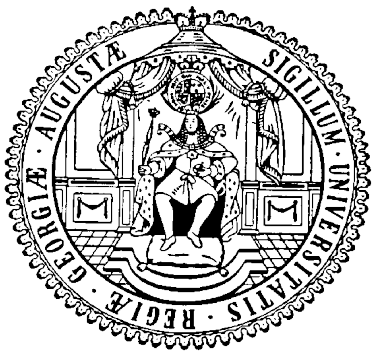
Genetic variation and inheritance of phytosterol and oil content in winter oilseed rape (*Brassica napus* L.)

Li Shia Teh



Göttingen 2014

Genetic variation and inheritance of phytosterol and oil content in winter oilseed rape (*Brassica napus* L.)

Dissertation
to obtain the Ph.D. degree
in the Faculty of Agricultural Sciences,
Georg-August-Universität Göttingen
Germany

presented by
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Göttingen, 20 November 2014

D7

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Date of dissertation: 20 November 2014

Dedicated to

my genetic contributors

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List of Abbreviations

ACP acyl carrier protein

AFLP amplified fragment length polymorphisms

C16:0 palmitic acid

C16:0-ACP palmitoyl-ACP

C18:0-ACP stearoyl-ACP

C18:1 oleic acid

C18:1-ACP oleoyl-ACP

C18:2 linoleic acid

C18:3 linolenic acid

CIM composite interval mapping

DArT Diversity Arrays Technology

DGAT diacylglycerol acyltransferase

DH double-haploid

FAD2 fatty acid desaturase 2

FAD3 fatty acid desaturase 3

FATA fatty acyl-ACP thioesterases A

FATB fatty acyl-ACP thioesterases B

FDR false-discovery rate

G3P glycerol-3-phosphate

GLM general linear model

GPAT glycerol-3-phosphate acyltransferase

HMG1 3-hydroxy-3-methylglutaryl-CoA reductase 1

HMG2 3-hydroxy-3-methylglutaryl-CoA reductase 2

HMGR 3-hydroxy-3-methylglutaryl-CoA reductase

HMGS 3-hydroxy-3-methylglutaryl-CoA synthase

IPP isopentyl pyrophosphate

KASII 3-ketoacyl-ACP synthase II

KASP KBioscience competitive allele-specific PCR

LD linkage disequilibrium

LDL low-density lipoprotein

LPAAT lysophosphatidic acid acyltransferase

MEP plastidial methylerythritol phosphate

MIM multiple interval mapping

MLM mixed-linear model

MVA cytosolic mevalonate

NIRS near-infrared reflectance spectroscopy

PAP phosphatidic acid phosphatase

PCR polymerase chain reaction

QTL quantitative trait loci

SAD stearoyl-ACP desaturase

SAT sterol acyltransferase

SMT1 sterol C24-methyltransferase 1

SMT2 sterol C24-methyltransferase 2

SNP single nucleotide polymorphisms

InDels insertion and deletion

SODH Sansibar $\times$ Oase double haploid

SSR simple sequence repeats

Chapter 1

General Introduction

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is the world's third-leading source of vegetable oil for human nutrition and industrial products. The term "double low" or "canola" is commonly used to refer to oilseed rape with <2% erucic acid in the oil and <25 $\mu mol\, g^{-1}$ glucosinolates in the seed (Bundessortenamt, 2014). Canola oil is highly recognized for its nearly ideal fatty-acid profile, that is, having low level of saturated fatty acids, high monosaturated fatty acids and a good proportion of omega-3 and omega-6 polyunsaturated fatty acids. More recently, minor salutary oil constituents like carotenoids, phytosterols, and tocopherols in canola oil have also received special attention owing to its various health benefiting properties.

Phytosterols or plant sterols are natural constituents of vegetable oil with serum cholesterol lowering properties (Best et al., 1954). They reduce the absorption of cholesterol from the intestine by effectively displacing cholesterol from micellar binding (Heinemann et al., 1991). An increasing appreciation of this beneficial effect on human health has resulted in the development of various food products enriched with phytosterols. Consumption of such food products, also known as functional foods, has been promoted as a dietary option to decrease serum cholesterol levels and consequently decrease the occurrence of coronary heart disease.

Plants have a characteristically complex sterol mixture in contrast to mamalians and fungi which contain only one major sterol, cholesterol and ergosterol, respectively. Phytosterols differ from cholesterol by the presence of an extra alkyl group at C24. In oilseed rape, 24-methyl sterols comprise mainly of campesterol and brassicasterol while 24-ethyl sterols comprise mainly of

sitosterol and avenasterol (Appelqvist et al., 1981).

Phytosterols are found in vegetable oils, nuts, seeds and some vegetables and fruits (Weihrauch and Gardner, 1978). The common sources of phytosterols added in functional food are obtained from byproduct in the refining of vegetable oils or tall oil obtained from the pulping of wood. It is estimated that these sources can only supply about 10% of the people in developed countries (Law, 2000). While most crude vegetable oils generally contain about 0.1-0.5% phytosterols, crude rapeseed oil contains about 0.5-1.0% phytosterols and is ranked second highest in phytosterol content, next to corn oil which contains about 0.8-1.6% phytosterols (Piironen et al., 2000; Hamama et al., 2003; Raymer, 2002). Therefore, oilseed rape is considered as one of the valuable sources of phytosterol for the food and nutraceutical industry.

Genetic variation of total phytosterol content in oilseed rape has been reported using different populations. The highest range of total phytosterol content was reported in a collection of 27 canola winter oilseed rape cultivars which ranged from 357 to 480 mg $100\ g_{seed}^{-1}$ (Amar et al., 2009) as compared with the 101 resynthesized rapeseed lines which ranged from 208 to 433 mg $100\ g_{seed}^{-1}$ (Amar et al., 2009) and the three different double-haploid (DH) populations which ranged from 257 to 415 mg $100\ g_{seed}^{-1}$ (Amar et al., 2008a). The high total phytosterol content observed in canola winter oilseed rape cultivars is attributed to the low erucic acid content in the seed oil as close negative correlations between erucic acid content and phytosterol content have been found in non-canola quality populations (Amar et al., 2008a,b, 2009). Furthermore, quantitative trait loci (QTL) analysis also revealed that two of the three QTL identified for total phytosterol content are collocated with two erucic acid genes on N8 and N13 in a DH population segregating for erucic acid (Amar et al., 2008b). While correlations between total phytosterol content and both protein content and glucosinolate content appear to be uncorrelated in three different DH populations, and conflicting correlations were observed between total phytosterol content and oil content among the populations (Amar et al., 2008a).

With the knowledge acquired from previous work (Amar et al., 2008a,b, 2009), the present study aimed to perform linkage mapping and association mapping to dissect the genetic basis for the large variation of seed phytosterol content and composition observed in canola oilseed rape cultivars and to investigate the correlations between phytosterol content and oil content as well as

other important traits such as fatty acid composition, protein content of defatted meal and seed weight.

For linkage mapping, a segregating DH population was constructed by crossing two winter oilseed rape cultivars, "Sansibar" and "Oase". These two parental lines were selected from a collection of 27 canola winter oilseed rape cultivars due to their contrasting phytosterol and oil content in seed; Sansibar contains the highest phytosterol content ($\sim$480 mg 100 g_{seed}^{-1}) and the lowest oil content (43%) while Oase contains the lowest phytosterol content ($\sim$360 mg 100 g_{seed}^{-1}) and the highest oil content (46%) (Amar et al., 2009). Field trials of SODH population were carried out in two mega-environments, Europe (Germany and Sweden) and East China (Hangzhou). For QTL mapping, a genetic map was constructed for SODH population with a combination of different types of molecular markers. The genetic map of SODH population will be described in **Chapter 3**. Genetic variation and QTL identified for all analysed traits in SODH population will be presented in **Chapter 3** based on evaluation from field trials conducted in Europe (Germany and Sweden) while **Chapter 4** will be based on evaluation from field trials conducted in East China (Hangzhou).

For association mapping, 81 canola quality winter oilseed rape varieties and breeding lines which have been cultivated in six environments in Germany were used. A total of 692 markers—685 mapped amplified fragment length polymorphisms (AFLP) markers and seven single nucleotide polymorphisms (SNP)/insertion and deletion (InDels) candidate gene-based markers—were analyzed for association with the phenotyped traits using two models (general linear model (GLM) and K models). The study will be presented in **Chapter 5**

Chapter 6 presents a general discussion based on the genetic variation observed in SODH population and the 81 canola quality winter oilseed rape varieties and breeding lines. The correspondence of QTL between linkage mapping and association mapping were examined for their stability across different genetic backgrounds and growing environments.

Chapter 2

Literature review

2.1 Importance of oilseed rape

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is the most important member of the Brassicaceae family. It is an amphidiploid species, originated as a result of natural interspecific hybridization between turnip rape (*Brassica rapa* L., syn *campestris*; genome AA, 2n = 20) and cabbage (*Brassica oleracea* L.; genome CC, 2n = 18). It was domesticated as an oilseed crop 400-500 years ago (Gómez-Campo, 1999) and through intensive breeding efforts over the past four decades, it has advanced to become a major international crop.

Today, oilseed rape is the world's third-leading source of vegetable oil and the second most important oilseed in the world after soybean (Figure 2.1). In 2013, oil production from oilseed rape amounts to 26.1 million tons, accounting for 15% of the world's vegetable oil supply (USDA, `http://apps.fas.usda.gov/psdonline/psdHome.aspx`). The main oilseed rape producing countries are Europe, China, Canada and India. In the EU-28 countries, 9.6 million tons of oilseed rape was produced, accounting for 37% of worldwide oilseed rape production. Cultivation acreage of oilseed rape is predicted to expand, particularly in European regions where demand is growing for renewable fuels such as biodiesel.

Oil produced from oilseed rape is valued as both edible and industrial oil. In Europe, almost all of the oilseed rape cultivation is "double low" or "canola" quality—that is, <2% erucic acid in the oil and <25 $\mu mol\, g^{-1}$ glucosinolates in the seed—because of its ideal properties for both human

nutrition and biofuel production. A small fraction of high erucic acid oilseed rape is also cultivated for oleochemical industry. Apart from being cultivated for oil production, oilseed rape meal is also one of the most widely used protein sources for animal feed.

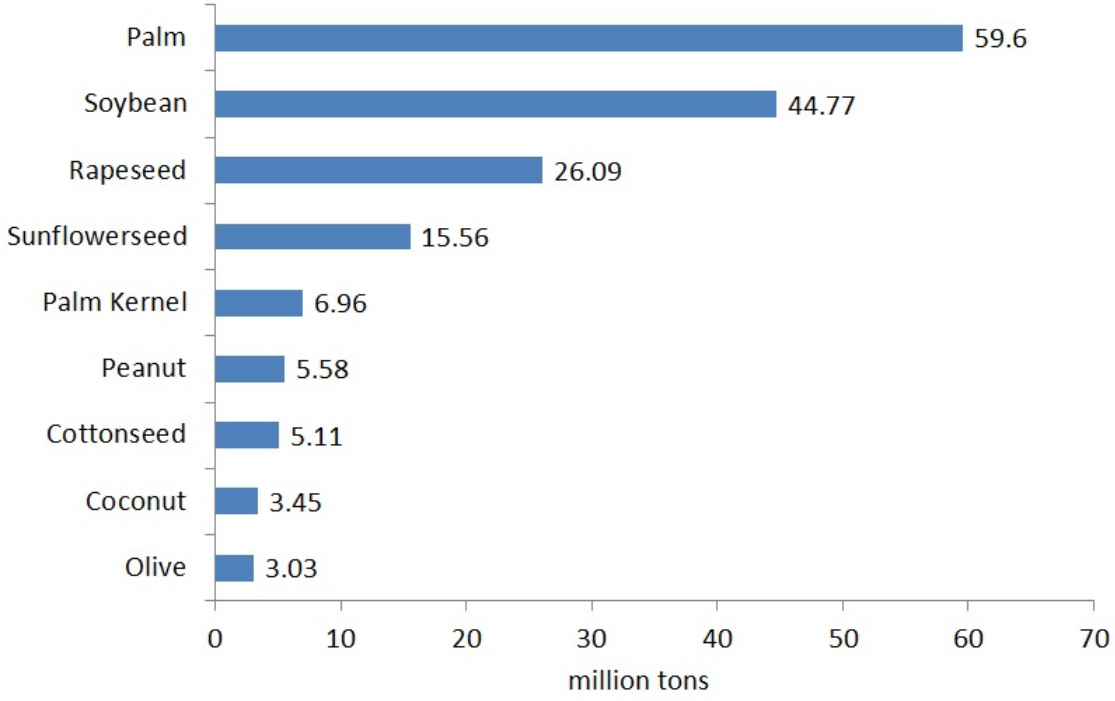

Figure 2.1: World production of vegetable oils in 2013/2014 (in million metric tons). Source: USDA Foreign Agricultural Service; `http://apps.fas.usda.gov/psdonline/psdHome.aspx`

2.2 Breeding for seed oil quality in oilseed rape

Oil is composed mainly of triacylglycerols with lesser amounts of phospholipids and glycolipids, and trace amounts of monoacylglycerols, diacylglycerols and free fatty acids. During oil extraction, the lipid soluble compounds of polyisoprenoid origin such as phytosterols, tocopherols, carotenoids, and chlorophyll, are also included into the oil fraction. Triacylglycerols are derived from three fatty acids esterified to a glycerol. Since triacylglycerols constitute about 90% of the oil, the fatty acid composition which represent the overall composition of the triacylglycerols is an important quality parameter determining the value and suitability of the oil for nutritional or industrial application.

Earlier oilseed rape cultivars contained high contents of erucic acid (up to 50%) glucosinolates in

seed. Erucic acid renders the oil to taste bitter and might cause heart problems when consumed in large quantities while glucosinolate made oilseed rape meal undesirable as a livestock feed due to the release of by-products that can cause liver and kidney damage in monogastric animals. As such, the oil was traditionally used as raw material by the chemical industry and only used as edible oil in time of crisis. These two limitations were overcome in the 1970s by the development of "0" and "00" varieties (Stefansson, 1983; Downey, 1990; Röbbelen and Downey, 1989). The first 0-quality variety, containing less than 1% erucic acid in the seed oil, came from a spontaneous mutant of the German spring cultivar "Liho". Subsequent identification of low glucosinolate content in Polish spring variety "Bronowski" was then used in an international backcrossing program to introduce this polygenic trait into 0-quality material. The result was the release of first 00-quality spring variety "Tower" in 1974 and since then, the term "canola" or "double low" quality has been used to refer to cultivars with zero erucic acid and low glucosinolates content. It was this remarkable breeding success that advanced oilseed rape into one of the major oil crops worldwide.

Following this, additional breeding effort to modify fatty acid composition has been to reduce the linolenic acid content from about 10% to less than 3% and to increase oleic acid content from about 50% to over 75%. Oil with high oleic acid and low linolenic acid content (HOLLi) is desirable to enhance shelf life and to reduce trans-fatty acids, an important characteristic determining the frying stability of oil (Warner and Mounts, 1993). Breeding for high C18:1 and low C18:3 mutants have been produced through mutagenesis, where at least three major genes are known to be responsible for the HOLLi phenotype. However, a major setback of the HOLLi phenotype is the impact of linkage drag associated with yield penalty (Auld et al., 1992). Therefore, breeding for high yielding HOLLi cultivars is one of the present breeding goals.

Recently, minor salutary oil constituents such as carotenoids, phytosterols, and tocopherols have also drawn the attention among plant breeders and researchers to study and improve its content and composition due to their conferred health benefiting properties that could further enrich the oil quality (Shewmaker et al., 1999; Yu et al., 2008; Wei et al., 2010; Amar et al., 2008b; Marwede et al., 2005; Fritsche et al., 2012).

2.3 Phytosterols

Phytosterols or plant sterols are natural occurring plant constituents with similar chemical structure as cholesterol. Whereas cholesterol is the unique sterol of vertebrates and ergosterol is the major sterol of fungi, plants possess a characteristically complex sterol mixture. The structural variations of phytosterols arise from different number of carbon atoms on C-24 in the side chain as well as the number and position of double bonds in the tetracyclic skeleton (Figure 2.2). With each plant species having a unique characteristic distribution of sterols, more than 250 types of sterols have been identified in the plant kingdom so far (Hartmann, 2004). In fact, the composition of sterol is sometimes used as a chemical fingerprint to detect admixtures for authentication of oil type to monitor trade and to ensure compliance with legislation (Gordon and Miller, 1997). In oilseed rape, the sterol profile consists mainly of sitosterol, campesterol, brassicasterol and avenasterol, while cholesterol and stigmasterol occur only in trace amounts (Figure 2.2) (Appelqvist et al., 1981). Brassicasterol is a characteristic sterol of *Brassicaceae* species and in oilseed rape, it amounts to about 13% of total phytosterol content.

Since 1950s, phytosterols are widely known for their cholesterol lowering properties. An effective dose of 1 - 3 $g\,d^{-1}$ leads to reduction between 8 - 15 % in low-density lipoprotein (LDL) cholesterol (Quilez et al., 2003). Other promising effects include anti-cancer (Woyengo et al., 2009), anti-atherosclerosis (Moghadasian et al., 1997), anti-inflammation (Bouic, 2001) and anti-oxidation (Van Rensburg et al., 2000). These health-promoting properties have led to the development of functional food enriched with phytosterol as bioactive ingredients. A variety of foods fortified with phytosterols, such as margarines, mayonnaises, salad dressings, milk, dairy products, and snack bars are now widely available in the market. The principal sources of phytosterols are tall-oil, the fat-soluble fraction of the hydrolysate obtained during wood pulping process, and deodorizer distillate fraction from vegetable oil refining. With oilseed rape ranked as second highest in phytosterol content among vegetable oils, it may serve as a valuable base stock for phytosterol enrichment of foods. In terms of cholesterol lowering ability, a meta-analysis of the human studies reported similar cholesterol-lowering effects using either plant stanol or sterol esters (Law, 2000). As suggested by Miettinen (2001), the hypothetical composition of plant product for lowering LDL cholesterol level would be either vegetable oil rich in plant stanols, particularly

in sitostanol, esterified with polyunsaturated fatty acids or plant sterols comprising mainly of sitosterol esters with low campesterol ester content. The preference for low campesterol is due to the higher absorption of campesterol than sitosterol in the intestinal tract which may pose risk to patients with phytosterolaemia during chronic consumption of plant sterols (Lees et al., 1977).

Like cholesterol in vertebrates, phytosterols are integral components of the cell membrane and as such, regulate the fluidity and permeability of phospholipid bilayer and modulate the activity of membrane bound proteins such as enzymes and signal transduction components (Hartmann, 1998). They are found primarily in the outer membrane of mitochondria, in the membranes of the endoplasmic reticulum, and in the plasma membrane. Because the fluidity of the membrane is to some extent governed by the content of sterols, the unique feature of plants in possessing a complex sterol mixture has been proposed to be an evolutionary response for adaptation to large temperature variation (Dufourc, 2008). In a study of membrane dynamics, Dufourc (2008) proposed that the presence of an additional ethyl group branched on C-24 may reinforce the attractive van der Waals interactions leading to increase membrane cohesion and therefore less sensitive to temperature. In addition, phytosterol such as campesterol serves as biosynthetic precursors of the plant-growth regulators called brassinosteroids which play important roles in controlling gene expression, cell division and expansion, responses to light and dark, and fertility (Yokota, 1997; Schumacher and Chory, 2000).

2.4 Overview of the metabolic pathways

2.4.1 Seed oil synthesis

Seed oil synthesis occurs in two stages, firstly through the production of acyl chains by the plastids, followed by their sequential assembly into triacylglycerol by the acyltransferases of the endoplasmic reticulum (Ohlrogge and Browse, 1995). The fatty acid synthesis in plastid is initiated by the condensation of acetyl-CoA and malonyl-CoA acyl carrier protein (ACP), a multistep process yielding a 4-carbon acyl-ACP. In *B. napus*, repeated cycles of 2-carbon elongation result in the synthesis of palmitoyl-ACP (C16:0-ACP) which can either be hydrolyzed by the fatty acyl-ACP thioesterases B (FATB) to release palmitic acid (C16:0) from ACP or further elongated

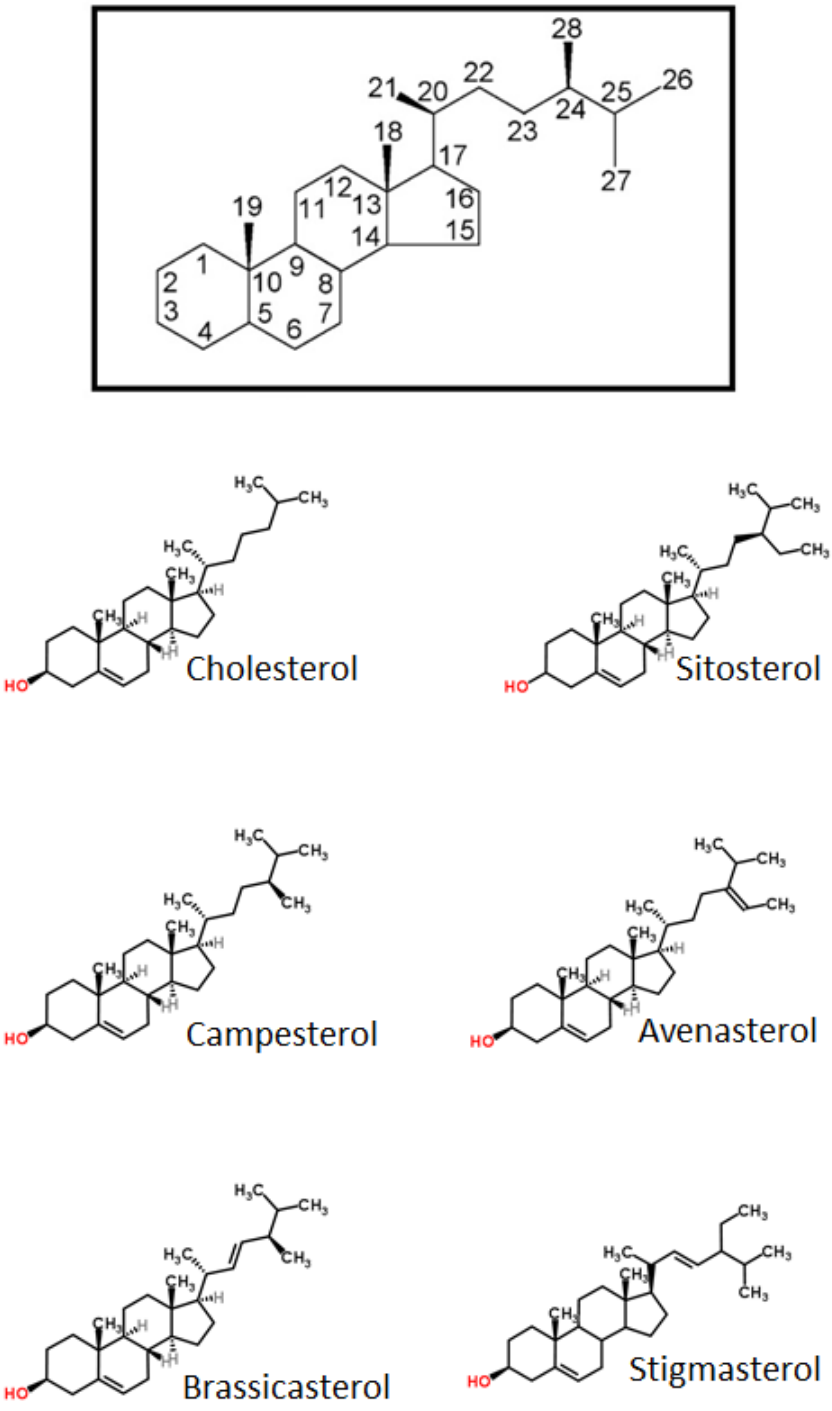

Figure 2.2: Chemical structure of phytosterols. Numbered carbon atoms of the sterol core rings and the chemical structure of phytosterols in *Brassica napus*.

by 3-ketoacyl-ACP synthase II (KASII) to stearoyl-ACP (C18:0-ACP), desaturated by stearoyl-ACP desaturase (SAD) to oleoyl-ACP (C18:1-ACP), and finally hydrolyzed by the fatty acyl-ACP thioesterases A (FATA) to release oleic acid (C18:1) from ACP. The resulting main products from plastid fatty acid synthesis are C16:0 and C18:1 free fatty acids. As their relative proportions are determined by the activities of FATA, FATB, SAD, and KASII, these enzymes have all been targeted in various studies aimed at increasing the saturated fatty acid composition of *B. napus* seed oil (reviewed by Stoll et al. (2005)).

Upon leaving the plastid, newly synthesized fatty acids are esterified to fatty acyl-CoA and assembled into glycerolipids at the endoplasmic reticulum. The *de novo* assembly of triacylglycerol from glycerol-3-phosphate (G3P) and acyl-CoA, also known as the Kennedy pathway, involves only four enzymatic steps: first, two acylations of G3P by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), followed by phosphatidic acid phosphatase (PAP), and a third acylation by diacylglycerol acyltransferase (DGAT). Additional more complex pathways have also been defined for triacylglycerol synthesis in which the membrane lipid phosphatidylcholine is a central intermediate in the flux of fatty acids or diacylglycerol, or both substrate into triacylglycerol (Bates et al., 2013).

Generally, flux into metabolic pathway can be manipulated either by increasing the supply of upstream substrates or by increasing the strength of sink or demand in the final steps of a pathway. In oilseed rape, experiment of a top-down control analysis has demonstrated that lipid assembly via acyltransferases exerts greater control over triacylglycerol accumulation than fatty acid synthesis (Ramli et al., 2002). Particularly, the DGAT activity which catalyzes the final committed step in the Kennedy pathway; its relatively low activity as compared with other enzymes in the lipid biosynthesis pathway as well as the accumulation of diacylglycerol in developing seeds have suggested that the DGAT catalyzed reaction represents a restriction point in seed oil formation (Perry and Harwood, 1993; Perry et al., 1999). In a transgenic approach, over-expression of two different DGAT1 gene sources (*A. thaliana* and *B. napus*) in *B. napus* have demonstrated enhancement of oil content in multiple field trials (Taylor et al., 2009). Besides the catalytic enzymes that are directly involved in metabolic process, transcription factors that regulate tissue specific oil accumulation have also attracted wide-spread interests. For example, the WRINKLED 1 (WRI1) gene in *A. thaliana* (Cernac and Benning, 2004) and several other WRI1-related transcription

factors, such as LEAFY COTYLEDON2 (LEC2) (Baud et al., 2007), LEAFY COTYLEDON1 (LEC1), FUSCA3 (FUS3) and ABA INSENSITIVE3 (ABI3) (Mu et al., 2008; Baud et al., 2009; Shen et al., 2010).

2.4.2 Phytosterol synthesis

The biosynthetic pathway leading from acetyl-coA to end-products of phytosterol has been characterized into details. Recent reviews include those of Bach and Benveniste (1997) and Benveniste (2002). An overview of the phytosterol biosynthesis pathway is described here with emphasis on several key genes that are known to influence modulation of phytosterol content and composition based on transgenic studies. In general, the phytosterol biosynthesis can be divided into two stages: (1) regulation of carbon flux into the isoprenoid pathway to cycloartenol and (2) transformation of cycloartenol to 24-alkyl sterols (Figure 2.3).

In the early stage, the regulation of carbon flux into the isoprenoid pathway mainly occur via the cytosolic mevalonate (MVA) pathway as opposed to the plastidial methylerythritol phosphate (MEP) pathway which seems to be responsible for the synthesis of terpenes of plastidial origin (McCaskill and Croteau, 1998). The MVA pathway in the cytosol begins with acetyl-CoA as the initial substrate and undergoes six enzymatic reactions to produce isopentyl pyrophosphate (IPP) . 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) is the second enzyme in the MVA pathway which catalyzes the condensation of acetoacetyl-CoA and acetyl-CoA to produce HMG-CoA (Ferguson and Rudney, 1959; Rudney and Ferguson, 1959; Stewart and Rudney, 1966; Lynen, 1967). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) then converts HMG-CoA to mevalonate, which then undergoes two phosphorylation and a decarboxylation reaction to form IPP.

In *Arabidopsis*, over-expression of wild-type and mutant HMGS up-regulate genes involved in MVA pathway, including HMGR and sterol C24-methyltransferase 2 (SMT2), leading to increased phytosterol content of up to 29 % in seedlings and enhanced stress tolerance response (Wang et al., 2011a). In *B. juncea*, co-ordinated regulation of HMGS and HMGR have also been observed upon germination and in response to salicylic acid (Alex et al., 2000). However, enhancement of phytosterol level in seed tissue has so far not been reported.

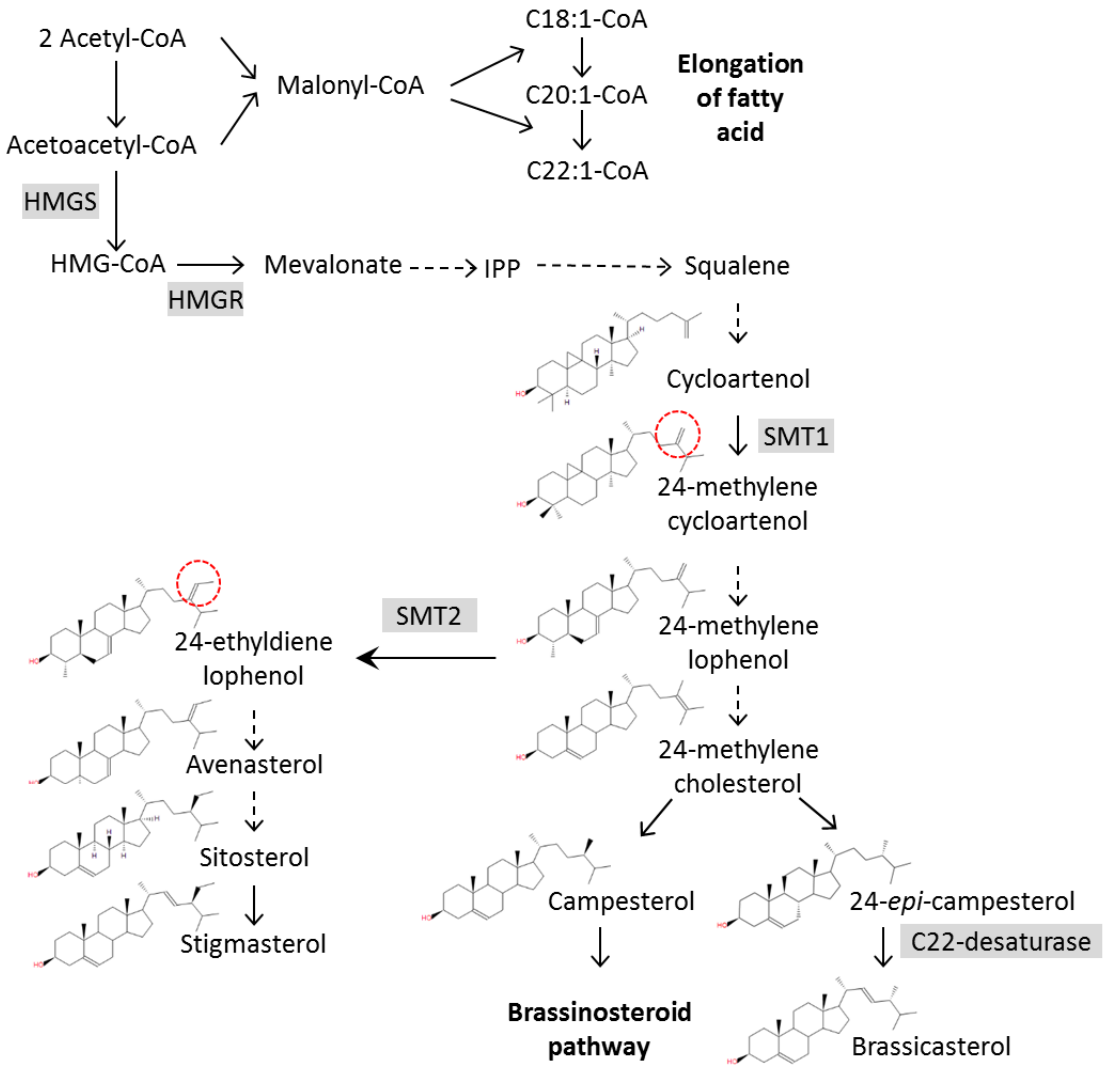

Figure 2.3: Simplified phytosterol biosynthetic pathway in plants. *Solid* and *dashed arrows* indicate single and multiple biosynthetic steps, respectively. Adapted from Benveniste (2002), Schaller (2003). *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *SMT1* C-24 sterol methyltransferase 1, *SMT2* C-24 sterol methyltransferase 2

As for the subsequent enzyme, transgenic tobacco with an ectopic expression of a N-terminal truncated *Hevea brasiliensis* HMGR gene have led to 3.2-fold increased of seed phytosterol level (Harker et al., 2003b) and a modification of *Arabidopsis* HMGR gene on a target site for phosphorylation by SNF1-related protein kinase (SnRK) have shown 2.4-fold enhancement of seed phytosterol level (Hey et al., 2006). In contrast, over-expression of an unmodified *Arabidopsis* HMGR gene in transgenic tabacco resulted in little increase in seed phytosterol accumulation while no change in phytosterol levels occurred in transgenic *Arabidopsis* despite the fact that the gene was expressed and high levels of transcript were detected (Re et al., 1995). These findings suggest that HMGR is regulated in part at transcriptional level and in part by other regulatory mechanisms at post-trasncriptional level. External factors such as light, pathogens and wounding have also shown to influence the HMGR activity (Chappell et al., 1995; Korth et al., 2000). In addition, a substantial proportion of the overproduced phytosterols in transgenic plants are in the form of sterol intermediates such as cycloartenol, which led to the suggestion that the next step in the pathway, namely the conversion of cycloartenol to 24-methylene cycloartanol, catalysed by sterol C24-methyltransferase 1 (SMT1), is a "slow step" in the synthesis of 4-desmethylsterols (end-product sterols).

In the second stage, cycloartenol is transformed into end-product sterols in a series of enzyme catalyzed methylations, demethylations, and desaturations. The conversion of cycloartenol into 24-methylene cycloartenol is principally catalyzed by SMT1. In tobacco, over-expression of SMT1 increase end-product sterols and decrease intermediate sterols, resulting in 44% increase of total phytosterol accumulation in seeds (Holmberg et al., 2003). In potato, over-expression of a soybean SMT1 gene also displayed total increased in phytosterol level for both leaves and tubers, derived mainly due to increased levels of the 24-ethyl sterols isofucosterol and sitosterol (Arnqvist et al., 2003). Enhancement of total phytosterol is greater under co-expression of both HMGR and SMT1 which have been proposed to be key steps in regulating carbon flux through the phytosterol biosynthetic pathway. For instance, in tobacco, co-expression of the catalytic domain of *H. brasiliensis* HMGR (tMHGR) and *Nicotiana tabacum* SMT1 significantly elevates seed phytosterol content up to 2.5-fold and reduces the amount of cycloartenol synthesized (Holmberg et al., 2003). The phytosterol biosynthesis pathway is essentially linear until reaching 24-methylene lophenol in which SMT2 enzyme acts at the branch point directing carbon flux towards C-24 ethyl sterols (avenasterol,

sitosterol, stigmasterol) and away from C-24 methyl sterol or brassinosteroid biosynthesis. In transgenic *Arabidopsis*, antisense SMT2 plants contain lower phytosterol levels, higher campesterol levels and exhibit dwarfism accompanied by reduced apical dominance, floral organ elongation, and fertility; while plants that overexpressed SMT2 contain higher levels of sitosterol, lower levels of campesterol and exhibit reduced plant stature that can be rescued with exogenous application of brassinosteroids (Schaeffer et al., 2001).

Another subtle difference in chemical structure of phytosterols is the specific occurrence of a double bond at C-22 in the sterol side chain such as stigmasterol and brassicasterol. Study on transgenic *Arabidopsis* has demonstrated that cytochrome P450 enzyme encoded by *CYP710A2* may be responsible for the C-22 desaturase activity which converts 24-*epi*-campesterol to brassicasterol (Morikawa et al., 2006).

In oilseed rape, about 35% of phytosterols in seed oil is in the form of steryl esters (Harker et al., 2003a). They are generally thought to serve as intracellular storage molecules for sterols and free fatty acids when the amounts are in excess of that required for the cells. For instance, characterization of tobacco mutant *sterov* (*ste*rol *ov*eraccumulation) and transgenic plants which exhibited higher HMGR enzyme and a dramatic increase in the mevalonate flux resulted in accumulation of sterols as cytoplasmic steryl esters found in lipid droplets (Maillot-Vernier et al., 1991; Gondet et al., 1994; Schaller et al., 1995; Bouvier-Navé and Benveniste, 1995). The esterification of sterols via the transfer of acyl groups from acyl donors to sterols are catalyzed by sterol acyltransferase (SAT) (Bouvier-Navé and Benveniste, 1995; Zimowski and Wojciechowski, 1981).

2.5 QTL mapping for oil and phytosterol content in *B. napus*

Mapping the genetic loci that control the quantitative variation is a preliminary step to disclose the complex regulation of a polygenic trait. Better knowledge of the genetic determinism of a trait could in turn aid breeders in advancing the crop. Linkage mapping is the traditional method for identifying QTL while association mapping, originally used in humans and animals, has recently been adopted in plants. Association mapping has at least two main advantages over traditional linkage mapping methods (Zhu et al., 2008). First, it mitigates the need to construct population

from crosses by using natural population which has a much broader germplasm context. Second, it can achieves a higher resolution mapping by exploiting linkage disequilibrium (LD) generated from historical recombination events. In all mapping approaches, however, a trade-off exists between statistical power and resolution. Association mapping is also associated with a higher risk of biased estimation or even false inference due to population structure. As such, an ideal analysis would be to reap the benefits of each method by complementary use of both linkage and association mapping to obtain both high power of detection and resolution.

Over the past few decades, numerous QTL for oil content in oilseed rape have been identified using different mapping methods and different populations. In linkage mapping studies, the segregating populations were developed either from crosses where both parental lines had a high oil content (Zhao et al., 2005), both had a moderate oil content (Ecke et al., 1995; Burns et al., 2003; Zhao et al., 2005; Qiu et al., 2006; Jiang et al., 2014), or one had a high oil and the other a moderate oil content (Delourme et al., 2006). The first genetic studies which set out to map QTL controlling the seed oil content variation in *B. napus* detected three discrete loci (Ecke et al., 1995). Of the three loci, two are closely associated with erucic acid content, indicating a direct effect of the erucic acid genes on oil content. Burns et al. (2003) identified seven QTL using an intervarietal subset of substitution lines. Subsequent study involving a European and a Chinese parental lines ("Sollux" and "Gaoyou", SG population) detected eight QTL with additive effects and nine pairs of loci with additive$\times$additive epistasis along with high genotype $\times$ environment interactions (Zhao et al., 2005). Another similar population generated between a Chinese and a European parental lines ("Tapidor" and "Ningyou7", TN population) identified 7 QTL for oil content in which three were found coincided with QTL for erucic acid (Qiu et al., 2006). Using two populations with different genetic backgrounds ("Darmor-*bzh* and Yudal", DY population; "Rapid" and "NSL96/25", RNSL population), Delourme et al. (2006) identified 14 and 10 genomic regions associated with seed oil content in which only one QTL was found potentially common to the two populations. The study reported three pairs of epistatic interactions and attributed additive effects as the main factors contributing to variation in oil content.

A larger number of QTL for oil content were reported using association mapping approach. In a first experiment on whole-genome association analysis in oilseed rape, Honsdorf et al. (2010) identified 22 QTL for oil content in a set of 84 canola quality winter. Using a new-type population

and a traditional oilseed rape population, Zou et al. (2010) identified 54 QTL associated with seed oil content, 6 of which were found collocated with QTL detected by Qiu et al. (2006) using interval mapping approach. Another association mapping study which included 17 SNPs derived from 9 candidate genes from the triacylglycerol biosynthetic pathway in a population of 685 diverse elite oilseed rape inbred lines demonstrated that in addition to main effects, both intergenic and intragenic epistasis also contributed a considerable amount of genotypic variation in oil content (Würschum et al., 2013). The identified interactions includes certain key enzymes involved in the main pathway of storage oil formation as well as the WRI1 transcription factor which is known to be involved in the control of storage compound biosynthesis in *Arabidopsis*.

In a recent study, Jiang et al. (2014) updated the number of QTL for seed oil content to 41 in the TN population with increased number of environments and marker density from the previous study reported by Qiu et al. (2006). With an additional TN reconstructed F_2 population, Jiang et al. (2014) detected 20 QTL with dominance effects in which a majority of them showed partially dominant effect and only four QTL showed positive complete-dominance or mild over-dominance, suggesting that oil content in oilseed rape has weaker heterosis compared with other traits such as seed yield (Radoev et al., 2008). In an attempt to account for full extent of the variation in seed-oil content, Jiang et al. (2014) also established a reference map by incorporating common markers from different genetic populations (SG, DY and RNSL populations) on the genetic map of TN population. The resulting reference map enabled QTL detected from SG, DY and RNSL populations as well as the significant markers detected by association study of Zou et al. (2010) to be aligned and compared with its own detected QTL. In total, the reference map identified 46 distinct QTL regions that control seed oil content on 16 of the 19 linkage groups of *B. napus*. Of the 46 QTL, 18 were identified in multiple populations.

So far, only one QTL mapping study for phytosterol has been reported in oilseed rape (Amar et al., 2008b). By using the population that was previously used by Ecke et al. (1995), Amar et al. (2008b) reported three QTL for total phytosterol content, two of which were found collocated with erucic acid genes on A08 and C03. Based on the fact that cytoplasmic acetyl-CoA is required as precursor for both synthesis of erucic acid and phytosterols (Figure 2.2) and that the alleles increasing phytosterol content exhibited negative relationship with erucic acid content, the authors further concluded that the two QTL identified for phytosterols were due to pleiotropic effect

exerted by the two erucic acid genes. As such, it can be anticipated that the utilization of a DH population that does not segregate for erucic acid would lead to increase of detection power for QTL with smaller effects.

Chapter 3

Genetic variation and inheritance of phytosterol and oil content in a doubled haploid population derived from the winter oilseed rape Sansibar × Oase cross cultivated in Europe

3.1 Abstract

Phytosterols are one of the minor seed constituents in oilseed rape that have received wide-interest in the food and nutrition industry due to its health benefit in lowering LDL cholesterol in humans. To understand the genetic basis of phytosterol content and its relationship with other seed quality traits in oilseed rape, quantitative trait loci (QTL) mapping was performed in a segregating double-haploid (DH) population derived from the cross of two winter oilseed rape varieties "Sansibar" and "Oase", termed SODH population. Both parental lines are of canola quality and were chosen due to their contrasting phytosterol and oil contents in seed. A genetic map was constructed for SODH population based on a total of 1642 markers (AFLP, candidate-gene based marker, DArT, Silico-DArT, SSR, and SNP), organized in 23 linkage groups and covering a map length of 2350 cM with a mean marker interval of 2.0 cM. The SODH population was cultivated at six environments in Europe and was phenotyped for phytosterol contents as well as some important seed quality traits such as oil content, fatty acid composition and protein content of defatted meal, and a yield related trait, seed weight. Multiple interval mapping identified 29 QTL for nine phytosterol traits, 16 QTL for four fatty acids, six QTL for oil content, four QTL for protein content of defatted meal and three QTL for seed weight. Colocalizations of QTL for different traits were more frequently observed than individual isolated QTL. Four genomic regions with major QTL ($R^2 \geq 25\%$) were found for brassicasterol on A04, campesterol:sitosterol and 24-methyl:24-ethyl sterol on A06, C18:1 and C18:3 on A01, and C16:0 on A09. Possible candidate genes that underlie these four QTL genomic regions were revealed by aligning locations of QTL with the reference sequence of *Brassica rapa*. A relatively good collinearity between genetic and physical map positions were observed in all four QTL genomic regions. QTL for brassicasterol on A04 was colocalized with *CYP710A1*, a gene that encodes the cytochrome P450 enzyme which might be responsible for converting 24-epi-campesterol to brassicasterol. QTL for campesterol:sitosterol and 24-methyl:24-ethyl sterol on A06 were colocalized with *SMT2*, a gene that encodes the sterol C24-methyltransferase 2 which converts 24-methylenelophenol to 24-ethylidene lophenol. QTL for C18:1 and C18:3 on A01 were colocalized with *FAD2*, a gene that encodes the endoplasmic delta-12 oleate desaturase which desaturate C18:1 into C18:2. QTL for C16:0 on A09 was colocalized with *FATB*, a gene that encodes the acyl-ACP thioesterase which hydrolyzes the thioester bond of C16:0-ACP and releases C16:0

and ACP.

