty acid synthesis in the QTL *qOC-A2-4-KN* (*KASI-BnaA02g24400D*; *KASII-BnaA02g17050D*; L*CAS-BnaA02g18280D*; *LCAS-BnaA02g21860D*; *LPAAT-BnaA02g17090D*; *LPAAT-BnaA02g17170D*; *LPCAT-BnaA02g19010D*). Subsequently, Guan et al. (2019) pinpointed quantitative trait nucleotides (QTN) associated with erucic acid in spring-type *B. napus* at a physical location of 6.9 Mbp on chromosome A02 in their associated mapping analysis, closely aligning with the QTL location discovered in this study (Guan et al., 2019). Zhao et al. (2019) also identified an erucic acid-related SNP (*BnaA02005167036SNV*) on chromosome A02 in their GWAS study, which explained 19.8 % of the phenotypic variation in erucic acid content. The physical location of this SNP was proximal to the QTL identified in the current at 8.9-11.3 Mbp.

Numerous studies have validated the presence of QTL on chromosome A05 in *B. napus* that control fatty acid composition (Zhao et al., 2022; Javed et al., 2016b; Yusuf et al., 2022) and oil content (Fu et al., 2017; Liu et al., 2016; Zhao et al., 2008)). In our experiment, we identified an erucic acid QTL (*qEA_CBER2_A05*) located at 6.1-9.9 cM on chromosome A05, with a physical position of 33.4-36.7 Mbp. In the GWAS study of fatty

acids in *B. napus* by Zhao et al. (2019), a SNP impacting C18:1 and C20:1 (*BnaA05033791095SNV*) was found on chromosome A05 at a physical position of 33.7 Mbp. The location discovered by Zhao et al. (2019) coincided with the consensus QTL position identified in our study. Furthermore, Gacek et al. (2017) in their GWAS study reported multiple potential genes on A05 influencing C18:1 and C18:2, which participated in the biosynthesis process of fatty acid elongation (Gacek et al., 2017). Yusuf et al. (2022) also detected a QTL affecting C18:2 (q18:2-A5) on A05 in their study on oil content and fatty acid QTL (Yusuf et al., 2022), aligning with the QTL identified in our study at 33.4-36.7 Mbp. Through this QTL, Yusuf et al. (2022) identified candidate genes controlling fatty acid elongation within the range of 38.3-40.6 Mbp on A05 (*FAD2* (*BnaA05G0427800ZS*) and *LPAAT5* (*BnaA05G0429000ZS*)), which also played a role in the formation of erucic acid (Jadhav et al., 2005; Jarvis et al., 2021; Yusuf et al., 2022). Tang et al. (2021) in their GWAS study on oil content in *B. napus*, identified multiple SNPs at 40.1-41.1 Mbp on A05 that directly affected oil content. This location was in proximity to the erucic acid QTL (33.4-36.7 Mbp) identified in our research, and candidate genes affecting oil content and fatty acid composition were identified (*BnaA05g16720D* (protein kinase & ATP binding) and *BnaA05g28570D* (methyltransferase activity)) (Tang et al., 2021).

A QTL found in the PT 2021 environment was also discovered on chromosome C06 at position of 13.7-28.4 Mbp. Both QTL and GWAS studies have indicated the presence of QTL on C06 controlling oil content (Fu et al., 2017; Yan et al., 2009; Zhao et al., 2022). Zou

et al. (2016) identified a marker (*Bn-scaff_16397_1-p21961*) at the physical location of 32.9 Mbp on C06, which was related to erucic acid content. Similarly, Körber et al. (2016) identified a SNP at a similar location (33 Mbp) and named this QTL *ERA.C06.s1*. Zhao et al. (2019) mentioned a SNP (*BnaC06022466785SNV*) on chromosome C06 related to erucic acid content, physically located at 22.5 Mbp, close to the location in our study (13.7-28.4 Mbp). Raboanatahiry et al. (2017) also identified 11 QTL related to oil content and pinpointed 8 candidate genes associated with oil content. The physical positions of these QTL and genes were proximal to the C06 QTL identified in our study, suggesting that these oil content candidate genes may be involved in the biosynthesis of erucic acid and play a role in influencing erucic acid content. Bilgrami et al. (2022) also discovered a QTL impacting oil content at a physical position of 28 Mbp, further substantiating the influence of these genomic regions on erucic acid content.