3.2 Introduction

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is the world's third-leading source of vegetable oil for human nutrition and industrial products. In terms of nutritional composition, oilseed rape oil possesses a nearly ideal fatty-acid profile as edible oil, that is, having low level of saturated fatty acids, high monosaturated fatty acids and good proportion of omega-3 and omega-6 polyunsaturated fatty acids. Recently, some minor salutary oil constituents such as carotenoids (Shewmaker et al., 1999; Yu et al., 2008; Wei et al., 2010), phytosterols (Amar et al., 2008b), and tocopherols (Marwede et al., 2005; Wang et al., 2012b; Fritsche et al., 2012) have also drawn the attention among plant breeders and researchers to study and improve its content and composition due to the conferred health benefiting properties that could further enrich the oil quality.

Since the 1950s, phytosterols are widely known for their cholesterol lowering properties. An effective dose of 1 - 3 g day^{-1} leads to reduction between 8 - 15% in LDL-cholesterol (Quilez et al., 2003). Other promising effects include anti-cancer (Woyengo et al., 2009), anti-atherosclerosis (Moghadasian et al., 1997), anti-inflammation (Bouic, 2001) and anti-oxidation (Van Rensburg et al., 2000). These health-promoting properties have led to the development of functional food enriched with phytosterols as bioactive ingredients. A variety of foods fortified with phytosterols, including margarines, mayonnaises, vegetable oils, salad dressings, milk, dairy products, beverages, and snack bars, are now widely available in the market (Berger et al., 2004). The most common sources of phytosterol added to foods are tall oil—a byproduct of the pulping industry that is rich in sitosterol and sitostanol (Jones et al., 1998)—and distillate fraction from vegetable oil refining. While most crude vegetable oils contain about 1 to 5 g kg^{-1} of phytosterol, corn oil contains about 8 to 16 g kg^{-1} and oilseed rape oil contains about 5 to 10 g kg^{-1} (Piironen et al., 2000). The exceptionally high amount of phytosterol in oilseed rape means that it may serve as one of the valuable base stock for the health and nutrition industry.

Phytosterols include a wide variety of molecules that are structurally similar to cholesterol. The structural variations of phytosterols arise from different number of carbon atoms on C-24 in the

side chain as well as the number and position of double bonds in the tetracyclic skeleton. In oilseed rape, the phytosterol profile consists mainly of sitosterol, campesterol, brassicasterol and avenasterol, while cholesterol and stigmasterol occur only in trace amounts (Appelqvist et al., 1981). Brassicasterol is a characteristic sterol of *Brassicaceae* species and in oilseed rape, it amounts to about 13% of total phytosterol content. In terms of cholesterol lowering ability, high sitosterol and low campesterol levels are preferable due to the higher absorption of campesterol than sitosterol in the intestinal tract which may pose risk to patients with phytosterolaemia during chronic consumption of phytosterols (Lees et al., 1977). A few studies which evaluate the brassicasterol-rich phytosterols mixtures obtained from oilseed rape have reported similar cholesterol lowering properties to those phytosterols obtained from other sources like tall oil (Demonty et al., 2007; Heggen et al., 2010), suggesting that oilseed rape can be considered as a suitable alternative source for phytosterol enrichment of foods.

Among the adapted winter oilseed rape populations, modern cultivars with canola quality contain higher amount of total phytosterol content than the genetically diverse or resynthesized lines that are of non-canola quality. This observation is due to the close negative correlation between total phytosterol content and erucic acid content (Amar et al., 2009). In a winter oilseed rape DH population segregating for erucic acid, QTL mapping shows that two of the three QTL identified for total phytosterol content are colocalized with two erucic acid genes Amar et al. (2008b). Based on the fact that cytoplasmic acetyl-CoA is required in the synthesis of both erucic acid and phytosterols, colocalizations of QTL are most likely attributed to pleiotropic effect exerted by erucic acid. To further investigate the inheritance of phytosterols and its relation to other important seed quality traits, a DH population constructed from two canola quality winter oilseed rape cultivars, Sansibar and Oase, was used in this study. The parental lines were selected based on previous screening which has been shown to differ with respect to phytosterol and oil content. It is anticipated that by the use of this DH population that does not segregate for erucic acid, higher detection power for QTL with smaller effects or novel alleles could be unravelled in the present study.

3.3 Materials and Methods

3.3.1 Plant material

The experimental population consisted of 226 F1 microspore-derived DH lines derived from the Sansibar $\times$ Oase cross. The two parental lines are among the 27 canola quality winter oilseed rape cultivars analyzed by Amar et al. (2009) and were chosen due to their contrasting total phytosterol content and oil content in seed; Sansibar contains the highest total phytosterol content ($\sim$480 mg 100 g_{seed}^{-1}) and lowest oil content (43%) while Oase contains the lowest total phytosterol content ($\sim$360 mg 100 g_{seed}^{-1}) and highest oil content (46%). The DH population was developed in the Division of Plant Breeding at Georg-August-Universität Göttingen and was named as SODH population.

3.3.2 Field experiments

The SODH population and the parental lines were cultivated in six environments: two environments at Göttingen, Germany during growing seasons 2009/11 and 2010/11; one environment at Einbeck, Germany during growing season 2010/11 by KWS Saat AG; one environment at Asendorf, Germany during growing season 2011/2012 by Deutsche Saatveredelung (DSV) AG; and two environments at Svalöv, Sweden during growing seasons 2010/11 and 2011/12 by Lantmännen SW Seed. The field trials were carried out in small plots in a complete randomized design without replication. Seeds of ten open pollinated plants from each line were harvested and bulked for analyses.

3.3.3 Molecular Markers

Genomic DNA of the SODH population and their parental lines were isolated from young leaves of 4 to 5 week-old greenhouse-grown seedlings using Nucleon PhytoPure plant extraction kits (GE Healthcare, Illustra^{TM}) according to manufacturers instructions. DNA was quantified using Bio-Rad Fluorescent DNA Quantification Kit (Bio-Rad Laboratories CA, USA).

SSR and AFLP markers

simple sequence repeats (SSR) analysis was carried out following the M13-tailing polymerase chain reaction (PCR) technique (Schuelke, 2000). PCR reactions were performed in 96-well PCR plates with a volume of 20 µl per reaction, containing 25 ng of genomic DNA, 0.05μM of forward primer with a M13 tail of 19 bp at the 5′ end, 0.05μM of reverse primer, 0.05 μM of M-13 primer, 2.5 mM $MgCl_2$, 0.2mM of each dNTP, 1$\times$ PCR buffer and 1 U of *Taq* DNA polymerase. A two-step touchdown PCR program was performed in a Biometra T1 Thermocycler (Biometra GmbH, Göttingen, Germany): 95 °C for 2 min; 5 cycles of 95 °C for 45 s; 68 °C (-2 °C/ cycle) for 5 min, 72 °C for 1 min; 5 cycles of 95 °C for 45 s, 58 °C (-2 °C/ cycle) for 1 min, 72 °C for 1 min; 27 cycles of 95 °C for 45 s, 47 °C for 30 s and 72 °C for 1 min; and 72 °C for 10 min. A total of 350 primer pairs obtained from various sources were screened for polymorphisms between the parents. Only SSR markers that were successfully mapped in SODH population are listed in Appendix A.1. The SSR primer pairs prefixed with "BRA" and "CB" were developed by Celera AgGen consortium, and prefixed with "MR" and "MD" were developed by Division of Plant Breeding at Georg-August-Universität Göttingen.

AFLP analysis was performed by adapting the method described by Vos et al. (1995). A total of 16 primer combinations (Table 3.1) made up from 8 *Eco*RI fluorescence labelled primers and 4 *Mse*I primers were used.

Table 3.1: Sixteen primer combination used in APLF analysis

E32M48	E37M50	E36M51	E36M59
E39M48	E38M50	E37M51	E37M59
E44M48	E40M50	E38M51	E38M59
E45M48	E44M50	E44M51	E44M59

The PCR products of AFLP and SSR were separated on the ABI PRISM 3100 genetic analyzer (Applied Biosystems) with GeneScan-500 ROX size standard (Applied Biosystems) using 36-cm capillary arrays. The results were analyzed with GeneScan software version 3.7 (Applied Biosystems) and scored using Genotyper software version 3.7 NT (Applied Biosystems).

SNP markers

A total of 125 polymorphic SNP were genotyped by the breeding company KWS Saat AG and were kindly provided to us for map construction.

DArT and SilicoDArT markers

The SODH population was genotyped with the *Brassica napus* v1.0 Diversity Arrays Technology (DArT) microarray comprising of 3072 markers, designated with the prefix "brPb". A subset of 183 lines from the SODH population was genotyped with 4787 Silico-DArT markers (`www.diversityarrays.com/dart-application-dartseq-data-types`), designated with the suffix "|F |0". DArT and Silico-DArT markers were performed by Diversity Array Technology Pty Ltd, Yarralumla, Australia. The sequences for DArT markers were retrieved from `http://www.diversityarrays.com/dart-map-sequences` while the sequences for Silico-DArT clones were provided by Diversity Array Technology Pty Ltd, Yarralumla, Australia.

KASP markers

From the Illumina Infinium Brassica 60K SNP array, a subset of 32 markers that were polymorphic between the parental lines, Sansibar and Oase, were selected for KBioscience competitive allele-specific PCR (KASP) genotyping (Trait Genetics GmbH). Of the 32 markers, 13 were physically closely linked to promising candidate genes for phytosterol biosynthesis and 19 were associated with oil content in SGDH14 $\times$ Express617 DH population (Nina Behnke, personal communication). The sequences for SNP markers were provided by Isobel Parkin (AAFC, Saskatoon, Canada). The physical positions were based on reference genome of *B. rapa* v1.5 genome database (BRAD; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* v1.0 genome database (Bolbase; `http://www.ocri-genomics.org/bolbase/`). KASP markers prefixed with "BNKS" and its corresponding name in Illumina Infinium Brassica 60K SNP array is shown in Appendix A.2

Candidate gene-based markers

Five candidates genes known to be involved in the regulation of phytosterol biosynthesis were selected to develop candidate gene-based markers: *3-hydroxy-3-methylglutaryl-CoA reductase 1 (HMG1), 3-hydroxy-3-methylglutaryl-CoA reductase 2 (HMG2),3-hydroxy-3-methylglutaryl-CoA synthase (HMGS),sterol C24-methyltransferase 1 (SMT1)* and *sterol C24-methyltransferase 2 (SMT2)*. The first step involved designing a locus-specific marker to differentiate between homologues based on locus-specific SNP, followed by sequencing of the amplicons to screen for allelic SNP between the parental lines. If an allelic SNP was found, an allele-specific marker was developed for the pertaining homologue.

To search for homologues for each candidate gene, BLAST searches were performed with *Arabidopsis* gene sequence against the reference sequence of *B. rapa* (BRAD v1.5; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* (Bolbase v1.0; `http://www.ocri-genomics.org/bolbase/`) genomes. *Arabidopsis* gene sequence and the corresponding homologues from *B. rapa* and *B. oleracea* were aligned using CLC main workbench 6.0 (CLC Bio, Aarhus, Denmark) program to elucidate the intron-exon structure and to identify locus-specific SNP. Locus-specific primer was designed such that the locus-specific SNP was placed at the 3' end of one of the primer pair and, wherever possible, in the exon region near the intron-exon boundaries to frame one or two introns that are potentially variable at allelic level.

To test for locus-specificity, each of the designed primer pairs was tested on *B. rapa* (Chiifu-401), *B. oleracea* (Rustico SG 2707), and *B. napus* (Sansibar and Oase). The PCR reaction was carried out in a total volume of 25 µl consisting of 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTP, 1.25 U of *Taq* DNA polymerase, and 0.4 mM of each forward and reverse primer. The annealing temperature for each primer pair was calculated with the following formula:

$$T_m(^\circ\mathrm{C}) = 69.3 + (0.41 \times \%\mathrm{GC}) - (650/\text{primer length})$$

The PCR thermal profile was set as following: initial denaturing at 94 °C for 3 min, 30 cycles of denaturing at 94 °C for 1 min, annealing at temperature calculated for the primer pair for 1 min,

extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. Amplified PCR products were visualized after electrophoresis on 1.5% agarose gel to check the amplicon size and to determine if the calculated annealing temperature could yield a clear and sharp band on the agarose gel. Gradient PCR or touchdown PCR was employed to optimize the annealing temperature if necessary. A primer pair was assumed to be locus-specific when one discrete band was observed only from its targeted genome, either *B. rapa* or *B. oleracea*, and from the amphidiploid species *B. napus* (See example: Figure 3.1).

When the primer pair was assumed to be locus-specific, sequencing was performed for PCR products derived from the template of Sansibar and Oase. A total volume of 50 µl (2 × 25 µl) of PCR products were purified with the innuPREP DOUBLEpure Kit (AnalytikJena AG, Jena, Germany) according to the manufacter's instructions and sequenced with BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) on an ABI-3100 Genetic Analyzer.

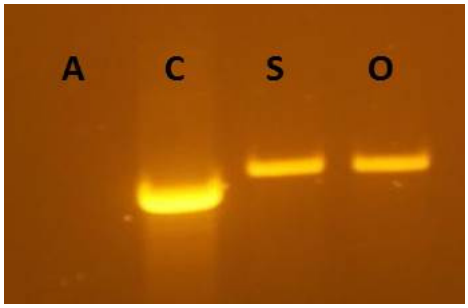

Figure 3.1: A typical example from locus-specific primer pair of SMT1C03-5 on gel electrophoresis screening. SMT1C03-5 primer pair was designed based on intergenomic SNP among four gene copies of *SMT1* to target amplification of gene fragment from the C genome. Amplicons of fragment size between 750 and 1000 bp was observed from its targeted genome *B. oleracea* ("**C**") and from the amphidiploid species *B. napus* (Sansibar "**S**"; Oase "**O**") while no amplicons was observed from *B. rapa* ("**A**").

The sequencing reads were trimmed to remove low quality sequences (quality limit = 0.02) and assemble to the reference sequence using CLC main workbench 6.0 (CLC Bio, Aarhus, Denmark). The sequencing reads were then assessed to identify allelic SNP between Sansibar and Oase.

If an allelic SNP was found between the parents, an allelic-specific primer pair was designed such that one contains an allelic SNP at the 3' end and another contains a locus-specific SNP at the 3' end. The allele-specific primer pair was then used to genotype the SODH population. Amplified PCR products were analyzed on 1.5% agarose gel to score for polymorphisms on the basis of presence/absence (dominant) of PCR products.

The schematic gene structure of phytosterol candidates genes are depicted in Appendix A.4 while the locus-specific and allele-specific primers are listed in Appendix A.5 and Appendix A.6, respectively. In addition, two candidate gene-based markers for *DGAT1*, D120E-3 and Dx-3 (Appendix A.6), were kindly provided by Dr. Renate Schmidt from IPK Gaterslaben and included in map construction. BLAST search with primer sequence against the reference sequence of *B. rapa* and *B. oleracea* genomes indicated that D120E-3 was located on A07 in *B. rapa*, annotated with gene ID Bra039003 while marker Dx-3 was detected in scaffolds of *B. oleracea*, annotated with gene ID Bol29796.

3.3.4 Linkage map of SODH population

Linkage map was constructed using MAPMAKER/EXP 3.0 (Lincoln et al., 1992) with the aid of a purpose-built Perl script (unpublished; Wolfgang Ecke, personal communication) that automates the mapping process. Segregation of each marker was tested by χ^2analysis (p = 0.05) to assess the goodness-of-fit for the expected segregation ratio (1:1). Markers which were significantly deviating from 1:1 segregation ratio were regarded as skewed segregated markers while markers which were not significantly different from 3:1 or beyond were defined as strongly skewed segregated markers. Markers that were strongly skewed segregated were initially excluded for map construction and were attempted for mapping after the initial map was built. Markers were assigned to linkage groups to construct a core map by the "*group*" command with the minimum LOD score parameter set to 4 and the maximum distance parameter set to 35 cM. The most probable marker order within each group was determined by the command "order" and the resulting high fidelity map was built upon by adding markers using the command "*try*". Markers having more than the predetermined number of crossovers were excluded in the high-fidelity map. Markers that were not supported by a LOD score of 3 in the high fidelity map were placed at their most likely position in the linkage group. Following this, the "*ripple*" command was used to find the optimal marker order in the linkage groups. Genetic distances between loci were calculated using the Kosambi mapping function (Kosambi, 1944). The resulting map consisted of high fidelity markers which are supported by a LOD score of at least three and placed markers which are supported with LOD score of less than three.

The map was further optimized by constructing each linkage group 200 times with a random subset of five highly informative markers according to MAPMAKER/EXP3.0 command order to obtain the possible variant of a high fidelity map. The optimal variant was selected to have as many markers as possible, as few double crossover as possible, and that the markers were as evenly distributed as possible. The map was aligned with common marker loci on established genetic maps based on SSR (Piquemal et al. (2005); Radoev et al. (2008); Sharpe and Lydiate, unpublished data), DArT (Raman et al., 2013) and SNP (KWS Saat AG, unpulished data). Linkage groups were named according to the nomenclature of Parkin et al. (1995) as A01 to A10 and C01 to C09.

For QTL mapping purpose, a subset of markers were selected from the high-fidelity markers on the basis that the distance between adjacent marker was about 5 - 10 cM. The term framework map was used to refer to the map used for QTL mapping.

3.3.5 Phenotypic analysis

Phytosterol content

Phytosterol content was analyzed by adapting the protocol of Amar et al. (2008a) and Fernández-Cuesta et al. (2012), following a direct alkaline hydrolysis method which involves three major steps: alkaline hydrolysis (saponification), extraction of the non-saponifiable matter, and derivatisation of the sterols to trimethylsilyl (TMS)-ether derivatives. The main advantage of using this method is that it bypasses the lipid extraction step, facilitating large number of seed samples to be analysed more economically. The downside of this method is that alkaline hydrolysis could only quantify free sterols and steryl esters, but not steryl glycosides. The hydrolysis of acetal bond between phytosterol and the carbohydrate moiety requires acidic condition which may be destructive to the compound and laborious for routine analysis. Hence, it is possible that the present analysis would underestimate the total phytosterol concentration in the seed sample.

For each sample analysis, 200 mg of seeds were weighted and placed in a polypropylene tube. Two mililiter of 2% potassium hydroxide (Carl Roth, Germany) in ethanol (w/v) was added for alkaline hydrolysis, followed by 200 µl of 2% cholesterol (99% purity, Sigma-Aldrich, Germany) in hexane-ethanol (3:2) solution, used as an internal standard to quantify phytosterol content. By

placing one stainless steel rod (1.1 cm length; 0.4 cm diameter) in each tube, seeds were crushed and homogenized using a custom-built vertical homogenizer (Institute of Applied Plant Nutrition, Georg-August-Universität Göttingen) for 3 min at a speed deemed sufficient to homogenize the seeds. The tubes were subsequently incubated for 15 min at 80 °C in a water bath and cooled at room temperature for 30 min. To extract the phytosterols, 1.0 ml of hexane and 1.5 ml of distilled water were added, briefly vortexed, and centrifuged for 10 min at 4000 rpm. The upper hexane layer was transferred to a new tube and left over night on a hot plate at 37.5 °C to evaporate. The residue obtained after evaporation was dissolved with 80 µl hexane and derivatized with 20 µl of silylating agent, composed of hexamethyldisilazane (Fluka analytical):trimethylchlorosilane (Sigma-Aldrich purum > 98%; GC grade) 3:1. The solution was pipetted into a GC vial, capped, and incubated at room temperature for 20 min. To settle the precipitate, the derivatized samples were centrifuged for 10 min at 3000 rpm prior GC analysis.

Analysis of derivatized sterols was performed using capillary gas-liquid chromatograph (Chrompack CP-9003), equipped with autosampler, split injector (320 °C; injection volume of 3 µl with a split ratio of 100:1) and flame ionization detector 320 °C, with fused silica capillary column of medium polarity (SE-54, 50 m long, 0.1 um film thickness, 0.25 mm i.d. coated with 5%-phenyl-1%-vinyl-methylpolysiloxane)(IVA Analysentechnik, Meerbusch, Germany). Hydrogen (carrier gas) pressure was set at 150 kPa. Initial oven temperature was set at 240 °C with an increment of 5 °C min^{-1} to final oven temperature at 275 °C and held for 20 min. Total analytical time was 25 min.

Phytosterol content was expressed as mg 100 g_{seed}^{-1}. The phytosterol traits evaluated in this study include contents of brassicasterol, campesterol, sitosterol, avenasterol, total phytosterol, 24-methyl sterol, 24-ethyl sterol and ratio of campesterol:sitosterol and 24-methyl:24-ethyl sterol. Total phytosterol content was calculated as the sum of brassicasterol, campesterol, sitosterol and avenasterol contents. 24-methyl sterol was calculated as the sum of brassicasterol and campesterol contents. 24-ethyl sterol was calculated as the sum of sitosterol and avenasterol contents.

Fatty acid composition

Fatty acid composition was analysed by gas chromatography using method adapted from Thies (1971). Approximately 200 mg of seed, 1 ml of Na-methylate-methanol (0.5 mol L^{-1}), and one

stainless steel rod (1.1 cm length; 0.4 cm diameter) were added in a propylene tube. The seeds were then homogenized using a custom-built vertical homogenizer (Institute of Applied Plant Nutrition, Georg-August-Universität Göttingen) for 3 min. Following incubation for 20 min at room temperature, 300 µl iso-octane and 100 µl 5% $NaHSO_4$ in water were added, briefly vortexed, and centrifuged for 3 min at 4000 rpm. About 200 µl of the upper phase was pipetted into a GC vial and 3 µl was injected into a gas chromatograph (Thermo Trace GC Ultra), equipped with autosampler, split injector (split ratio 70:1), flame ionization detector (320 °C), and capillary FFAP-phase (0.25 mm × 25 m; Macherey & Nagel). Hydrogen (carrier gas) pressure was set at 100 kPa. Oven temperature was set at 210 °C. Total analytical time was 6 min.

The fatty acid content reported in this study include palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), expressed as percentage of total fatty acids in mature seeds.

Oil and protein content of defatted meal

Oil and protein content in seeds were estimated by near-infrared reflectance spectroscopy (NIRS) using calibration raps2012.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel, Germany). Oil content and protein content of defatted meal were expressed as a percentage of seed dry matter content at 9% moisture.

Protein content of defatted meal was calculated by using the estimated seed oil content and seed protein content obtained from the NIRS prediction as follows:

$$\%\text{Protein of defatted meal} = \%\text{Seed protein} - (100 - \%\text{Seed oil}) \times 100\%$$

Seed weight

Thousand seed weight was obtained from weight conversion of 500 seeds. The seeds were counted using a seed counter (Model:Contador, Pfeuffer GmbH, D-97318 Kitzingen, `http://www.pfeuffer.com`).

3.3.6 Statistical analysis

Variance components, heritability and means were estimated by using PLABSTAT software version 3A (Utz, 2011). The model implemented in ANOVA anaylsis was as follow:

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

where Y_{ij} is the trait value of genotype i in environment j, μ is the general mean, g_i is the effect of ith genotype, e_j is the effect of jth environment, and ge_{ij} is the interaction between ith genotype and jth environment. The genotype was treated as fixed effect, whereas environment was treated as random effect.

Broad sense heritability ($\hat{h}^2$) was estimated as follow:

$$\hat{h}^2 = \frac{\hat{\sigma}^2_G}{\hat{\sigma}^2_G + \frac{\hat{\sigma}^2_{GE}}{E}}$$

where $\hat{\sigma}^2_G$ and $\hat{\sigma}^2_{GE}$ are variance components for g and e; E refers to number of environment. Mean values across all environments were used to calculate Spearman's rank correlation coefficients between traits.

3.3.7 QTL mapping

QTL detection was performed with WinQTL Cartographer software ver. 2.5 (Wang et al., 2012a). QTL were initially detected with composite interval mapping (CIM) using model 6. For each trait, the LOD significance threshold (α=0.05) were estimated by 1000 permutation tests. Five markers selected by a forward and backward regression method were used as cofactors. CIM tests were performed at 1-cM steps with a 10-cM window size. Peaks were treated as separate QTL when the distance is more than 5 cM and the minimum LOD value exceeds one between any two adjacent peaks.

Subsequently, multiple interval mapping (MIM) was performed to refine the QTL position, to search for more QTL, and to investigate epistatic effects among the detected QTL. The MIM model was build upon a priori model from CIM analysis and progressively refined using the BIC-M2 = 2ln(n) criterion. QTL positions that did not remain significant when fitted with others were then dropped from the model. QTL effects and their percentage of phenotypic variance explained by individual and all the QTL were estimated with the final model fitted in MIM. A one-LOD drop from the peak position was used as a confidence interval for each QTL.

3.3.8 Identification of possible candidate genes for major QTL

To identify the underlying candidate genes for major QTL, reference sequence of *B. rapa* (BRAD v1.5; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* (Bolbase v1.0; `http://www.ocri-genomics.org/bolbase/`) genomes were utilized. Sequence-informative markers (SSR, DArT, Silico-DArT, and KASP) that are within the QTL genomic region were aligned with the reference physical map of *B. rapa* or *B. oleracea* to search for colocalization with candidate gene. The physical position of each marker sequence was located by performing a BLAST search using CLC main workbench 6.0 program (CLC Bio, Aarhus, Denmark). The position of the best hit was recorded only when the marker sequence from a particular linkage group fell onto the same corresponding chromosome in *B. rapa* or *B. oleracea*.

3.4 Results

3.4.1 Polymorphism of molecular markers

Different types of molecular markers were used in the construction of the genetic map for SODH population: AFLP, SSR, DArT, Silicor-DArT, SNP, KASP and candidate-gene based markers. With 16 AFLP primer combinations, a total of 75 polymorphic markers could be scored in the SODH population. Of the 350 SSR primer pairs screened, 23 (0.07 %) were found polymorphic between the parents and exhibited clear and unambiguous amplification. Seven of the 23 SSR primer pairs amplified more than one polymorphic locus, resulting in 32 SSR loci. Approximately 13%

(407/3072) of DArT and 42%(2005/4787) of silico-DArT markers were polymorphic between the parents.

Five candidate genes involved in phytosterol biosynthesis were selected to develop candidate gene-based markers. By performing BLAST searches with gene sequence of *A. thaliana* against the reference sequence of *B. rapa* (BRAD v1.5 ; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* genomes (Bolbase v1.0; `http://www.ocri-genomics.org/bolbase/`), between two to four homologues were found for each gene (Appendix A.3). The alignments of the homologues generally exhibited similar intron-exon structure (Appendix A.4). In total, 63 primer pairs were designed for 16 homologues of the five candidate genes and were screened for locus specificity. From the initial screening with gel electrophoresis, 26 primer pairs were assumed to be locus-specific and were subjected to Sanger sequencing for confirmation. Of the 26 primer pairs, 21 primer pairs (Appendix A.5) showed high-quality sequences with no secondary peaks (Appendix A.7). Among them, one locus-specific primer pair (HMG2A10-2) did not amplify from Oase template, allowing genotyping to be performed directly on the SODH population. For the rest of the 19 primer pairs, the amplified sequences were examined for allelic variation between Sansibar and Oase. Only one partial fragment amplified by primer pair HMG1A07-4 was found with allelic differences (Appendix A.6). The sequence fragment covered two partial exons and one intron of the *HMG1* gene, revealing 6 SNPs between Sansibar and both *B. rapa* and Oase. With a fragment size of 674 bp, this corresponded to a density of 1 SNP/113 bp. Of the six SNPs, five were found at the exon region and one was found at the intron region. By translating the nucleotide sequence, it appeared that the third SNP (A/T) altered the amino acid from tyrosine to phenylalanine while no amino acid differences were found from the other 4 SNPs at the exon region (Appendix A.6). Following this, an allele-specific marker, HMG1A07-O1, was developed for this gene and was used to genotype the SODH population (Appendix A.6). In addition , two candidate gene-based marker for *DGAT1* gene (D120E-3 and Dx-3) were used for genotyping and included in map construction.

3.4.2 Linkage map of SODH population

After removal of markers with a minor allele frequency of less than 10%, a total of 2555 marker loci were available for map construction. The resulting linkage map for SODH population has 1642 markers mapped onto 23 linkage groups and covered 2350.2 cM with a mean interval distance of 2.0 cM between markers. The unmapped markers were either ambiguously linked to various linkage groups, unlinked, or formed small linkage groups that were excluded for estimation of the linkage map length. About 50% (457/913) of the unmapped markers showed skewed segregation of which 47% (217/457) showed strong skewed segregation. The number of markers, map size, marker density and mean distance between markers are summarized in Table 3.2 and the genetic map is shown in Appendix A.8. All linkage groups could be assigned with chromosome names according to the nomenclature of Parkin et al. (1995) as A01 to A10 and C01 to C09. The 23 linkage groups represented 19 chromosomes in *B. napus*, additional four linkage groups (A08-II, C02-II, C03-II, and C04-II) were formed due to loose or no linkage to their main linkage groups. The map has an average density of 0.70 marker per cM with distribution of markers varied from 0.20 to 1.37 marker/cM across the linkage groups (Table 3.2). The A genome comprised more markers (987) as compared to the C genome (655), with a mean interval distance between markers of 1.6 cM in the A genome and 2.4 cM in the C genome. The number of markers mapped in an individual linkage group ranged from 7 (A08-II) to 164 (A07).

About 44% of the mapped markers (718) showed significant ($P = 0.05$) segregation distortion with the majority (76%) of the markers favouring the "Sansibar" allele. Loci with skewed segregation favouring the "Sansibar" allele were mostly found on linkage groups A07, A10, C03, and C05; while loci with skewed segregation favouring the "Oase" allele were clustered mainly on linkage groups A05, C01, C03-II and C04-II.

Three candidate gene-based markers (HMG1A07-O1, HMG2A10-2S and D120E-3) were mapped on A07 and another (Dx-3) was mapped on C09 (Figure 3.3, Appendix A.8).

Table 3.2: Marker distribution, size, density and mean distance between markers of each linkage group in the linkage map of SODH population.

Linkage group	No. of markers per linkage group								Size (cM)	Marker density (cM^{-1})	Mean distance btw. markers (cM)[b]
	AFLP	CG[a]	DArT	KASP	Silico-DArT	SNP	SSR	Total			
A01	2		6	1	58	4	4	75	98.0	0.77	1.50
A02	3		5		26	3		37	40.7	0.91	1.30
A03	7		19	3	107	15	1	152	224.1	0.68	1.70
A04	2		14		84	5	2	107	194.2	0.55	2.10
A05	2		7	2	63	5	3	82	163.8	0.50	2.20
A06	4		18	2	95	5		124	128.0	0.97	1.20
A07	1	3	9	2	134	15		164	133.7	1.23	0.90
A08	2		9	1	31	2		45	37.8	1.19	1.10
A08-II			1		6			7	9.7	0.72	1.90
A09	3		8	2	49	2	1	65	130.2	0.50	2.50
A10	8		11		107	3		129	94.1	1.37	0.90
C01			4		24	4	2	34	77.5	0.44	2.70
C02			1	1	11	1		14	48.2	0.29	4.40
C02-II	1		1		16	1	1	20	97.9	0.20	5.40
C03	2		5	2	72	3		84	111.3	0.75	1.40
C03-II	3		5	1	45	6	1	61	134.8	0.45	2.40
C04			1		19	2	2	24	79.5	0.30	3.60
C04-II	5		3	4	75	6		93	101.0	0.92	1.20
C05	2		1	1	45	4		53	92.1	0.58	1.90
C06	2		5	1	49	7		64	95.2	0.67	1.60
C07	8		8	2	91	7	5	121	142.4	0.85	1.40
C08	4				27	2		33	52.5	0.63	1.80
C09	1	1	3		44	4	1	54	63.5	0.85	1.20
A genome	34	3	107	13	760	59	11	987	1254.3	0.79	1.57
C genome	28	1	37	12	518	47	12	655	1095.9	0.60	2.42
Whole genome	62	4	144	25	1278	106	23	1642	2350.2	0.70	2.01

[a] CG: candidate gene-based markers

[b] Co-segregating markers are represented as a single marker in the calculation of mean distance between markers.

3.4.3 Phenotypic analysis

Highly significant genotype and environment effects were found for all traits in the SODH population (Table 3.3). Broad-sense heritability (H^2) estimates were high, ranging from 0.80 to 0.90, indicating that much of the phenotypic variance were genetically determined.

The phenotypic traits showed normal or near-normal distributions, with extreme values at both ends of the distributions exceeded the extreme values of both parental distributions, suggesting transgressive segregation (Figure 3.2). The total phytosterol content ranged from 311.2 to 486.9 mg 100 g_{seed}^{-1}, with a mean of 401.9 mg 100 g_{seed}^{-1} (Table 3.4). Among the four quantified end-products of sterol pathway, sitosterol was the most prominent sterol, followed by campesterol, brassicasterol and avenasterol. The 24-ethyl sterol content, which include sitosterol and avenasterol, was higher than the 24-methyl sterol content, which comprise of campesterol and brassicasterol. Between the parents, Sansibar consistently showed higher phytosterol content than Oase while Oase had a higher ratio of 24-methyl:24-ethyl sterol than Sansibar and only small difference was observed for the ratio of campesterol:sitosterol. The oil content was high in this population, ranging from 41.2 to 48.6%, with a mean of 46.3 %. Between the parents, Oase had a higher oil content than Sansibar.

Highly significant correlations (P = 0.01) were observed between total phytosterol and the four individual sterols (Table 3.5). All nine phytosterol traits were positively correlated to palmitic acid (C16:0) while brassicasterol in particular was correlated to all the major fatty acids. Oil was positively correlated with total phytosterol and oleic acid and negatively correlated with linoleic and linolenic acids. Except for brassicasterol, no significant correlation was observed between phytosterols and protein of defatted meal.

Table 3.3: Variance components and heritability of the SODH population ($n = 226$)

Trait	Variance components (σ^2)			Heritability
	Genotype (G)	Environment (E)	G×E	(h^2)
Phytosterols (mg 100 g_{seed}^{-1})				
Brassicasterol	14.28**	5.06**	16.12	0.84
Campesterol	315.99**	160.43**	150.54	0.92
Sitosterol	267.43**	36.23**	310.05	0.83
Avenasterol	48.38**	94.09**	52.44	0.84
Total phytosterol	1139.02**	706.69**	934.69	0.88
24-methyl sterol	330.13**	188.89**	192.91	0.91
24-ethyl sterol	412.95**	206.00**	368.78	0.87
Campesterol:sitosterol[a]	89.77**	24.56**	41.65	0.92
24-methyl:24-ethyl sterol[a]	62.90**	8.29**	34.98	0.92
Other traits				
C16:0 (%)	0.10**	0.04**	0.07	0.89
C18:1 (%)	2.57**	0.52**	1.48	0.91
C18:2 (%)	1.23**	0.10**	2.19	0.92
C18:3 (%)	0.43**	0.15**	0.31	0.89
Oil (%)	1.65**	3.52**	1.94	0.83
Protein content of defatted meal (%)	1.69**	8.01**	2.31	0.80
Seed weight (g)	0.20**	0.23**	0.26	0.84

[a] original values (ratio) $\times$ 100
** denotes significance at $P = 0.05$

Table 3.4: Descriptive statistic of the parents and the SODH population ($n = 226$)

Trait	Parents Sansibar	Parents Oase	Double haploid population ($n = 226$)				
	Mean		Min	Max	Mean	*F*-value	LSD 5%
Phytosterol (mg 100 g_{seed}^{-1})							
Brassicasterol	50.4	46.4	32.7	59.5	48.8	6.3**	4.6
Campesterol	157.2	114.9	87.8	192.7	136.5	13.6**	13.9
Sitosterol	226.7	167.5	154.9	251.6	193.4	6.2**	20.0
Avenasterol	23.8	19.7	9.8	52.3	25.4	6.5**	8.2
Total phytosterol	461.7	352.4	311.2	486.9	401.9	8.3**	34.6
24-methyl sterol	207.7	161.3	130.2	214.0	185.3	11.3**	15.7
24-ethyl sterol	250.4	187.2	170.1	252.7	218.8	7.7**	21.8
Campesterol:sitosterol[a]	69.4	68.7	47.3	99.7	71.0	13.9**	7.3
24-methyl:24-ethyl sterol[a]	82.9	86.2	62.3	108.2	85.3	12.1**	6.6
Other traits							
C16:0 (%)	5.0	4.6	3.8	5.6	4.8	9.6**	0.3
C18:1 (%)	58.8	63.1	57.3	65.4	61.6	11.5**	1.4
C18:2 (%)	21.0	18.7	17.1	24.1	19.9	14.1**	0.9
C18:3 (%)	9.8	9.0	7.5	11.8	9.6	9.3**	0.6
Oil (%)	43.7	46.3	41.2	48.6	45.4	6.1**	1.6
Protein of defatted meal (%)	29.3	32.9	27.3	35.2	30.5	5.1**	1.7
Seed weight (g)	5.5	5.6	4.4	7.8	5.8	5.8**	0.6

[a] original values (ratio) $\times$ 100

** denotes significance at $P = 0.05$

LSD 5%: least significant difference at the level of $P = 0.05$

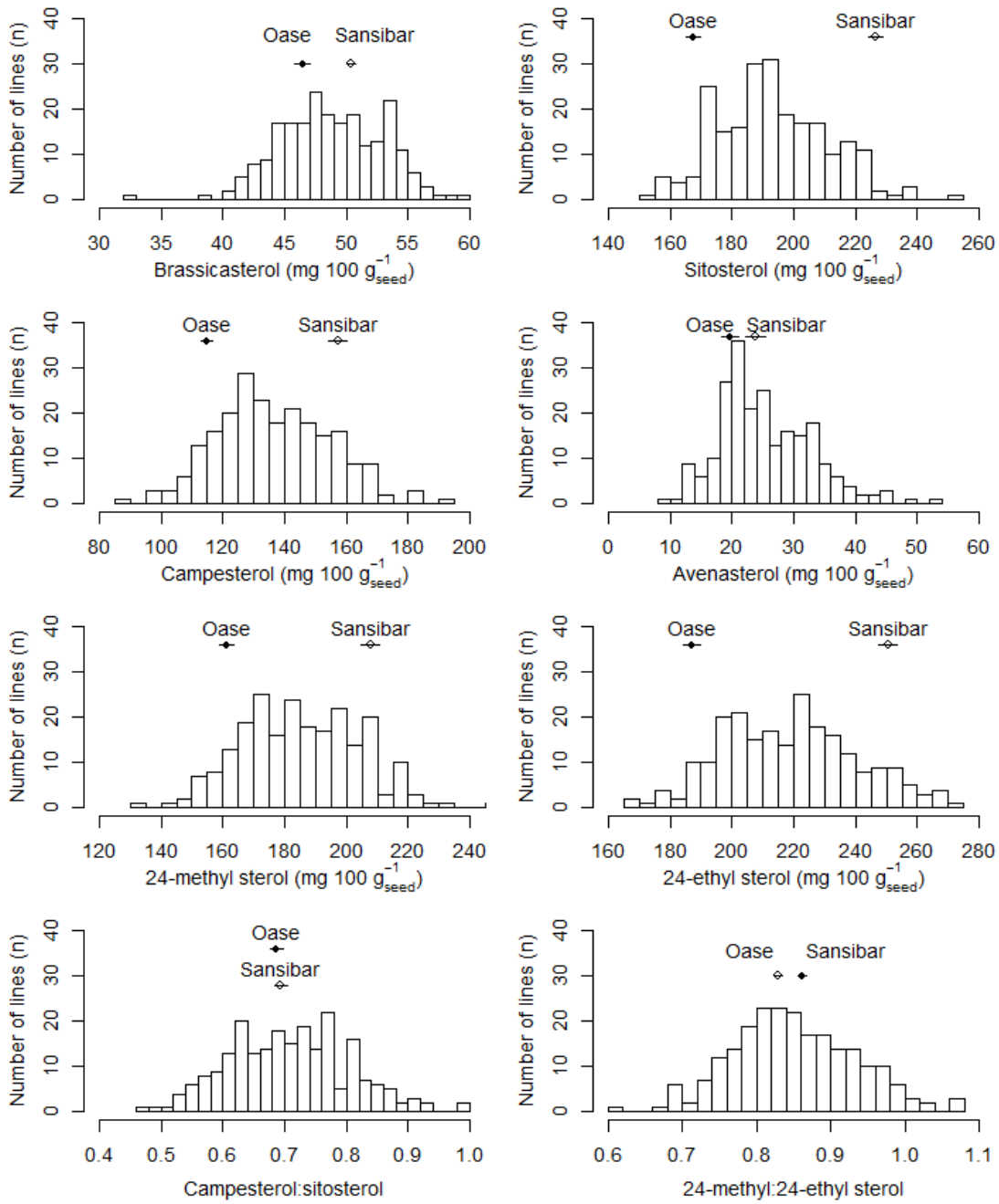

Figure 3.2: Frequency distribution of phytosterol contents, fatty acid composition, oil content, protein content of defatted meal and seed weight in SODH population. Parental mean values are indicated by the symbol ○ for Sansibar and ● for Oase. The standard error of the parental mean is indicated by the line (−) on the symbol.

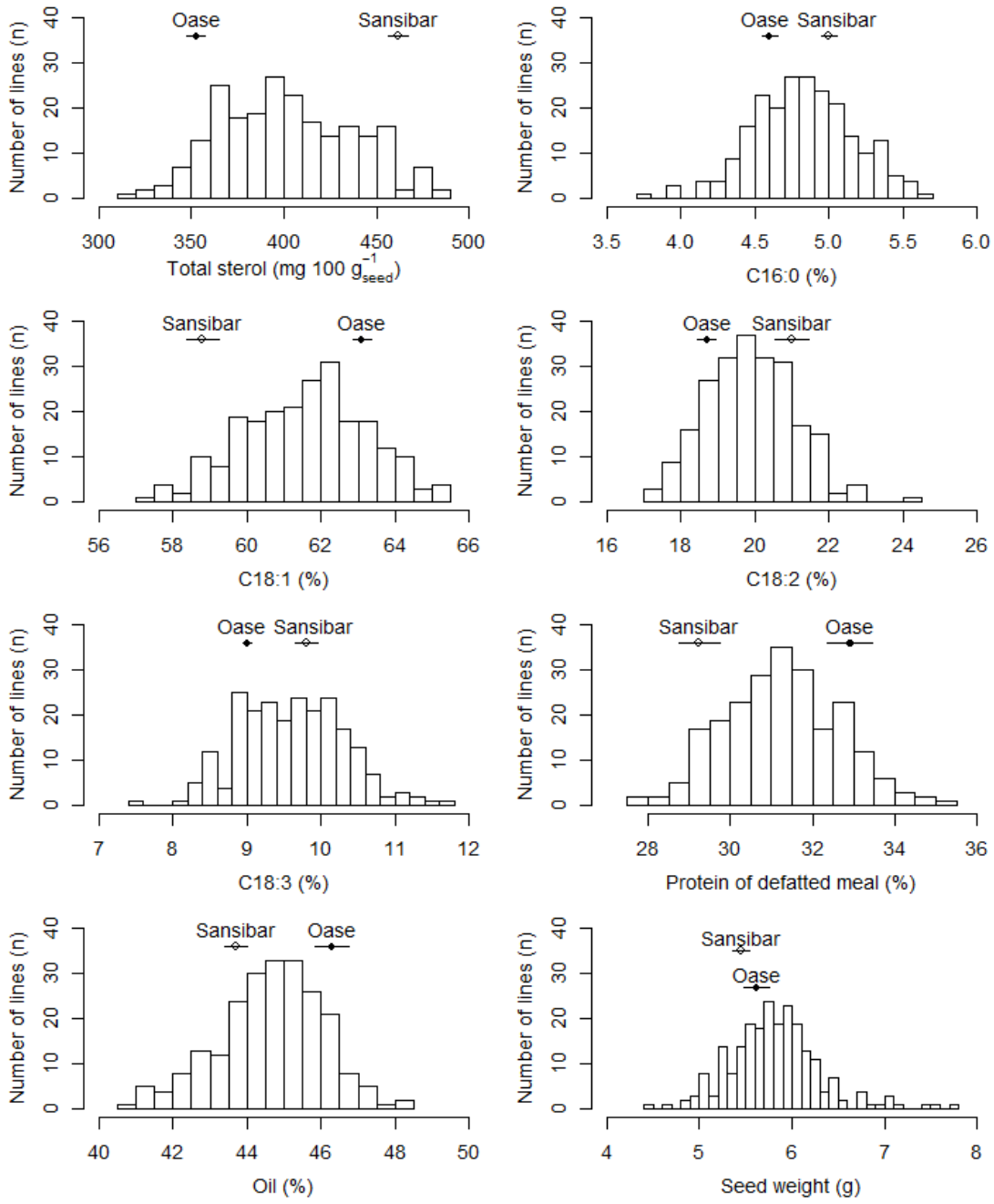

Figure 3.2: (*continued from previous page*) Frequency distribution of phytosterol contents, fatty acid composition, oil content, protein content of defatted meal and seed weight in SODH population. Parental mean values are indicated by the symbol ○ for Sansibar and ● for Oase. The standard error of the parental mean is indicated by the line (−) on the symbol.

Table 3.5: Spearman's rank correlation of traits in the SODH population ($n = 226$)

	Brassicasterol	Campesterol	Sitosterol	Avenasterol	Total phytosterol	24-methyl sterol	24-ethyl sterol	Campesterol: sitosterol	24-methyl: 24-ethyl sterol	C16:0	C18:1	C18:2	C18:3	Oil	Protein of defatted meal
Campesterol	0.03														
Sitosterol	0.15*	0.32**													
Avenasterol	0.04	0.78**	0.36**												
Total phytosterol	0.20**	0.84**	0.74**	0.80**											
24-methyl sterol	0.24**	0.98**	0.34**	0.77**	0.86**										
24-ethyl sterol	0.14*	0.53**	0.95**	0.65**	0.88**	0.54**									
Campesterol:sitosterol	−0.08	0.77**	−0.35**	0.53**	0.34**	0.73**	−0.11								
24-methyl:24-ethyl sterol	0.12	0.49**	−0.61**	0.15*	0.01	0.50**	−0.45**	0.89**							
C16:0	0.29**	0.31**	0.18**	0.26**	0.33**	0.36**	0.24**	0.18**	0.15*						
C18:1	−0.43**	−0.06	−0.09	0.04	−0.11	−0.15*	−0.06	−0.01	−0.12	−0.53**					
C18:2	0.27**	0.02	0.02	−0.09	0.03	0.08	−0.01	0.02	0.11	0.31**	−0.83**				
C18:3	0.37**	0.10	0.01	0.09	0.12	0.18**	0.04	0.08	0.13*	0.29**	−0.68**	0.34**			
Oil	−0.10	0.20**	0.16*	0.30**	0.24**	0.17**	0.23**	0.09	−0.06	−0.02	0.48**	−0.51**	−0.23**		
Protein of defatted meal	−0.27**	0.06	−0.01	−0.05	−0.01	0.00	−0.02	0.06	0.03	−0.18**	−0.09	0.06	0.16*	−0.43**	
Seed weight	0.12	−0.08	−0.19**	−0.13	−0.15*	−0.05	−0.20**	0.05	0.15*	−0.07	−0.11	0.14*	0.06	−0.38**	0.16*

* and ** denotes significance at $P < 0.05$ and 0.01

3.4.4 QTL mapping

To identify genetic loci controlling the phenotypic traits, multiple interval mapping was performed with means of phenotypic data obtained from 6 environments and a framework map consisting of 273 markers. Here, the framework map refers to a subset of high-fidelity markers that were distributed on the map at every interval of 5 - 10 cM.

A total of 58 QTL were identified: 29 QTL for the nine different phytosterol traits, 16 QTL for the four fatty acid compositions, six QTL for oil content, four QTL for protein of defatted meal and three QTL for seed weight. These QTL were distributed on 13 linkage groups as shown in Figure 3.3. Colocalizations of QTL for different traits were more frequently observed than individual isolated QTL.

Phytosterols

The 29 QTL identified for nine phytosterol traits were distributed on nine linkage groups (A01, A02, A03, A04, A06, A07, C03-II, C05, and C08). Between one and six QTL were detected for each phytosterol trait, which collectively explained between 7.2 and 71.5% of the total phenotypic variation. Of the 27 QTL, three were major QTL ($R^2 \geq 25\%$) located on linkage group A04 (*DE-Bra.3*), A06 (*DE-CSratio.3* and *DE-MEratio.4*). On A04, the major QTL for brassicasterol (*DE-Bra.3*) showed overlapping confidence interval with QTL for campesterol, campesterol:sitosterol and 24-methyl:24-ethyl sterols. The additive effect of *DE-Bra.3* was negative as opposed to the other three QTL. On A06, the two major QTL identified for campesterol:sitosterol (*DE-CSratio.3*) and 24-methyl:24-ethyl (*DE-MEratio.4*) sterol were found collocated with QTL for campesterol (*DE-Camp.2*) and 24-methyl sterol (*DE-Methyl.1*). These four QTL showed negative additive effects, indicating that the alleles increasing the trait values were derived from "Oase". For total phytosterol content, two QTL with positive additive effects were detected on A07 and C08; QTL on A07 (*DE-TPC.1*) was located at the top of the linkage group, close to the genomic region with many colocalized QTL while QTL on C03 (*DE-TPC.2*) was found colocalized with 8 QTL for different traits (phytosterols, fatty acids, and oil content).

Fatty acids

The 16 QTL identified for four different fatty acid compositions were distributed on nine linkage groups (A01, A03, A04, A07, A09, C05, C07, C08, C09). Between two and six QTL were detected for each fatty acid constituent, which collectively explained between 30.6 and 59.0% of the total phenotypic variance. Three major QTL were detected for C16:0, C18:1 and C18:3. For palmitic acid (C16:0), the major QTL (*DE-16:0.2*) which explained 28.8% of the phenotypic variation was located on A09 with additive effect of -0.30%. The major QTL for oleic acid (C18:1) and linolenic acid (C18:3) were found clustered on A01 along with seven minor QTL within a genomic region of 27 cM (65-92 cM). The seven minor QTL corresponded to QTL for brassicasterol, campesterol:sitosterol, 24-methyl:24-ethyl sterol, C16:0, C18:2, oil and protein of defatted meal.

Oil content

Six QTL detected for oil content were distributed on five linkage groups (A01, A02, A07, A08, C03-II, C08). Individual QTL explained between 4.3 and 6.7% of the phenotypic variance and collectively accounted for 32.6% of the total phenotypic variance. All of the six QTL showed negative additive effects, indicating that the alleles increasing oil content were derived from "Oase". Five of the six QTL showed overlapping confidence intervals with different traits. The QTL DE-Oil.3 which accounted for the largest effect (6.7% of the phenotypic variance) was located on A07 within the confidence interval of QTL for brassicasterol. A candidate gene marker for *HMGI* (HMG1A07-O1) was also mapped within the confidence intervals of these two QTL (Figure 3.3).

Protein content of defatted meal

Four QTL detected for protein content of defatted meal were distributed on three linkage groups (A01, C03-II, A07). Individual QTL explained between 8.0 and 12.9% of the phenotypic variance and collectively accounted for 38.1% of the phenotypic variance. All of the four QTL were found colocalized with QTL for different traits.

Seed weight

Three QTL detected for seed weight were distributed on three linkage groups (A02, A07, C03-II). Individual QTL explained between 6.1 and 10.7% of the phenotypic variance and collectively accounted for 27.1% of the total phenotypic variance. Additive effect was positive for QTL located on A02 and C03-II and negative for QTL located on A07. On A02, *DE-SW.1* was collocated with a QTL for oil content with opposite negative effect. On A07, *DE-SW.2* was colocalized with three positive-effect QTL for phytosterols (brassicasterol, campesterol, and 24-methyl sterols) and two negative-effect QTL for protein of defatted meal and oleic acid. On C03-II, *DE-SW.3* was colocalized with positive-effect QTL for brassicasterol and negative-effect QTL for protein of defatted meal.

Table 3.6: QTL detected for phytosterol contents (mg 100 g_{seed}^{-1}), fatty acid composition (%), oil content (%), protein content of defatted meal (%) and seed weight (g) in SODH population

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
Brassicasterol	DE-Bra.1	A01	79	74–85	5.3	0.92	4.5	62.6
	DE-Bra.2	A03	172	167–178	6.8	1.03	5.1	
	DE-Bra.3	A04	95	91–97	31.7	−2.61	38.3	
	DE-Bra.4	A07	47	41–53	5.4	0.94	4.7	
	DE-Bra.5	A07	116	100–131	3.3	−0.77	2.6	
	DE-Bra.6	C03-II	82	79–88	8.1	1.13	7.5	
Campesterol	DE-Camp.1	A04	95	87–99	6.5	5.83	11.7	37.8
	DE-Camp.2	A06	66	59–70	8.6	−6.59	13.8	
	DE-Camp.3	A07	46	38–52	3.9	4.56	5.1	
	DE-Camp.4	C08	12	0–20	4.7	4.74	7.3	
Sitosterol	DE-Sito.1	A06	94	92–99	6.5	6.06	12.0	
	DE-Sito.2	C05	87	78–89	6.0	6.15	11.3	23.2
Avenasterol	DE-Ave.1	C08	14	1–20	5.9	2.59	11.8	11.8
Total phytosterol	DE-TPC.1	A07	27	13–38	3.5	10.49	8.2	14.2
	DE-TPC.2	C08	14	0–35	3.1	8.79	6.1	
24-methyl sterol	DE-Methyl.1	A06	64	59–69	9.9	−7.73	15.9	31.1
	DE-Methyl.2	A07	46	38–52	4.7	5.40	6.9	
	DE-Methyl.3	C08	13	1–20	5.2	5.44	8.3	
24-ethyl sterol	DE-Ethyl.1	A06	94	91–100	3.6	5.92	7.2	7.2
Campesterol:sitosterol	DE-CSratio.1	A01	68	65–86	3.2	1.46	2.2	71.5
	DE-CSratio.2	A04	93	85–98	12.8	3.16	14.7	
	DE-CSratio.3	A06	64	62–77	30.4	−5.45	33.3	
	DE-CSratio.4	C05	83	77–86	16.5	−3.82	16.4	
	DE-CSratio.5	C08	17	2–20	6.1	2.03	4.9	
24-methyl: 24-ethyl sterol	DE-MEratio.1	A01	83	79–92	5.2	1.68	3.6	70.3

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Table 3.6 – *continued from previous page*

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
	DE-MEratio.2	A02	25	0–5	4.9	1.59	4.5	
	DE-MEratio.3	A04	92	83–102	4.2	1.53	5.6	
	DE-MEratio.4	A06	63	61–66	32.3	−5.05	38.7	
	DE-MEratio.5	C05	84	79–89	16.5	−3.33	17.8	
C16:0	DE-16:0.1	A01	73	71–77	6.2	0.08	4.8	59.0
	DE-16:0.2	A09	100	99–103	21.7	−0.30	28.8	
	DE-16:0.3	C05	87	73–89	3.9	−0.07	4.0	
	DE-16:0.4	C08	8	2–17	12.2	0.12	10.4	
	DE-16:0.5	C09	26	24–30	8.1	0.10	10.9	
C18:1	DE-18:1.1	A01	84	82–89	16.5	−0.87	26.3	43.6
	DE-18:1.2	A07	47	41–54	3.7	−0.39	5.6	
	DE-18:1.3	C08	4	0–15	8.1	−0.60	11.7	
C18:2	DE-18:2.1	A01	74	72–77	11.2	0.53	18.8	30.6
	DE-18:2.2	A09	42	34–52	5.0	0.43	11.8	
C18:3	DE-18:3.1	A01	86	81–89	20.0	0.36	27.3	57.0
	DE-18:3.2	A03	104	88–123	3.9	0.15	4.8	
	DE-18:3.3	A04	35	21–49	3.2	−0.16	7.4	
	DE-18:3.4	C05	86	80–89	4.7	−0.17	3.5	
	DE-18:3.5	C07	82	81–92	5.0	−0.16	5.3	
	DE-18:3.6	C08	14	11–19	8.2	0.21	8.7	
Oil	DE-Oil.1	A01	73	68–79	3.1	−0.31	4.3	32.6
	DE-Oil.2	A02	21	17–26	4.7	−0.39	6.3	
	DE-Oil.3	A07	124	120–127	4.6	−0.44	6.7	
	DE-Oil.4	A08	16	0–23	2.2	−0.27	5.1	
	DE-Oil.5	C03-II	50	34–66	3.0	−0.37	4.7	
	DE-Oil.6	C08	17	0–34	2.9	−0.30	5.5	
Protein of defatted meal	DE-Pro.1	A01	76	68–88	5.2	0.40	8.7	38.1
	DE-Pro.2	A07	44	44–48	6.7	−0.44	8.5	

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Table 3.6 – *continued from previous page*

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
	DE-Pro.3	C03-II	33	27–37	4.6	−0.36	8.0	
	DE-Pro.4	C03-II	92	89–97	6.9	−0.46	12.9	
Seed weight	DE-SW.1	A02	23	21–29	5.5	0.15	10.3	27.1
	DE-SW.2	A07	47	44–54	5.5	−0.16	10.7	
	DE-SW.3	C03-II	85	78–93	3.0	0.12	6.1	

[a] 1-LOD Confidence interval

[b] Additive effect is the substitution effect of one "Oase" allele by one "Sansibar" allele.

[c] R^2 is the percentage of variation explained by each QTL

[d] TR^2 is the percentage of variation explained by all QTL

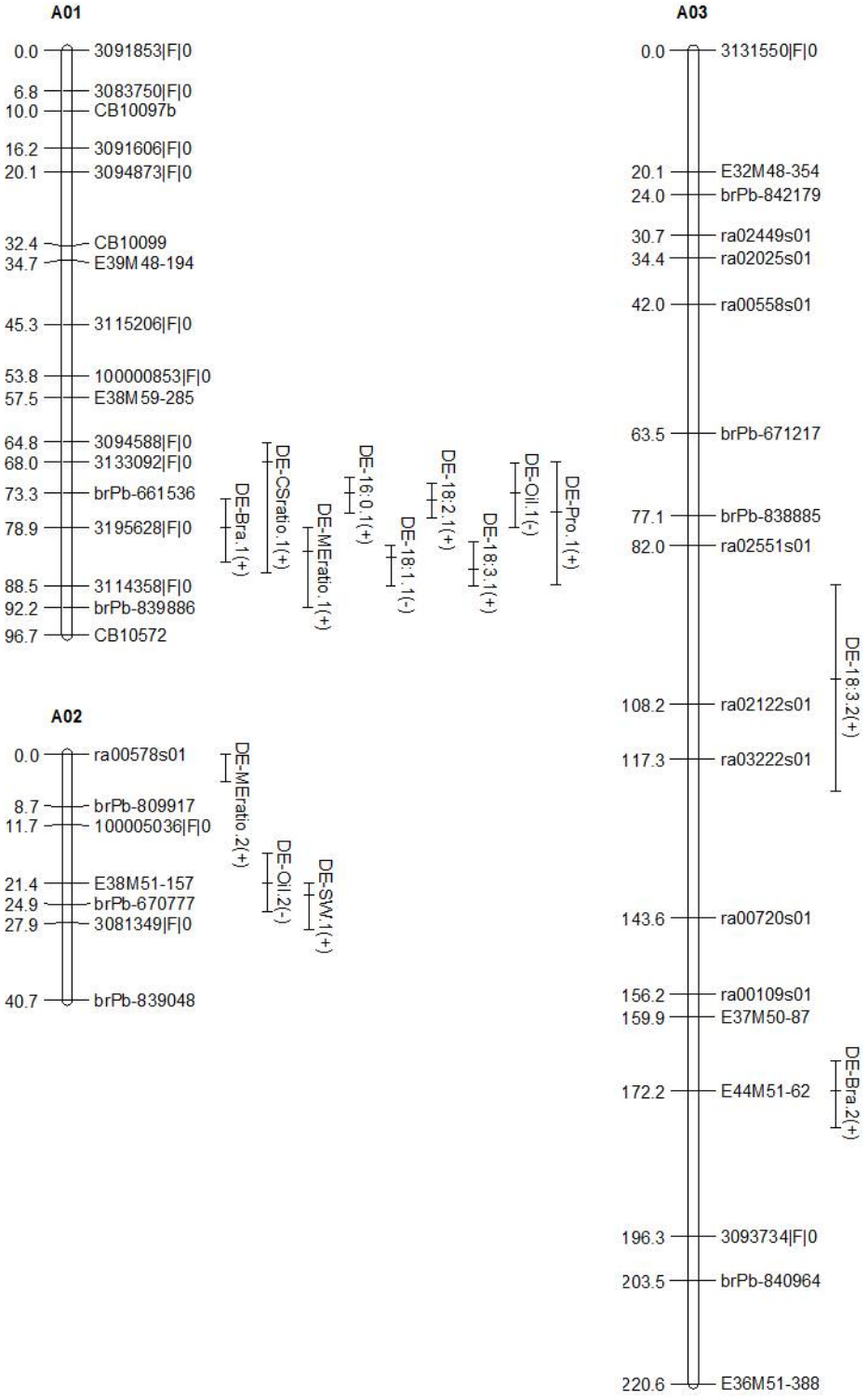

Figure 3.3: QTL associated with phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. {*} on marker name indicates candidate gene-based marker. *Italic* font of marker name indicates placed marker. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

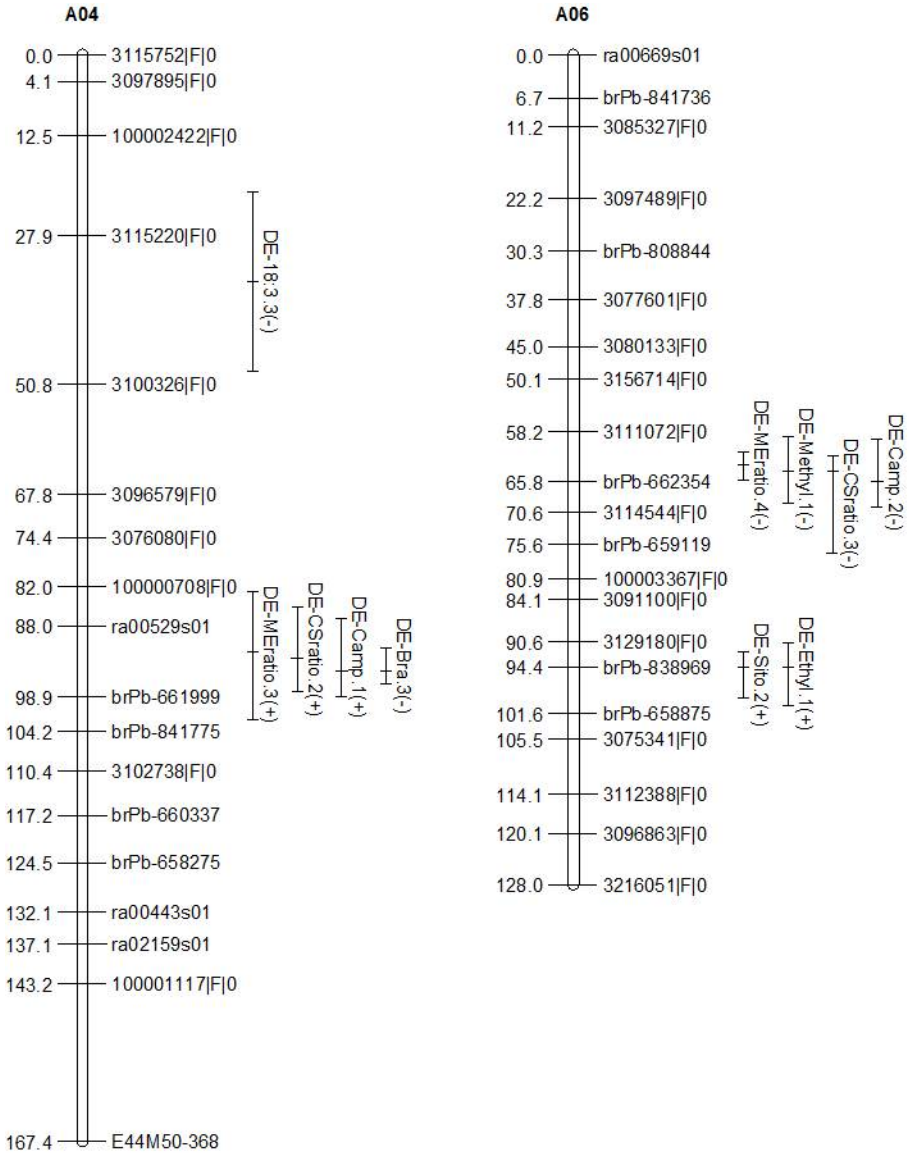

Figure 3.3: (*continued from previous page*) QTL associated with phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. {*} on marker name indicates candidate gene-based marker. *Italic* font of marker name indicates placed marker. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

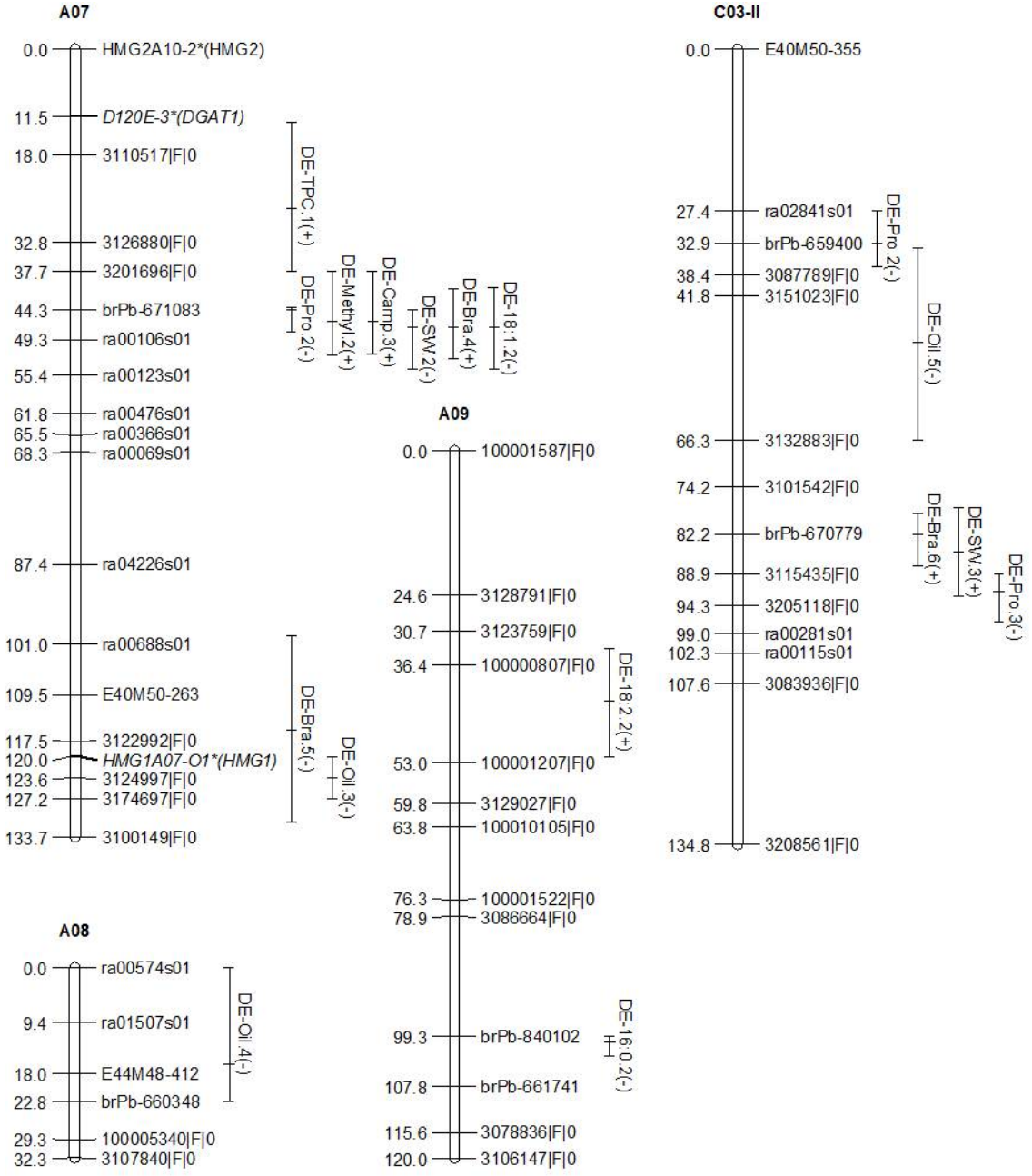

Figure 3.3: (*continued from previous page*) QTL associated with phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. * on marker name indicates candidate gene-based marker. *Italic* font of marker name indicates placed marker. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

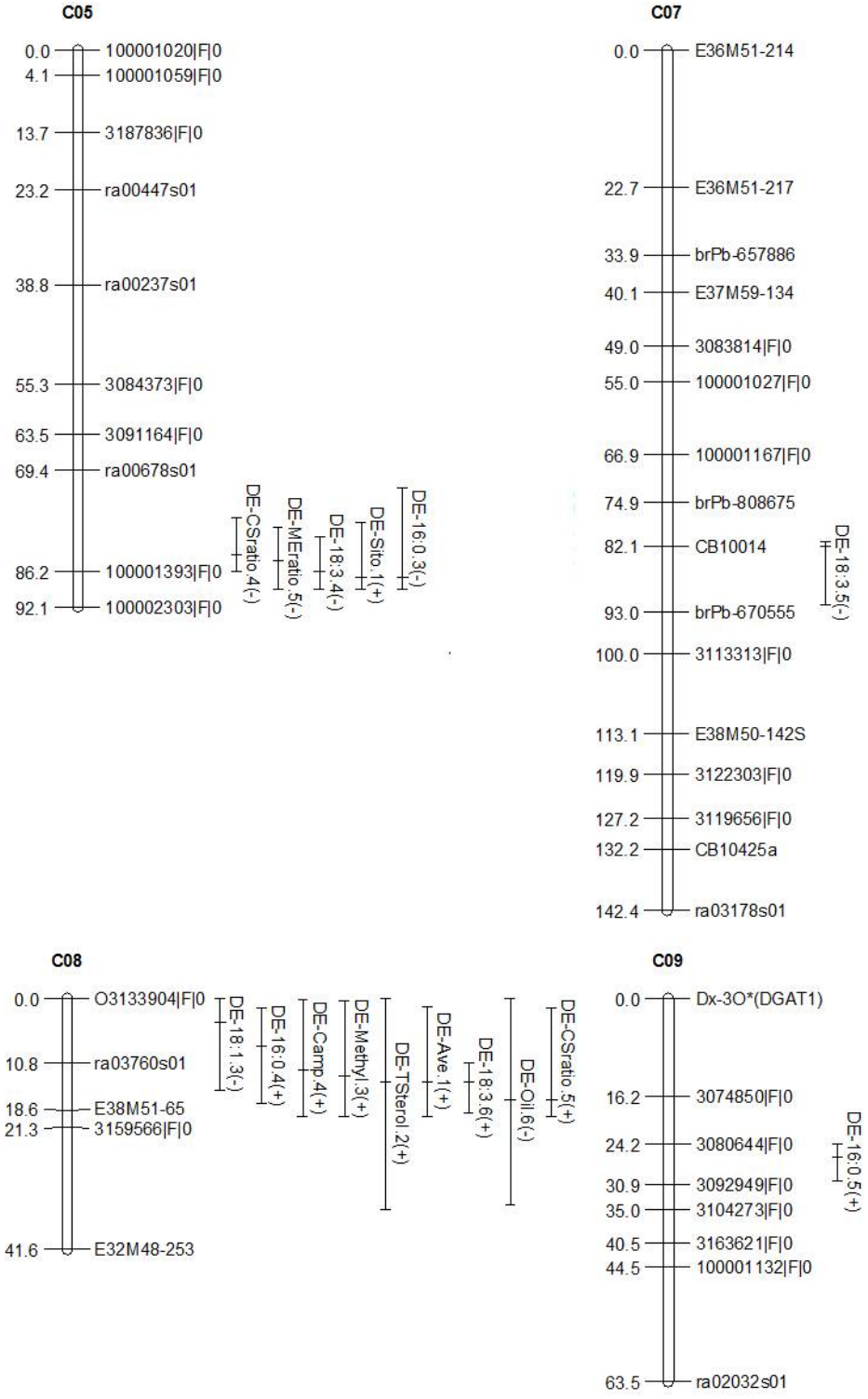

Figure 3.3: (*continued from previous page*) QTL associated with phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. * on marker name indicates candidate gene-based marker. *Italic* font of marker name indicates placed marker. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively.

3.4.5 Identification of possible candidate genes for major QTL

Phytosterols

Based on the phytosterol biosynthetic pathway, the predicted genes involved in two major QTL regions were *CYP710A2* on A04 and *SMT2* on A06. In *Arabidopsis*, *CYP710A2* encodes the enzyme that catalyzes the conversion of both 24-*epi*-campesterol and sitosterol to brassicasterol and stigmasterol, respectively (Morikawa et al., 2006) while the enzyme SMT2 catalyzes the second methylation reaction, converting 24-methylene lophenol to 24-ethylidene lophenol (Schaeffer et al., 2001).

A BLAST search conducted with *CYP710A2* gene of *Arabidopsis* (At2g34490) against the reference sequence of *B. rapa* genome resulted in two homologues (Bra021916 and Bra021917) found on chromosome A04, annotated as *CYP10A1*, while a BLAST search with *SMT2* gene of *Arabidopsis* (At1g20330) resulted in one homologue on A06. To investigate if the predicted genes were between the marker intervals flanking the major QTL, sequence-informative markers (DArT, SilicoDArT, SSR, and SNP) that were mapped within the QTL genomic region in the SODH population were located on the physical reference map of *B. rapa* and *B. oleracea* genomes. The marker order along the QTL genomic regions on A04 and A06 of the SODH map was rather collinear with the physical maps of chromosomes (Figure 3.4a, Figure 3.5a). On A04, the marker intervals of the major QTL spanned a genetic region of 22 cM (82.0-104.2 cM) and a physical region of 2.1 Mbp (13.9-16.0 Mbp), corresponding to approximately 94 kbp per cM (Figure 3.4b). The two homologues of *CYP710A1* gene were located approximately 61 kbp from the closest flanking marker (O3112445 | F | 0). On A06, the marker intervals spanned a genetic region of 22.7 cM (58.2-80.9 cM) and a physical region of 12.8 Mbp (6.3-19.1 Mbp), corresponding to approximately 562 kbp per cM (Figure 3.5b). The homologue of *SMT2* gene was located approximately 664 kbp from the closest flanking marker (S3155791 | F | 0).

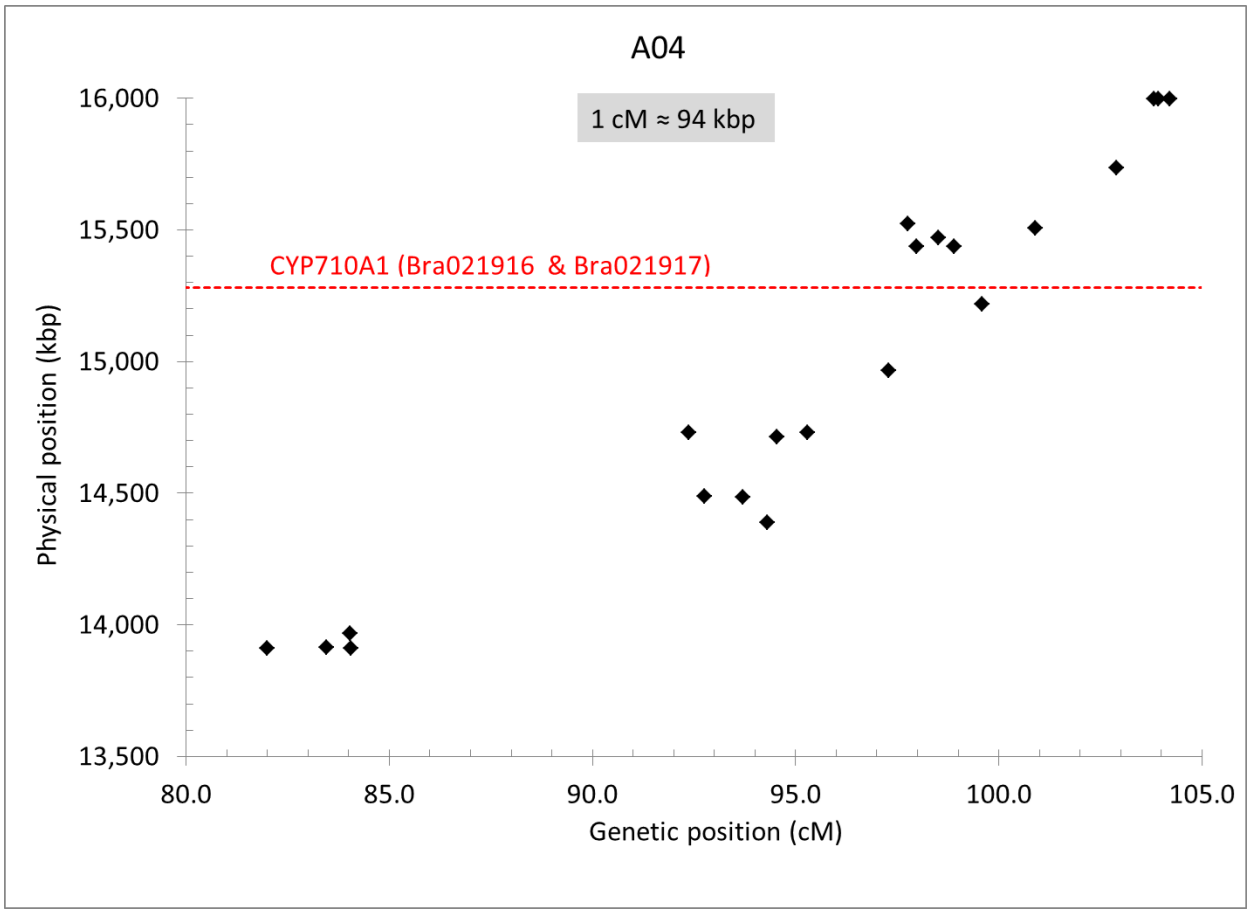

Figure 3.4: (a) Alignment of genetic and physical map positions of markers within the QTL genomic region (82-104.2 cM) on A04. The physical position of candidate gene (*CYP710A1*) is indicated by the *red dotted line*.

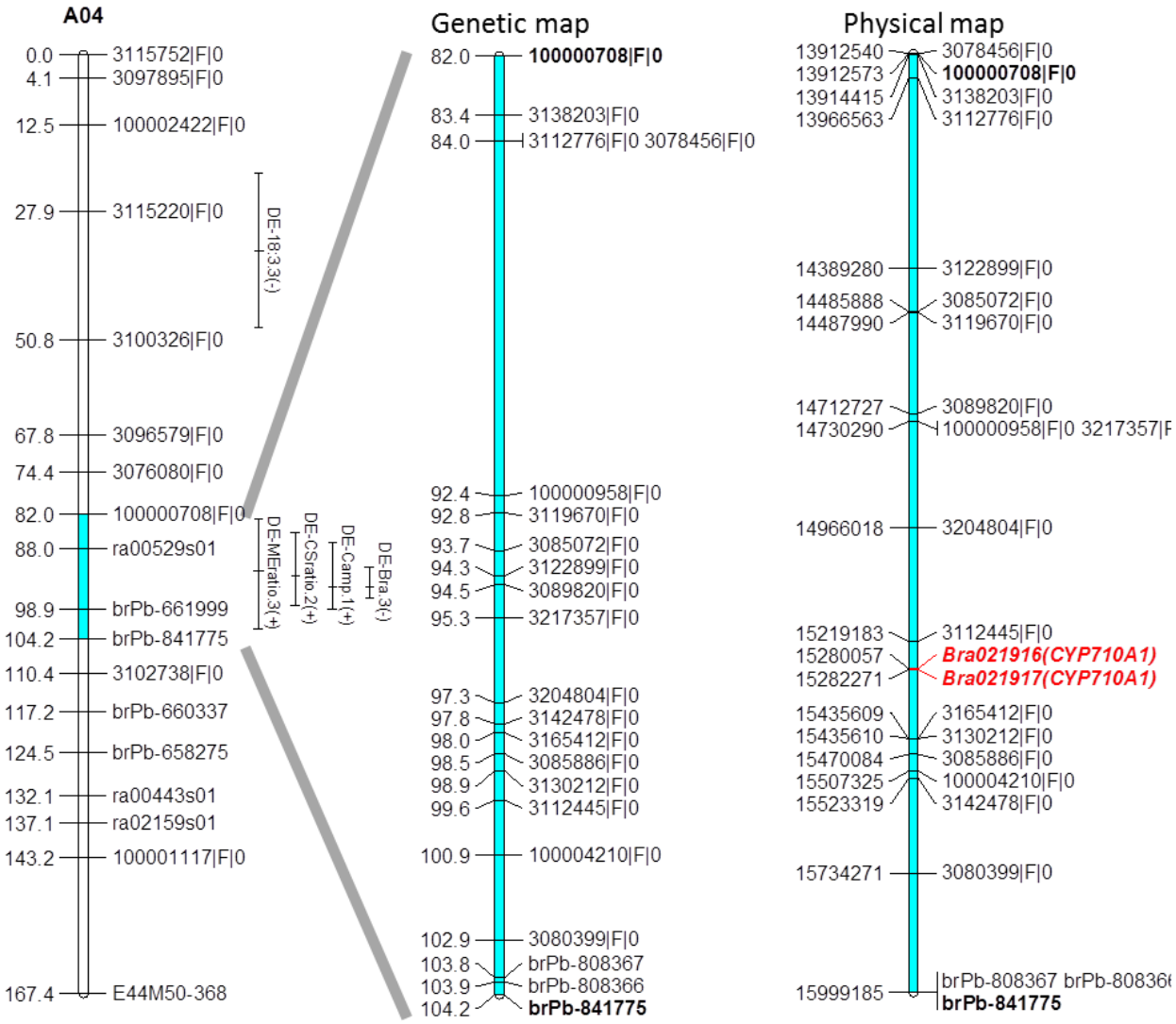

Figure 3.4: (b) Genetic and physical map positions of markers within the QTL genomic region (82-104.2 cM) on A04. *Left*: QTL mapped on A04 in framework map of SODH population. *Middle*: Additional markers mapped within the QTL genomic region (82-104.2 cM) in full map of SODH population *Right*: The corresponding physical positions of additional markers and the candidate gene (*CYP710A1*) in *B. rapa* genome.

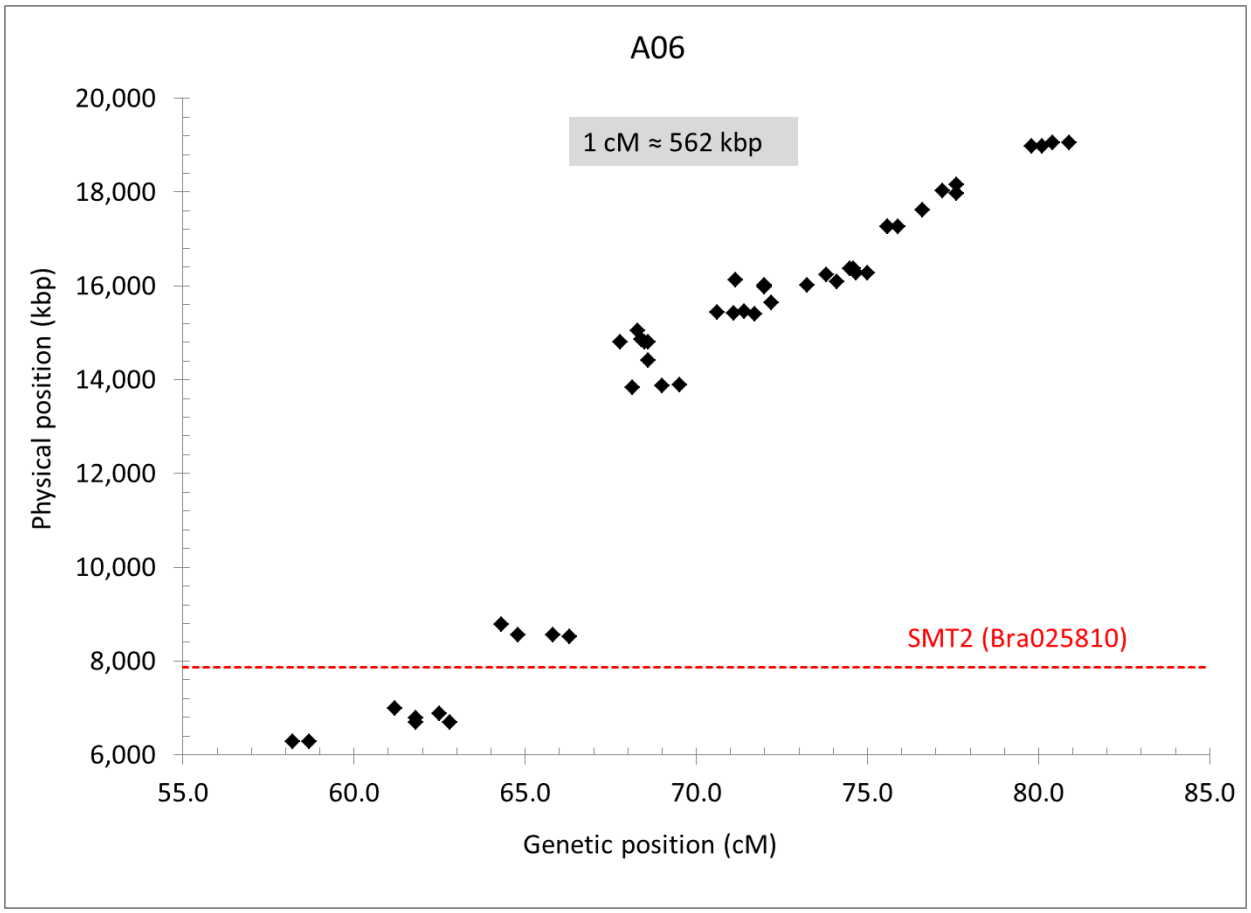

Figure 3.5: (a) Alignment of genetic and physical map positions of markers within the QTL genomic region on A06. The physical position of the candidate gene (*SMT2*) is indicated by the *red dotted line*.

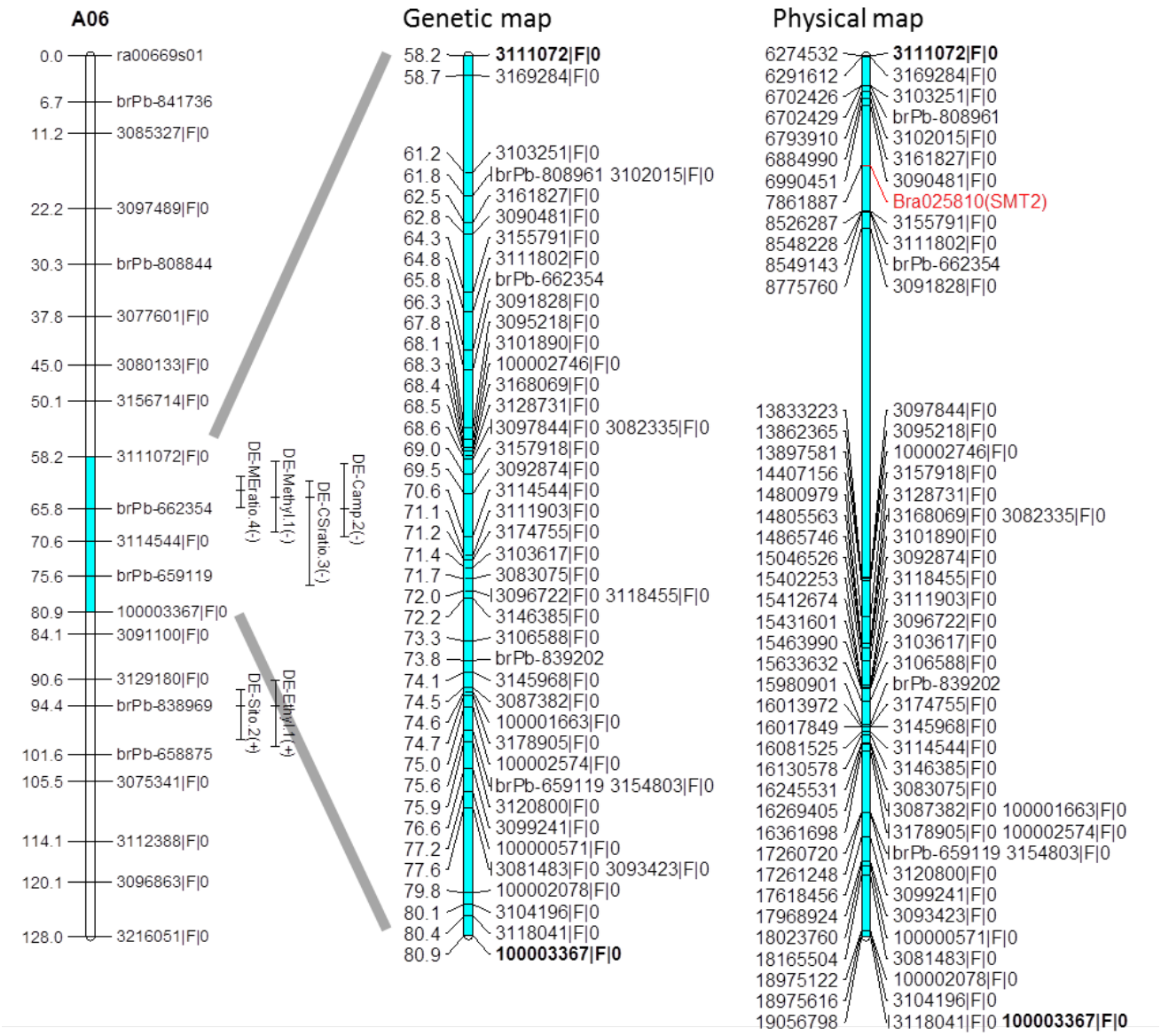

Figure 3.5: (b) Genetic and physical map positions of markers within the QTL genomic region (58.2-80.9 cM) on A06. *Left*: QTL mapped on A04 in framework map of SODH population. *Middle*: Additional markers mapped within the QTL genomic region in full map of SODH population *Right*: The corresponding physical positions of additional markers and the candidate gene (*SMT2*) in *B. rapa* genome

Fatty acids

On A01, nine QTL were found clustered within a genomic region of 27 cM (65-92 cM). Among these were major QTL for C18:1 and C18:3 and minor QTL for C16:0, C18:2 and oil content. Candidate genes known to be involved in fatty acid and oil synthesis on A01 are *fatty acid desaturase 2 (FAD2)* and *LPAAT*. Alignment of genetic and physical map positions of markers within the genomic region of 64.8 - 92.2 cM on A01 exhibited good collinearity and the major QTL was found collocated with *FAD2* while minor QTL for C16:0, C18:2 and oil content were found collocated with *LPAAT* (Figure 3.6a & b).