On the C09 chromosome, a newly identified QTL was unique to one environment and adds to the understanding of genetic factors influencing erucic acid content. While previous studies have noted QTL affecting oil content near this region (Fu et al., 2017; Körber et al., 2016; Tang et al., 2019; Wang et al., 2021), none were associated with erucic acid, possibly due to different genetic backgrounds (Rio et al., 2020; Vazquez et al., 2015) or environmental conditions not previously explored (Cao et al., 2001; Liu et al., 2014; Wu et al., 2005). The discovery underscored the importance of considering a broader genetic base and environmental factors in QTL research, offering new insights for crop

improvement and breeding strategies focused on erucic acid content enhancement (Li et al., 2008; Visscher & Goddard. 2004).

In the population, analysis identified correlations among agronomic traits, revealing that erucic acid content was largely independent of other agronomic traits (FLR, PH, LODG, PRO). This suggested that selecting for higher erucic acid content in *B. napus* might not adversely affect these agronomic characteristics. Notably, a weak but significant positive correlation between oil content and erucic acid levels hinted at a potential interaction between their biosynthetic pathways, underlining the importance of considering oil content in breeding strategies aimed at enhancing erucic acid production in *B. napus*.

4.6. Conclusions

The primary focus of this research was to explore the minor QTL affecting erucic acid content in crosses between two HEAR parents. By strategically selecting HEAR genotypes, we effectively masked the known major genes impacting erucic acid content and identified QTL on chromosomes A01, A02, and A05 that demonstrated consistent expression across diverse environments. Furthermore, this research unveiled environment-specific QTL on chromosomes C06 and C09. The primary impetus for delving into this study was to elevate the erucic acid content in *B. napus* to meet industrial demands. A comprehensive genetic understanding of these traits, in conjunction with the

development of molecular markers using for marker-assisted selection, holds the potential to boost erucic acid content in *B. napus*. Several genetic regions identified in this study are considered novel and warrant thorough investigation.

5. General Discussion and Conclusions

Brassica napus L. is ranked as the world's second-highest yielding oilseed crop (FAO, 2023) and was considered the most valuable crop in Canada (Wells & Slade, 2021). To maintain the competitiveness of Canada's *B. napus* industry, enhancing the value of this crucial crop and exploring the diversification of its end uses is imperative. Erucic acid, produced by *B. napus*, meets a broad range of industrial demands, and has significant potential for increased production capacity (Eskin et al., 2020). Enhancing the content of erucic acid and developing new cultivars of erucic acid with high erucic acid content *B. napus* not only fulfills specific industrial applications but also offers new market opportunities for agricultural producers (Guan et al., 2014; Lu et al., 2020; Sanyal et al., 2015).

The QTL mapping conducted in this study provided novel and comparable information on genomic regions associated with erucic acid content in *B. napus* seed. The primary aim was to analyze populations derived from high erucic acid parental genotypes, enriching the understanding of minor QTL influencing erucic acid content. Additionally, this research included correlation analyses with other agronomic traits.

Brassica napus could not have achieved its economic success without its improved seed quality characteristics (Hannoufa et al., 2014; Malabat et al., 2003; Schilbert et al., 2022). This research successfully showcased numerous genotypes with special seed quality traits. It also demonstrated that erucic acid content was largely uncorrelated with

most agronomic traits other than oil content in the two populations, suggesting that breeding for agronomic performance can occur without compromising erucic acid levels (Wijsman. 2005). This research also supported the negative correlations between seed oil content and protein content in the two populations, a factor that cannot be overlooked during breeding efforts (Shi et al., 2017).