The gene encoding FATB was postulated to be candidate for the major QTL of palmitic acid (*DE-16:0.2*) on A09. A BLAST search with *FATB* gene sequence of *Arabidopsis* (At1g08510) against the reference genome of *B. rapa* resulted in three homologues located on chromosome A06, A08, and A09. Alignment of genetic and physical map positions of markers within the genomic region of 99.3 - 107.8 cM on A09 exhibited good collinearity and the major QTL was found collocated with the homologue of *FATB* (Bra031631) on A09 (Figure 3.7a & b).

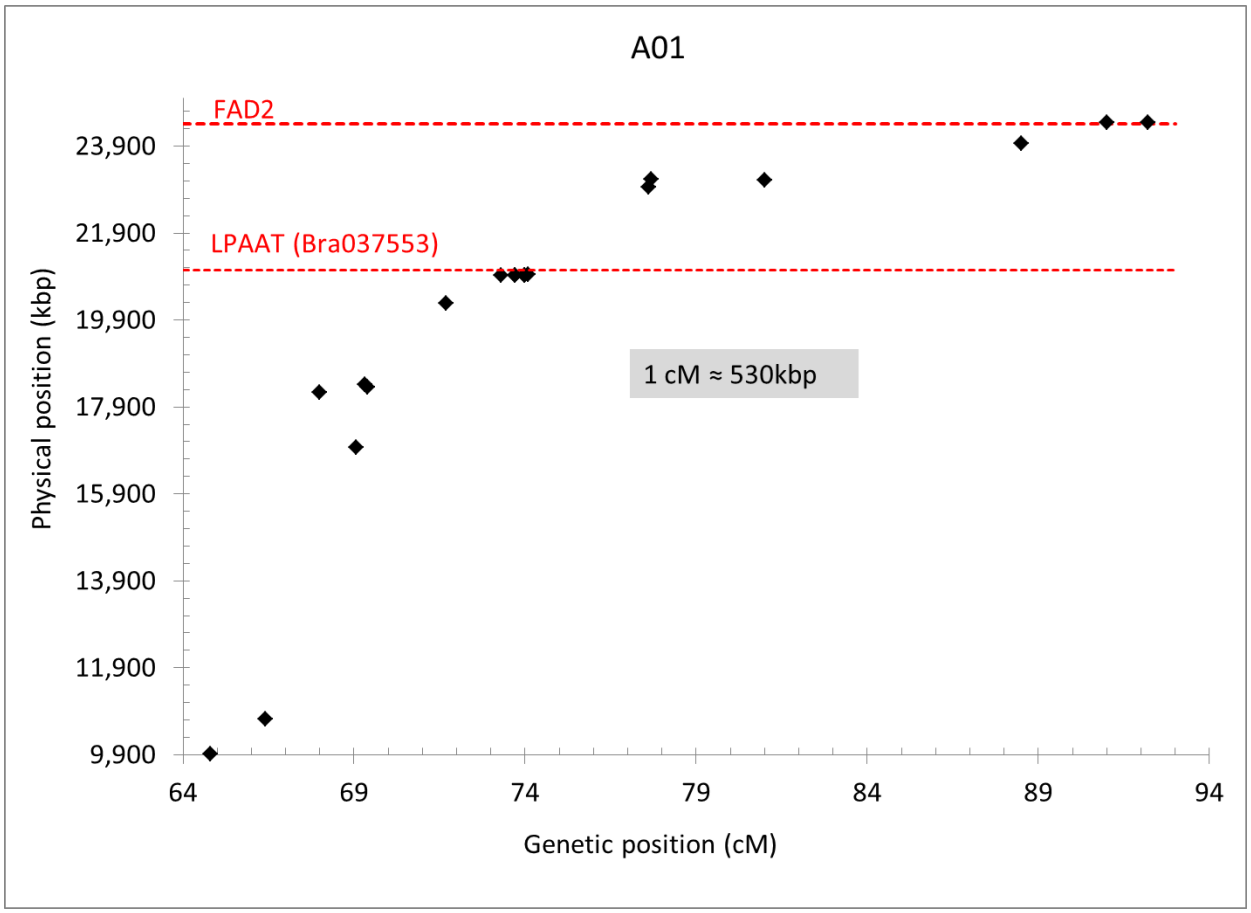

Figure 3.6: (a) Alignment of genetic and physical map positions of markers within the QTL genomic region on A01. The physical position of candidate genes (*FAD2*) and (*LPAAT*) are indicated by the *red dotted line*.

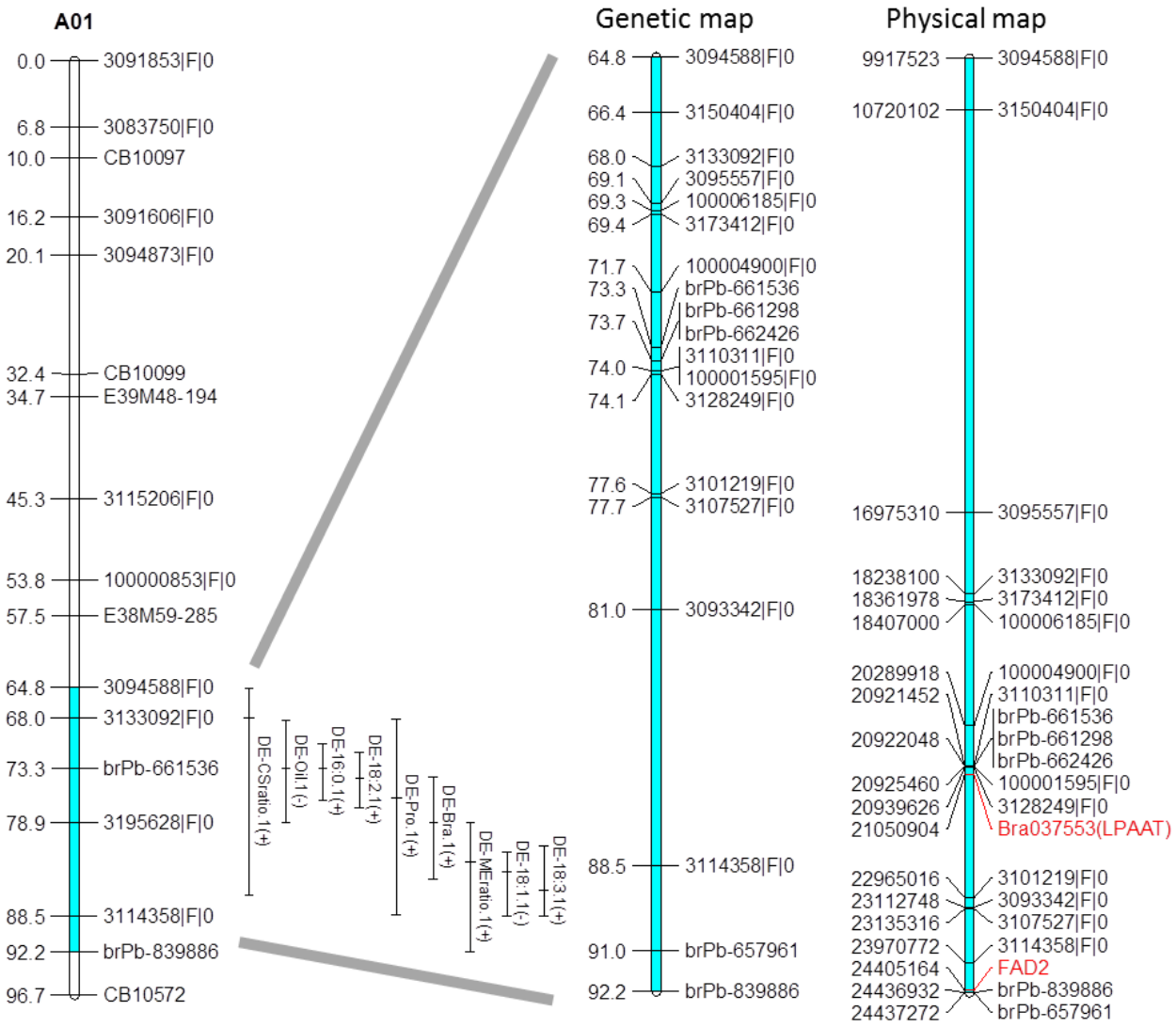

Figure 3.6: (b) Genetic and physical map positions of markers within the QTL genomic region (64.8-92.2 cM) on A01. *Left*: QTL mapped on A01 in framework map of SODH population. *Middle*: Additional markers mapped within the QTL genomic region in full map of SODH population *right*: The corresponding physical positions of additional markers and the candidate genes (*FAD2*) and (*LPAAT*) in *B. rapa* genome

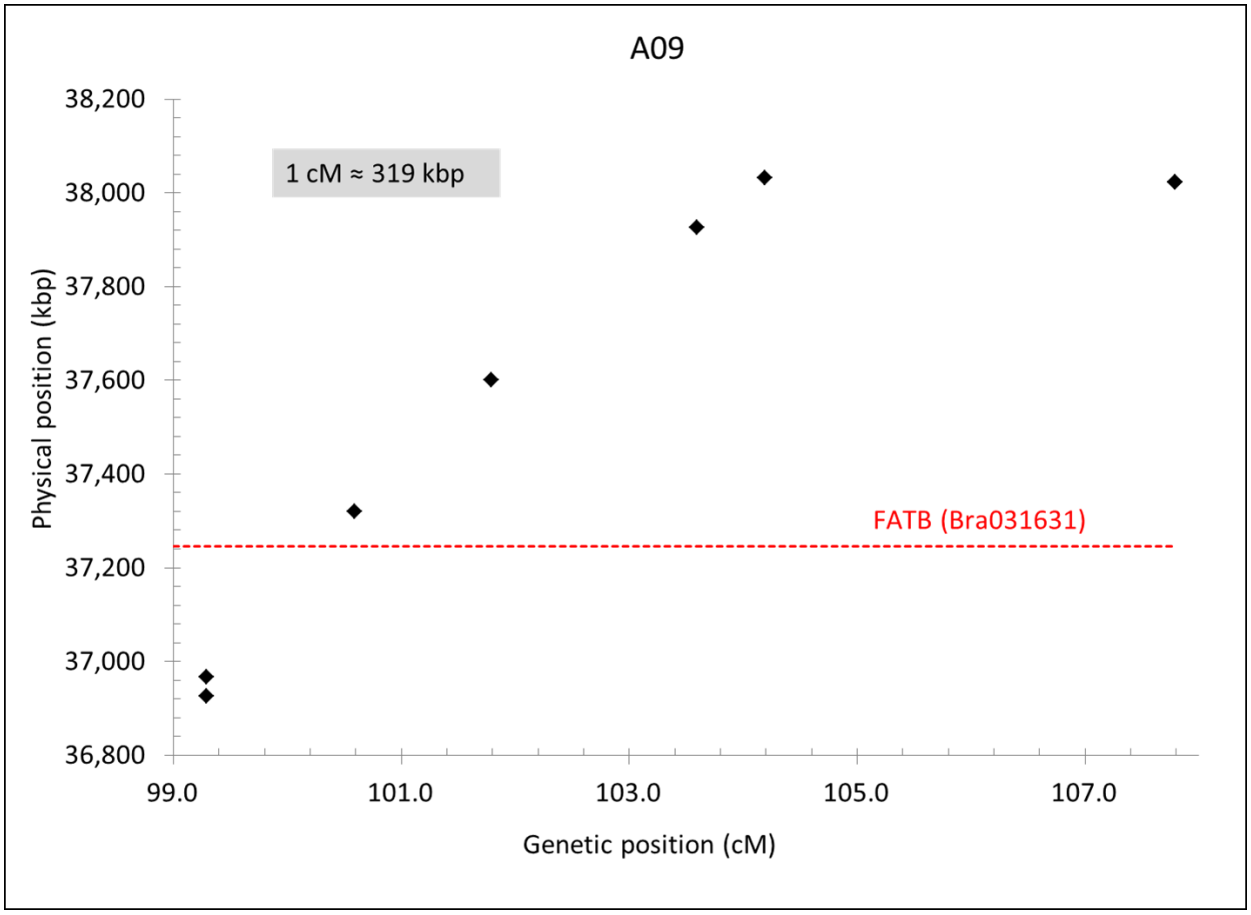

Figure 3.7: (a): Alignment of genetic and physical map positions of markers within the QTL genomic region (99.3-107.8 cM) on A09. The physical position of predicted gene (*FATB*) is indicated by the *red dotted line*.

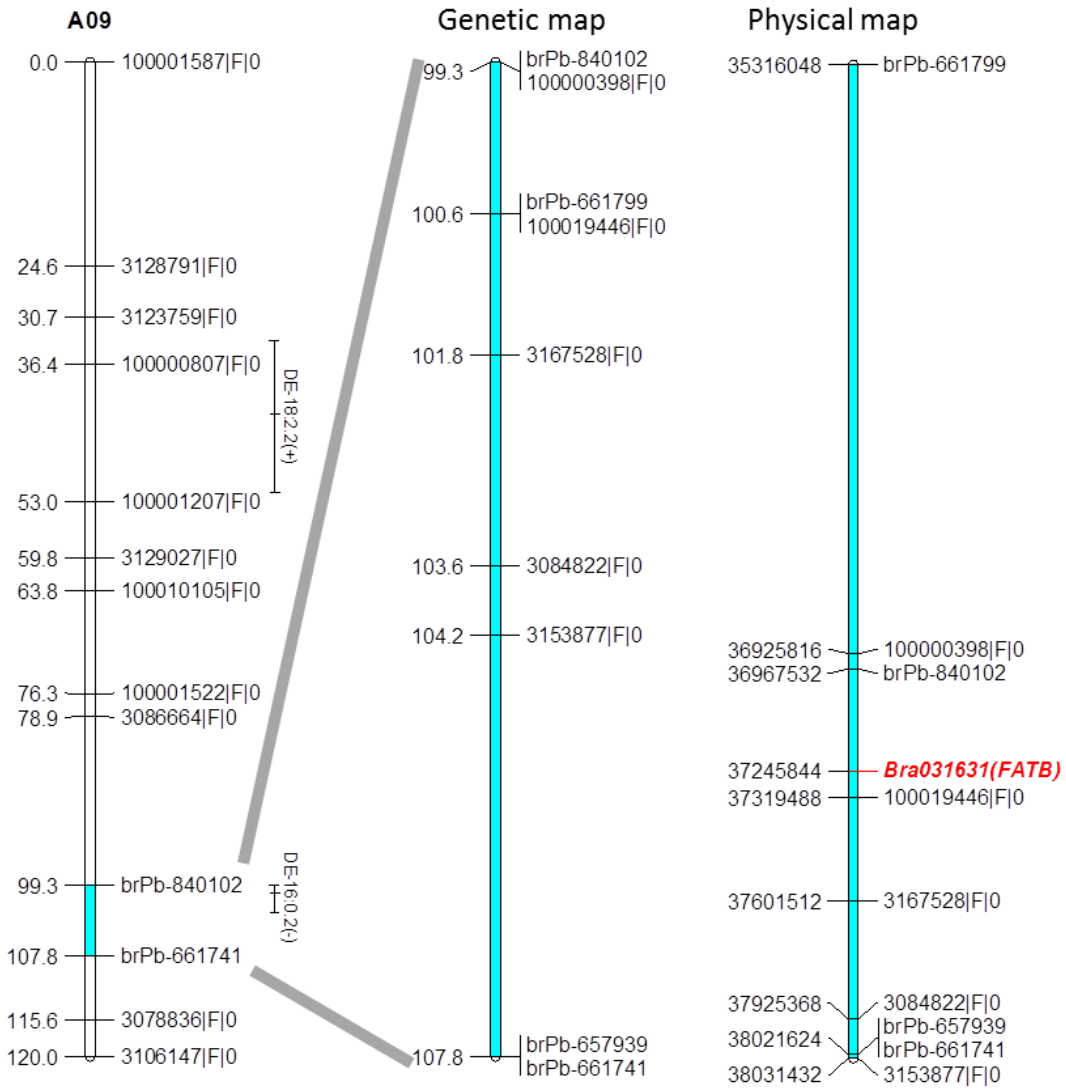

Figure 3.7: (b) Genetic and physical map positions of markers within the QTL genomic region (99.3-107.8 cM) on A09. *Left*: QTL mapped on A09 in framework map of SODH population. *Middle*: Additional markers mapped within the QTL genomic region in full map of SODH population *Right*: The corresponding physical positions of additional markers and the candidate gene (*FATB*) in *B. rapa* genome

3.5 Discussion

3.5.1 Polymorphism of molecular markers

Overall, the two parental lines showed low level of polymorphisms in most marker types. This was not at all surprising because both parental lines are canola quality winter oilseed rape cultivars which presumably have a narrow genetic background. The numbers of polymorphic markers were greatly increased with array-based high-throughput DArT and silico-DArT markers which at the same time were also sequence informative. A greater effort was needed in the development of candidate gene-based markers due to the allotetrapoid nature of *B. napus*. As shown in Appendix A.3, between two to four copies were found for each gene. Although the reference genome sequences of *B. rapa* and *B. oleracea* have eased the task in identifying locus-specific SNP, they are not the exact diploid progenitors of *B. napus*. As such, only about one-third of the markers designed were shown to be locus-specific. With the narrow genetic background of the two parental lines, only two allele-specific markers for *HMG1* and *HMG2* were developed.

3.5.2 Linkage map of SODH population

In this study, a genetic map was constructed for SODH population based on combinations of AFLP, SSR, DArT, Silico-DArT, SNP, KASP and candidate-gene based markers. All 23 linkage groups could be assigned with chromosome names after aligning with other published maps. As a 10 cM interval between marker loci is commonly used for QTL analysis, the SODH map can be considered suitable for performing QTL analysis. Among the mapped markers, 718 (44%) showed significant ($P = 0.05$) segregation distortion, with the majority of alleles favoring parent "Sansibar" clustered on four linkage groups while the remaining alleles favoring parent "Oase" clustered on another four linkage groups. High level of segregation distortion has also been observed in previously reported *B. napus* maps (Kaur et al., 2009; Zhang et al., 2011; Raman et al., 2013; Delourme et al., 2013). Such phenomenon appears to be common in maps of microspore derived DH population, which may be due to the differential responsiveness between the two parental lines to microspore culture during in vitro androgenesis and seedling regeneration (Foisset and Delourme, 1996). The

SODH map has a higher number of markers mapped on the A genome than on the C genome, similar to a few reported studies (Raman et al., 2013; Delourme et al., 2013; Bancroft et al., 2011). The varying marker density observed across the linkage groups may indicate uneven distribution of recombination events along the chromosomes or variation in polymorphic genomic regions between the parental lines.

3.5.3 Phenotypic analysis

The nutritive value of oil from oilseed rape can be further improved by enhancing the phytosterol content. Due to the pleiotropic effect of the erucic acid genes on phytosterol content (Amar et al., 2008b), two canola quality winter oilseed rape cultivars with contrasting total phytosterol content and oil content were crossed to produce a DH population for genetic studies on phytosterol content. Results from the phenotypic analysis revealed a relatively large and significant phenotypic variation for all the traits. Total phytosterol content which ranged from 311.2 to 486.9 mg 100 g_{seed}^{-1} was comparable to the range from 356.6 to 480.0 mg 100 g_{seed}^{-1} reported in 27 modern rapeseed cultivars (Amar et al., 2009) and higher than the range from 257.0 to 410.4 mg 100 g_{seed}^{-1} reported in a DH population segregating for erucic acid content (Amar et al., 2008b). By taking oil content into consideration, the theoretical phytosterol content in oil ranged from 718.2 to 1122.9 mg 100 g_{oil}^{-1} in the SODH population, which was lower than the range from 765.9 to 1402.3 mg 100 g_{oil}^{-1} in 12 different spring canola varieties (Abidi et al., 1999) but higher than the range from 464.0 to 807.0 mg 100 g_{oil}^{-1} in nine canola lines (Vlahakis and Hazebroek, 2000) and the range from 447.5 to 928.0 mg 100 g_{oil}^{-1} in three different DH populations of winter oilseed rape (Amar et al., 2008a). The high total phytosterol content found in SODH population may be attributed to the low or zero erucic acid content in the seed oil as a negative correlation between the two traits has been reported by Amar et al. (2008a). Among the individual sterols, sitosterol was the most prominent sterol, followed by campesterol, brassicasterol and avenasterol, which is in accord with the relative contents reported from literatures (Vlahakis and Hazebroek, 2000; Verleyen et al., 2002; Amar et al., 2008a,b, 2009). The range of seed oil content from 41.2 to 48.6% was within the range of commercial cultivars which usually contain about 40-50% of oil. Significant genotypic variation and high heritablity observed in all traits suggest that SODH population is suitable for QTL analysis.

Correlation analysis revealed a particular trend between phytosterols and fatty acids. Positive correlations were observed between all nine phytosterol traits and palmitic acid while brassicasterol in particular was more highly correlated with all the major fatty acids than with other phytosterols. Although free sterol and steryl ester fractions were not separately analyzed in the present study, it has been reported that about 35% of phytosterols exist as steryl ester form and most importantly, the concentrations of sterols and fatty acids in the steryl ester fraction do not reflect their concentrations in the total sterols and fatty acids obtained by saponification of the oil (Gordon and Miller, 1997). In particular, brassicasterol is present at higher concentration in free sterol fraction and palmitic acid is present at higher concentrations in the steryl ester fraction (Gordon and Miller, 1997; Verleyen et al., 2002). Therefore, it can be postulated that the observed correlations between phytosterols and fatty acids were in part due to the affinities between particular pairs of phytosterol and fatty acid in forming steryl esters. Negative correlations between oil content and polyunsaturated fatty acids such as linoleic and linolenic acids and positive correlations between oil content and both oleic acid and total phytosterol content were in the favor of breeding for better nutritive oilseed rape as reduced levels of polyunsaturated fatty acids and increased levels of oleic acid will increase oxidative stability of oil while phytosterols can lower LDL-cholesterols.

3.5.4 QTL mapping

In this study, QTL were detected by multiple interval mapping (MIM) which uses model selection technique to detect the epistatic interactions among the QTL detected by composite interval mapping (CIM) method (Kao et al., 1999). The approach thereby combines QTL mapping analysis with the analysis of the genetic architecture of quantitative traits. Results from MIM indicate that additive effects are the main factors contributing to variation in all traits as no significant epistatic interaction was detected in any case. Compared with previous study of Amar et al. (2008b), the present study detected less QTL for all the four individual phytosterols and total phytosterol contents. While Amar et al. (2008b) detected two major QTL on A08 and C03 and a minor QTL on C08 for total phytosterol content, the present study identified only two minor QTL located on A07 and C08. The disappearance of two major QTL in the present study corroborate the findings of Amar et al. (2008b) which states that the two major QTL for total phytosterol content were most likely due to pleiotropic effects exerted by the erucic acid genes. As a matter of fact, the present

study did not detect any QTL on A08 and C03 for all the nine phytosterol traits except for the one minor QTL for brassicasterol identified on C03-II (*DE-Bra.6*). By disregarding the QTL on A08 and C03 from the study of Amar et al. (2008b), the number of QTL was almost the same as detected in the present study except for avenasterol in which 4 additional QTL were detected in the study of Amar et al. (2008b). Similarly, the present study shows that more QTL were detected for individual phytosterol content than total phytosterol content. Of the nine linkage groups that harboured QTL for phytosterols in this study, only two linkage groups (A02 and A07) were not found present with QTL in Amar's study.

As the complete genome sequence of *B. napus* has only been made available after the analysis, reference sequence of *B. rapa* and *B. oleracea* genomes were utilized to represent the subgenomes of *B. napus* in identification of possible candidate genes underlying the major QTL. Of the total 16 traits analyzed, QTL with major effect were found for brassicasterol on A04, campesterol:sitosterol and 24-methyl:24-ethyl sterol on A06, C18:1 and C18:3 on A01, and C16:0 on A09. Marker sequences within the four genomic regions with major QTL were aligned with the physical map of *B. rapa* to inspect for colocalization with candidate genes. A good colinearity between genetic and physical map positions was observed for all four genomic regions.

Based on the phytosterol biosynthetic pathway, the possible candidate genes for major QTL on A04 and A06 were *CYP710A2* and *SMT2*, respectively. Overlapping QTL between brassicasterol and campesterol on A04 as well as the opposite additive effects were similarly observed in the study of Amar et al. (2008b), indicating that they may be the same loci in both populations. The *CYP710A* genes have been known to encode cytochrome P450 enzyme that catalyzes the C-22 desaturation reaction, converting both 24-*epi*-campesterol and sitosterol to brassicasterol and stigmasterol, respectively (Morikawa et al., 2006). In *Arabidopsis*, three C-22 sterol desaturases encoded by *CYP10A1*, *CYP10A2* and *CYP710A4* (Morikawa et al., 2006; Arnqvist et al., 2008) are able to catalyze the synthesis of stigmasterol while only one C22-desaturase encoded by *CYP710A2* is able to produce brassicasterol (Morikawa et al., 2006). Therefore, a BLAST search was conducted with *CYP710A2* gene of *Arabidopsis* (At2g34490) against the reference sequence of *B. rapa* genome. The results were two homologues (Bra021916 and Bra021917) found on chromosome A04, both annotated as *CYP10A1*. In comparison with *Arabidopsis* which contain 3-fold higher of stigmasterol content than brassicasterol content (Benveniste, 2002), the predominant Δ_{22}-sterol in *B. napus* is

brassicasterol while stigmasterol only occurs as trace amount. In regard to that, it is possible that the enzyme CYP710A1 may have different role or substrate specificity in *B. napus*. Through alignment of the genomic region with the *B. rapa* physical map, the two homologues of *CYP710A1* were shown to locate within the QTL genomic region, supporting the hypothesis that *CYP710A1* gene is the candidate gene for *DE-Bra.3*. Furthermore, colocalization of QTL *DE-Bra.3* with three other correlated QTL (*DE-Camp.1*, *DE-CSratio.2* and *DE-MEratio.3*) in opposite direction of additive effects seems to suggest that *CYP710A1* gene may exert pleiotropic effect on the composition of phytosterols. Given that brassicasterol is synthesized via two enzymatic steps from 24-methylene cholesterol and campesterol is synthesized directly from 24-methylene cholesterol, a trade off between campesterol and brassicasterol is usual in the case of parallel biosynthetic pathways. However, this explanation does not seem to apply on the correlated QTL for 24-methyl:24-ethyl sterol which accounted for both campesterol and brassicasterol as 24-methyl sterol. As QTL for 24-methyl:24-ethyl sterol only explained a small fraction of the phenotypic variation (R^2 = 5.6) compared to the other three QTL which individually explained between 38.3 and 14.7% of the phenotypic variation, the modulation of 24-methyl:24-ethyl sterol could be an indirect effect from C-22 desaturase reaction.

On A06, two major QTL associated with campesterol:sitosterol and 24-methyl:24-ethyl sterol were collocated together with two other minor QTL for campesterol and 24-methyl sterol. About 14 cM below this genomic region lies a collocation of two minor QTL for sitosterol and 24-ethyl sterol with positive additive effects as opposed to the upper genomic region. Compared with the result of Amar et al. (2008b), only one major QTL was detected for sitosterol on A06. Alignment between the genomic region and physical map of *B. rapa* revealed that *SMT2* gene was indeed between markers flanking the major QTL. The *SMT2* gene encodes the enzyme sterol methyltransferase 2 which catalyzes the second methylation reaction, converting 24-methylene lophenol to 24-ethylidene lophenol. In another words, *SMT2* gene controls the composition between campesterol and sitosterol or between 24-methyl sterol and 24-ethyl sterol. Campesterol to sitosterol ratio is of interest because it is important in plant growth and development (Schaeffer et al., 2001) and in humans, it determines the efficacy of cholesterol lowering ability (Miettinen, 2001). In plants, a low ratio of campesterol:sitosterol ratio is associated with overall size reduction of vegetative organs while high ratio of campesterol:sitosterol ratio is associated with additional size reduction,

modified apical dominance, novel flower morphology, and drastically reduced fertility (Schaeffer et al., 2001). In terms of cholesterol lowering ability, a low ratio of campesterol:sitosterol ratio is preferred due to the higher absorption of campesterol than sitosterol in the intestinal tract (Lees et al., 1977). Considering the fact that no unfavorable pleiotropic effect or close linkage with other quality traits were observed, independent effect of this major QTL or *SMT2* gene on A06 could be of interest for modifying phytosterol composition.

On A01, collocation of major QTL for C18:1 and C18:3 were found among a cluster of QTL that spanned a genomic region of 27 cM (65-92 cM). The additive effect of C18:1 was negative (-0.87%) as opposed to C18:3 (0.36%), indicating that *fatty acid desaturases*, *FAD2* and *fatty acid desaturase 3 (FAD3)*, may be responsible for the variations of these traits. The *FAD2* gene encodes the enzyme endoplasmic delta-12 oleate desaturase which desaturate C18:1 into C18:2 while *FAD3* encodes the enzyme endoplasmic delta-15 linoleate desaturase which desaturate C18:2 into C18:3. In *B. napus*, four loci located on A01, A05, C01 and C05 have been reported for *FAD2* (Schierholt et al., 2000) while five of the six QTL for *FAD3* have been mapped on A03, A04, A05 and two loci on C04 (Scheffler et al., 1997). Thus, *FAD2* could be the underlying gene for major QTL for C18:1 and C18:3. About 2-3 cM above the major QTL were a collocation of minor QTL for C16:0, C18:2 and oil content, indicating that other genes related to oil or fatty acid synthesis could be around the vicinity of *FAD2* gene. One of the genes that is located on A01 is *LPAAT* which plays an essential role in the synthesis of phosphatidic acid, a key intermediate in the biosynthesis of membrane phospholipids in all tissues and storage lipids in developing seeds. In *Arabidopsis*, expression of the oilseed rape microsomal LPAAT isozymes has resulted in enhancement of seed oil content and seed mass (Maisonneuve et al., 2010). As shown by the alignment of genomic region with the physical map of *B. rapa* (Figure 3.6), *FAD2* was between markers flanking major QTL for C18:1 *DE-18:1.1* and C18:3 *DE-18:3.1* while LPAAT gene was between markers flanking QTL for oil content *DE-Oil.1*.

Similarly, the genomic region of major QTL for palmitic acid (*DE-16:0.2*) on A09 was shown to collocate with *FATB* gene which encodes the enzyme that hydrolyzes the thioester bond of C16:0-ACP and releases C16:0 from acyl-ACP (Bonaventure et al., 2003). Acyl-ACP thioesterases are known to be responsible for regulating the chain termination during de novo fatty acid synthesis and in channeling carbon flux between the plastid and cyctosol in plants. The *FATB* gene belongs

to one of the two isoforms of acyl-ACP thioesterase which primarily hydrolyze C8 to C16 saturated acyl-ACPS (Jones et al., 1995). Given that no other QTL was found overlapping with *DE-16:0.2*, this further support the hypothesis that *FATB* is the underlying gene for the major QTL for C16:0.

In contrast to the large effects of QTL identified for phytosterols and fatty acid compositions, six minor QTL distributed on six linkage groups were identified for oil content. The alleles increasing oil content were all derived from "Oase", the parent with a high oil content, which explained why only slight transgressive segregation was observed in the SODH population. The SODH population is similar to the RNSL population used in the study of (Delourme et al., 2006) as the parents were also chosen in the elite winter oilseed rape germplasm due to their contrasting oil content. A total of 10 genomic regions distributed on 10 linkage groups were identified in the RNSL population (Delourme et al., 2006). A comparison between the two populations showed that QTL were similarly detected on five linkage groups (A01, A07, A08, C03, and C08) but it could not be confirmed if they were the same loci in both populations as the genetic maps do not share any common markers. In this study, the QTL with the largest effect was located on A07 (*DE-Oil.3*) and was found collocated with QTL for brassicasterol (*DE-Bra.5*) and the candidate gene-based marker of *HMG1* (HMG1A07O) at 120 cM. Given that *HMG1* gene is responsible for regulating the carbon flux into the isoprenoid pathway and there is no direct relationship between phytosterol and triacylglycerol biosynthesis, the collocation of both QTL with *HMG1* may be caused by a downstream effect of *HMG1* gene or alternatively, it might be due to close linkage between the causative genes and *HMG1*. Besides the *HMG1* gene, two other candidate genes, *HMG2* and *DGAT1*, were also mapped on A07 but were not found collocated with any QTL.

Above the overlapping QTL for oil and brassicasterol on A07 lies a genomic region (38 to 54 cM) which is noteworthy to inspect as it harbored six QTL associated with different traits (brassicasterol, campesterol, 24-methyl sterol, oleic acid, protein of defatted meal and seed weight). All of the six QTL showed minor effects; however, QTL for protein content of defatted meal and seed weight were the individual QTL which have the largest effect in their respective trait. Particularly for seed weight, numerous studies have consistently detected QTL on A07 in different populations with diverse genetic backgrounds (Quijada et al., 2006; Udall et al., 2006; Shi et al., 2009; Basunanda et al., 2010; Cai et al., 2012). In the latest study, 12 candidate genes underlying 8 QTL for seed weight were identified through comparative mapping among *Arabidopsis* and *Brassica* species but

no candidate genes could be inferred for the two major QTL detected on A07 (Cai et al., 2012). In this study, a collinear relationship could not be established for this region with the reference map of *B. rapa* and hence, impeded the investigation of candidate genes underlying the QTL. As have been reported from previous comparative genomic studies, some small-scale genomic changes such as translocations, insertions/deletions, inversions and rearrangements exist between *B. rapa* and *B. napus* (Osborn et al., 2003; Udall et al., 2005; Xu et al., 2010). A non-collinear relationship on A07 has also been reported by Raman et al. (2014) and Cai et al. (2012). Despite that, a clue was provided by a KASP marker, BNKS003013 placed at 45.4 cM (refer to full map on Appendix A.8), that one possible candidate gene underlying this QTL region could be *HMG2*. Marker BNKS003013 was previously selected due to its close physical proximity (4462 bp apart) to *HMG2*. The *HMG2* belongs to the gene family of HMGR which encodes the enzyme that catalyzes the synthesis of mevalonate from HMG-CoA and is generally considered to be a regulatory enzyme in phytosterol biosynthesis. Although intensive studies on HMGR have been carried out due to its pivotal role in the isoprenoid metabolism in plant cells, the role of each HMGR gene in plant development and metabolic regulation of isoprenoid biosynthesis remains unclear. It has been proposed that *HMG2* is important for cell division based on the restriction of *HMG2* expression to meristematic and floral tissues (Enjuto et al., 1995). Given that marker BNKS003013 was mapped close to *HMG2* gene and within the confidence interval of the three QTL associated with phytosterol, it can be speculated that *HMG2* on A07 is a functional gene in *B. napus* and the collocation of six QTL may be caused by the downstream effect of *HMG2* gene. Alternatively, collocation of QTL could also be caused by tight linkage between multiple gene that were involved.

3.6 Conclusion

Using a multiple interval mapping approach, the present study has identified QTL associated with phytosterol content as well as other seed quality traits and seed weight. The availability of *B. rapa* reference genome sequence has greatly facilitated the investigation of candidate genes involved in the genomic regions of major QTL located on A01, A04, A06 and A09. Within the QTL genomic regions, the marker orders of SODH genetic map were relatively collinear with the physical map of *B. rapa*. For phytosterols, the genomic region of major QTL identified for brassicasterol on A04

and campesterol:sitosterol and 24-methyl:24-ethyl sterol on A06 were shown to collocate with the candidate genes *CYP710A1* and *SMT2*, respectively. For fatty acids, major QTL for C18:1 and C18:3 on A01 were shown to collocate with *FAD2* gene and near to *LPAAT* which underlies minor QTL for C16:0, C18:2 and oil content while the major QTL for C16:0 on A09 coincided with *FATB* gene.

Chapter 4

Genetic variation and inheritance of phytosterol and oil content in a doubled haploid population derived from the winter oilseed rape Sansibar × Oase cross cultivated in East China

4.1 Abstract

Demand for oilseed rape as edible oil is growing in China due to its recognizable nutritional properties. Phytosterol is one of the minor salutary oil constituents that have received special attention in recent years due to its LDL-cholesterol lowering properties that could add value to oilseed rape. In this study, a DH population derived from two winter oilseed rape cultivars, "Sansibar" and "Oase", were evaluated over two years of replicated field experiments in Hangzhou, East China. Both parental lines are of canola quality and were chosen due to their contrasting phytosterol and oil content in seed. The traits evaluated include phytosterol content and composition as well as other important seed quality traits such as fatty acid composition, oil content, protein content of defatted meal, and a yield related trait, seed weight. Genotypic effects were predominant and highly significant compared with genotype $\times$ environment interaction effects, resulting in high heritabilities which ranged from 0.53 to 0.93. Multiple interval mapping identified 29 QTL for nine phytosterol traits, 13 QTL for four fatty acids, 7 QTL for oil content, 3 QTL for protein content of defatted meal and 2 QTL for seed weight. QTL results were compared with previous results based on field trials evaluated in Europe (Germany and Sweden) to discern environment-specific QTL from stable QTL by means of mega-environments, Europe (Germany and Sweden) and East China (Hangzhou). Most traits contain between 1 and 4 stable QTL except for total phytosterol content which only contain environment-specific QTL. Major QTL for brassicasterol on A04 and a collocation of two major QTL for campesterol:sitosterol and 24-methyl:24-ethly sterol on A06 were repeatedly detected across two mega environments, suggesting that there is genetic potential for altering phytosterol composition. In the case of enhancing phytosterol content, environment specific QTL would have to be used for marker assisted selection.

4.2 Introduction

Phytosterols or plant sterols are natural constituents of vegetable oil with serum cholesterol lowering properties (Best et al., 1954). An increasing appreciation of this beneficial effect on human health has led to the development of functional food enriched with phytosterols as bioactive ingredients. The major sources of phytosterols for current functional foods and dietary supplements

are tall oil and vegetable oil deodorizer distillate (Moreau, 2004). Among the common commercial vegetable oils, corn and oilseed rape contain the highest levels of phytosterols, varying from 8 to 16 g kg^{-1} and 5 to 10 g kg^{-1}, respectively (Piironen et al., 2000).

Phytosterols are isoprenoid derivatives with a four-ring steroid nucleus (Edwards and Ericsson, 1999). Higher plants contain a mixture of sterols in marked contrast to vertebrates and fungi which generally contain one major sterol—cholesterol and ergosterol, respectively. The mixture of phytosterols differ mainly in the number of carbon atoms on C-24 in the side chain as well as the number and position of double bonds in the tetracyclic skeleton. In oilseed rape, the phytosterol profile consists mainly of sitosterol, campesterol, brassicasterol and avenasterol, while cholesterol and stigmasterol occur only in trace amounts (Appelqvist et al., 1981). Brassicasterol is a characteristic sterol of *Brassicaceae* species and in oilseed rape, it amounts to about 13% of total phytosterol content (Appelqvist et al., 1981). Studies have shown that brassicasterol-rich phytosterols mixtures obtained from oilseed rape exhibit similar cholesterol lowering properties to those phytosterols obtained from other sources like tall oil (Demonty et al., 2007; Heggen et al., 2010), suggesting that oilseed rape is a suitable alternative source for phytosterol enrichment of foods.

Oilseed rape is a crop of major importance in both Europe and China. In Europe, winter oilseed rape is the dominating type whereas in China, both winter and spring varieties are grown depending on the climatic conditions. The winter variety has a longer vegetation period and give a better yield, but can only be grown in areas with a mild winter climate. In this study, the SODH population, derived from two European canola quality winter oilseed rape cultivars, was evaluated in East China (Hangzhou) over two years of replicated field experiments. Compared with Europe (Germany), the daily temperature of oilseed rape growing period in East China (Hangzhou) is about 1 to 4 °C higher and total growth period is about 80 days shorter (Zhao et al., 2005). Such contrasting growing conditions have been evaluated using a DH population derived from a cross between German winter oilseed rape cultivar "Sollux" and the Chinese semi-winter/spring variety "Gaoyou"; significant environmental influence was reported for oil content and fatty acid composition (Zhao et al., 2005, 2008) whereas for phytosterol content, large environmental effect was only observed for avenasterol and total phytosterol content (Amar et al., 2008a)

Evaluation of foreign gene pool such as this is of practical importance for breeding as new favourable alleles may be discovered and later incorporated into local adapted material. In addition, comparing QTL identified across mega environments could discern stable QTL from environment-specific QTL. While stable QTL has a wider use in breeding program, environment-specific QTL are also valuable for breeding program targeted on specific environment. As such, the present study will also compare the QTL identified based on evaluation of field trials in East China (Hangzhou) with QTL identified based on evaluation of field trials in Europe (Germany and Sweden) from previous study.

4.3 Materials and methods

4.3.1 Plant material

The plant material used was as described in section 3.3.1

4.3.2 Field experiments

Field trials were carried out at Hangzhou, East China for two consecutive years (in the growing season 2011/12 and 2012/13) with two replicates in a complete randomized design by Prof. Jianyi Zhao (Zhejiang Academy of Agricultural Sciences, Hangzhou) The parental lines were included thrice in each replicate as checks. Seeds were harvested from 5 to 10 open pollinated plants for each line and bulked for analysis.

4.3.3 Phenotypic analysis

Phenotypic analysis were performed for phytosterol content, fatty acid composition, oil content, protein content of defatted meal, and seed weight as described in Section 3.3.5

4.3.4 Statistical analysis

Variance components, heritability, and means were estimated by using PLABSTAT software version 3A (Utz, 2011). The model implemented in ANOVA analysis was as follow:

$$Y_{ij} = \mu + g_i + e_j + r_{jk} + ge_{ij} + \varepsilon_{ijk}$$

where Y_{ij} is the trait value of genotype i in environment j in replicate k; μ is the general mean; g_i is the effect of ith genotype, e_j is the effect of jth environment, r_{jk} is effect of replicate k in the environment j; ge_{ij} is the interaction between ith genotype and jth environment; and ε_{ijk} is the within environment error associated with genotype i, environment j, and replicate k. The genotype

was treated as fixed effect, whereas environment and replicate were treated as random effects. The corresponding broad sense heritability is estimated following Hill and Weir (1988) as follow:

$$\hat{h}^2 = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \frac{\hat{\sigma}_{GE}^2}{E} + \frac{\hat{\sigma}_\varepsilon^2}{ER}}$$

where $\hat{\sigma}_G^2$, $\hat{\sigma}_{GE}^2$ and $\hat{\sigma}_\varepsilon^2$ are variance components for g, e, ε, respectively; E and R refers to number of environment and number of replicates. Mean values of the genotypes across the environments were used to calculate Spearman's rank correlation coefficients between traits.

4.3.5 QTL mapping

QTL detection was performed as described in Section 3.3.7

4.4 Results

4.4.1 Phenotypic analysis

The SODH population was evaluated over two years of replicated trials in Hangzhou, East China. Analysis of variance revealed highly significant genotype effects for all traits while environment effect was significant only for oil, C18:2, C18:3 and seed weight and genotype $\times$ environment interaction was significant for C18:1, C18:2, C18:3, and seed weight (Table 4.1). Broad-sense heritability (H^2) estimates ranged from 0.53 to 0.93 (Table 4.1). A relatively large variation was observed for all traits (Table 4.2). Total phytosterol content ranged from 340.5 to 507.7 mg 100 g_{seed}^{-1}, with a mean of 421.3 mg 100 g_{seed}^{-1}. Among the four quantified end-products of sterol pathway, sitosterol was the most prominent sterol, followed by campesterol, brassicasterol and avenasterol. The 24-ethyl sterol content, which include sitosterol and avenasterol, was higher than the 24-methyl sterol content, which comprised of campesterol and brassicasterol. The oil content was high in this population, ranging from 39.9 to 45.8%, with a mean of 42.9% (Table 4.2). Trait variation exhibited normal or near-normal distributions for all traits, with extreme values at both ends of the distributions exceeding the extreme values of both parental lines, suggesting transgressive segregation (Figure 4.1).

Highly significant positive correlations (P = 0.01) were observed between the four individual phytosterols and total phytosterol (Table 4.3). Brassicasterol showed higher correlations to all the major fatty acids than with individual phytosterols. Oil was positively correlated with total phytosterol and oleic acid and negatively correlated with linoleic and linolenic acids.

Table 4.1: Variance components and heritability of the DH population ($n = 226$)

Trait	Variance components (σ^2)			Heritability
	Genotype (G)	Environment (E)	G×E	(h^2)
Phytosterol (mg 100 g_{seed}^{-1})				
Brassicasterol	11.93**	0.96	0.31	0.78
Campesterol	290.06**	−6.25	4.68	0.89
Sitosterol	401.77**	20.52	−3.38	0.83
Avenasterol	5.16**	0.06	0.46	0.53
Total phytosterol	1113.20**	18.26	−8.49	0.82
24-methyl sterol	305.22**	−7.52	4.89	0.86
24-ethyl sterol	454.18**	26.09	−6.54	0.83
Campesterol:sitosterol[a]	51.33**	1.06	1.66	0.93
24-methyl:24-ethyl sterol[a]	54.52**	1.08	1.30	0.92
Other traits				
C16:0 (%)	0.10**	−0.05	0.01	0.64
C18:1 (%)	2.31**	0.35	0.20**	0.84
C18:2 (%)	1.18**	0.18+	0.09*	0.85
C18:3 (%)	0.18**	0.08+	0.08**	0.72
Oil (%)	0.67**	0.10+	0.19**	0.70
Protein of defatted meal (%)	1.32**	−0.01	0.01	0.82
Seed weight (g)	0.12**	0.02*	0.03**	0.84

[a] original values (ratio) ×100

+, *, and ** denotes significance at $P < 0.10, 0.05$, and 0.01

Table 4.2: Descriptive statistic of the parents and the DH population ($n = 226$)

Trait	Parents		Double haploid population ($n = 226$)				
	Sansibar	Oase					
	Mean		Min	Max	Mean	*F*-value	LSD 5%
Phytosterol (mg 100 g_{seed}^{-1})							
Brassicasterol	48.5	49.1	39.3	60.0	48.2	4.6**	5.1
Campesterol	142.8	121.2	90.0	187.6	134.1	9.3**	16.5
Sitosterol	270.0	204.8	172.6	281.9	226.1	6.0**	24.9
Avenasterol	11.3	11.7	5.2	24.0	12.9	2.1**	6.0
Total phytosterol	472.5	386.8	340.5	507.7	421.3	5.6**	43.2
24-methyl sterol	191.3	170.3	140.5	238.0	182.3	7.4**	19.2
24-ethyl sterol	281.3	216.5	182.0	298.9	239.0	5.9**	27.0
Campesterol:sitosterol[a]	59.2	52.9	43.4	81.4	59.6	15.4**	5.3
24-methyl:24-ethyl sterol[a]	78.8	68.1	60.4	96.2	76.8	13.1**	5.9
Other traits							
C16:0 (%)	5.8	5.3	4.4	7.1	5.5	2.8**	0.7
C18:1 (%)	62.2	64.8	59.5	69.3	64.2	1.3**	2.9
C18:2 (%)	20.6	18.5	14.9	22.7	18.9	6.7**	1.3
C18:3 (%)	7.1	6.7	5.5	8.1	6.8	3.6**	0.7
Oil (%)	41.3	44.6	39.9	45.8	42.9	3.3**	1.5
Protein of defatted meal (%)	29.6	32.3	27.7	35.7	30.6	5.6**	1.5
Seed weight (g)	3.1	2.8	2.5	4.9	3.3	6.2**	0.4

[a] original values (ratio) $\times 100$
** denotes significance at $P = 0.01$
LSD 5%: least significant difference at the level of $P < 0.05$

Table 4.3: Spearman's rank correlation of traits in the DH population ($n = 226$)

	Brassicasterol	Campesterol	Sitosterol	Avenasterol	Total phytosterol	24-methyl sterol	24-ethyl sterol	24-methyl: 24-ethyl sterol	Campesterol: sitosterol	C16:0	C18:1	C18:2	C18:3	Oil	Protein of defatted meal
Campesterol	0.06														
Sitosterol	0.04	0.47**													
Avenasterol	0.07	0.59**	0.41**												
Total sterol	0.18**	0.82**	0.86**	0.62**											
24-methyl sterol	0.26**	0.97**	0.47**	0.59**	0.83**										
24-ethyl sterol	0.05	0.53**	0.99**	0.51**	0.90**	0.53**									
Campesterol:sitosterol	0.00	0.67**	−0.30**	0.30**	0.17*	0.64**	−0.23**								
24-methyl:24-ethyl sterol	0.18**	0.46**	−0.51**	0.11	−0.05	0.48**	−0.45**	0.94**							
C16:0	0.31**	0.11	0.17*	0.08	0.20**	0.17**	0.18**	−0.03	−0.01						
C18:1	−0.53**	−0.02	−0.08	−0.10	−0.13	−0.12	−0.09	0.07	0.00	−0.55**					
C18:2	0.50**	−0.06	0.03	0.03	0.05	0.04	0.04	−0.12	−0.02	0.37**	−0.91**				
C18:3	0.36**	0.04	0.05	0.14*	0.10	0.11	0.06	−0.01	0.03	0.20**	−0.61**	0.43**			
Oil	−0.24**	0.29**	0.13	0.20**	0.21**	0.23**	0.15*	0.24**	0.12	−0.20**	0.36**	−0.39**	−0.07		
Protein of defatted meal	−0.17*	0.08	0.08	0.14*	0.07	0.04	0.09	0.02	−0.05	−0.24**	0.11	−0.19**	0.17**	0.12	
Seed weight	0.01	0.12	−0.09	0.03	0.02	0.13*	−0.08	0.20**	0.22**	−0.05	0.19**	−0.22**	−0.10	−0.02	0.12

* and ** denotes significance at $P < 0.05$ and 0.01

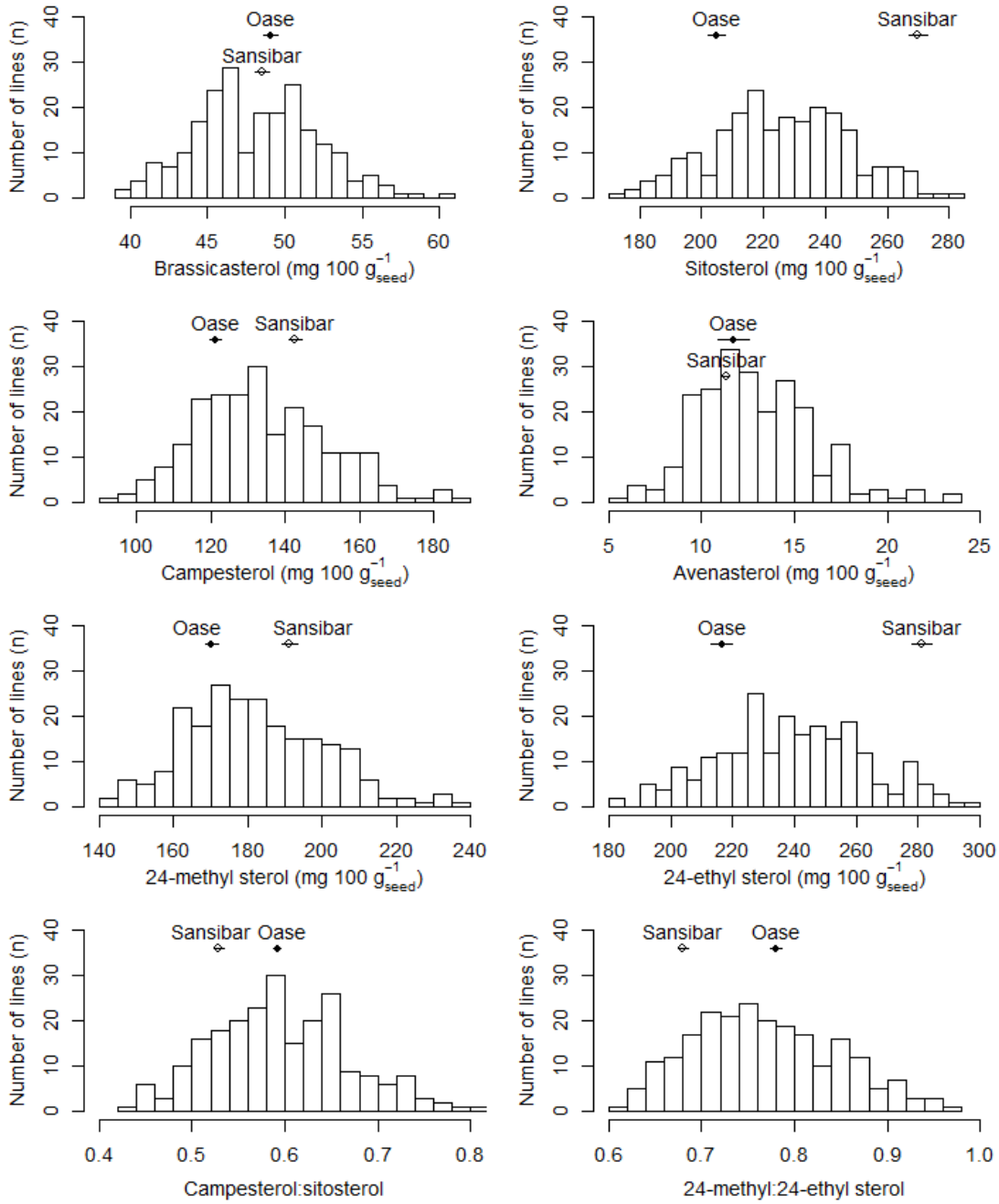

Figure 4.1: Frequency distribution of phytosterol contents, fatty acid composition, oil content, protein content of defatted meal and seed weight in SODH population. Parental mean values are indicated by the symbol ○ for Sansibar and ● for Oase. The standard error of the parental mean is indicated by the line (−) on the symbol. (*continued on next page*)

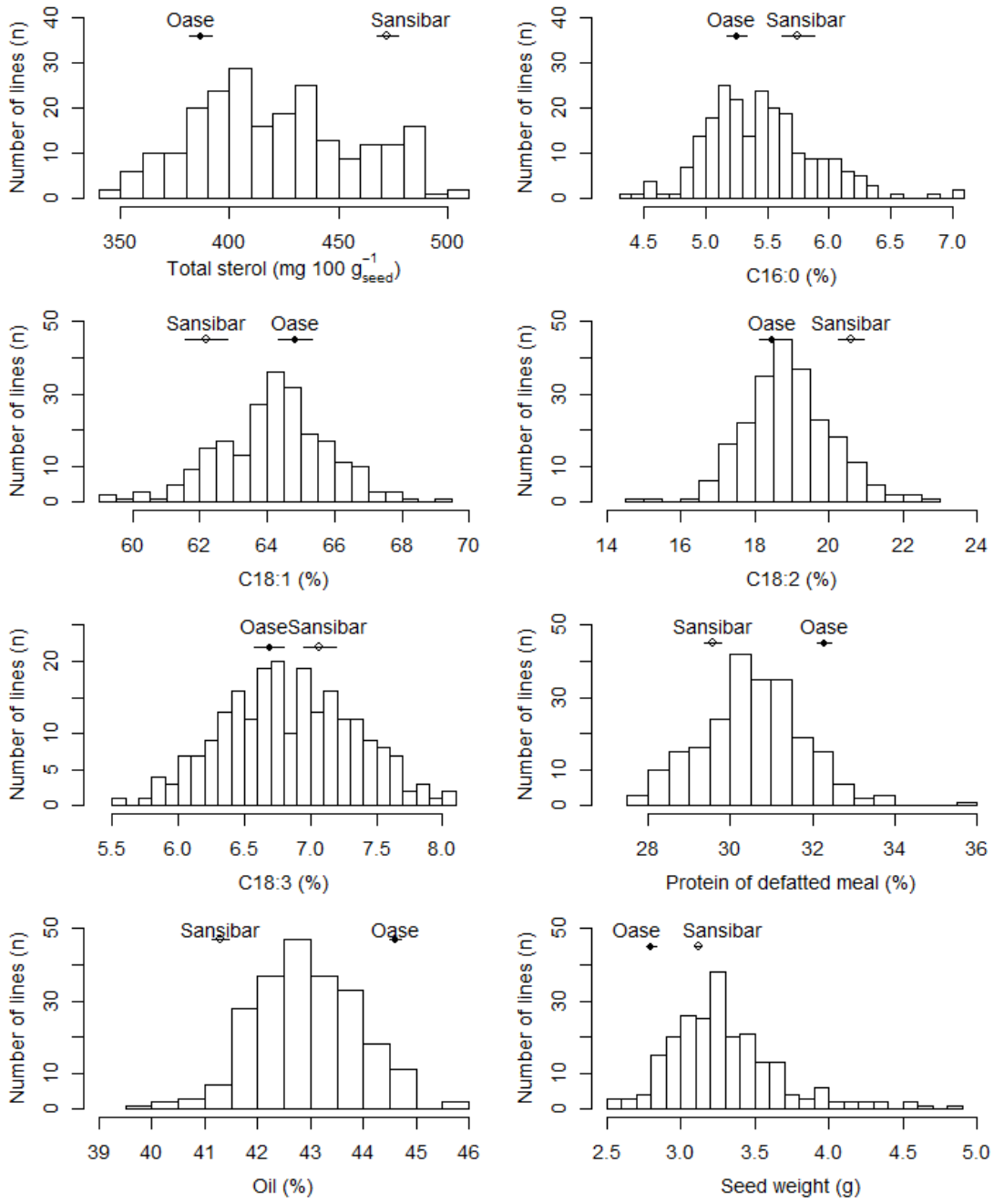

Figure 4.1: (*continued from previous page*) Frequency distribution of phytosterol contents, fatty acid composition, oil content, protein content of defatted meal and seed weight in SODH population. Parental mean values are indicated by the symbol ○ for Sansibar and ● for Oase. The standard error of the parental mean is indicated by the line (−) on the symbol.

4.4.2 QTL mapping

Multiple interval mapping was performed with the adjusted phenotypic means and a framework map of SODH population consisting of 273 markers (Chapter 3). A total of 54 QTL were identified: 29 QTL for the nine phytosterol traits, 13 QTL for the four fatty acids, 7 QTL for oil content, 3 QTL for protein content of defatted meal and 2 QTL for seed weight (Table 4.4, Figure 4.2).

Phytosterols

A total of 29 QTL identified for nine phytosterol traits were distributed on 10 linkage groups (A01, A02, A03, A04, A05, A06, A07, C03, C05, and C08). Between one and six QTL were detected for each phytosterol trait, which collectively explained between 6.5 and 71.9% of the total phenotypic variation. Of the 29 QTL, three were major QTL ($R^2 \geq 25\%$) located on linkage group A04 (*HZ-Bra.2*) and A06 (*HZ-CSratio.3* and *HZ-MEratio.2*). On A04, the major QTL for brassicasterol (*HZ-Bra.3*) showed overlapping confidence interval with QTL for campesterol, campesterol:sitosterol, oil content and linolenic acid. Negative additive effects were observed for brassicasterol, oil content and linolenic acid as opposed to the positive additive effects for campesterol and campesterol:sitosterol. On A06, the two major QTL identified for campesterol:sitosterol (*HZ-CSratio.3*) and 24-methyl:24-ethyl sterol (*HZ-MEratio.2*) were found collocated with QTL for campesterol (*HZ-Camp.3*), 24-methyl sterol (*HZ-Methyl.2*), and sitosterol (*HZ-Sito.1*). All the five QTL collocated within a region of 21 cM (52 - 73 cM) showing negative additive effects except for sitosterol. About 16 - 26 cM further down the genomic region, two additional minor QTL were identified for 24-ethyl sterol (*HZ-Ethyl.1*) with negative additive effect and 24-methyl:24-ethyl sterol (*HZ-MEratio.3*) with positive additive effect. Only one QTL for total phytosterol content *HZ-TPC.1* was identified on C03 and was found collocated with QTL for oil content (*HZ-Oil.5*). Both QTL showed negative additive effects, indicating that allele derived from "Oase" increasing 9.4 mg 100 g_{seed}^{-1} of phytosterol and 0.26% of oil

Fatty acids

Thirteen QTL identified for four fatty acid composition were distributed on 9 linkage groups (A01, A03, A04, A05, A09, C03-II, C07, C09). Between two and five QTL were detected for each fatty acid constituent, which collectively explained between 24.3 and 42.5% of the total phenotypic variance. For palmitic acid, the individual QTL which had the largest effect (16.5% of the phenotypic variance) was located on A09; whereas for oleic, linoleic, and linolenic acids, the individual QTL which had the largest effect were all located on A01. On A01, QTL for oleic acid (*HZ-18:1.1*) was located 11 cM above the two overlapping QTL for linoleic (*HZ-18:2.1*) and linolenic (*HZ-18:3.1*) acids.

Oil content

Seven QTL detected for oil content were distributed on seven linkage groups (A04, A07, A09, C01, C03, C03-II and C06). Individual QTL explained between 4.2 and 10.2% of the phenotypic variance and collectively accounted for 47.1% of the total phenotypic variance. Five of the seven QTL showed negative additive effects, indicating that the allele increasing oil content were mostly derived from "Oase". Individual QTL which had the largest effect (10.2% of the phenotypic variance) was located on A07 and was found collocated with three QTL related to phytosterols and one QTL for protein content of defatted meal. Additionally, a candidate gene marker for *HMG1* (HMG1A07-O1) was also mapped within this QTL region (Figure 4.2). Three QTL which appeared as isolated QTL (no collocation with other QTL) were located on A09, C01 and C06.

Protein content of defatted meal

Three QTL detected for protein content of defatted meal were distributed on two linkage groups (A07 and C03-II). Individual QTL explained between 6.6 and 11.6% of the phenotypic variance and collectively accounted for 26.0% of the total phenotypic variance. Two of the three QTL were located on C03-II in which one QTL (*HZ-Pro.2*) was found collocated with QTL for oil content and linolenic acid while another QTL *HZ-Pro.3* which had the largest effect was located 47 cM below *HZ-Pro.2*.

Seed weight

Two QTL for seed weight were found on linkage group A07 and C08, which collectively accounted for 21.8% of the total phenotypic variance. The additive effect was negative for QTL on A07 and positive for QTL located on C08.

Table 4.4: QTL detected for phytosterol contents (mg 100 g_{seed}^{-1}), fatty acid composition (%), oil content (%), protein content of defatted meal (%) and seed weight (g) in SODH population. QTL detected specifically in Hangzhou, East China are highlighted in *bold* whereas QTL that were detected on the same linkage groups in both Europe (Chapter 3) and Hangzhou are shown in normal font.

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
Brassicasterol	HZ-Bra.1	A03	172	168–182	3.0	0.81	3.4	56.3
	HZ-Bra.2	A04	94	90–97	4.0	-2.55	41.7	
	HZ-Bra.3	**A05**	**115**	**95–120**	**5.0**	**0.87**	**6.3**	
	HZ-Bra.4	A07	115	102–121	7.0	-0.88	4.8	
Campesterol	**HZ-Camp.1**	**A01**	**35**	**26–42**	**4.1**	**4.34**	**6.5**	34.8
	HZ-Camp.2	A04	102	92–113	5.3	5.10	9.3	
	HZ-Camp.3	A06	68	60–73	7.4	-6.14	12.1	
	HZ-Camp.4	C08	20	3–32	4.0	4.36	6.8	
Sitosterol	HZ-Sito.1	A06	64	52–71	2.2	4.57	10.1	17.4
	HZ-Sito.2	C05	87	73–89	3.5	5.94	7.3	
Avenasterol	HZ-Ave.1	C08	20	2–29	3.7	0.85	7.4	7.4
Total phytosterol	**HZ-TPC.1**	**C03**	**101**	**89–109**	**3.0**	**-9.40**	**6.5**	**6.5**
24-methyl sterol	**HZ-Methyl.1**	**A01**	**35**	**26–42**	**4.5**	**4.82**	**7.3**	34.2
	HZ-Methyl.2	A06	69	59–73	8.0	-6.73	12.6	
	HZ-Methyl.3	**C05**	**76**	**65–86**	**3.2**	**-4.43**	**5.9**	
	HZ-Methyl.4	C08	20	12–29	5.1	5.22	8.4	
24-ethyl sterol	HZ-Ethyl.1	A06	95	89–102	4.7	7.27	9.3	9.3
Campesterol:sitosterol	**HZ-CSratio.1**	**A02**	**0**	**0–6**	**4.9**	**1.41**	**4.2**	71.4
	HZ-CSratio.2	A04	94	88–109	7.1	1.80	8.7	
	HZ-CSratio.3	A06	64	62–69	30.5	-4.15	32.3	
	HZ-CSratio.4	**A07**	**121**	**101–130**	**3.8**	**-1.28**	**5.5**	
	HZ-CSratio.5	C05	89	81–90	17.3	-2.97	4.0	
	HZ-CSratio.6	C08	19	13–20	6.6	1.62	16.6	
24-methyl: 24-ethyl sterol	HZ-MEratio.1	A02	1	0–5	6.0	1.66	5.5	71.9

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Table 4.4 – *continued from previous page*

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
	HZ-MEratio.2	A06	64	61–67	19.5	-3.66	27.1	
	HZ-MEratio.3	**A06**	**103**	**99–109**	**5.1**	**-1.73**	**9.3**	
	HZ-MEratio.4	**A07**	**112**	**102–121**	**6.1**	**-1.64**	**5.1**	
	HZ-MEratio.5	C05	87	84–89	21.1	-3.49	20.9	
	HZ-MEratio.6	**C08**	**19**	**13–20**	**5.0**	**1.44**	**4.0**	
C16:0	HZ-16:0.1	A09	98	90–103	8.9	-0.18	16.5	24.3
	HZ-16:0.2	C09	27	17–33	4.0	0.12	7.8	
C18:1	HZ-18:1.1	A01	61	55–67	5.	-0.55	11.40	25.8
	HZ-18:1.2	**A05**	**106**	**86–111**	**5.7**	**-0.56**	**9.6**	
	HZ-18:1.3	C08	0	0–17	3.2	-0.42	4.7	
C18:2	HZ-18:2.1	A01	84	79–91	9.1	0.44	13.90	42.5
	HZ-18:2.2	**A03**	**125**	**109–140**	**3.4**	**0.28**	**6.3**	
	HZ-18:2.3	**A04**	**90**	**83–97**	**3.9**	**-0.28**	**6.5**	
	HZ-18:2.4	**A05**	**106**	**102–111**	**6.1**	**0.35**	**8.7**	
	HZ-18:2.5	**C03-II**	**39**	**35–42**	**5.2**	**0.33**	**7.2**	
C18:3	HZ-18:3.1	A01	84	76–88	13.9	0.24	23.3	37.2
	HZ-18:3.2	**A05**	**72**	**62–80**	**3.1**	**0.12**	**5.7**	
	HZ-18:3.3	C07	82	79–90	6.0	-0.15	8.1	
Oil	**HZ-Oil.1**	**A04**	**99**	**95–103**	**4.8**	**0.24**	**6.3**	47.1
	HZ-Oil.2	**A07**	**125**	**119–129**	**6.2**	**-0.33**	**10.2**	
	HZ-Oil.3	A09	43	33–58	3.0	-0.23	6.4	
	HZ-Oil.4	**C01**	**42**	**25–50**	**3.4**	**-0.22**	**3.8**	
	HZ-Oil.5	**C03**	**104**	**94–109**	**5.1**	**-0.26**	**7.2**	
	HZ-Oil.6	C03-II	42	38–50	6.5	-0.30	9.0	
	HZ-Oil.7	**C06**	**3**	**0–22**	**3.6**	**0.27**	**4.2**	
Protein of defatted meal	HZ-Pro.1	A07	124	120–131	4.0	-0.36	6.60	26.0
	HZ-Pro.2	C03-II	38	30–42	3.6	-0.33	7.8	

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Table 4.4 – *continued from previous page*

Trait	QTL name	Linkage group	Peak (cM)	CI[a] (cM)	LOD	Additive effect[b]	R^{2c}	TR^{2d}
	HZ-Pro.3	C03-II	92	89–97	5.2	-0.41	11.6	
Seed weight	HZ-SW.1	A07	47	45–54	8.5	-0.16	15.9	21.8
	HZ-SW.2	**C08**	**5**	**0–17**	**3.1**	**0.09**	**5.80**	

[a] 1-LOD Confidence interval

[b] Additive effect is the substitution effect of one "Oase" allele by one "Sansibar" allele.

[c] R^2 is the percentage of variation explained by each QTL

[d] TR^2 is the percentage of variation explained by all QTL

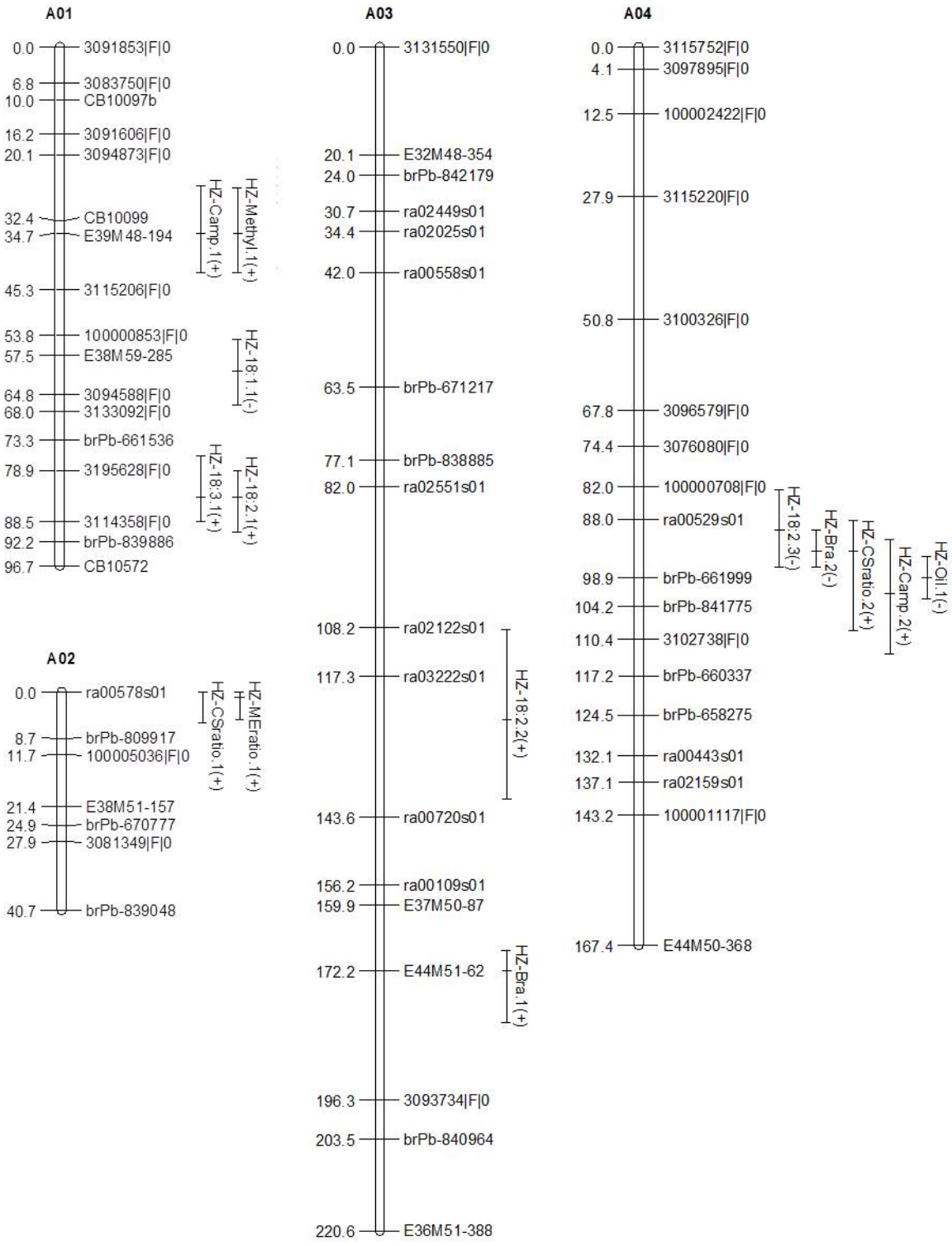

Figure 4.2: QTL associated to phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

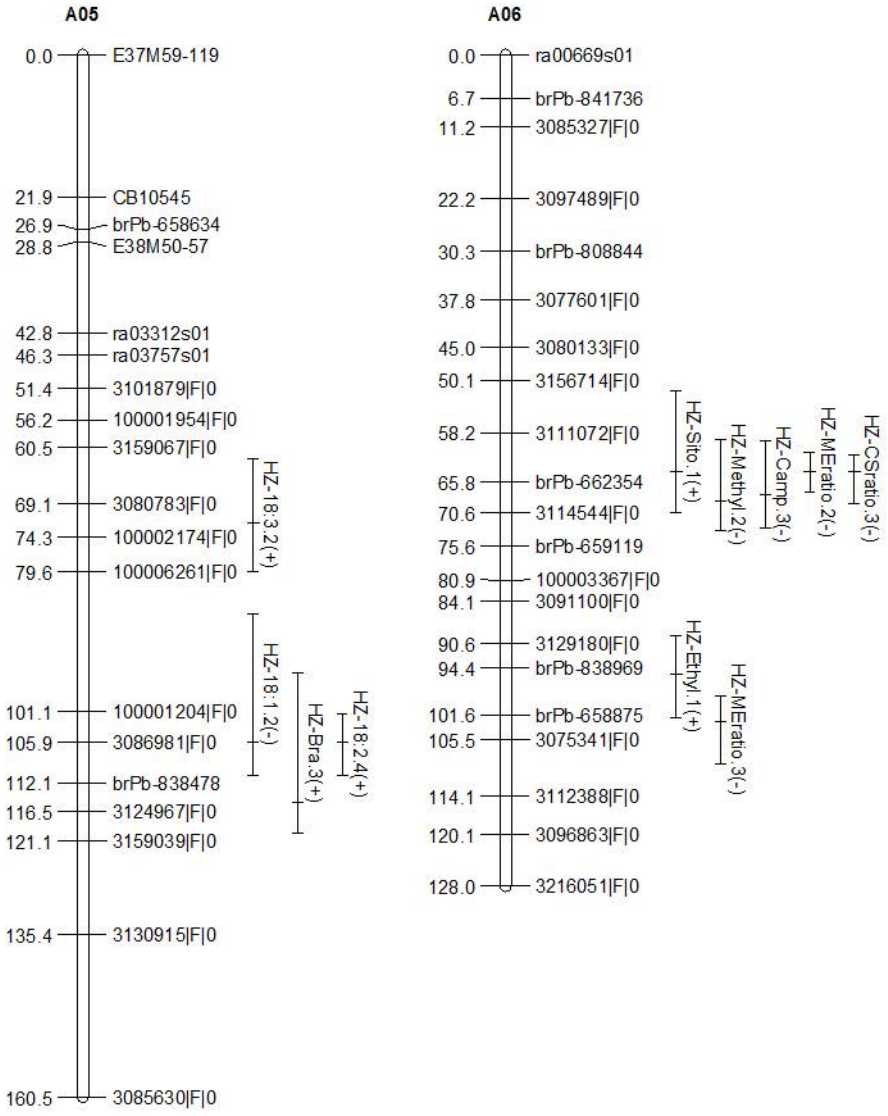

Figure 4.2: (*continued from previous page*) QTL associated to phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

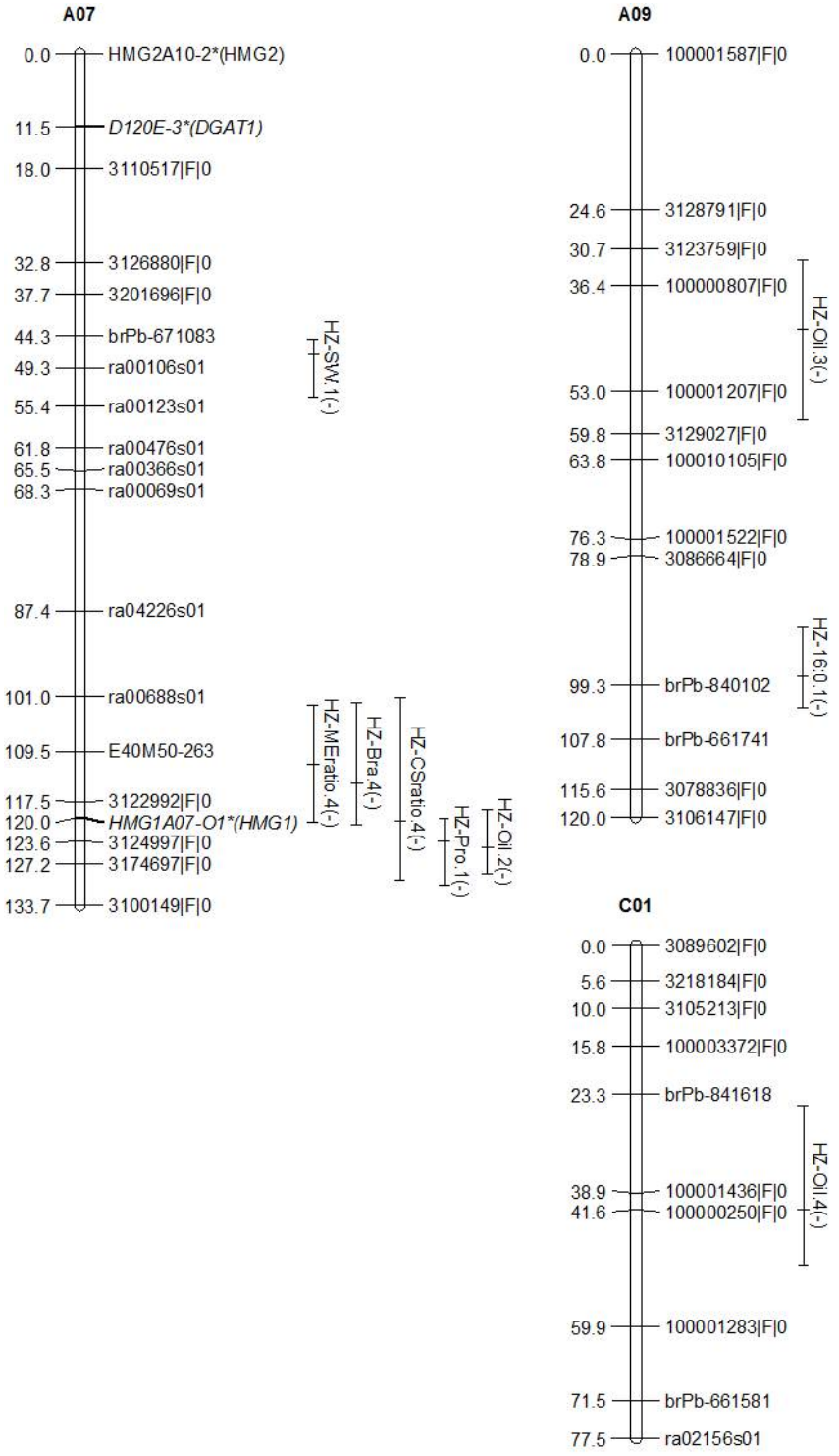

Figure 4.2: (*continued from previous page*) QTL associated to phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population.{+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*).

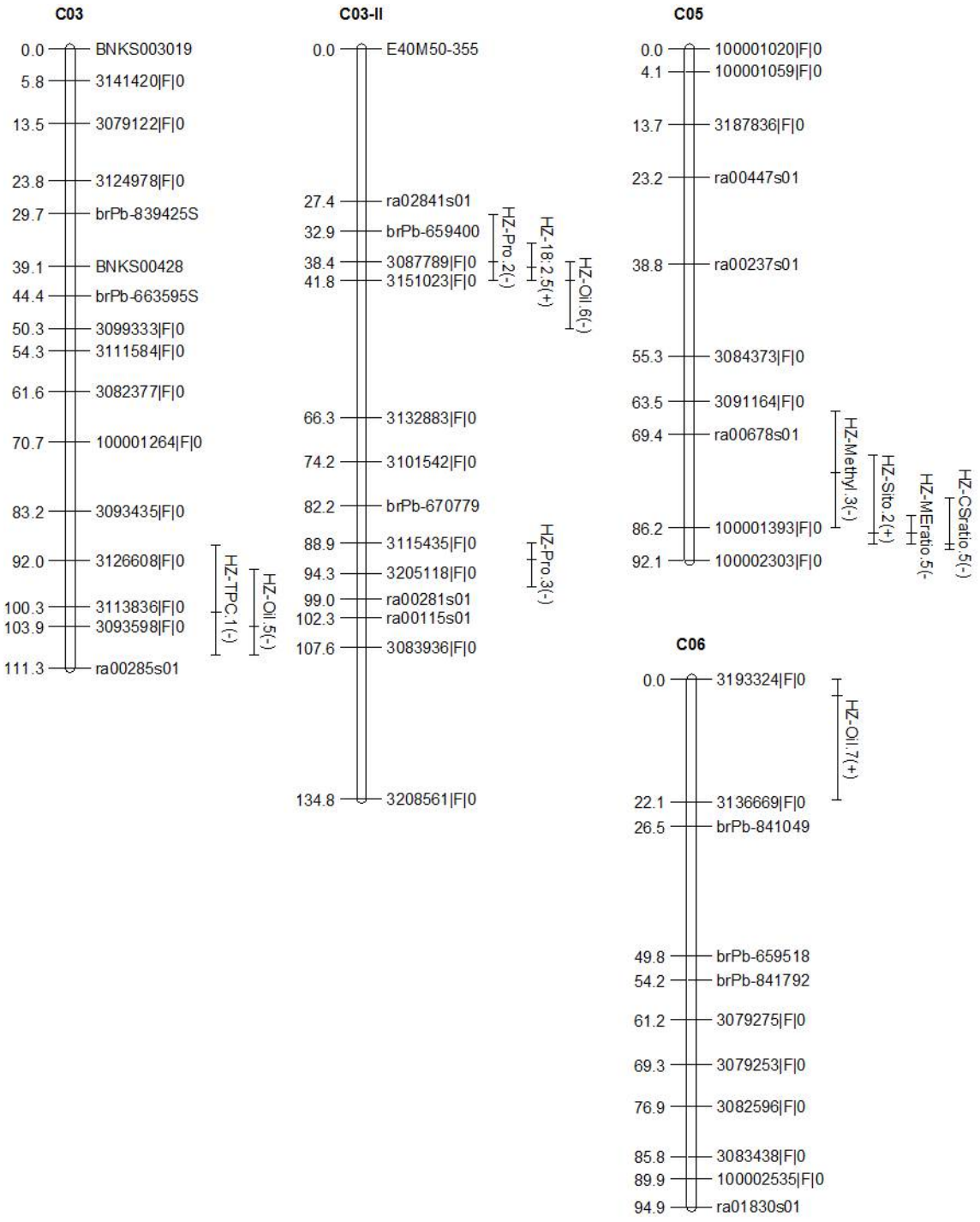

Figure 4.2: (*continued from previous page*) QTL associated to phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population. {+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively. (*continued on next page*)

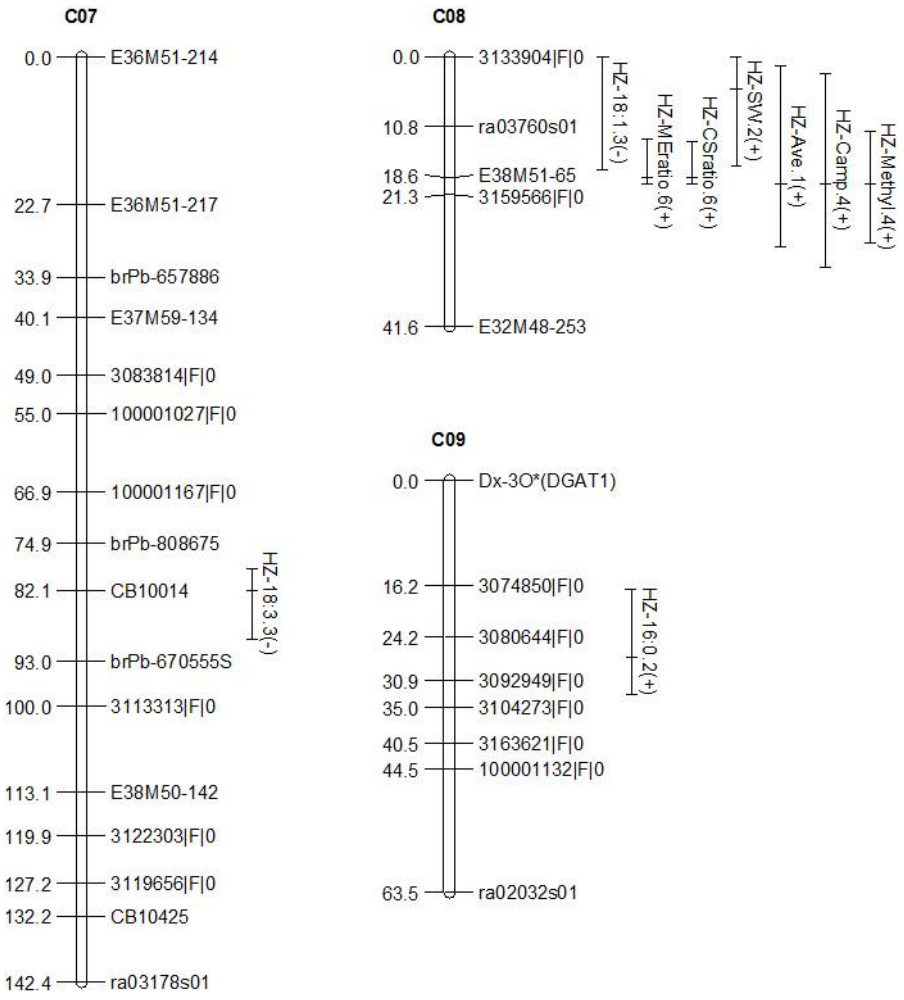

Figure 4.2: (*continued from previous page*) QTL associated to phytosterol traits, fatty acid compositions, oil content, protein of defatted meal, and seed weight in SODH population.{+} and {-} indicate that the trait value is increased by the allele "Sansibar" and "Oase", respectively.

4.5 Discussion

4.5.1 Phenotypic analysis

Oilseed rape is one of the most important oil crops in both Europe and China. To investigate the influence of environmental effect on phytosterol content and other seed quality traits, field trials were conducted in Hangzhou, East China over two years of replicated experiments in addition to the field trials in Europe (Chapter 3). To compare the results from both trials in the following discussion, field experiments conducted in China will be referred as "CN trial" in order to distinguish field experiments conducted in Europe from previous study (Chapter 3), which will be referred as "EU trial".

Comparison of results from both trials revealed that most traits were influenced by the two mega environments. For example, the SODH population showed a higher mean of total phytosterol content in CN trial (421.3 mg 100 g_{seed}^{-1}) as compared with EU trial (401.9 mg 100 g_{seed}^{-1}) whereas the seed oil content in EU trial (45.4%) was higher than that obtained in CN trial (42.9%). In contrast to previous studies which analyzed phytosterol content and oil content in Germany and China, both phytosterol content and oil content showed higher mean in Germany than in China (Amar et al., 2008a; Zhao et al., 2005); total phytosterol content was 340 mg 100 g_{seed}^{-1} in Germany and 299 mg 100 g_{seed}^{-1} in China (Amar et al., 2008a) while oil content was 51.5% in Germany and 44.5% in China (Zhao et al., 2005). In terms of phytosterol composition, brassicasterol and campesterol content were about the same in both trials whereas higher sitosterol and lower avenasterol content were observed in the CN trial. The influence of specific environmental factors on phytosterol content has been demonstrated in one study which cultivated 12 commercial soybean lines under three different temperature regimes (Vlahakis and Hazebroek, 2000). The study reported a 2.5-fold variation in total phytosterol content, a positive correlation between total phytosterol content and higher temperature, and a modulation of phytosterol composition due to elevated temperature, with proportionally more campesterol at the expense of sitosterol and stigmasterol. Although the fact that CN trial has a slighly higher total phytosterol content, which presumably also has a warmer growing condition, than in EU trial corroborate the finding of Vlahakis and Hazebroek (2000), modulation of phytosterol composition was only observed between sitosterol and avenasterol in

CN trial. As for fatty acids, low temperature has been known to induce fatty acid desaturation in oilseed rape (Canvin, 1965; Williams et al., 1992). In accordance with that, CN trial showed a higher C18:1 and lower linoleic acid (C18:2) and linolenic acid (C18:3) contents than in EU trial. High broad sense heritability was similarly estimated for each of the traits in the CN trial, except for avenasterol and C16:0 which showed a lower heritability compared to the EU trial. The low heritability of avenasterol and C16:0 might partly be due to analytical error in determining the small amount of content from incomplete separation of peaks in gas chromatographic analysis.