In contrast to previous studies that predominantly utilized canola parents to investigate the impact of the *FAE1* gene on erucic acid content, this research adopts an approach by employing crosses between two high erucic acid rapeseed (HEAR) genotypes from distinct populations. Early research efforts have indeed highlighted the significance of the *FAE1* gene; however, they largely replicated existing methodologies, thereby reaffirming established effects (Cao et al., 2010; Das et al., 2002; Gill et al., 2021; Saini et al., 2019; Wang et al., 2022). Our study aims to broaden genetic exploration by identifying minor QTL that influences erucic acid content beyond the extensively studied *FAE1* gene. This approach is intended to enhance our comprehension of the genetic factors contributing to erucic acid production in *B. napus* species, thereby addressing gaps left by prior studies and avoiding the redundancy of repeating similar observations in multiple sections of the research (Wen et al., 2016; Yusuf et al., 2022; Zhao et al., 2019).

The analysis of erucic acid QTL results from the two populations (CBLD2 and CBER2) revealed that research on erucic acid content in *B. napus* involves complex genetic interactions and environmental factors, highlighting the complex nature of this trait. The CBLD2 population revealed seven QTL related to erucic acid content, with significant loci

detected on chromosomes A04, A07, A10, C01, C02, C04, and C08, showcasing a broad genetic basis for trait variance. Notably, the QTL qEA_CBLD2_A04 and qEA_CBLD2_A07 were consistent across all environments, highlighting their potential stability and influence on erucic acid content (Bao et al., 2018; Li et al., 2018). The CBER2 analysis identified five key QTL located on chromosomes A01, A02, A05, C06, and C09. These findings were consistent across different environments, indicating a similar genetic complexity and environmental interaction in erucic acid content determination (Shi et al., 2009; Yu et al., 2016). The presence of QTL across diverse chromosomal regions in both populations underscores the polygenic nature of erucic acid synthesis and its regulation within *B. napus* (Luo et al., 2017; Singh et al., 2007). A comparative analysis between the two populations reveals that while both share a complex genetic architecture for erucic acid content, the specific QTL identified differs, with no direct overlap in chromosomal locations. This variation may be attributed to the distinct genetic backgrounds of the two populations and the environmental conditions under which they were evaluated (Malabat et al., 2003; Yusuf et al., 2022; Zou et al., 2016).

The novelty in this study lies in the detection of QTL not previously reported in literature (like *qEA_CBLD2_A10; qEA_CBLD2_C04; qEA_CBLD2_C08; qEA_CBER2_A01; qEA_CBER2_C09*), providing fresh insight into the genetic architecture of erucic acid content in *B. napus*. This research advances our genetic knowledge and supports the development of marker-assisted selection strategies, ultimately facilitating the breeding of *B. napus* cultivars with optimized erucic acid levels for industrial applications.

The research did have several limitations. Firstly, the two populations revealed varying marker density and distribution across chromosomes. This uneven distribution could lead to gaps in the genetic map, potentially missing significant QTL or inaccurately estimating their effects (Yang et al., 2012; Devey et al., 2004). Secondly, the QTL identified accounted for only a portion of the phenotypic variance in erucic acid content, suggesting that other genetic factors, possibly minor QTL or epistatic interactions, were not captured (Koide et al., 2019). This implies a limited understanding of the genetic architecture underlying erucic acid content (Koide et al., 2019; Vazquez et al., 2015). Thirdly, the resolution of QTL mapping depends on population size, marker density, and the recombination events captured within the population. These studies may have faced limitations in resolution, affecting the precise location of QTL and the identification of candidate genes (Silva-Junior & Grattapaglia, 2015; Zou et al., 2016). Enhancing marker density, expanding population sizes, conducting functional validation of candidate genes, or exploring the genetic basis of environment-specific QTL effects could provide a constructive perspective for advancing the field.