As shown also in the EU trial, correlation analysis in CN trial revealed highly significant correlations between brassicasterol and all the major fatty acids than with other phytosterols. Significant correlation between total phytosterol and oil content remained positive, indicating that an increase in oil content can also result in increased total phytosterol content. On the other hand, less significant correlations were observed between palmitic acid and all the nine phytosterol traits in the CN trial. A more pronounced effect of environmental influence can be observed between oil content and protein content of defatted meal in which no significant correlation was found in CN trial while significant negative correlation (R^2 = -0.43) was found in EU trial. Specific environmental factors such as rainfall and temperature are known to be significantly associated with oil and protein content. Pritchard et al. (2000a) reported that seed oil content was higher with high rainfall and low temperature while the reverse was true for protein content. As such, the different relationship observed between oil content and protein content of defatted meal across the two trials strongly reflects the contrasting growing conditions of the two mega environments.

4.5.2 QTL mapping

The present discussion will compare the QTL detected in CN trial to the QTL previously identified in EU trial to discern stable QTL from environment specific QTL. In table Table 4.4, QTL detected specifically in CN trial are highlighted in bold whereas QTL that were detected on the same linkage group across two trials are shown in normal font. Table 4.5 shows the confidence intervals and additive effects of QTL that were located on the same linkage group across two trials. Of the 29 QTL identified for nine phytosterol traits in CN trial, 19 were commonly detected across the two trials and were distributed in seven genomic regions at seven linkage groups (A02, A03, A04,

A06, A07, C05 and C08) while 10 environment-specific QTL for CN trial were distributed in eight genomic regions at eight linkage groups (A01, A02, A05, A06, A07, C03, C05, and C08).

As found in EU trial, CN trial also identified one major QTL for brassicasterol on A04 and a collocation of two major QTL for campesterol:sitosterol and 24-methyl:24-ethly sterol on A06. The underlying genes for the major QTL on A04 and A06 were *CYP710A1* and *SMT2*, respectively (Chapter 3: Figures 3.4 and 3.5). As suggested by Tanksley (1993), QTL with major effects are more likely to be stable across different environments. Although more than half of the QTL were commonly detected in both trials, no common QTL for total phytosterol content was identified, suggesting that breeding for high phytosterol content through marker assisted selection would have to utilize environment-specific QTL. In the EU trial, two minor QTL for total phytosterol content were located on A07 (*DE-TPC.1*) and C08 (*DE-TPC.2*) with positive additive effects; *DE-TPC.1* appeared as independent QTL while *DE-TPC.2* was collocated with QTL for oil content with opposite additive effect. Since breeding for oil content has a higher priority, this means that only *DE-TPC.1* on A07 is more practical to be used for marker assisted selection. Whereas in the CN trial, QTL identified for total phytosterol content on C03 (*HZ-TPC.1*) was found collocated with QTL for oil content (*HZ-Oil.5*) with similar negative additive effect, indicating that allele derived from "Oase" increased both total phytosterol content and oil content. Compared with EU trial, this environment-specific QTL is more valuable to be used for marker assisted selection as pyramiding QTL that control traits of interest from different genomic regions into one background is a challenging and time consuming task in plant breeding.

For oil content, five of the seven QTL in CN trial showed that alleles increasing oil content were derived from the high oil parent "Oase" in contrast to the EU trial which showed that all alleles increasing oil content were derived from "Oase". Comparison of QTL across two trials identified two stable QTL, 5 environment-specific QTL in CN trial, and four environment-specific QTL in EU trial. As reported in many genetic studies, seed oil content is a complex quantitative trait which is strongly affected by environment and most of the QTL identified exhibit relatively minor effects (Zhao et al., 2006; Delourme et al., 2006; Qiu et al., 2006; Zou et al., 2010). QTL that are environment-specific could be utilized by the relevant breeding programs while stable QTL that have a broader use could be first fixed in breeding programs since they will make positive contribution to the phenotype in diverse environments. In this study, two stable QTL for oil content were identified

Table 4.5: QTL repeatedly identified on the same linkage groups in both locations (Europe and Hangzhou, East China)

Trait	LG	Europe (EU trial)			Hangzhou, East China (CN trial)		
		QTL name	Additive effect[b]	CI[a] (cM)	QTL name	Additive effect[b]	CI[a] (cM)
Brassicasterol	A03	DE-Bra.2	1.03	167–178	HZ-Bra.1	0.81	168–182
	A04	DE-Bra.3	−2.61	91–97	HZ-Bra.2	−2.55	90–97
	A07	DE-Bra.5	−0.77	100–131	HZ-Bra.4	−0.88	102–121
Campesterol	A04	DE-Camp.1	5.83	87–99	HZ-Camp.2	5.10	92–113
	A06	DE-Camp.2	−6.59	59–70	HZ-Camp.3	−6.14	60–73
	C08	DE-Camp.4	4.74	0–20	HZ-Camp.4	4.36	3–32
Sitosterol	A06	DE-Sito.2	6.06	92–99	HZ-Sito.1	4.57	52–71
	C05	DE-Sito.1	6.15	78–89	HZ-Sito.2	5.94	73–89
Avenasterol	C08	DE-Ave.1	2.59	1–20	HZ-Ave.1	0.85	2–29
24-methyl sterol	A06	DE-Methyl.1	−7.73	59–69	HZ-Methyl.2	−6.73	59–73
	C08	DE-Methyl.3	5.44	1–20	HZ-Methyl.4	5.22	12–29
24-ethyl sterol	A06	DE-Ethyl.1	5.92	91–100	HZ-Ethyl.1	7.27	89–102
Campesterol:	A04	DE-CSratio.2	3.16	85–98	HZ-CSratio.2	1.80	88–109
sitosterol	A06	DE-CSratio.3	−5.45	62–77	HZ-CSratio.3	−4.15	62–69
	C05	DE-CSratio.4	−3.82	77–86	HZ-CSratio.5	−2.97	81–90
	C08	DE-CSratio.5	2.03	2–20	HZ-CSratio.6	1.62	13–20
24-methyl:	A02	DE-MEratio.2	1.59	0–5	HZ-MEratio.1	1.66	0–5
24-ethyl sterol	A06	DE-MEratio.4	−5.05	61–66	HZ-MEratio.2	−3.66	61–67
	C05	DE-MEratio.5	−3.33	79–89	HZ-MEratio.5	−3.49	84–89
C16:0	A09	DE-16:0.2	−0.30	99–103	HZ-16:0.1	−0.18	90–103
	C09	DE-16:0.5	0.10	24–30	HZ-16:0.2	0.12	17–33
C18:1	A01	DE-18:1.1	−0.87	82–89	HZ-18:1.1	−0.55	55–67
	C08	DE-18:1.3	−0.60	0–15	HZ-18:1.3	−0.42	0–17
C18:2	A01	DE-18:2.1	0.53	72–77	HZ-18:2.1	0.44	79–91
C18:3	A01	DE-18:3.1	0.36	81–89	HZ-18:3.1	0.24	76–88
	C07	DE-18:3.5	−0.16	81–92	HZ-18:3.3	−0.15	79–90
Oil	A07	DE-Oil.3	−0.44	120–127	HZ-Oil.3	−0.23	119–129
	C03-II	DE-Oil.5	−0.37	34–66	HZ-Oil.6	−0.30	38–50
Protein of	A07	DE-Pro.1	−0.44	44–48	HZ-Pro.1	−0.36	120–131
defatted meal	C03-II	DE-Pro.2	−0.36	27–37	HZ-Pro.2	−0.33	30–42
	C03-II	DE-Pro.3	−0.46	89–97	HZ-Pro.3	−0.41	89–97
Seed weight	A07	DE-SW.2	−0.16	44–54	HZ-SW.1	−0.16	45–54

[a] 1-LOD Confidence interval

[b] Additive effect is the effect of substitution of one "Oase" allele by one "Sansibar" allele.

on A07 and C03-II; QTL on A07 was consistently collocated with QTL for brassicasterol while QTL on C03-II was repeatedly collocated with QTL for protein content of defatted meal. In addition, the similar direction of additive effects in both collocations of QTL are favourable in the aspect of breeding for quality traits. Although not much is known about the specific role of brassicasterol in plants and its relative effect on lowering LDL cholesterol compared to sitosterol and campesterol, clinical studies have shown that brassicasterol-rich phytosterol mixtures obtained from oilseed rape has similar cholesterol lowering properties to those phytosterols obtained from other sources like tall oil (Demonty et al., 2007; Heggen et al., 2010). On C03-II, collocation of QTL for oil content and protein content of defatted meal with similar direction of additive effects indicates that both compounds can be increased simultaneously. According to Si et al. (2003), a simultaneous increase of both oil content and protein content of defatted meal would occur at the expense of seed residue. As such, this QTL might possibly be related to reduce fiber content as has been demonstrated by Suprianto (2014).

For fatty acid composition, CN trial detected more QTL for C18:2 while EU trial detected more QTL for C16:0 and C18:3. At least 1 to 2 QTL for each of the fatty acid composition was/were stably identified across the two environments. Major QTL that were detected in EU trial only showed moderate effect in CN trial. For example, QTL for C16:0 on A09 which showed an effect of -0.30 in EU trial was detected in CN trial with -0.18 around the same location. However, a collocation of two major QTL for C18:1 and C18:3 in EU trial not only showed a lower effect in CN trial but were also slightly shifted in position. As such, these two QTL in CN trial were not coincided with *FAD2* gene as shown previously (Figure 3.6a & b) but were only located close to the *FAD2* gene. One linkage group that may be of practical importance in Chinese breeding program is A05 which contain QTL for C18:1, C18:2, and brassicasterol which were absent in EU trial.

For protein content of defatted meal, QTL detected on A07 in CN trial was shifted about 70 cM below from the QTL detected in EU trial while two other QTL detected on C03-II were around the same regions across two trials. Both QTL on C03-II showed similar direction of additive effect and were about 50 cM apart from each other. As mentioned before, collocation of QTL for oil content and protein content of defatted meal was observed on the upper region of C03-II across both trials. The additional QTL detected on the lower region of C03-II, however, showed close linkage with seed weight and brassicasterol in EU trial while no collocation with other QTL was detected in CN

trial. Nevertheless, the stability of these two QTL on C03-II across two diverse environments could be of interest for breeding programs aim at increasing protein content without affecting other seed quality traits.

Besides improving oil content, seed yield is also one of the major breeding goals in oilseed rape. Of the three components that determine seed yield (siliques per plant, seeds per silique and seed weight), seed weight has a relatively high heritability compared with other seed yield component traits (Shi et al., 2009). In CN trial, QTL for seed weight detected on A07 remained exactly at the same position like in EU trial except that collocation of QTL with other traits was not observed in CN trial. As mentioned in previous chapter, numerous studies have consistently detected QTL for seed weight on A07 in different populations with diverse genetic backgrounds but no candidate genes could be inferred for this QTL so far (Quijada et al., 2006; Udall et al., 2006; Shi et al., 2009; Basunanda et al., 2010; Cai et al., 2012). In addition, one environment-specific QTL was detected on C08 in CN trial and two environment-specific QTL were detected on A02 and C03-11 in EU trial. These QTL could be utilized by relevant breeding programs for marker assisted selection aim at improving seed weight or yield.

4.6 Conclusion

For all the 16 traits analyzed, genotypic effects were predominant in the SODH population that was cultivated in two years of replicated trials in Hangzhou, China. The stability of QTL across environments was determined by comparison of QTL identified at two mega environments, Europe and East China. Most traits contain QTL that were consistently detected across two trials except for total phytosterol content, indicating that environmental effect greatly influences the variation of total phytosterol content. In comparison, QTL for phytosterol composition were more consistently detected across both trials, suggesting a greater potential for modifying phytosterol composition in oilseed rape. Particularly, the major QTL for brassicasterol on A04 and the two major QTL for campesterol:sitosterol and 24-methyl:24-ethly sterol on A06 that were consistently detected across the two trials.

Chapter 5

Genetic variation and association mapping for phytosterol and oil content in canola quality winter oilseed rape (*Brassica napus* L.)

5.1 Abstract

Phytosterols are natural constituents of vegetable oils that are known for their cholesterol-lowering properties. Oilseed rape (*Brassica napus* L.) is ranked the second richest source of phytosterols among vegetable oils. Improving the phytosterol content or composition could give an added value to the seed quality of oilseed rape cultivars. The present study was carried out to dissect the genetic basis of seed phytosterol content and composition through association mapping in a collection of 81 canola quality winter oilseed rape. Other important seed quality traits such as oil content, protein content of defatted meal, fatty acids and a yield related trait, seed weight, were also included in the analysis to examine their relationship with phytosterols. Field trials were performed at six environments in Germany. The phenotypic variations in all of the traits were predominantly due to genotypic effects as demonstrated by high broad-sense heritabilities which ranged from 0.86 to 0.97. Association analysis was performed with 692 markers (685 AFLP and 7 SNP/InDels candidate gene-based markers) and phenotypic means of 6 environments using the K model, a MLM incorporating kinship matrix (*K*) as a random effect. By testing against a false discovery rate (FDR) of 0.20, between 1 and 11 markers were found significantly associated with five of the nine phytosterol traits, 11 markers were found significantly associated with oil content while no significant association was found for fatty acids, protein content of defatted meal and seed weight. With the consideration of linkage disequilibrium between pairs of significantly associated markers, these markers represented between one and seven QTL for five of the nine phytosterol traits and six QTL for oil content. The minimal phenotypic variance explained by the QTL ranged from 14% to 47%. For phytosterol traits, one QTL was identified for brassicasterol on A04, a collocation of QTL for campesterol, 24-methyl sterol and total phytosterol was observed on C09, while seven QTL for 24-methyl:24-ethyl sterol were distributed on another seven different linkage groups (A02, A05, A06, A07, C03, C05, C07). Significant marker associated with brassicasterol on A04 was shown to coincide with *CYP710A1* while significant marker associated for 24-methyl:24-ethyl sterol on A06 was shown to coincide with *SMT2*. QTL for oil content were distributed on five linkage groups (A01, A03, C01, C04 and C09). Comparison of associated markers between phytosterol traits and oil content revealed only one significant marker that was associated with campesterol, 24-methyl sterol, total phytosterol and oil content, suggesting that increasing both total phytosterol content

and oil content are possible in canola quality winter oilseed with the increase of total phytosterol content contributed by the increase of campesterol content.

5.2 Introduction

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is one of the most important oil crops in the temperate regions of the world. In 2012, the production of rapeseed oil amounted to 24.2 million tons, accounting for 18% of the world's vegetable oil supply (US Department of Agriculture). The oil is used for human consumption, biodiesel production, and industrial purposes while the meal is used in animal feeding. Canola quality of oilseed rape, containing zero erucic acid and low levels of glucosinolates, is well-regarded as prime edible oil since its development in the 1970s. It has a nearly ideal fatty-acid profile—that is, low in saturated fatty acids, high in monosaturated fatty acids and a good proportion of omega-3 and omega-6 polyunsaturated fatty acids. Other minor salutary oil constituents like carotenoids, phytosterols, and tocopherols are also present in appreciable amounts in canola oil.

Recently, an emerging trend in food industry has been the shift towards developing "functional" foods by deliberate fortification to make them more nutritious and healthier. One example is the incorporation of phytosterols to margarine and dairy products to reduce LDL cholesterol level. An effective dose of 1 to 3 g d^{-1} could lead to 8 to 15% reduction in LDL-cholesterol (Quilez et al., 2003). The major sources of phytosterols are derived from tall oil, a by-product of the Kraft process of wood pulp manufacture, and deodorizer distillate fraction from vegetable oil refining. While most crude vegetable oils contain about 1 to 5 g kg^{-1} of sterols, corn and rapeseed contain an exceptional high amount of sterols, that is, 8 to 16 g kg^{-1} for corn and 5 to 10 g kg^{-1} for rapeseed (Piironen et al., 2000). Hence, oilseed rape may serve as one of the valuable base stock for the health and nutrition industry.

In plants, phytosterols are known to play at least two essential roles: as bulk components of membrane regulating membrane fluidity and permeability and as presursors for growth-promoting brassinosteroids. Plants possess their own characteristic distribution of phytosterols in genetically defined proportions (Nes, 1977) as opposed to vertebrates and fungi which generally contain one

major sterol—cholesterol and ergosterol, respectively. The variety of phytosterols are structurally similar to cholesterol, differing by the presence of an extra methyl or ethyl group on the cholesterol side chain. For instance, at C-24, sitosterol has an ethyl group while campesterol has a methyl group.

In oilseed rape, the sterol profile consists mainly of sitosterol, campesterol, brassicasterol and avenasterol, while cholesterol and stigmasterol occur only in trace amounts (Appelqvist et al., 1981). Among the adapted winter rapeseed lines and varieties, Amar et al. (2009) found that the modern rapeseed cultivars contain already higher amount of total phytosterol than the genetically diverse winter rapeseed or resynthesized lines. This occurrence may be explained by the fact that total phytosterol is negatively correlated with erucic acid (Amar et al., 2008b), a compound that has long been eliminated in modern rapeseed cultivars. In a linkage mapping study by Amar et al. (2008b), two of the three QTL identified for total phytosterol content were also found within the confidence intervals of the two erucic acid genes in a winter rapeseed DH population segregating for erucic acid. Competition for cytoplasmic acetyl-CoA, an early essential precursor, to synthesize erucic acid and phytosterols further suggested that pleiotropy is in play between these two traits.

An alternative approach to linkage mapping in studying the genetic basis of complex traits is association mapping. Association mapping utilizes LD from historical recombination between QTL and marker alleles in a much broader germplasm context for finer resolution mapping. It harnesses the genetic diversity of natural populations, or in the case of crop plants, collections of varieties and breeding lines, thereby mitigating the need to construct new experimental population with the inherent limitations such as time consuming and low resolution mapping. In oilseed rape, association mapping has been performed to identify QTL for several seed quality traits (Zou et al., 2012; Li et al., 2014; Honsdorf et al., 2010), yield-related traits (Cai et al., 2013), phenological and morphological traits (Honsdorf et al., 2010), and antinutritive seed meal compounds (Snowdon et al., 2010). So far, association mapping for phytosterol content has not been reported.

In this study, a population of 81 canola quality winter rapeseed varieties and breeding lines were used for association mapping. The 81 genotypes were a subset of the 85 genotypes which have been characterized with 845 AFLP markers in a LD study by Ecke et al. (2010). The study reported an overall low level of LD with a mean r^2 of 0.027 and that high level of LD between linked markers

extended only for about 1-2 cM. Despite the limitations in population size and insufficient marker density for a comprehensive genome-wide association analysis, a number of QTL were identified for a range of traits (Honsdorf et al., 2010). Therefore, the present study aimed to analyze the genetic variation of phytosterol content in the 81 canola quality winter rapeseed varieties and to uncover novel QTL for phytosterols.

5.3 Materials and Methods

5.3.1 Plant material

The seed material consisted of 81 Northern European canola quality winter rapeseed varieties and breeding lines (Appendix A.9). The 81 genotypes were a subset of the 85 genotypes used in a LD study by Ecke et al. (2010) and also a subset of the 84 genotypes used in an association study by Honsdorf et al. (2010).

5.3.2 Field experiments

Field trials were performed at six environments: three environments during growing season 2007/2008—Reinshof, Thüle, and Hohenlieth—and three environments during growing season 2008/2009—Reinshof, Thüle, Rauischholzhausen. The field trials were carried out without replication. Seeds from ten open pollinated plants from each line were harvested and bulked for analyses.

5.3.3 Molecular markers

The 81 genotypes have previously been characterized with 845 AFLP markers by Ecke et al. (2010). Of these markers, a subset of 685 AFLP markers which have no absolute LD ($r^2 < 1.0$) between pairs were selected for association analysis. These AFLP markers have been mapped in a segregating double haploid population derived from the cross Express $\times$ R53. Details about genotypes, marker analysis and genetic map are described in Ecke et al. (2010). Additionally, the population was also

genotyped with seven SNP/InDels candidate gene-based markers developed by Ecke et al. (2007) (Table 5.1).

Table 5.1: Candidate gene loci for SNP/InDels markers. Source: Ecke et al. (2007)

Gene coding for	Locus
Biotin carboxyl carrier protein (BCCP2)	B62
Pyruvate dehydrogenase kinase E1a (PDHK)	P57
Pyruvate kinase (PK)	K141
Wrinkled (WRI)	WRI102
	WRI80
Pyruvate dehydrogenase (PDH)	H40
β-Ketoacyl-ACP-synthase III (KAS III)	S13

5.3.4 Phenotypic analysis

Phytosterol content

Analysis of phytosterol content was as described in Section 3.3.5.

Fatty acid composition

Oleic acid (C18:1), linolenic acid (C18:3) in seed were estimated by NIRS using the calibration raps2012.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel, Germany). Oleic acid (C18:1) and linolenic acid (C18:3) content are expressed as percentage (%) of the total fatty acid content.

Oil and protein content of defatted meal

Analysis of oil and protein content of defatted meal was as described in Section 3.3.5

Seed weight

Analysis of seed weight was as described in Section 3.3.5

5.3.5 Statistical analysis

Variance components, heritability, means, and phenotypic correlations were estimated by using PLABSTAT software version 3A (Utz, 2011). The model implemented in ANOVA anaylsis was :

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

where Y_{ij} is the trait value of genotype i in environment j, μ is the general mean, g_i is the effect of ith genotype, e_j is the effect of jth environment, and ge_{ij} is the interaction between ith genotype and jth environment. The genotype and environment were treated as random effect.

Broad sense heritability ($\hat{h}^2$) was estimated as follow:

$$\hat{h}^2 = \frac{\hat{\sigma}^2_G}{\hat{\sigma}^2_G + \frac{\hat{\sigma}^2_{GE}}{E}}$$

where $\hat{\sigma}^2_G$ and $\hat{\sigma}^2_{GE}$ are variance components for g and e; E refers to the number of environment. Mean values across all environments were used to calculate Spearman's rank correlation coefficients between traits.

5.3.6 Association analysis

Association analysis was performed with TASSEL standalone version 3.1 (Bradbury et al., 2007) for a total of 14 traits. Since population structure was not detected in this poulation (Ecke et al., 2010; Honsdorf et al., 2010), only two models were evaluated: (1) GLM and (2) K model, a mixed-linear model (MLM) incorporated with kinship matrix (K) as a random effect. The phenotypic data were the means obtained over six environments while the molecular marker data consisted of 685 AFLP and 7 SNP/InDels candidate gene-based markers. The relative kinship coefficient K_{ij} between inbreds i and j were estimated using R statistical software (R Core Team 2012) based on the total molecular marker data as follows:

$$K_{ij} = \frac{Q_{ij} - Q_m}{1 - Q_m}$$

where Q_{ij} is the probability of identity by state for random loci from i and j, and Q_m is the average probability of identity by state for loci from random individuals from the sample (Ritland, 1996). Negative kinship values between lines were set to 0.

The statistical significance of marker-trait associations was tested against the false-discovery rate (FDR) of 20%. Quantile–quantile (QQ) plots of the expected versus observed P values in the negative $\log_{10}$ scale were inspected for an indication of inflation by false positive associations.

In cases where multiple significant markers were found on the same linkage group, the LD between marker pairs were examined if they were indications of the same QTL. A group of significant markers on one linkage group were considered as a single QTL when the LD between pairs of these markers were significant at a threshold of $P \leq 1.4 \times 10^{-7}$ (derived by a Bonferroni correction from a global α-level of 0.05 over 356,590 possible marker pairs of 845 AFLP markers (Ecke et al., 2010)). Therefore, the marker which has the lowest P-value was selected to represent the indicative QTL. The phenotypic variance explained by all the QTL identified in each trait was estimated by multiple linear regression using R statistical software (R Core Team 2012).

5.3.7 Identification of possible candidate genes for the associated marker/QTL

To identify candidate genes for associated marker, reference sequence of *B. rapa* (BRAD v1.5; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* (Bolbase v1.0; `http://www.ocri-genomics.org/bolbase/`) genomes were utilized. Additionally mapped markers on Express $\times$ R53 genetic map (W. Ecke, personal communication) that are sequence informative such as DArT and SSR were used as reference points to inspect if the associated AFLP marker was collocated with the candidate gene. The physical position of each marker sequence was located by performing a BLAST search using CLC main workbench 6.0 program (CLC Bio, Aarhus, Denmark). The position of the best hit was recorded only when the marker sequence from a particular linkage group fell onto the same corresponding chromosome in *B. rapa* or *B. oleracea*.

5.4 Results

5.4.1 Phenotypic analysis

The phenotypic data for the 81 genotypes used in this study were obtained from six environments. Highly significant genotype and environment effects were found for all 14 traits (Table 5.2). Environmental effect was larger than genotype effect in avenasterol, campesterol:sitosterol, C18:3, oil, and protein of defatted meal whereas genotypic $\times$ environmental effect (G$\times$ E) was smaller than genotypic effect in all traits. Estimates of heritability was high for all traits, ranging from 0.86 to 0.97. Table 5.3 shows the means and ranges for all the 14 traits analyzed. Total phytosterol content ranged from 298.8 to 491.5 mg 100 g_{seed}^{-1}, with a mean of 388.7 mg 100 g_{seed}^{-1}. Sitosterol was the most prominent sterol, followed by campesterol, brassicasterol and avenasterol. The 24-ethyl sterol content, which include sitosterol and avenasterol, was higher than the 24-methyl sterol content, which comprised of campesterol and brassicasterol. The range of campesterol:sitosterol ratio was slightly larger than the 24-methyl:24-ethyl sterol ratio.

Highly significant positive correlations (P = 0.01) were observed between total phytosterol and the individual phytosterols except for brassicasterol (Table 5.4). Brassicasterol, a characteristic sterol of *Brassicaceae* family, appeared to have a different correlation trend than the rest of the individual phytosterols. It was found negatively correlated to campesterol, sitosterol, total sterol, 24-ethyl sterol, campesterol:sitosterol, and oil. While some phytosterol traits were found correlated to fatty acids (C18:1 and C18:3) and oil, no significant correlation was observed between phytosterol traits and protein of defatted meal. Less significant negative correlations were found between some phytosterol traits and seed weight.

Table 5.2: Variance components and heritability of the 81 canola quality winter oilseed rape cultivars

Trait	Variance components (σ^2)			Heritability
	Genotype (G)	Environment (E)	G×E	(h^2)
Phytosterols (mg 100 g_{seed}^{-1})				
Brassicasterol	103.79**	4.38**	16.07	0.97
Campesterol	514.74**	128.77**	118.44	0.96
Sitosterol	312.14**	299.60**	130.73	0.93
Avenasterol	49.81**	203.49**	44.37	0.87
Total phytosterol	1082.47**	400.26**	398.54	0.94
24-methyl sterol	329.62**	141.30**	110.46	0.95
24-ethyl sterol	381.59**	106.89**	132.26	0.95
Campesterol:sitosterol[a]	136.87**	162.80**	45.13	0.95
24-methyl:24-ethyl sterol[a]	73.96**	21.85**	17.65	0.96
Other traits				
C18:1 (%)	4.71**	3.40**	1.36	0.95
C18:3 (%)	0.56**	0.68**	0.25	0.93
Oil (%)	1.56**	5.84**	1.47	0.86
Protein of defatted meal (%)	1.93**	7.38**	1.78	0.87
Seed weight (g)	0.24**	0.06**	0.17	0.89

[a] original values (ratio) ×100

+, *, and ** denotes significance at $P < 0.10, 0.05$, and 0.01

Table 5.3: Descriptive statistic of the 81 canola quality winter oilseed rape cultivars

Trait	Min	Max	Mean	*F*-value	LSD 5%
Phytosterols (mg 100 g_{seed}^{-1})					
Brassicasterol	22.1	60.6	36.8	39.8**	4.6
Campesterol	76.3	200.7	145.4	27.1**	12.4
Sitosterol	139.9	230.0	178.1	15.3**	13.0
Avenasterol	14.3	53.3	28.4	7.7**	7.6
Total phytosterol	298.8	491.5	388.7	17.3**	22.7
24-methyl sterol	125.1	225.9	182.2	18.9**	11.9
24-ethyl sterol	167.3	265.6	206.4	18.3**	13.1
Campesterol:sitosterol[a]	51.6	117.0	82.9	19.2**	7.6
24-methyl:24-ethyl sterol[a]	65.6	108.7	88.7	26.2**	4.8
Other traits					
C18:1 (%)	56.6	71.1	62.6	21.7**	1.3
C18:3 (%)	9.9	14.0	11.9	14.8**	0.6
Oil (%)	39.1	46.8	43.9	7.4**	1.4
Protein of defatted meal (%)	28.5	35.8	31.3	7.5**	1.5
Seed weight (g)	4.5	7.2	5.3	9.3**	0.5

[a] original values (ratio) $\times 100$
** denotes significance at $P = 0.01$
LSD 5%: least significant difference at the level of $P < 0.05$

Table 5.4: Spearman's rank correlation of traits ($n = 81$)

	Brassicasterol	Campesterol	Sitosterol	Avenasterol	Total phytosterol	24-methyl sterol	24-ethyl sterol	24-methyl: 24-ethyl sterols	Campesterol: sitosterol	C18:1	C18:3	Oil	Protein of defatted meal
Campesterol	-0.61^{**}												
Sitosterol	-0.41^{**}	0.50^{**}											
Avenasterol	-0.09	0.25^{*}	0.04										
Total phytosterol	-0.32^{**}	0.81^{**}	0.74^{**}	0.41^{**}									
24-methyl sterol	-0.15	0.85^{**}	0.37^{**}	0.28^{*}	0.84^{**}								
24-ethyl sterol	-0.37^{**}	0.53^{**}	0.90^{**}	0.43^{**}	0.84^{**}	0.45^{**}							
Campesterol:sitosterol	-0.46^{**}	0.74^{**}	-0.13	0.20	0.37^{**}	0.66^{**}	-0.04						
24-methyl:24-ethyl sterol	0.08	0.35^{**}	-0.44^{**}	-0.16	0.04	0.50^{**}	-0.45^{**}	0.76^{**}					
C18:1	-0.07	-0.22^{*}	-0.16	0.09	-0.26^{*}	-0.29^{**}	-0.10	-0.09	-0.15				
C18:3	-0.09	0.32^{**}	0.22^{*}	-0.12	0.32^{**}	0.36^{**}	0.16	0.18	0.16	-0.68^{**}			
Oil	-0.27^{*}	0.36^{**}	0.15	0.11	0.29^{**}	0.32^{**}	0.19	0.31^{**}	0.19	0.17	0.03		
Protein of defatted meal	0.05	-0.03	-0.12	-0.12	-0.05	0.05	-0.16	0.06	0.18	-0.15	0.29^{**}	-0.29^{**}	
Seed weight	0.09	-0.25^{*}	-0.08	-0.28^{*}	-0.25^{*}	-0.26^{*}	-0.21	-0.19	0.03	-0.12	0.04	-0.22	0.10

* and ** denotes significance at $P < 0.05$ and 0.01

5.4.2 Association mapping

Association analysis was performed with 692 molecular markers and phenotypic means of 6 environments using two models: (1) GLM and (2) K model which incorporated kinship matrix (*K*) as a random effect. The average relative kinship between any two genotypes was 0.057, with 76% of the pairwise kinship estimates ranged between 0 to 0.05, indicating a low level of relatedness (Figure 5.1). Numbers of significant markers detected in both models at FDR of 20% are shown in Table 5.5. With GLM model, between 3 and 154 significant markers were found for 12 of the 14 traits. No significant marker was found associated with oleic acid content and seed weight. By taking kinship coefficient between individuals into account, the K model reduced the number of significant markers to between 1 and 11 for 6 of the 14 traits. The significant markers identified in K model were all detected in GLM. Under the assumption that the observed phenotype is not associated with the tested markers, the quantile-quantile plot should depict a uniform [0,1] distribution from the *P* values of the association tests. As shown in Figure 5.2, the distribution was strongly skewed towards significance for GLM as compared with K model, indicating that K model would be more suitable for association analysis.

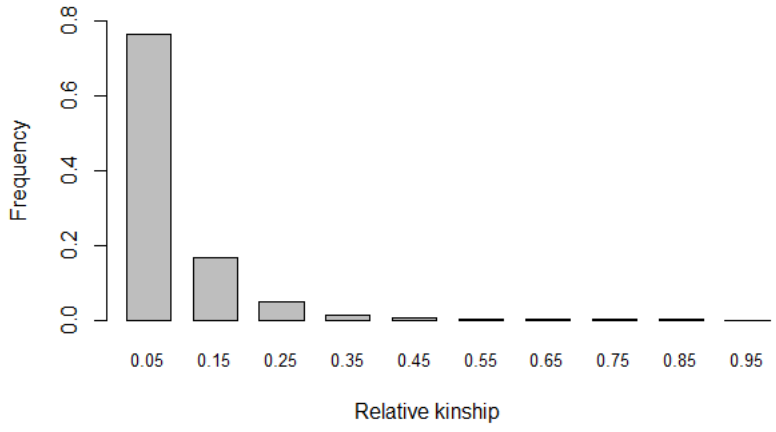

Figure 5.1: The distribution of pairwise relative kinship values between 81 canola quality winter rapeseed varieties and breeding lines.

Results of association analyses from K model are shown in detail in Table 5.6 and summarized in Table 5.7. The number of significant markers ranged from 1 (brassicasterol and total phytosterol) to 11 (24-methyl:24-ethyl sterol and oil content). The significant markers are distributed on 13 linkage

Table 5.5: Number of significant markers at FDR = 0.20

Trait	No. of significant markers	
	GLM model	K model
Brassicasterol	5	1
Campesterol	41	9
Sitosterol	12	-
Avenasterol	4	-
Total phytosterol	27	1
24-methyl sterol	52	6
24-ethyl sterol	5	-
Campesterol:sitosterol	71	-
24-methyl:24-ethyl sterol	154	11
C18:1	-	-
C18:3	3	-
Oil	26	11
Protein of defatted meal	5	-
Seed weight	-	-

groups: A01, A02, A03, A04, A05, A06, A07, C01, C03, C04, C05, C07 and C09. In cases where more than one marker was found on the same linkage group in a trait, significant LD between marker pairs ($P \leq 1.4 \times 10^{-7}$) were used as an indication that the marker pairs represent the same QTL. For instance, 9 markers associated with campesterol that were located between 5.2 to 26.4 cM on linkage group C09 were considered to represent the same QTL because the LD was significant between the marker pairs (Table 5.6). Among the 9 associated markers on C09, marker E36M50-184 which has the lowest *P*-value was selected to represent QTL AM-Camp.1.

In total, between one and seven QTL were revealed by the significant markers for the 6 respective traits (Table 5.7). More QTL were detected for 24-methyl:24-ethyl sterol ratio (seven QTL) and oil content (six QTL) as compared to one QTL detected for brassicasterol, campesterol, total phytosterol, and 24-methyl sterol contents. By using one representative marker per QTL in multiple linear regression analysis, the minimum phenotypic variance explained by the QTL ranged from 14% for total phytosterol content to 47% for 24-methyl:24-ethyl sterol (Table 5.7). From a total of 27 associated markers identified, 18 showed significant associations with one trait while 9 were found associated with more than one trait (Table 5.8). Markers that were associated with more than one trait were all located on C09 and the phenotypic effects of the shared markers were all positive for campesterol, 24-methyl sterol, total phytosterol, and oil content. Most notably, marker E45M53-229 was found associated with all the four traits.

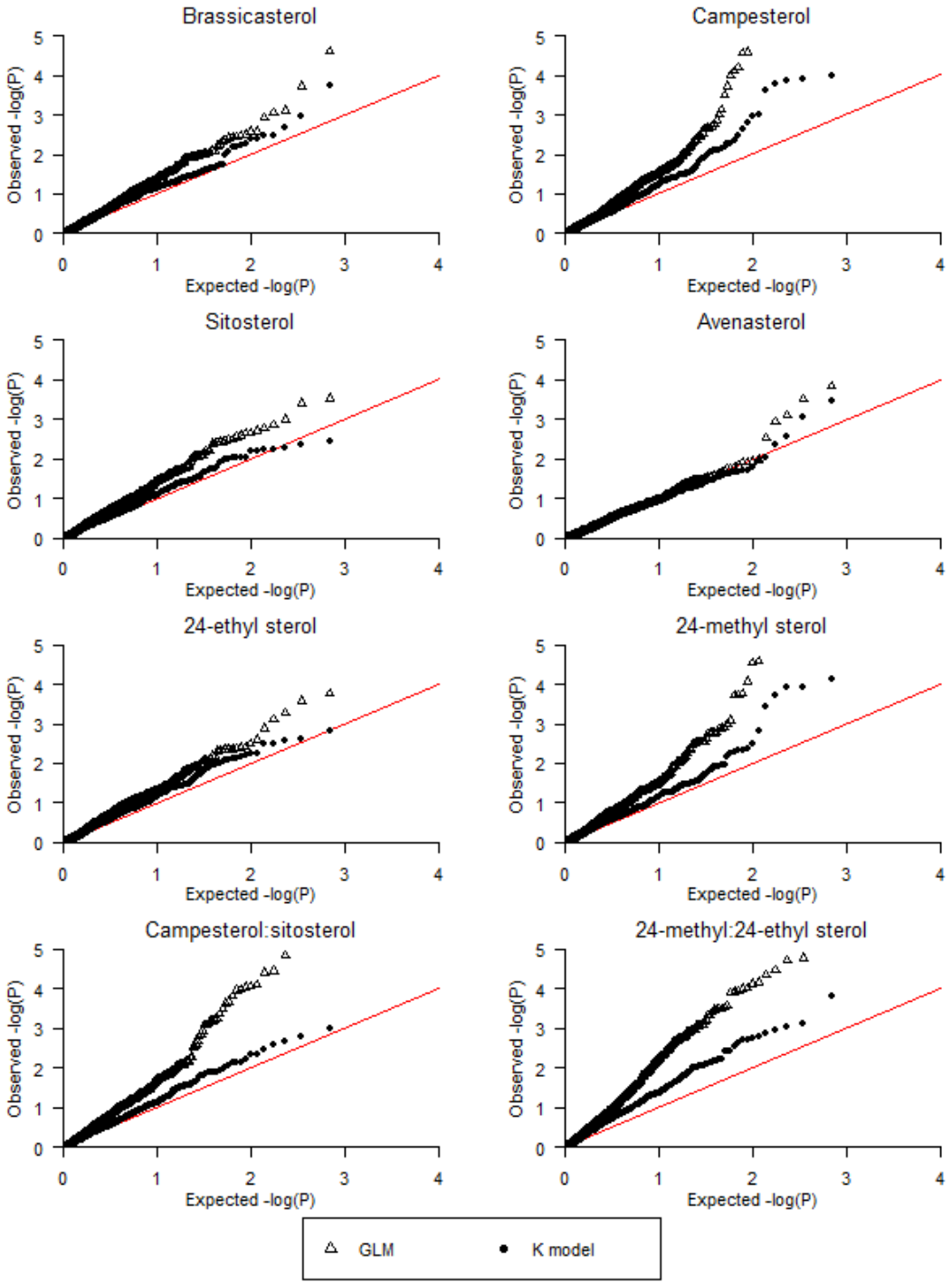

Figure 5.2: Quantile-quantile plots of both GLM and K model for all traits (*continued on next page*)

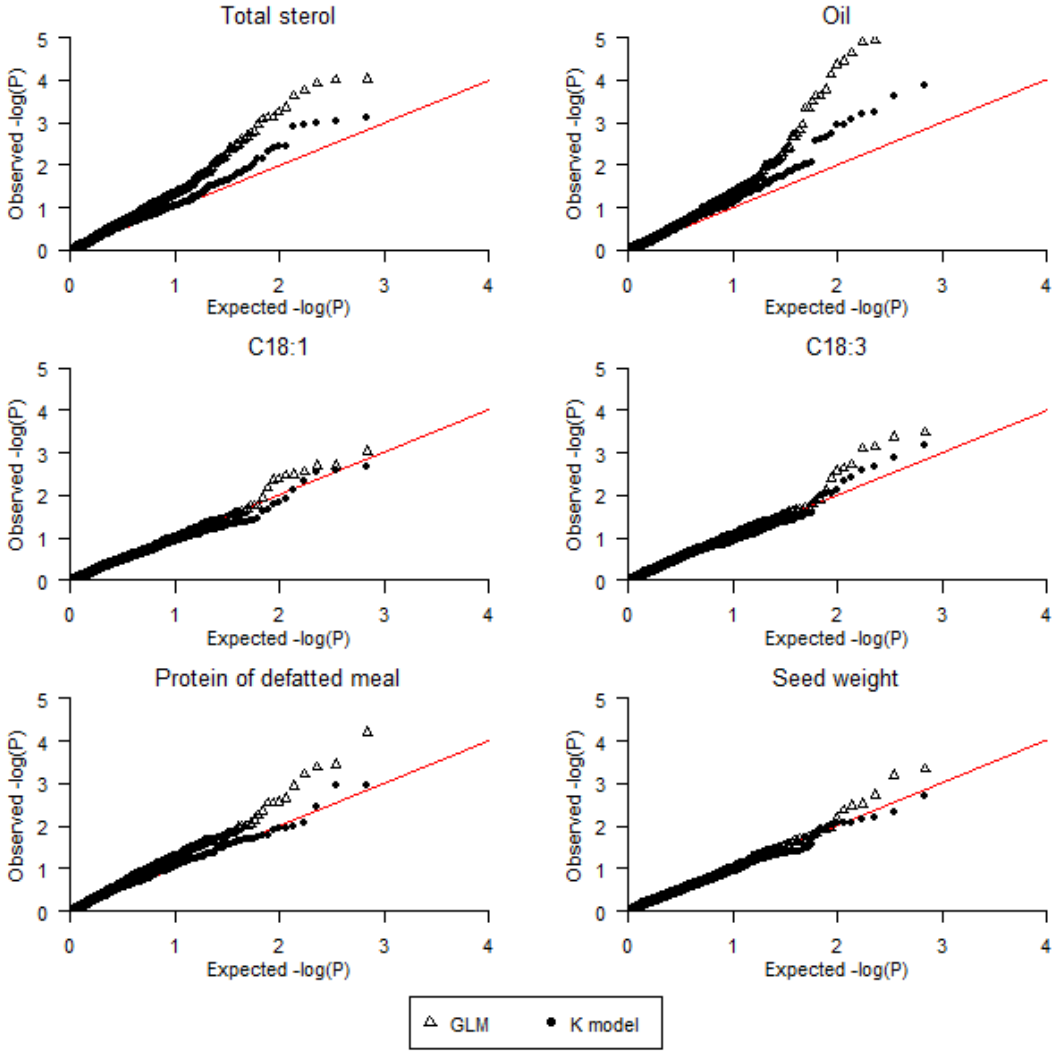

Figure 5.2: (*continued from previous page*) Quantile-quantile plots of both GLM and K model for all traits