In summary, this study has provided further insight into the genetic control of erucic acid quality traits in *B. napus* seed. Moreover, QTL observed in two different populations through two years of field and mapping analysis were identified on chromosomes A04, A07, A10, C01, C02, C04, C08 in the CBLD2 population, and A01, A02, A05, C06, and C09 in the CBER2 population. This research will aid in the development of new cultivars with optimized erucic acid content. By identifying the key QTL and gaining a deeper

understanding of their impact on erucic acid content, we can strategically improve selection, thereby enhancing the industrial value of HEAR seed.

6. Future Research Recommendations

This study lays the groundwork for future research on erucic acid in *B. napus*, highlighting the need for advanced genetic analysis and innovative breeding strategies based on QTL analysis. Moving forward, several improvements and suggestions can enhance the efficacy and depth of future experiments in this area.

Advanced sequencing technologies, such as long-read sequencing (like Pacific Biosciences (PacBio) and Oxford Nanopore Technologies) (Chen et al., 2021; Lee et al., 2020), or other methods like the *Brassica* 60K Illumina® Infinium™ SNP chips (Mason et al., 2017), could be utilized to enhance marker density and strengthen the genomic analyses of these populations. This would not only improve the resolution of QTL mapping but also facilitate the discovery of minor QTL and allow for precise effect estimations (Hatzig et al., 2015).

Expanding the diversity and scale of populations used in QTL mapping is considered crucial (Harwood, 1980; Korir et al., 2013; Van Ooijen, 1999). Thus, it could be important to incorporate genetic materials from various geographical origins to enable the identification of QTL that are robust across different environmental conditions, thereby advancing the identification of minor QTL (Guan et al., 2019).

Another important step is the molecular characterization of key QTL regions through fine mapping. This could be followed by functional validation of candidate genes using CRISPR/Cas9 (Okuzaki et al., 2018) or RNA interference techniques (Qi et al., 2018). This

will explain the molecular mechanisms of erucic acid production and pave the way for further genetic enhancement (Zhao et al., 2019). Integrating omics data, such as transcriptomics, proteomics, and metabolomics, with QTL findings will elucidate the biosynthetic pathways and regulatory mechanisms governing erucic acid content. Specifically, transcriptomics can be applied to detect transcriptional data, identifying genes that are actively expressed in regions associated with high erucic acid content as identified by QTL analysis. This allows for the mapping of gene expression patterns that are directly linked to erucic acid synthesis. Furthermore, proteomics offers an approach to detect the proteins produced in plant tissues, including those directly involved in the synthesis or regulation of erucic acid. By analyzing the proteome, researchers can link protein activity to specific QTL, uncovering the proteins that play a critical role in the pathway and potentially identifying novel targets for genetic manipulation to enhance erucic acid content. Lastly, metabolomics enables the examination of metabolite profiles related to different genetic backgrounds or environmental conditions. By measuring the levels of erucic acid and its related compounds directly, metabolomics provides a validation of the functional impacts of QTL on the erucic acid pathways (Tang et al., 2019; Weinstein et al., 2008). This comprehensive understanding will support the development of HEAR cultivars with optimized erucic acid content, improving the total production of erucic acid per hectare.

The findings from this research will also aid in the development of molecular markers for erucic acid content. Insights from this study, coupled with the outlined

recommendations for future research, will lay the groundwork necessary for the advancement of molecular marker development in this domain. Applying this knowledge in subsequent breeding programs is anticipated to yield *B. napus* cultivars characterized by their tailored erucic acid levels.

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8. Abbreviations

QTL - Quantitative Trait Loci

HEAR - High Erucic Acid Rapeseed

DH - Doubled Haploid

GBS - Genotyping-By-Sequencing

SNPs - Single Nucleotide Polymorphisms

FAD2 - Fatty Acid Desaturase 2

FAE1 - Fatty Acid Elongase 1

RNAi - RNA interference

LPAAT - Lysophosphatidic Acid Acyltransferase

CMS - Cytoplasmic Male Sterility

RF - Recombination Frequency

RFLPs - Restriction Fragment Length Polymorphisms

AFLPs - Amplified Fragment Length Polymorphisms

SSRs - Simple Sequence Repeats

DGAT - Diacylglycerol Acyltransferase