Table 5.6: Results of association analysis with K model

LG[a]	Marker no.[b]	Marker	Pos.[c] (cM)	PE[d]	*P*-value	R^2	Multi regr.[e]	QTL	Linkage disequilibrium (r^2)[f] Marker. no 1	2	3	4	5	6	7	8
Brassicasterol (mg 100 g_{seed}^{-1})																
A04	1	E40M51-198	21.0	−9.30	2.41e−5	0.20	x	AM-Bra.1								
Campesterol (mg 100 g_{seed}^{-1})																
C09	1	E33M47-288	5.2	35.56	2.49e−4	0.18		AM-Camp.1	-							
C09	2	E36M51-141	9.2	35.41	1.68e−4	0.20		AM-Camp.1	**0.90**	-						
C09	3	E45M53-229	10.6	37.39	1.24e−4	0.20		AM-Camp.1	**0.81**	**0.90**	-					
C09	4	E36M50-184	10.6	35.83	1.05e−4	0.21	x	AM-Camp.1	**0.72**	**0.80**	**0.90**	-				
C09	5	E33M59-100	14.2	34.22	1.33e−4	0.20		AM-Camp.1	**0.82**	**0.90**	**0.81**	**0.90**	-			
C09	6	E32M49-403	19.5	26.88	2.26e−3	0.12		AM-Camp.1	**0.53**	**0.60**	**0.68**	**0.60**	**0.53**	-		
C09	7	E38M49-127	22.4	28.95	1.63e−3	0.13		AM-Camp.1	0.45	**0.51**	**0.57**	**0.66**	**0.59**	**0.75**	-	
C09	8	E32M60-396	25.2	35.88	9.92e−4	0.15		AM-Camp.1	0.34	0.39	0.44	**0.53**	0.48	**0.52**	**0.74**	-
C09	9	E34M53-139	26.4	37.74	1.14e−3	0.14		AM-Camp.1	0.40	0.44	0.50	0.44	0.40	**0.60**	**0.66**	**0.89**
Total sterol (mg 100 g_{seed}^{-1})																
C09	1	E45M53-229	10.6	44.44	1.29e−3	0.14	x	AM-Total.1								
24-methyl sterol (mg 100 g_{seed}^{-1})																
C09	1	E33M47-288	5.2	28.89	1.26e−4	0.20		AM-Methyl.1	-							
C09	2	E36M51-141	9.2	28.81	7.85e−5	0.22	x	AM-Methyl.1	**0.90**	-						
C09	3	E45M53-229	10.6	30.53	1.22e−4	0.20		AM-Methyl.1	**0.81**	**0.90**	-					
C09	4	E36M50-184	10.6	26.86	3.57e−4	0.17		AM-Methyl.1	**0.72**	**0.80**	**0.90**	-				
C09	5	E33M59-100	14.2	25.63	1.98e−4	0.19		AM-Methyl.1	**0.82**	**0.90**	**0.81**	**0.90**	-			
C09	6	E32M49-403	19.5	22.71	1.47e−3	0.14		AM-Methyl.1	**0.53**	**0.60**	**0.68**	**0.60**	**0.53**			
24-methyl:24-ethyl sterol																
A02	1	E32M51-122	161.7	7.63	1.07e−3	0.14	x	AM-MEratio.1								
A05	1	E42M55-165	90.1	−8.98	1.96e−3	0.13	x	AM-MEratio.2								

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Table 5.6 : *(Continued from previous page)* Results of association analysis with K model

LG[a]	Marker no.[b]	Marker	Pos.[c] (cM)	PE[d]	*P*-value	R^2	Multi regr.[e]	QTL	Linkage disequilibrium (r^2)[f] Marker. no 1	2	3	4	5	6	7	8
A06	1	E37M62-236	56.4	10.72	1.60e−3	0.13		AM-MEratio.3	-							
A06	2	E32M59-334	56.9	11.52	1.52e−4	0.20	x	AM-MEratio.3	**0.71**	-						
A06	3	E32M49-205	56.9	−11.28	1.34e−3	0.14		AM-MEratio.3	**0.68**	0.45						
A06	4	E39M49-368	56.9	−11.27	1.98e−3	0.13		AM-MEratio.3	**0.75**	**0.51**	**0.90**	-				
A06	5	E33M51-174	63.1	10.16	3.92e−3	0.11		AM-MEratio.3	**0.55**	**0.46**	**0.53**	0.46				
A07	1	E40M50-127	93.0	−8.22	2.98e−3	0.12	x	AM-MEratio.4								
C03	2	E36M56-172	15.0	−5.92	1.84e−3	0.13	x	AM-MEratio.5								
C05	1	E36M57-089	50.8	−7.38	2.29e−3	0.12	x	AM-MEratio.6								
C07	2	E39M62-176	50.6	7.19	3.72e−3	0.11	x	AM-MEratio.7								
Oil (%)																
A01	1	E39M62-071	74.0	1.72	1.86e−3	0.13	x	AM-Oil.1								
A03	1	E38M59-213	61.9	1.72	2.87e−3	0.12	x	AM-Oil.2	-							
A03	2	E38M51-252	100.4	−1.02	1.13e−3	0.14	x	AM-Oil.3	0.05							
C01	1	E36M51-355	106.7	0.92	2.53e−3	0.12	x	AM-Oil.4								
C04	1	E32M47-182	35.7	−1.08	8.40e−4	0.15	x	AM-Oil.5								
C09	1	E45M53-229	10.6	1.86	1.12e−3	0.14		AM-Oil.6	-							
C09	2	E36M50-184	10.6	2.05	1.31e−4	0.20	x	AM-Oil.6	**0.90**	-						
C09	3	E38M49-127	22.4	1.70	5.93e−4	0.16		AM-Oil.6	**0.57**	**0.66**	-					
C09	4	E32M60-396	25.2	2.10	2.41e−4	0.18		AM-Oil.6	0.44	**0.53**	**0.74**	-				
C09	5	E34M53-139	26.4	1.91	2.20e−3	0.13		AM-Oil.6	0.50	0.44	**0.66**	**0.89**	-			
C09	6	E39M55-408	31.3	1.97	6.66e−4	0.16		AM-Oil.6	0.44	**0.53**	**0.74**	**0.79**	**0.68**			

[a] Linkage group;

[b] Marker number on each linkage group

[c] Position of the marker on the respective linkage group;

[d] Phenotypic effect (See Table 5.3 for units of measurements); positive sign: visible marker allele increases trait; negative sign: visible marker allele decreases trait;

[e] Markers selected (marked with 'x')to represent the QTL in muliple regression analysis;

[f] Linkage disequilibrium between marker pairs on the same linkage group. Significant r^2 values are indicated in bold

Table 5.7: Summary of association analysis

Trait	K model				Multiple regression	
	No. of markers[a]	No. of LG[b]	Phenotypic effect[c] Min	Max	No. of QTL[d]	Adj. R^2
Phytosterols (mg 100 g_{seed}^{-1})						
Brassicasterol	1	1	9.30	9.30	1	0.19
Campesterol	9	1	26.88	37.74	1	0.21
Sitosterol	-	-	-	-	-	-
Avenasterol	-	-	-	-	-	-
Total sterol	1	1	44.44	44.44	1	0.14
24-methyl sterol	6	1	22.71	30.53	1	0.22
24-ethyl sterol	-	-	-	-	-	-
Campesterol:sitosterol[e]	-	-	-	-	-	-
24-methyl:24-ethyl sterol[e]	11	7	5.92	11.52	7	0.47
Other traits						
C18:1 (%)	-	-	-	-	-	-
C18:3 (%)	-	-	-	-	-	-
Oil (%)	11	5	0.92	2.10	6	0.39
Protein of defatted meal (%)	-	-	-	-	-	-
Seed weight (g)	-	-	-	-	-	-

[a] Number of significantly associated markers

[b] Number of linkage groups with significant markers

[c] Absolute value of minimal and maximal phenotypic effect of significant markers

[d] Number of representative markers used to determine the minimal phenotypic variance explained by the QTL

[e] original value (ratio) $\times$ 100

Table 5.8: The phenotypic effect of the associated markers detected in 6 traits.

LG[a]	Markers	Pos.[c]	Traits											
			Brassica-sterol		Campe-sterol		Total sterol		24-methyl sterol		24-methyl:24-ethyl sterol		Oil	
			PE[c]	QTL	PE	QTL	PE	QTL	PE	QTL	PE	QTL	PE	QTL
A01	E39M62-071	74.00											1.72	AM-Oil.1
A02	E32M51-122	161.70									7.63	AM-MEratio.1		
A03	E38M59-213	61.90											1.72	AM-Oil.2
	E38M51-252	100.40											−1.02	AM-Oil.3
A04	E40M51-198	21.05	−9.30	AM-Bra.1										
A05	E42M55-165	90.10									−8.98	AM-MEratio.2		
A06	E37M62-236	56.40									10.72	AM-MEratio.3		
	E32M49-205	56.90									−11.28	AM-MEratio.3		
	E32M59-334	56.90									11.52	AM-MEratio.3		
	E39M49-368	56.90									−11.27	AM-MEratio.3		
	E33M51-174	63.10									10.16	AM-MEratio.3		
A07	E40M50-127	93.00									−8.22	AM-MEratio.4		
C01	E36M51-355	106.7											0.92	AM-Oil.4
C03	E36M56-172	15.00									−5.92	AM-MEratio.5		
C04	E32M47-182	35.7											−1.08	AM-Oil.5
C05	E36M57-089	50.80									−7.38	AM-MEratio.6		
C07	E39M62-176	50.60									7.19	AM-MEratio.7		
C09	E33M47-288	5.20			35.56	AM-Camp.1			28.89	AM-Methyl.1				
	E36M51-141	9.20			35.41	AM-Camp.1			28.81	AM-Methyl.1				
	E36M50-184	10.60			35.83	AM-Camp.1			26.86	AM-Methyl.1			2.05	AM-Oil.6
	E45M53-229	10.60			37.39	AM-Camp.1	44.44	AM-Total.1	30.53	AM-Methyl.1			1.86	AM-Oil.6
	E33M59-100	14.20			34.22	AM-Camp.1			25.63	AM-Methyl.1				
	E32M49-403	19.50			26.88	AM-Camp.1			22.71	AM-Methyl.1				
	E38M49-127	22.40			28.95	AM-Camp.1							1.70	AM-Oil.6

Continued on next page

Table 5.8 –*Continued from previous page*

LG[a]	Markers	Pos.[b]	Traits											
			Brassica-sterol		Campe-sterol		Total sterol		24-methyl sterol		24-methyl:24-ethyl sterol		Oil	
			PE[b]	QTL	PE	QTL	PE	QTL	PE	QTL	PE	QTL	PE	QTL
	E32M60-396	25.20			35.88	AM-Camp.1							2.10	AM-Oil.6
	E34M53-139	26.40			37.74	AM-Camp.1							1.91	AM-Oil.6
	E39M55-408	31.30											1.97	AM-Oil.6

[a] LG: linkage group

[b] Marker position on the respective linkage group

[c] PE Phenotypic effect. Positive sign: visible marker allele increases trait; negative sign: visible marker allele decreases trait

5.4.3 Identification of possible candidate genes for the associated marker/QTL

Investigations were performed to search for candidate genes that may underlie QTL for phytosterol traits. Number of homologous copies and their chromosomal locations were obtained based on sequence similarity search using *A. thaliana* gene against the reference sequence of *B. rapa* (BRAD v1.5; `http://www.brassicadb.org/brad/`)(Wang et al., 2011b) and *B. oleracea* (Bolbase v1.0; `http://www.ocri-genomics.org/bolbase/`) genomes. Since AFLP markers used in this association analysis are not sequence informative, additionally mapped markers on Express $\times$ R53 genetic map (W. Ecke, personal communication) that are sequence informative such as DArT and SSR were used as reference points to inspect if the associated marker was collocated with the candidate gene.

For brassicasterol, the predicted gene is *CYP710A1* which encode cytochrome P450 enzyme that catalyzes the C-22 desaturation reaction, converting both 24-epi-campesterol and sitosterol to brassicasterol and stigmasterol, respectively (Morikawa et al., 2006). BLASTN search against the *B. rapa* genome indicated two homologous copies of *CYP710A1* on A04 which coincided with the associated marker for brassicasterol on A04 (Figure 5.4). Marker orders on A04 exhibit good collinearity in the alignment between Express $\times$ R53 genetic map and *B. rapa* genome. Associated marker (E40M51-194) located at 21 cM on the genetic map was flanked by markers that were also flanking the two copies of the predicted gene *CYP710A1* (Bra021916 & Bra021971) in *B. rapa* physical map. The closest flanking markers were just $\sim$300 kbp from the two copies of the predicted gene and 0.1 cM away from the associated marker.

The predicted gene for 24-methyl:24-ethyl sterol are SMT2 and SMT3 which can each catalyse the second methylation reaction, converting 24-methylene lophenol to 24-ethylidene lophenol. Based on BLASTN search in *B. rapa* and *B. oleracea* genomes, two homologous copies of *SMT2* are located on A06 and C05 while two homologous copies of *SMT3* are located on A07 and C05. Three of the seven QTL identified for 24-methyl:24-ethyl sterol were located on A06, A07 and C05. On A06 (Figure 5.3), the SSR/DArT markers spanning from 56.4 to 57.6 cM in the genetic map corresponded to a large physical distance of approximately 5 Mbp in *B. rapa* genome, suggesting a region of suppressed recombination from 5 - 15 Mbp (as shaded in grey in the figure). Both associated marker (E32M59-334) and the *SMT2* gene (Bra025810) were in the suppressed

recombination region which implies that *SMT2* may possibly underlie QTL for 24-methyl:24-ethyl sterol (*AM-MEratio3*). As for QTL on A07 and C05, no correspondence between associated marker and *SMT3* was found on A07 while *SMT2* and *SMT3* on C05 could not be ascertained to be the underlying genes for QTL on C05 due to the disruption of marker order around the region (results not shown).

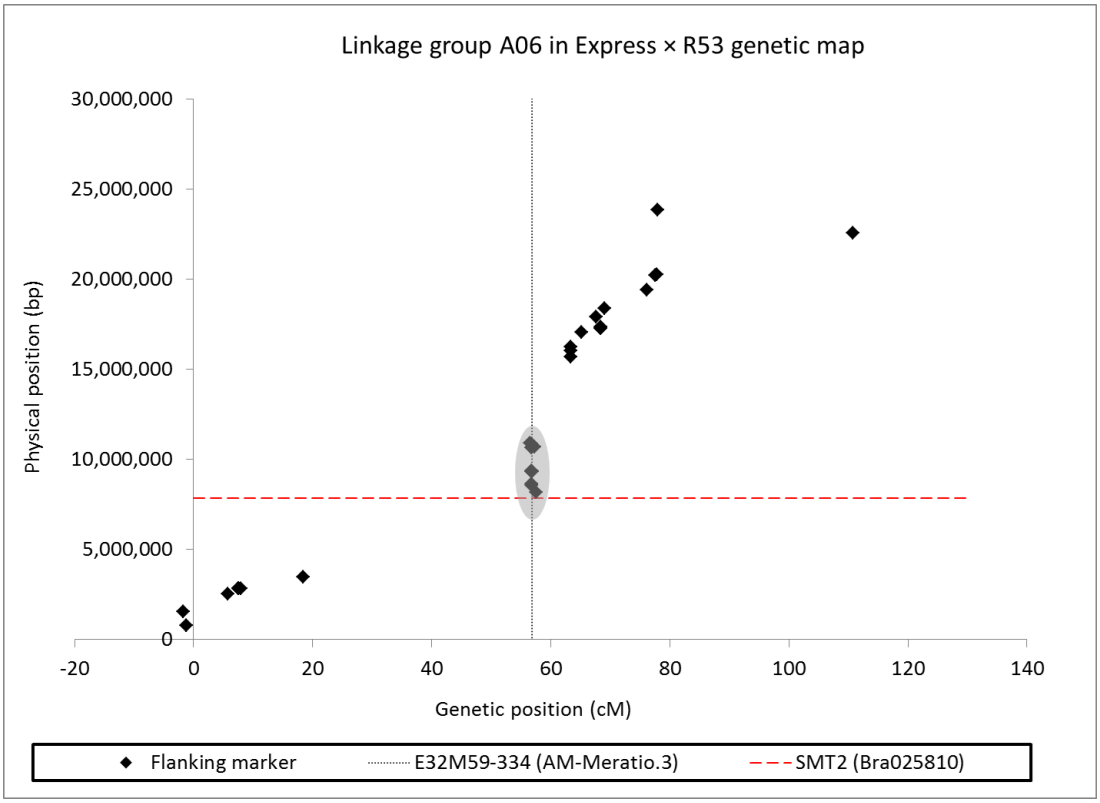

Figure 5.3: Alignment of physical positions (bp) of chromosome A06 in *B. rapa* genome on genetic positions (cM) of linkage group A06 in *B. napus* Express × R53 genetic map. Flanking markers consisted of either SSR or DArT markers are indicated by the diamond symbol. Marker E32M59-334 which represents QTL AM-MEratio.3 located at 56.9 cM on linkage group A06 in *B. napus* is indicated by the dotted line. *SMT2* (Bra025810), located at 7,861,887 bp on chromosome A06 of *B. rapa* is indicated by the dashed line. Region of suppressed recombination is indicated by shaded grey, suggesting that associated marker may be linked to the *SMT2* gene (Bra025810).

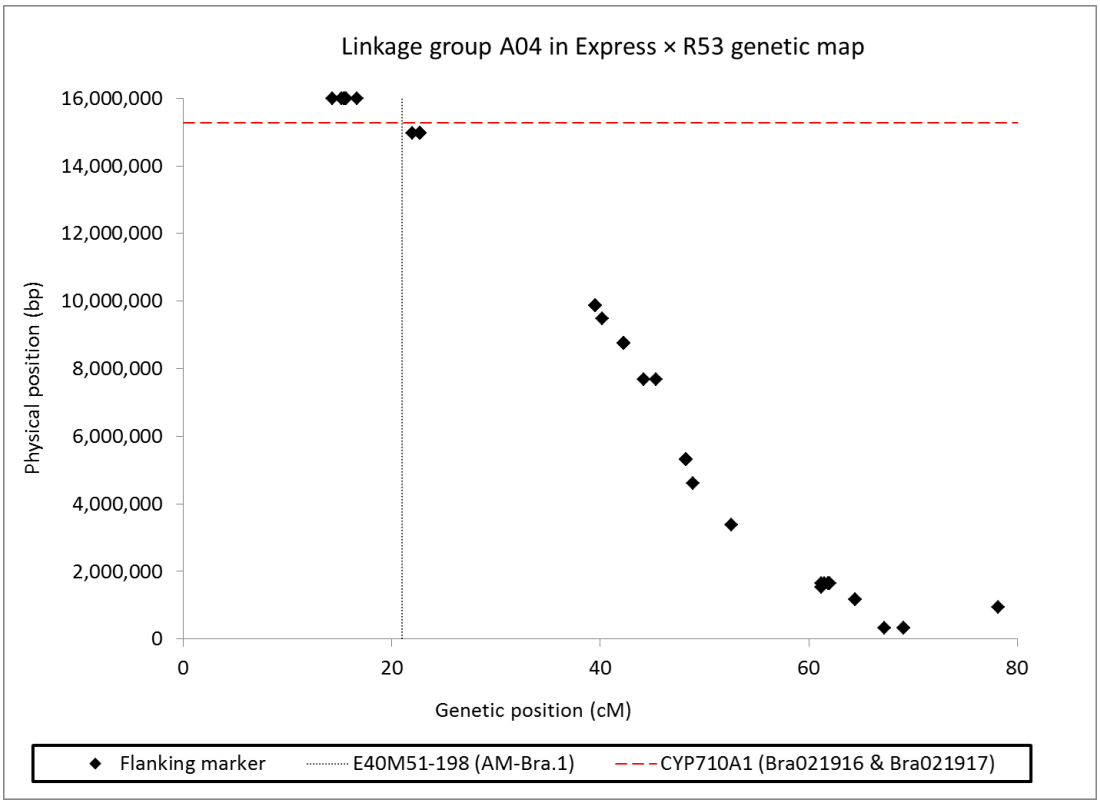

Figure 5.4: Alignment of physical positions (bp) of chromosome A04 in *B. rapa* genome on genetic positions (cM) of linkage group A07 in *B. napus* Express × R53 genetic map. Flanking markers consisted of either SSR or DArT markers are indicated by the diamond symbol. Marker E40M51-198 which represents QTL AM-Bra.1 located at 21.0 cM on linkage group A04 in *B. napus* is indicated by the dotted line. *CYP710A1* (Bra021916 & Bra021971) located at 15,280,057 bp and 15,282,271 on chromosome A04 of *B. rapa* is indicated by the dashed line.

5.5 Discussion

5.5.1 Phenotypic analysis

The 81 canola quality winter oilseed rape varieties and breeding lines revealed a relatively large variation in phytosterol content. Total phytosterol content ranged from 298.8 to 491.5 mg 100 g_{seed}^{-1} was larger than the range reported using 27 modern rapeseed cultivars, varying from 356.6 to 480.0 mg 100 g_{seed}^{-1} (Amar et al., 2009). Oil content ranging from 39.1 to 46.8% was as expected for commercial cultivars which usually contain about 40-50% oil content. Positive correlation between oil content and total phytosterol content was consistent with the results from SODH population (Tables 3.5 and 4.3) but not in agreement with the study from Amar et al. (2008a, 2009) who reported conflicting correlations between the two traits with different populations. Phenotypic analysis showed significant genotypic variation and high heritablity for all the traits, suggesting that this population is suitable for identifying marker-trait associations.

5.5.2 Association mapping

Association mapping is a useful tool for identifying marker-trait associations in existing cultivars and breeding lines. The population's genetic diversity, extent of genome-wide LD, and relatedness determine the mapping resolution, marker density, statistical methods, and mapping power (Zhu et al., 2008). The present 81 genotypes used in this study were a subset of the 85 genotypes that have been characterized with 845 AFLP markers in a LD study by (Ecke et al., 2010). The population has a very low level of LD (r^2 = 0.027) and that high levels of LD between linked markers extended for about 1-2 cM only. Although the rapid LD decayed implies that association mapping approach could give a higher mapping resolution as compared to linkage mapping in segregating populations, it also indicates that the marker density is insufficient and the mapping power is low. As estimated by Ecke et al. (2010), several thousand markers are required to saturate the rapeseed genome for a comprehensive genome-wide association analysis. With only 692 markers tested in this study, the number of QTL detected would be strongly influenced by chance for traits with a simple genetic architecture, while a certain fraction of the QTL would be detected

for traits with a complex genetic architecture. Another factor that influences the mapping power is sample size. At intermediate allele frequencies, the power to detect QTL is similar for both linkage and association analyses. But allele frequencies are highly variable in association mapping design, the number of samples needed to map QTL increases as allele frequencies depart from 0.5 (Mackay et al., 2009). Clearly, the sample size of 81 genotypes was rather small for association mapping. With low detection power due to insufficient marker density and small sample size, the numbers of QTL detected here have to be considered as a minimum number of QTL segregating in the 81 genotypes tested.

One main consideration in association mapping is confounding by population structure and cryptic relatedness which may cause spurious correlations, leading to an elevated false-positive rate. While population structure generally describes remote common ancestry of large groups of individuals, cryptic relatedness refers to recent common ancestry among smaller groups of individuals (Astle and Balding, 2009). Depending on the degrees of population structure and relatedness, the confounding effects can be corrected by statistical approaches like genomic control (Devlin and Roeder, 1999), structured association (Pritchard et al., 2000b), principal components (Price et al., 2006), and mixed-model (Yu et al., 2005). Because no clear stratification was detected in this population (Ecke et al., 2010), a mixed-model which include pairwise relatedness as random effect (termed K model in this study) was used to investigate the performance of reducing false positive rate in comparison to a naive GLM which does not account for population structure. The key to mixed-model approach is by using a random effect to estimate the fraction of the phenotypic variation that can be explained by genome-wide correlations or kinship. Estimation of relative kinship based on marker data have proven useful for quantitative inheritance studies in different populations (Loiselle et al., 1995; Lynch and Ritland, 1999). The *K* estimate approximates identity by descent, by adjusting the probability of identity by state between two individuals with the average probability of identity by state between random individuals (Yu et al., 2008). As depicted in Figure 5.2 and table 5.5, the K model performed better than the GLM in reducing false-positive rate, indicating that kinship can cause confounding effects on associations in the 81 genotypes. The large number of significant markers under the GLM approach were most likely due to spurious correlations rather than true associations. This argument relies partly on a priori notions about the genetic architecture of the trait, and partly on the limitations of the study as pointed out earlier.

With K model, significant associations were only detected for five phytosterol traits and oil content. Among the five phytosterol traits, the highest number of QTL was identified for 24-methyl:24-ethyl sterol, indicating that high allelic variation for modulating the phytosterol compositoion is present in current varieties and breeding materials. The QTL which explained the largest phenotypic variation for 24-methyl:24-ethyl sterol was located on A06 and was shown to coincide with the *SMT2* gene. The SMT2 is responsible for the second methylation reaction which converts 24-methylene lophenol to 24-ethylidene lophenol. In addition to SMT2, there exists another isoform, SMT3, which has a similar function like SMT2 (Husselstein et al., 1998; Bouvier-Navé et al., 1997; Schaller et al., 1998; Nes and Venkatramesh, 1999). Based on BLASTN search in *B. rapa* and *B. oleracea* genomes, two homologous copies of *SMT2* are located on A06 and C05 while two homologous copies of *SMT3* are located on A07 and C05. Although QTL were identified for 24-methyl:24-ethyl sterol on A07 and C05, no correspondence between QTL and *SMT3* was found on A07 while *SMT2* and *SMT3* on C05 could not be ascertained to be the underlying genes for the associated marker on C05 due to disruption of marker orders around the region. There are several reasons that more than four QTL were identified for 24-methyl:24-ethyl sterol even though four copies were expected in *B. napus* genome from sequence similarity search. A central finding from all the sterol mutant studies is that sterol balance cannot be predicted based on a simple linear pathway. SMT transcription may be regulated through a sterol feedback mechanism (Diener et al., 2000; Carland et al., 2002, 2010). Additionally, there exists some promiscuity in substrate specificity among the SMTs (Diener et al., 2000). For instance, even with the biosynthetic block at the initial step of methyl addition at C-24 (C_1 addition) in *smt1* mutant, methylated product is still present at equivalent or increased levels relative to wild type, suggesting that SMT2 and SMT3 can promote both C_1 and C_2 additions (Carland et al., 2010). In the case of mutants with complete deficiency of C-24 ethylidene SMT function, residual levels of downstream end product sitosterol were still detected (Carland et al., 2010). These findings suggest that alternative, not usually active, pathways are up-regulated in sterol mutant backgrounds. As found in a separate study, the lanosterol pathway, commonly found in yeast and mammals (Baker et al., 1995; Corey et al., 1996), exists in *Arabidopsis* as a minor branch and utilizes lanosterol rather than cycloartenol in its initial step and contribute a small fraction (1.5%) of total sitosterol (Ohyama et al., 2009). Taken together, the highly regulated sterol pathway and the redundancy among enzymes and pathways are in agreement with the fact that sterol biosynthetic pathway is more complex than previously thought

(Schrick et al., 2002), suggesting that 24-methyl:24-ethyl sterol could be a complex trait as well.

For brassicasterol, the associated marker was shown to coincide with *CYP710A1* which is responsible for converting both 24-epi-campesterol and sitosterol to brassicasterol and stigmasterol, respectively (Figure 5.4). The flanking markers that were close to the associated markers were 0.1 cM apart from the associated marker on genetic map and ∼300 kbp from the two copies of the predicted gene on *B. rapa* physical map, strongly supporting the hypothesis that *CYP710A1* is responsible for controlling the variation of brassicasterol. Moreover, the close linkage between the associated marker and *CYP710A1* gene was also reflected by its lowest *P*-value among all the identified associated markers for different traits.

In *B. napus*, association mapping has been used to dissect the genetic architecture of oil content and fatty acid composition (Honsdorf et al., 2010; Zou et al., 2010). With GLM and phenotypic means obtained from seven environments, Honsdorf et al. (2010) identified 22 QTL for seed oil content located on 14 different linkage groups and one QTL for oleic acid in a similar population as in the present study. Of the 11 markers associated with oil content in this study, 10 have previously been identified by Honsdorf et al. (2010) with E38M59-213 on linkage group A02 being the newly found associated marker. This discrepancy is most likely due to the different models and phenotypic means used in the association analysis. With the inclusion of phytosterol traits in this study, it was found that the associated markers for oil content on C09 were positively correlated with campesterol, 24-methyl sterol, and total phytosterol contents, suggesting that increasing both total phytosterol content and oil content are possible in canola quality winter oilseed rape with the increase of total phytosterol content contributed by the increase of campesterol content.

5.6 Conclusion

The 81 canola quality winter oilseed rape cultivars showed large variations and high broad-sense heritabilites for phytosterol content and composition, fatty acid composition, oil content, protein content of defatted meal and seed weight. In spite of that, association analysis performed with K model identified between one and seven QTL for five phytosterol traits and six QTL for oil content only. The small number of QTL identified could be due to the small sample size and insufficient

genome coverage in this study. With regards to phytosterol, there seems to be a greater potential in modulating phytosterol composition than enhancing phytosterol content as seven QTL were identified for 24-methyl:24-ethyl sterol compared with one QTL identified for total phytosterol content. The marker associated with total phytosterol content was similarly associated with campesterol, 24-methyl sterol, and oil content, suggesting that increasing both total phytosterol content and oil content are possible in canola quality winter oilseed rape with the increase of total phytosterol content contributed by the increase of campesterol content.

Chapter 6

General discussion

Phytosterols are natural constituents of vegetable oils with serum cholesterol lowering properties (Best et al., 1954). Enhancing phytosterol content in oilseed rape could further increase its nutritive value to human health. Due to the close negative correlation between erucic acid and phytosterol content, oilseed rape with canola quality inherently contain higher phytosterol content than the non-canola quality rapeseed (Amar et al., 2008a,b, 2009). This means that enhancing phytosterol content in oilseed rape would have to utilize the genetic diversity in oilseed rape with canola quality. Therefore, two populations were used in this study: (1) a DH population which derived from two canola winter oilseed rape cultivars, "Sansibar" and "Oase" (termed SODH population) and (2) a collection of 81 cultivars and breeding lines which largely represent the variability of canola quality winter oilseed rape in Northern Europe.

6.1 Genetic variation of phytosterol content and composition

Relatively large and significant genotypic variations were observed for all the traits in all three studies (Chapter 3-5). Total phytosterol content in the SODH population which ranged from 311.2 to 486.9 mg 100 g_{seed}^{-1} in the EU trial (Table 3.4) and 340.5 to 507.7 mg 100 g_{seed}^{-1} in the CN trial (Table 4.2) are comparable to the collection of 81 cultivars and breeding lines which ranged from 298.8 to 491.5 mg 100 g_{seed}^{-1} (Table 5.3). Compared with previous studies which reported 257 to 415 mg 100 g_{seed}^{-1} in three DH populations (n = 482) (Amar et al., 2008a) and 356.6 to 480.0 mg 100 g_{seed}^{-1}

in 27 canola winter oilseed rape cultivars (Amar et al., 2009), the present study showed a higher and larger range of total phytosterol content. Although the parental lines of SODH population were selected based on their contrasting total phytosterol content and oil content in just 27 canola winter oilseed rape cultivars (Amar et al., 2009), the magnitude of variation between the two populations used in this study were largely similar. For example, 24-methyl:24-ethyl sterol in the SODH population which ranged from 0.62 to 1.08 in the EU trial and 0.60 to 0.96 in the CN trial are similar to the collection of 81 cultivars and breeding lines which ranged from 0.66 to 1.09. Likewise, oil content in the SODH population which ranged from 41.2 to 48.6% in the EU trial and 39.9 to 45.8% in the CN trial are similar to the collection of 81 cultivars and breeding lines which ranged from 39.1 to 46.8%. In all three studies, analysis of variance revealed predominant and highly significant genotypic effects for phytosterol content and composition as well as other traits. High broad-sense heritabilities were consistently estimated in all three studies, suggesting that selection for genotypes with these traits could be performed efficiently.

6.2 Correlation between traits

Highly significant positive correlation (P = 0.01) between total phytosterol content and oil content was observed in all three studies in contrast to previous study by Amar et al. (2008a) who reported conflicting relationships between these two traits in three DH populations. No significant correlation was observed between total phytosterol content and protein content of defatted meal while no correlation to weak negative significant correlations (P = 0.05) were observed between total phytosterol content and seed weight. In both populations, brassicasterol appeared to have a different correlation trend compared with the rest of the individual phytosterols. For example, in SODH population, brassicasterol was negatively correlated with C18:1 and positively correlated with C16:0, C18:2, and C18:3 whereas other individual sterols were mostly not significantly correlated with fatty acids except for C16:0; in 81 cultivars and breeding lines, brassicasterol was negatively correlated with total phytosterol content whereas other individual phytosterols were positively correlated with total phytosterol content. Different types of relationships were also observed between the two populations. For instance, in SODH population, brassicasterol was negatively correlated with oleic acid in EU trial (r^2 = -0.43) and in CN trial (r^2 = -0.53) , whereas in

81 cultivars and breeding lines, no significant correlation was observed.

6.3 QTL mapping with linkage analysis and association analysis

Comparison of results between linkage mapping (Chapter 3 & 4) and association mapping (Chapter 5) showed that more QTL were detected in the SODH population than in 81 cultivars and breeding lines (Table 6.1). As discussed in Chapter 5, the low numbers of QTL detected in association mapping are mostly due to insufficient marker density and small sample size.

By aligning common markers between the genetic maps of Sansibar × Oase and Express × R53 as well as the alignment of additional sequence-informative markers on the two genetic maps to the reference sequence of *B. rapa* and *B. oleracea*, five QTL were found detected at similar position in both linkage mapping and association mapping (Table 6.1). In all three studies (Chapter 3 - 5), QTL for brassicasterol on A04 and QTL for 24-methyl:24-ethyl sterol on A06 have consistently explained a large portion of phenotypic variance and the underlying candidate genes were found to be *CYP710A1* and *SMT2*, respectively (Table 6.2). Whereas QTL for oil content on A01 explained only a small portion of the phenotypic variance in the EU trial of SODH population and in 81 cultivars and breeding lines. In Chapter 3, *LPAAT* gene was drawn to attention due to its colocalization with QTL for oil content and other traits and its close proximity to *FAD2* gene which colocalized with major QTL for C18:1 and C18:3. In association mapping, however, the associated marker for oil content was not shared with other traits and there was no other QTL on A01. Such an observation depict a common phenomenon between linkage mapping and association mapping. Due to the few recombination events in DH population, intervals to which QTL are mapped are large and may contain multiple genes. As such, it is usual to observe that sometimes different traits of QTL co-segregate together (Amar et al., 2008b; Zhao et al., 2008; Basunanda et al., 2010) and the central question has always been whether the clusters represent linked but otherwise independent QTL or pleiotropic effects of one locus. In association mapping, however, the low level of linkage disequilibrium can better unravel the ambiguity of this observation. As shown in Figure 6.1, the confidence interval of QTL for oil content in SODH population spanned a region of 11 cM on genetic map and about 6,160 kbp on the physical map of *B. rapa* genome which was found to harbour about 719 genes; whereas Ecke et al. (2010) have shown that LD decay

within 1-2 cM in the present association mapping population and the alignment of additional mapped markers flanking the associated marker were 3 cM apart on the genetic map and 260 kbp apart on the physical map of *B. rapa*, strongly supporting the hypothesis that *LPAAT* underlies QTL for oil content. Consequently, it can be postulated that the other QTL that were collocated with oil content in linkage mapping are most likely due to close linkage than pleiotropic effect of *LPAAT* gene. Since *LPAAT* gene was just 11.8 kbp from the closest mapped DArT marker (brPb-840880), suggesting that this marker could be used for marker assisted selection following proper validation. In Arabidopsis, expression of the oilseed rape microsomal LPAAT isozymes has shown enhancement of seed oil content and seed mass (Maisonneuve et al., 2010). Further work could also be performed to dissect the allelic diversity of *LPAAT* gene in the present canola cultivars to develop functional marker for marker-assisted selection. On a slightly different note, another glycerolipid acyltransferases, *DGAT* genes, which encodes the enzyme catalyzing the final committed step in the Kennedy pathway have received much attention in oilseed rape lately due to the enhancement of oil content achieved through transgenic approach (Taylor et al., 2009). In maize, ectopic expression of the high-oil *DGAT1-2* allele increases oil and oleic acid content by up to 41% and 107% (Zheng et al., 2008). The present study shows that positive correlation between oil and oleic acid content could also be due to close linkage between *FAD2* and *LPAAT* on the same chromosome as depicted in Figure 6.1. It should be pointed out that aside from insufficient marker coverage where the causal polymorphism is not in perfect LD with the genotyped markers, the fact that no QTL were detected for fatty acids in association mapping could partly be due to the discrepancy of analytical method used in fatty acid compositions between the two populations. In SODH population, fatty acids were quantified by gas-chromatography while in the 81 cultivars and breeding lines, fatty acids were only estimated by NIRS.

Table 6.1: Number of QTL detected with linkage analysis in SODH population (EU trial and CN trial) and association analysis in 81 cultivars and breeding lines.

Trait	Linkage mapping (LM)			Association mapping (AM)	QTL at similar position[a]	Total no. of QTL[b]
	EU trial	CN trial	Same QTL			
Phytosterols						
Brassicasterol	6	4	3	1	1	7
Campesterol	4	4	3	1	0	6
Sitosterol	2	2	2	0	0	2
Avenasterol	1	1	1	0	0	1
Total phytosterol	2	1	0	1	0	4
24-methyl sterol	3	4	2	1	0	6
24-ethyl sterol	1	1	1	0	0	1
Campesterol:sitosterol	5	6	4	0	0	7
24-methyl:24-ethyl sterol	5	6	3	7	3	12
Other traits						
C16:0	5	2	2	NA[c]	-	5
C18:1	3	3	1	0	0	5
C18:2	2	5	1	NA	-	6
C18:3	6	3	2	0	0	7
Oil	6	7	2	6	1	16
Protein of defatted meal	4	2	2	0	0	4
Seed weight	3	3	1	0	0	5

[a] QTL detected at similar position in both linkage mapping (Sansibar × Oase) and association mapping (based on the genetic map of Express × R53)

[b] Total number of QTL identified by both association mapping and linkage mapping

[c] NA Not available

Table 6.2: QTL detected at similar position in both linkage mapping and association mapping. Comparison of QTL was based on alignment of common markers between the genetic maps of Express × R53 and Sansibar × Oase as well as the alignment of additional sequence-informative markers on the two genetic maps to the reference sequence of *B. rapa* and *B. oleracea*

Trait		Linkage mapping			Association mapping			Candidate gene
		QTL	aa[a]	CI[b]	QTL	PE[c]	Pos.[d]	
Brassicasterol	A04	DE-Bra-3	2.61	91-97	AM-Bra.1	-9.3	21	*CYP710A1*
		HZ-Bra.2	2.55	90-97				(Bra021916 & Bra021917)
24-methyl:24-ethyl sterol	A02	DE-MEratio.2	1.59	0-5	AM-MEratio.1	7.63	162	
		HZ-MEratio.1	1.66	0-5				
	A06	DE-MEratio.3	-5.05	61-66	AM-MEratio.3	11.52	56-63	*SMT2* (Bra025810)
		HZ-MEratio.2	-3.66	61-67				
	C05	DE-MEratio.4	-3.33	79-89	AM-MEratio.6	-7.38	51	
		HZ-MEratio.5	-3.49	84-89				
Oil	A01	DE-Oil.1	-0.31	68-79	AM-Oil.1	1.72	74	*LPAAT* (Bra037553)

[a] Additive effect: the substitution effect of one "Oase" allele by one "Sansibar" allele.

[b] 1-LOD Confidence interval

[c] Phenotypic effect: Positive sign: visible marker allele increases trait value; negative sign: visible marker allele decreases trait value

[d] Position of associated markers were based on Express × R53 genetic map (Ecke et al., 2010)

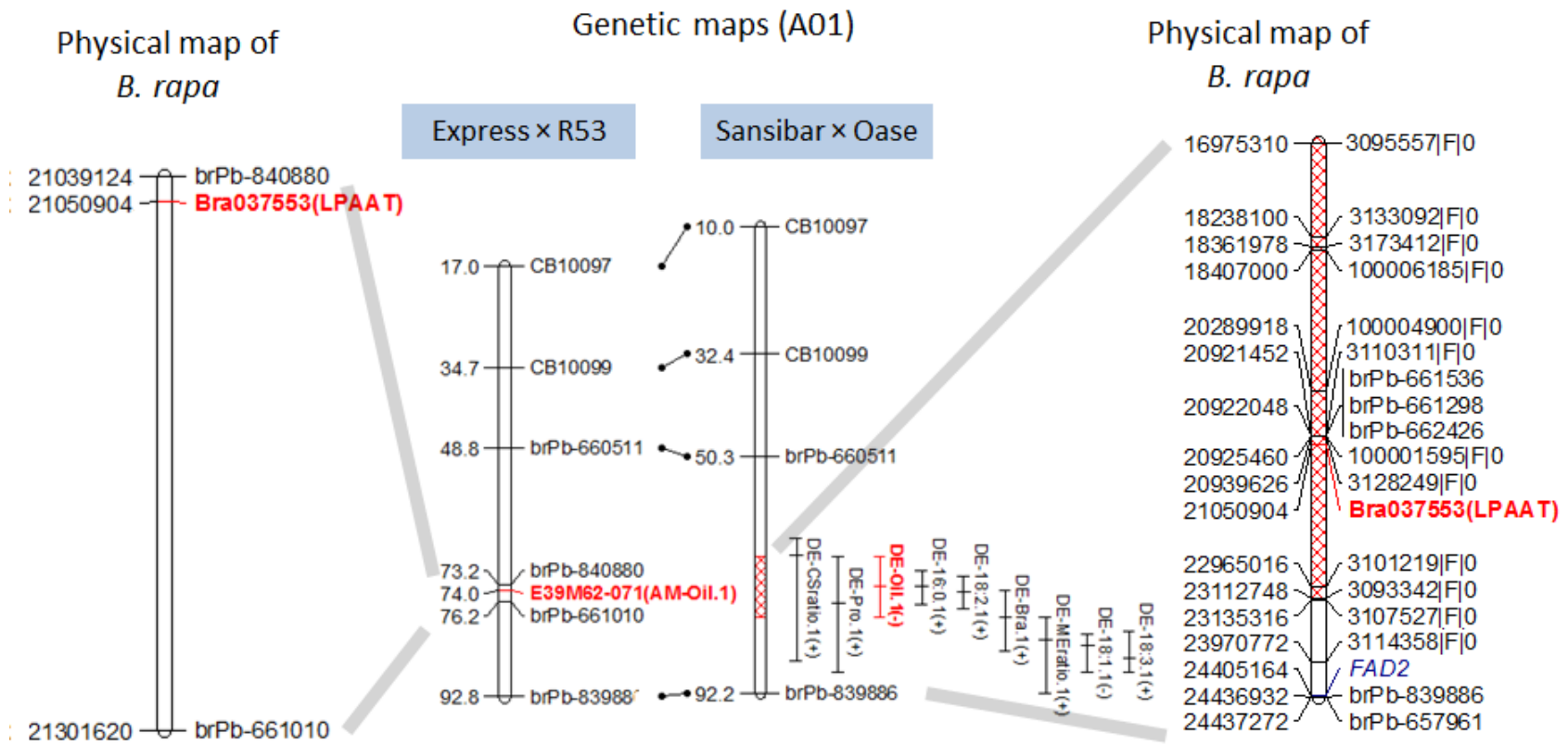

Figure 6.1: Similar QTL for oil content on A01 identified by linkage mapping and association mapping. Associated marker for oil content (E39M62-071) identified by association mapping was flanked by two DArT markers (brPb-840880—brPb-661010) in Express × R53 genetic map which were flanking *LPAAT* when located on physical map of *B. rapa*. QTL for oil content (DE-Oil.1) identified by linkage mapping collocated with *LPAAT* as shown by the alignment of additional markers mapped within the QTL confidence interval (68–79 cM) on physical map of *B. rapa*.

With the use of a broader germplasm that largely encompasses genetic variation of canola quality winter oilseed rape in Northern Europe, association mapping identified additional QTL for phytosterol and oil content that were different from linkage mapping in SODH population (Table 6.1). By integrating the QTL results obtained from three studies, the highest number of QTL was identified for oil content (Table 6.1), confirming the polygenic determinism of this trait as reported in many studies (Burns et al., 2003; Ecke et al., 1995; Zhao et al., 2005; Qiu et al., 2006; Jiang et al., 2014). Further work could be performed by comparing results of QTL identified in this study with other genetic populations since oil content is determined by many genes with small effects. Accounting for full extent of the variation could potentially lead to a successful pyramiding approach in realizing the goal to improve oil content in oilseed rape. Additionally, the recent availability of reference sequence *B. napus* genome could also be utilized to locate identified QTL on the physical map for better comparison and analysis of the underlying genes (Chalhoub et al., 2014). The second highest number of QTL was detected for 24-methyl:24-ethyl sterol, a trait that is less complex than oil content since it is mainly determined by the SMT2 enzyme only (Schaeffer et al., 2000). As can be seen by the effect of the QTL, a large proportion of QTL variation could be explained by QTL on A06, suggesting that this maybe the principal locus controlling the composition of phytosterols. It has been proposed that the ability of plants to synthesize sterols with branched ethyl groups (as in sitosterol and stigmasterol) may be part of an evolutionary adaptation process to cope up with wider temperature fluctuations, and to maintain the essential membrane associated metabolic processes, as compared to animals (Dufourc, 2008). Further work could be performed to investigate whether QTL controlling phytosterol composition are correlated to QTL for physiological or morphological traits in oilseed rape to benefit from the current findings.

Considering the fact that strong pleiotropic effect of erucic acid genes on total phytosterol content were eliminated in the two canola quality winter oilseed rape populations used in this study, it was expected that the resulting higher power of QTL detection power would translate to high number of QTL identified. Conversely, each of the three studies only detected 1-2 QTL for total phytosterol content (Table 6.1), implying that it is a quantitative trait controlled by many genes with small effects. Altogether, the three studies identified four QTL on four different linkage groups (A07, C03, C08, C09). Previous study by (Amar et al., 2008b) has identified two QTL with large effects that were due to pleiotropic effects of erucic acid genes on A08 and C03 and a QTL with small effect on

C08. By broad comparison of chromosome arms on C08, it is likely that the QTL on C08 detected in Amar's study correspond to the QTL detected by linkage mapping (EU trial) in this study. This means that three novel QTL were uncovered for total phytosterol content from this study. With respect to its relationship with oil content, three of the four QTL for total phytosterol were found collocated with QTL for oil content on C03, C08, and C09. Of these, QTL on C03 detected in CN trial by linkage mapping and C09 detected by association mapping showed alleles with similar direction of effect, implying that increasing both phytosterol and oil content are possible in canola winter oilseed rape. However, the collocation of QTL observed were most likely due to close linkage than pleiotropic effect because the biosynthetic pathways of oil and phytosterols are not directly related (Hartmann, 1998; Stoll et al., 2005).

6.4 Conclusion

Owing to its rapid LD decay in association mapping population, a small number of QTL were only identified for some phytosterol traits and oil content. The limitation of association mapping in oilseed rape in terms of marker density has already been lifted by the recent rapid development of SNP markers (Bus et al., 2011; Delourme et al., 2013; Durstewitz et al., 2010; Tollenaere et al., 2012) and various high-throughput platforms such as GoldenGate, Infinium and second-generation sequencing, enabling rapid genotyping of several thousand markers in parallel (Ganal et al., 2012). Therefore, it can be foreseen that by employing these recent marker technologies in the joint use of linkage mapping and association mapping, more novel QTL could be unravelled to improve the genetic dissection of phytosterol and oil content.

Chapter 7

Summary

Phytosterols are natural constituents of vegetable oils that are among the dietary option to reduce serum LDL-cholesterol. Oilseed rape (*Brassica napus* L.) is ranked the second richest source of phytosterols among vegetable oils. Improving the phytosterol content or composition could enhance the nutritive value of oil produced from oilseed rape. Due to the close negative correlation between total phytosterol content and erucic acid content, oilseed rape with canola quality inherently contain higher amount of phytosterols than non-canola quality oilseed rape.

To understand the genetic basis of phytosterol content and its relationship with other traits, QTL analysis was performed with both linkage mapping and association mapping. For interval mapping, a segregating DH population, termed SODH population, was constructed from the cross of two winter oilseed rape cultivars "Sansibar" and "Oase"; both parental lines are of canola quality. They were chosen due to their contrasting phytosterol and oil content in seeds based on previous screening in 27 canola quality winter oilseed rape cultivars; Sansibar contains the highest phytosterol content ($\sim$480 mg 100 g_{seed}^{-1}) and the lowest oil content (43%) while Oase contains the lowest phytosterol content ($\sim$360 mg 100 g_{seed}^{-1}) and the highest oil content (46%). A genetic map was constructed based on a total of 1642 markers (AFLP, candidate-gene based marker, DArT, Silico-DArT, SSR, and SNP), organized in 23 linkage groups and covering a map of 2350 cM with a mean marker interval of 2.0 cM. Field trials of SODH population were carried out in two mega-environments, Europe (Germany & Sweden; EU trial) during growing seasons 2009/10, 2010/11 and 2011/12 and East China (Hangzhou; CN trial) during growing season 2011/12 and 2012/13, in which the genetic variation of traits and multiple interval mapping were evaluated

separately.

For association mapping, a collection of 81 canola quality winter oilseed rape cultivars and breeding lines which have previously been characterized with 845 mapped AFLP markers in a LD study were used. The study reported an overall low level of LD with a mean r^2 of 0.027 and that high level of LD between linked markers extended only for about 1-2 cM, implying that high resolution mapping could be expected in this population. Of the 845 AFLP markers, a subset of 685 which have no absolute LD ($r^2 < 1.0$) between pairs were selected for association analysis. These AFLP markers have been mapped in a segregating double haploid population derived from the Express $\times$ R53 cross. Additionally, seven SNP/InDels candidate gene-based markers were also included in the analysis, resulting in a total number of 692 molecular markers that is deemed insufficient to saturate the oilseed rape genome compared to the estimated number of several thousands. Despite the limitation of marker density, the population still present a valuable resource for genetic studies as it largely represents the genetic variation of winter oilseed rape cultivars and breeding lines in Northern Europe. Field trials have previously been performed at 6 environments during growing seasons 2007/2008 and 2008/2009 in Germany. Association analysis was performed using K model, a mixed-linear model which incorporated kinship matrix as a random effect to account for relatedness.

Phytosterol content was quantified using gas-chromatography following a direct alkaline hydrolysis method which bypasses the lipid extraction step, facilitating large number of seed samples to be analysed more economically. The individual phytosterols that were quantified include two 24-methyl sterols, brassicasterol and campesterol, and two 24-ethyl sterols, sitosterol and avenasterol. In total, nine phytosterol traits were included in both QTL analysis: brassicasterol, campesterol, sitosterol, avenasterol, total phytosterol, 24-methyl sterol, 24-ethyl sterol, campesterol:sitosterol, and 24-methyl:24-ethyl sterol. Other traits include seed quality traits such as oil content, fatty acid composition, protein content of defatted meal and a yield related trait, seed weight. Oil content and protein content of defatted meal were estimated by NIRS. Fatty acids (C16:0, C18:1, C18:2 and C18:3) were quantified using gas-chromatography in SODH population while fatty acids (C18:1, C18:3) were estimated using NIRS in 81 cultivars and breeding lines.

Analysis of variance revealed relatively large variation and highly significant genotypic effects for

all the traits in SODH population and 81 cultivars and breeding lines. Total phytosterol content in the SODH population which ranged from 311.2 to 486.9 mg 100 g_{seed}^{-1} in the EU trial and 340.5 to 507.7 mg 100 g_{seed}^{-1} in the CN trial are comparable to the collection of 81 cultivars and breeding lines which ranged from 298.8 to 491.5 mg 100 g_{seed}^{-1}. Oil content in the SODH population ranged from 41.2 to 48.6% in the EU trial and 39.9 to 45.8% in the CN trial are similar to the collection of 81 cultivars and breeding lines which ranged from 39.1 to 46.8%. In all three studies, broad-sense heritabilities for total phytosterol content ranged from 0.88 to 0.94 while oil content ranged from 0.70 to 0.86. High broad-sense heritabilities were similarly estimated for all the other traits, suggesting that selection for genotypes with these traits could be performed efficiently. In all three studies, highly significant positive correlations ($P = 0.01$) were consistently observed between total phytosterol content and oil content; In SODH population, $r_s = 0.24$ in EU trial and $r_s = 0.24$ in CN trial, and in 81 cultivars and breeding lines, $r_s = 0.29$. No significant correlation was observed between total phytosterol content and protein content of defatted meal while no correlation to weak negative significant correlations ($P = 0.05$) were observed between total phytosterol content and seed weight.

In the SODH population, multiple interval mapping identified between 1 and 6 QTL for nine phytosterol traits in both EU trial and CN trial. The minimal phenotypic variance explained by these QTL ranged from 7.2% to 70.3% in EU trial and 6.5% to 71.9% in CN trial. Comparison of QTL results revealed that between 1 and 4 QTL were repeatedly identified for eight of the nine phytosterol traits across the two mega-environments. These stable QTL were located in seven genomic regions at seven linkage groups (A02, A03, A04, A06, A07, C05 and C08). QTL for total phytosterol content were environment-specific; two QTL were identified on A07 and C08 in EU trial and one QTL was identified on C03 in CN trial. In both trials, major QTL ($R^2 \geq$ 25%) were repeatedly identified for brassicasterol on A04 which collocated with *CYP710A1* and campesterol:sitosterol and 24-methyl:24-ethyl sterol on A06 which collocated with *SMT2*. For other traits, between 2 and 6 QTL were identified in EU trial, and between 2 and 7 were identified in CN trial. The minimal phenotypic variance explained by these QTL ranged from 27.1% to 59.0% in EU trial and 21.8% to 47.1% in CN trial. In EU trial, major QTL for C18:1 and C18:3 on A01 collocated with *FAD2* which is located relatively close to *LPAAT* gene (about 3,354 kbp apart based on *B. rapa* genome) that coincided with minor QTL for C16:0, C18:2 and oil content; while another

major QTL was identified for C16:0 on A09 which coincided with *FatB* gene. Whereas in the CN trial, only small to moderate effects of QTL were identified for other traits. Comparison of QTL results between both trials revealed that between 1 and 2 QTL were repeatedly identified for other traits. For oil content, two stable QTL were identified out of six QTL in EU trial and seven in CN trial. Collocation of QTL between total phytosterol content and oil content were observed on C08 in opposite direction of additive effect in EU trial while on C03 in the same direction of additive effect in CN trial, suggesting that increasing both phytosterol and oil content depends on regions of cultivation.

In 81 cultivars and breeding lines, association analysis using K model identified between one and seven QTL for five of the nine phytosterol traits and six QTL for oil content. The minimal phenotypic variance explained by these QTL ranged from 14% to 47%. For phytosterol traits, one QTL was identified for brassicasterol, campesterol, 24-methyl sterol and total phytosterol while seven QTL were identified for 24-methyl:24-ethyl sterol. The associated marker for brassicasterol QTL on A04 has the lowest *P*-value among all the identified associated markers for different traits and was shown to coincide with *CYP710A1*. Similarly, the QTL which explained the largest phenotypic variation for 24-methyl:24-ethyl sterol was located on A06 and was shown to coincide with the *SMT2*. For oil content, six QTL were identified. Between phytosterol and oil content, shared significant markers were only observed on C09 in which one of them was shared among campesterol, 24-methyl sterol, total phytosterol, and oil content with the same direction of phenotypic effect. This suggests that increasing both total phytosterol content and oil content are possible in canola quality winter oilseed rape with the increase of total phytosterol content contributed by the increase of campesterol content.

Comparison of QTL results between linkage mapping and association mapping showed that more QTL were detected in SODH population than in 81 cultivars and breeding lines. This is because association mapping is under-powered both in terms of marker density and sample size. Nonetheless, association mapping identified additional QTL for phytosterol and oil content that were different from linkage mapping in SODH population; one QTL for campesterol, 24-methyl sterol and total phytosterol, four QTL for 24-methyl:24-ethyl sterol and five QTL for oil content. QTL that were identified at similar location in both linkage mapping and association mapping were QTL for brassicasterol on A04, 24-methyl:24-ethyl sterol on A02, A06 and C05 and oil content

on A01. Of particular interest is the QTL for oil content on A01 as it coincided with *LPAAT* which plays an essential role in the synthesis of phosphatidic acid, a key intermediate in the biosynthesis of membrane phospholipids in all tissues and storage lipids in developing seeds. Moreover, alignment of additional mapped marker from Express $\times$ R53 to the reference genome of *B. rapa* identified a DArT marker that was located just 11.8 kbp apart from *LPAAT* gene, suggesting that this QTL could be used for marker assisted selection following proper validation.

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Appendix A

Appendix

A.1 SSR primers

SSR primer sequences

SSR primer	Forward primer (5′ - 3′)	Reverse primer (5′ → 3′)
BRAS014	CCCATTGACAACTCTTCTCTT	CTGTGTTCGCCCATTATG
CB10014	TGATACTAAAGGCAAGTCTA	CTGACCATAACCCATCG
CB10026	TCGTTCTGACCTGTCGTTAT	GGAAATGGCTGCTCATGTT
CB10028	CTGCACATTTGAAATTGGTC	AAATCAACGCTTACCCACT
CB10036	ATTCATCTCCTGCTCGCTTAG	AAACCCAAACCAAAGTAAGAA
CB10080	GCCCTCAACCTGTAAAGT	TTGTTGGTGTGTGAATCATA
CB10097	ACTTCGGTGGTTCTATTTCT	CGACGGTTAATCAAGTTTCT
CB10099	CTTCCCCTTTCATCGAACT	TAGAAGCATTTGGAAACGCA
CB10103	GACGGATGCCTAATAATGAT	TCCTCAAAACTGCCTGTAAG
CB10109	GTGTAGCCAGCTTGATCCT	CTTCTTCTGATGCAGCAGTG
CB10208	ACTACTGTTGCGGTTGGA	GGCATTCATTACGTCTGC
CB10415	GAACTCGTCGCGGTAGTA	TCTCTTTCCTCGCAGATG
CB10425	GGTGGCTTGTAGGGACTT	GCTCCCGTAAACTCTTCC
CB10431	GGGTTTACTGGGTTCGTT	GCAGAAGGGGAAACACTT
CB10493	TGACGTGTGAGCAACAGA	CTGAGTCACAAGCCGAGT
CB10534	AGCTGCAACCACAACTCT	GGAGCGCAAGAAAAG
CB10545	CTCGCAATAGTCGCAGAT	TGCCCTACTGTCTCCTCA
CB10572	AGTCACACAATGGCGTCT	TTACGGTCTGAGCCTTGA
MD38	AGCCTTACATATTCACCTACC	TTAAGCTCCAACGTGTTC
MR155	AGGCACTATATCAGACAACTG	GACATATGCGATGACACTT

A.2 KASP markers

Markers associated with oil content in SGDH14 × Express617 (Nina Behnke, personal communication).

No.	Marker name	
	KASP	Illumina Infinium Brassica 60K SNP
1	BNKS00092	Bn-A03-p14306440
2	BNKS00151	Bn-A05-p20020286
3	BNKS00264	Bn-A08-p14597858
4	BNKS00281	Bn-A09-p36402581
5	BNKS00428	Bn-scaff_24631_1-p422231
6	BNKS00459	Bn-scaff_16534_1-p1901223
7	BNKS00572	Bn-scaff_16069_1-p2323317
8	BNKS00589	Bn-scaff_17740_1-p834125
9	BNKS003001	Bn-A01-p7137897
10	BNKS003003	Bn-A03-p11053758
11	BNKS003005	Bn-A05-p22943215
12	BNKS003006	Bn-A06-p2617079
13	BNKS003011	Bn-A09-p4436808
14	BNKS003012	Bn-A09-p7047022
15	BNKS003017	Bn-scaff_15783_1-p350376
16	BNKS003018	Bn-scaff_16547_1-p59615
17	BNKS003019	Bn-scaff_16755_1-p1208174
18	BNKS003023	Bn-scaff_17869_1-p115797
19	BNKS003028	Bn-scaff_24859_1-p44428

Markers that were physically closely linked to candidate genes for phytosterol biosynthesis.

No.	Marker name		Candidate gene	Gene ID
	KASP	Illumina Infinium Brassica 60K SNP		
1	BNKS003008	Bn-A07-p21078249	HMG1	Bra015739
2	BNKS003020	Bn-scaff_16903_2-p305070	HMG1	Bol039236
3	BNKS003022	Bn-scaff_17799_1-p1214222	HMG1	Bra015739
4	BNKS003026	Bn-scaff_21034_1-p10697	HMG1	Bra008261
5	BNKS003013	Bn-A10-p11918400	HMG2	Bra002053
6	BNKS003025	Bn-scaff_19614_1-p119351	HMG2	Bol017681
7	BNKS003009	Bn-A07-p5713057	HMGS	Bra014870
8	BNKS003027	Bn-scaff_23401_1-p188311	HMGS	Bol045227
9	BNKS003002	Bn-A02-p3507057	SMT1	Bra023430
10	BNKS003004	Bn-A03-p2521128	SMT1	Bra006211
11	BNKS003014	Bn-scaff_15714_1-p1787439	SMT1	Bra023430
12	BNKS003024	Bn-scaff_18936_1-p277517	SMT1	Bol034248
13	BNKS003007	Bn-A06-p7726350	SMT2	Bra025810

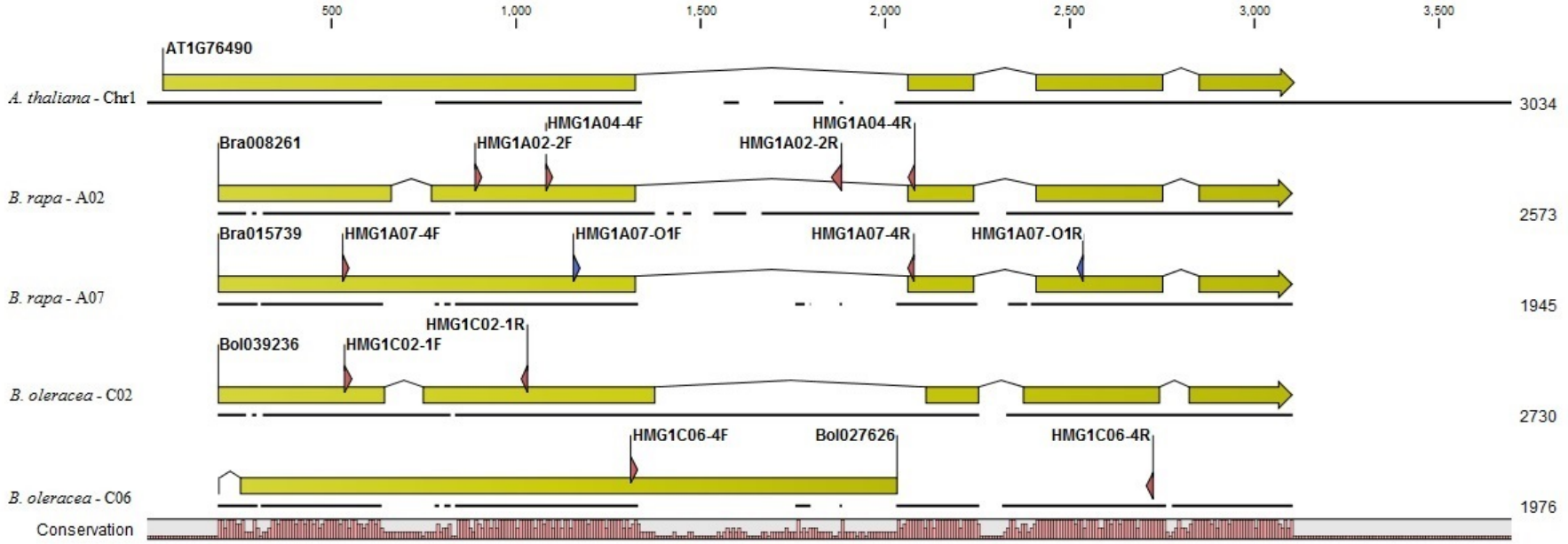
A.3 Candidate genes

List of candidate genes involved in phytosterol biosynthesis. BLAST search was performed with *A. thaliana* gene sequence against *B. rapa* v1.5 genome database (BRAD) and *B. oleracea* v1.0 genome database (Bolbase)

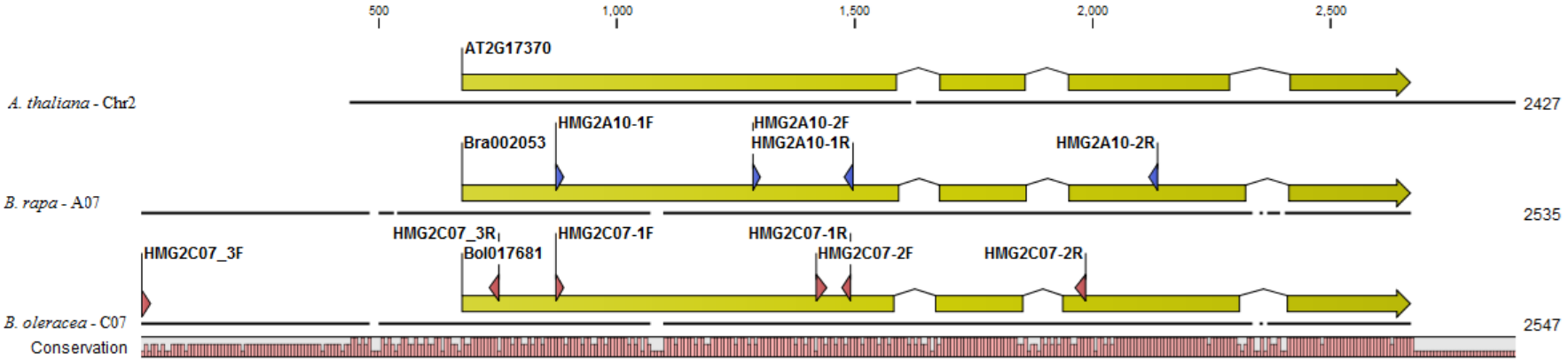
Gene annotation	Species	Gene ID	Chr.	Sequence length	E value
HMG1	*A. thaliana*	AT1G76490	1	3034	
3-hydroxy-3-methylglutaryl CoA reductase 1	*B. rapa*	Bra008261	A02	2573	7e-22
		Bra015739	A07	1945	2e-87
	B. oleracea	Bol039236	C02	998	e-129
		Bol027626	C06	2730	6e-85
HMG2	*A. thaliana*	AT2G17370	2	2427	
3-hydroxy-3-methylglutaryl-CoA reductase 2	*B. rapa*	Bra002053	A07	1910	2e-87
	B. oleracea	Bol017681	C07	1916	8e-93
HMGS	*A. thaliana*	AT4G11820	4	3566	
3-hydroxy-3-methylglutaryl-CoA synthase	*B. rapa*	Bra033126	A02	2835	1e-76
		Bra014870	A07	2885	1e-70
	B. oleracea	Bol045227	C06	2849	2e-79
		Bol008312	UNC[a]	2820	9e-69
SMT1	*A. thaliana*	AT5G13710	5	3364	
Sterol C24-methyltransferase 1	*B. rapa*	Bra023430	A02	6937	e-156
		Bra006211	A03	3102	e-136
	B. oleracea	Bol034248	C03	2746	e-135
		Bol004256	UNC	2652	6e-48
SMT2	A. thaliana	AT1G20330	1	1472	
Sterol C24-methyltransferase 2	*B. rapa*	Bra205810	A06	1071	0
	B. oleracea	Bol026945	C05	1071	0

[a] UNC Unknown chromosome number

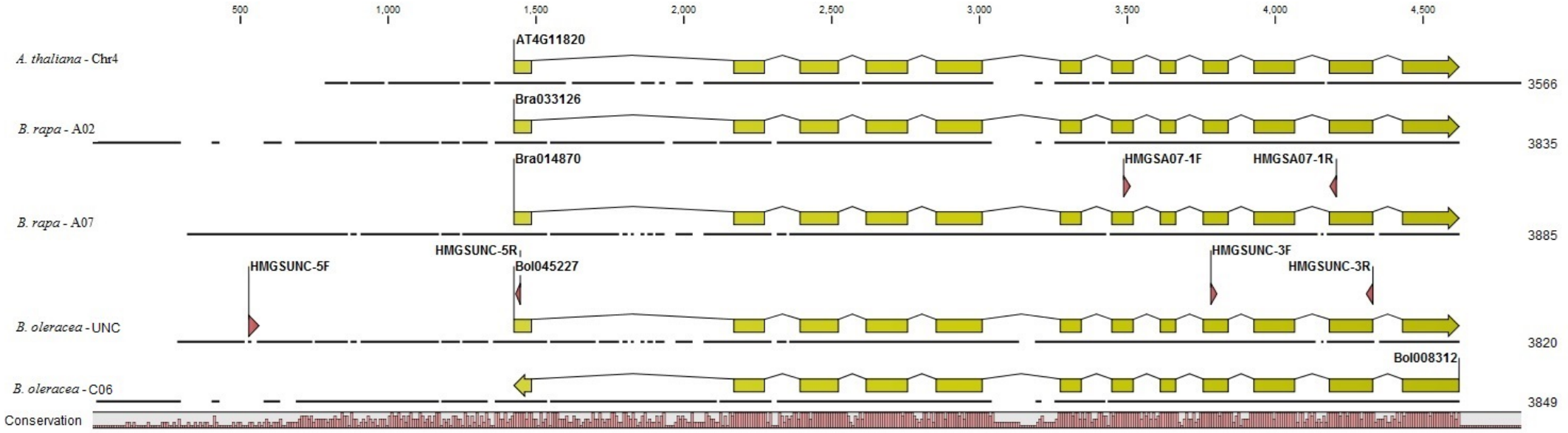
A.4 Schematic gene stucture of phytosterol candidate genes



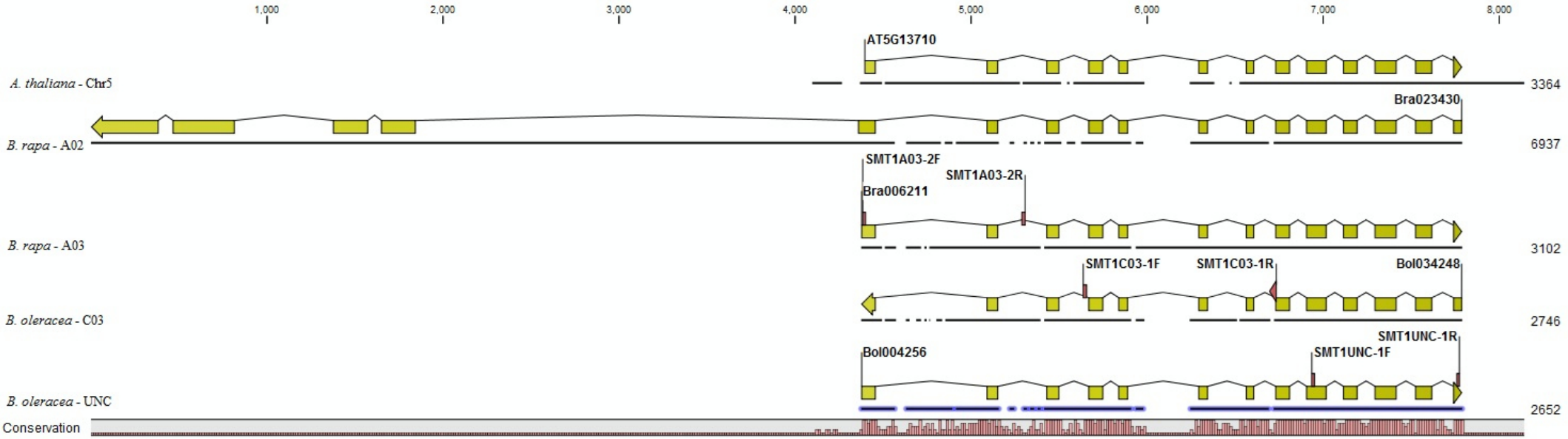
Alignment of *HMG1* gene sequence from *A. thaliana* with homologous copies from *B. rapa* and *B. oleracea* . *Yellow bar* indicates coding region while *line* between bars indicates non-coding region. *Red arrow* indicates the locus-specific primer pair. *Blue arrow* indicates the allele-specific primer pair. Additional 978 bp at the untranslated region was retrieved for locus Bol017626 from *B. oleracea* genome sequence.



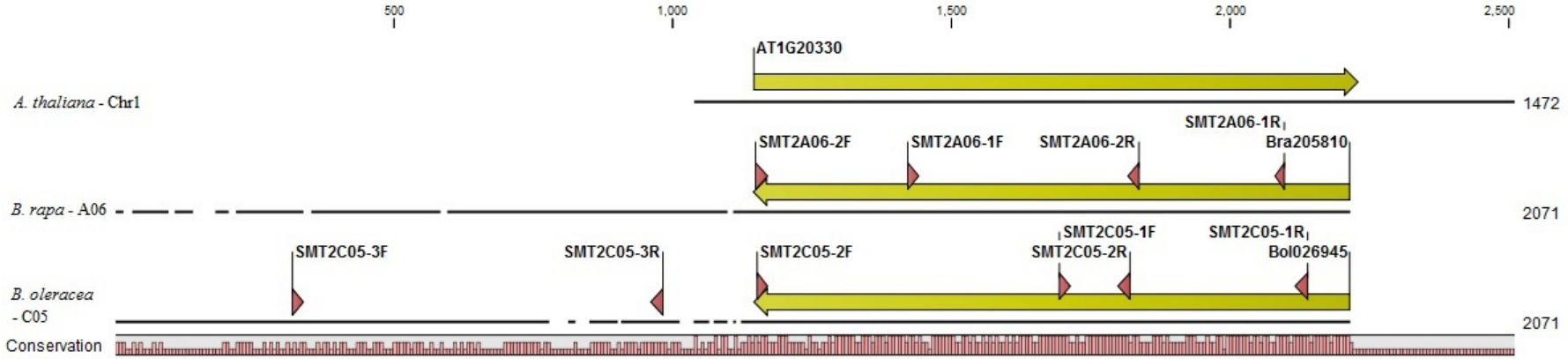
Alignment of *HMG2* gene sequence from *A. thaliana* with homologous copies from *B. rapa* and *B. oleracea* . *Yellow bar* indicates coding region while *line* between bars indicates non-coding region. *Red arrow* indicates the locus-specific primer pair. *Blue arrow* indicates the allele-specific primer pair.



Alignment of *HMGS* gene sequence from *A. thaliana* with homologous copies from *B. rapa* and *B. oleracea* . *Yellow bar* indicates coding region while *line* between bars indicates non-coding region. *Red arrow* indicates the locus-specific primer pair. Additional 1 kbp were retrieved from the untranslated region to search for more interspecific SNP.



Alignment of *SMT1* gene sequence from *A. thaliana* with homologous copies from *B. rapa* and *B. oleracea*. *Yellow bar* indicates coding region while *line* between bars indicates non-coding region. *Red arrow* indicates the locus-specific primer pair.



Alignment of *SMT2* gene sequence from *A. thaliana* with homologous copies from *B. rapa* and *B. oleracea* . *Yellow bar* indicates coding region while *line* between bars indicates non-coding region. *Red arrow* indicates the locus-specific primer pair. Additional 1 kbp were retrieved from the untranslated region to search for more interspecific SNP.

A.5 Locus-specific markers

Locus-specific markers for phytosterol candidate genes

Chr.	Gene ID	Primer name	Primer sequence (5′ → 3′)		T_m (°C)	Sequenced fragment size (bp)	SNP
HMG1							
A02	Bra008261	HMG1A02-2	Fwd	CCTTCCTACTCCCTCGAGTCC	61	803	0
			Rev	TTCTCTAAACACGAAAACCCAAAAA			
		HMG1A02-4	Fwd	CCTTTGTTGCTTGACGGGTAT	62	797	0
			Rev	CAGCCTCGCGAATCTACTTGA			
A07	Bra015739	HMG1A07-4	Fwd	GCATCGATTTCGTCCAGTCT	61	674	6
			Rev	GCCTCGCAAATCTACTCGAC			
C06	Bol027626	HMG1C06-4	Fwd	CTGTTGTTTTCAACAGGTCTGG	60	672	0
			Rev	TATGGATATCTTTCCCGTCGTT			
C02	Bol039236	HMG1C02-1	Fwd	GACTTCGTCCAATCGTTCATCTT	60	410	0
			Rev	AGCATTGCCCCAATATGGAT			
HMG2							
A07	Bra002053	HMG2A10-1	Fwd	GACGCCTCTTCACGTCGTC	62	697	NA[a]
			Rev	CAACGGGAGCTCTAGTCATGG			
		HMG2A10-2	Fwd	TGGGACAGTGCTGCGAGA	63	799	NA[a]
			Rev	CCACCAAGAGAACCAGCCATA			
C07	Bol017681	HMG2C07-1	Fwd	GACGCCTCTTCACGTCGTT	63	566	0
			Rev	GGAGCTCTAGTCATCGCATCC			
		HMG2C07-2	Fwd	CAGAGGATGCAAGGCTATTCACTTA	64	557	0

Continued on next page

Appendix A.5: *continued from previous page*

Chr.	Gene ID	Primer name	Primer sequence (5′ → 3′)		T_m (°C)	Sequenced fragment size (bp)	SNP
			Rev	AGTTTATAGCCGAAGCCTTCTTGTC			
		HMG2C07-3	Fwd	CAACGGACATGACAACTTGC	60	676	0
			Rev	CGAGGAGGAGCAGTGACAAA			
HMGS							
A07	Bra014870	HMGSA07-1	Fwd	GCAGACTACTTTGTATTCCATTCTCC	64	544	0
			Rev	TGAGAACATAACCACCCTCTTCTCT			
UNC	Bol008312	HMGSUNC-3	Fwd	AGTTCACTCCTTATTCGTCTTTGT	65	770	0
			Rev	CCTCATGTCTAGCCTTCAGTTTACCA			
		HMGSUNC-5	Fwd	GCACAAATCAAATCGGTTCA	56	674	0
			Rev	CCAATATCCCGACGTTCTT			
SMT1							
A03	Bra006211	SMT1A03-2	Fwd	AAGTCTGGAGCTATGGATCTCG	61	712	0
			Rev	AGCTCATTCAAAGGGTGAGAAG			
UNC	Bol004256	SMT1UNC-1	Fwd	CTAAAGCCCGGACAATGTTTC	60	775	0
			Rev	CGGGCCAAGAAGAAATACAT			
C03	Bol034248	SMT1C03-1	Fwd	AATTAGCATCCCTGTTTTGGTC	61	752	0
			Rev	GAAGTCAGCCTACAAATTCATCAC			
SMT2							
A06	Bra205810	SMT2A06-1	Fwd	TTCCACTTCTCTCCTTCCATCC	64	657	0
			Rev	CTTAGGCGCAACTCCAACG			
		SMT2A06-2	Fwd	GACAGTGTCGCACTCTTCTTCACT	64	668	0
			Rev	GGTGACCCACTCGTACGATACT			
C05	Bol026945	SMT2C05-1	Fwd	CCAGATGCCCTTCGATGATAAT	64	448	0

Continued on next page

Appendix A.5: *continued from previous page*

Chr.	Gene ID	Primer name	Primer sequence (5′ → 3′)		T_m (°C)	Sequenced fragment size (bp)	SNP
			Rev	ATCAGCAGTCTTAAACAACATCTCG			
		SMT2C05-2	Fwd	GGCGTAGCATTCTTCTTCACT	63	650	0
			Rev	GATACGTACAAAGATCCGGGTTTC			
		SMT2C05-3	Fwd	CATTGGAGGTCGTTGATCATTT	58	565	0
			Rev	AATGAGAAGAAGCCTGATTTGG			

[a] No amplification was observed from Oase. Only fragment amplified from Sansibar was sequenced.

A.6 Allele-specific markers

Allele-specific markers for *HMG1* and *HMG2*.

Chr.	Gene ID	Primer name	Primer sequence (5′ → 3′)	T_m (°C)	Fragment size (bp)
A07	Bra015739	HMG1A07-O1	Fwd CAGAGGGTGCAAGGCTATGTA[a]	62	618
			Rev CAAAGAAGCCACGCTCGTC[b]		
A07	Bra002053	HMG2A10-2[c]	Fwd TGGGACAGTGCTGCGAGA	63	799
			Rev CCACCAAGAGAACCAGCCATA		

[a] Designed based on intragenomic SNP at the 3′ end. See Appendix A.6
[b] Designed based on intergenomic SNP at the 3′ end.
[c] HMG2A10-2 was designed based on intergenomic SNP but was shown to be also allele-specific

Allele-specific marker for *dgat1*.

Primer name	Primer sequence (5′ → 3′)	T_m (°C)
D120E-3	Fwd CTGCCTTTACCGTCGAGAAAC	60
	Rev AGATCAAGCGGGCAAAAATGG	
Dx-3	Fwd GTCTTCAGCTAATAGCATCAAACATTC	60
	Rev GTGGAGGGTCAAAGCTAAATTTC	

Alignment of the partial gene sequence of *hmg1* from *B. rapa* (Chiifu) with fragments amplified from Sansibar and Oase by locus-specific marker HMG1A07-4. *Yellow bars* indicate coding region. *Brown bar* indicates the allele-specific primer designed based on intragenomic SNP at the 3′ end. *Dots* indicates consensus nucleotides.

```
                1                                                          60
B. rapa         FISRADSNDSDRDYLVNDDHHRLVTCPPPIVAKLPNPEPPLPEEDEEIVKSVLDGVVPSY
Oase            FISRADSNDSDRDYLVNDDHHRLVTCPPPIVAKLPNPEPPLPEEDEEIVKSVLDGVVPSY
Sansibar        FISRADSNDSDRDYLVNDDHHRLVTCPPPIVAKLPNPEPPLPEEDEEIVKSVLDGVVPSY

               61                                                         120
B. rapa         ALESRLGDCKRAASIRREALQRMTGRSIEGLPLDGFDYDSILGQCCEMPVGYVQIPVGIA
Oase            ALESRLGDCKRAASIRREALQRMTGRSIEGLPLDGFDYDSILGQCCEMPVGYVQIPVGIA
Sansibar        ALESRLGDCKRAASIRREALQRMTGRSIEGLPLDGFDYDSILGQCCEMPVGYVQIPVGIA

               121                      ↓  ↓    ↓    ↓               ↓    180
B. rapa         GPLLLDGYEYSVPMATTEGCLVASTNRGCKAMYVSGGATSTVLKDGMTRAPVVRFASARR
Oase            GPLLLDGYEYSVPMATTEGCLVASTNRGCKAMYVSGGATSTVLKDGMTRAPVVRFASARR
Sansibar        GPLLLDGYEYSVPMATTEGCLVASTNRGCKAMFVSGGATSTVLKDGMTRAPVVRFASARR

               181               202
B. rapa         ASELKFFLESPENFETLAVVFN
Oase            ASELKFFLESPENFETLAVVFN
Sansibar        ASELKFFLESPENFETLAVVFN
```

Comparison of the partial amino acid sequences of *B. rapa*, Oase and Sansibar. *Arrowhead* indicates the position of amino acid with SNP. The third SNP altered the amino acid from tyrosine (Y) in *B. rapa* and Oase to phenylalanine (F) in Sansibar

A.7 Sequencing results

HMG1A02-2

CTCGAGTCCCGTCTTGGAGACTGCAAGAGAGCGGCGACGATACGGCGCGAGGCGCTGCAGAGGATCACCGGGAGGTCGATC
GAAGGGTTACCGTTGGATGGGTTCGATTACGAGTCGATATTGGGGCAATGCTGCGAGATGCCTGTCGGGTACGTGCAGATA
CCCGTGGGGATCGCTGGGCCTTTGTTGCTTGACGGGTATGAGTACTCTGTTCCGATGGCTACGACGGAAGGGTGTTTGGTT
GCTAGCACTAATAGAGGATGCAAGGCTATGTATGTCTCTGGTGGAGCGACGAGTACGGTGGTTAAGGATGGTATGACGAGA
GCGCCTGTGGTTAGGTTCGCGTCGGCGAGGAGGGCTTCGGAGCTTAAGTTTTTCTTGGAGGATCCTGAGAACTTTGACACG
TTGGCTGTTGTCTTCAACAGGTCCATGTCTCATCTCTTTTGATTTGACTTTTTGTGCATATGCATGATGATGACTGATGAG
TCTAGAATGAAGTAGTGTTAGGACATTTGGGTTGGGGTATTTAGGACCAGTGTTAGGACATTTGTCGGATCGAGTCGGTTC
GGACGGGTTTCGATTTTTATAAAACCCGAAGTGGACAAAAGCAAAAAAACCGAAAAAATCACAAAAACCCAAGTTAAACCT
GGAGAGACCGAAAATGATTCAGATAATTTTGGGTTTTTCTGGTTATTTTTTATTTATAAATTATATTATATGTATAGAAAT
TAAAAATAATTAATATTTTTTTTATTATATTTCAAATATTCTGATATCTGTTTGCTTCTTGGTTTTTTGGGTTT

HMG1A02-4

GGTATGAGTACTCTGTTCCGATGGCTACGACGGAAGGGTGTTTGGTTGCTAGCACTAATAGAGGATGCAAGGCTATGTATG
TCTCTGGTGGAGCGACGAGTACGGTGGTTAAGGATGGTATGACGAGAGCGCCTGTGGTTAGGTTCGCGTCGGCGAGGAGGG
CTTCGGAGCTTAAGTTTTTCTTGGAGGATCCTGAGAACTTTGACACGTTGGCTGTTGTCTTCAACAGGTCCATGTCTCATC
TCTTTTGATTTGACTTTTTGTGCATATGCATGATGATGACTGATGAGTCTAGAATGAAGTAGTGTTAGGACATTTGGGTTG
GGGTATTTAGGACCAGTGTTAGGACATTTGTCGGATCGAGTCGGTTCGGACGGGTTTCGATTTTTATAAAACCCGAAGTGG
ACAAAAGCAAAAAAACCGAAAAAATCACAAAAACCCAAGTTAAACCTGGAGAGACCGAAAATGATTCAGATAATTTTGGGT
TTTTCTGGTTATTTTTTATTTATAAATTATATTATATGTATAGAAATTAAAAATAATTAATATTTTTTTATTATATTTCAA
ATATTCTGATATCTGTTTGCTTCTTGGTTTTTTGGGTTTTCGTGTTTAGAGAAATAGAAACAGTTTGAAATTTTTTGTAAA
TTCTGGTCTGGTTTTGGATTTTTTTGTTTGGTTGTTTTCAGGTTTAGGATAATAGTGACTGTGTTACAAAAAAAAGGATAA
TAGTGATTTGATTTAAAGATTTGAAATTTGATCCACTGACGAAGTGTTCTTGTGGTTTGCAGGTCAAG

HMG1A07-4 (Oase)

TTCATCTCACGCGCCGACTCCAACGACTCCGATCGAGATTACCTCGTGAACGACGACCACCACCGCCTCGTCACGTGCCCC
CCTCCGATCGTCGCCAAGCTGCCGAATCCGGAGCCTCCTCTCCCGGAGGAAGACGAGGAGATCGTGAAATCGGTGCTCGAC
GGGGTCGTCCCTTCGTACGCGCTCGAATCTCGCCTCGGGGATTGCAAACGCGCGGCGTCGATAAGGAGAGAAGCGTTGCAG
AGGATGACCGGGAGGTCGATTGAAGGATTGCCGTTGGATGGATTCGATTACGATTCGATCTTGGGGCAATGCTGCGAGATG
CCTGTGGGATACGTGCAGATCCCCGTGGGGATCGCTGGACCGTTGCTGCTCGACGGTTACGAGTACTCTGTTCCCATGGCG
ACGACGGAAGGGTGTTTGGTGGCGAGTACTAACAGAGGGTGCAAGGCTATGTATGTATCCGGTGGTGCGACGAGTACTGTG
CTTAAGGATGGGATGACGAGAGCGCCTGTTGTGAGGTTTGCTTCGGCGAGGAGAGCTTCTGAGCTTAAGTTTTTCTTGGAG
AGTCCTGAGAACTTTGAGACTCTGGCTGTTGTTTTCAACAGGTACAATTGCATGGGAACTGTTTATGGTGTGATTAGGGAC
TAAAGAGGTGATTTTGTGGTTTGCAG

HMG1A07-4 (Sansibar)

TCCAGTCCTTCATCTCACGCGCCGACTCCAACGACTCCGATCGAGATTACCTCGTGAACGACGACCACCACCGCCTCGTCA
CGTGCCCCCCTCCGATCGTCGCCAAGCTGCCGAATCCGGAGCCTCCTCTCCCGGAGGAAGACGAGGAGATCGTGAAATCGG
TGCTCGACGGGGTCGTCCCTTCGTACGCGCTCGAATCTCGCCTCGGGGATTGCAAACGCGCGGCGTCGATAAGGAGAGAAG
CGTTGCAGAGGATGACCGGGAGGTCGATTGAAGGATTGCCGTTGGATGGATTCGATTACGATTCGATCTTGGGGCAATGCT
GCGAGATGCCTGTGGGATACGTGCAGATCCCCGTGGGGATCGCTGGACCGTTGCTGCTCGACGGTTACGAGTACTCTGTTC
CCATGGCGACGACGGAAGGGTGTTTGGTGGCGAGTACTAATAGAGGGTGTAAGGCTATGTTTGTATCCGGTGGAGCGACGA

GTACTGTGCTTAAGGATGGGATGACGAGAGCGCCTGTTGTTAGGTTTGCTTCGGCGAGGAGAGCTTCTGAGCTTAAGTTTT
TCTTGGAGAGTCCTGAGAACTTTGAGACTCTGGCTGTTGTTTTCAACAGGTACAATTGCATGGGAACTGTTTATGGTGTGA
TTAGGGACTAAAGAGGTTATTTTGTGGTTTGCAGGTCGA

HMG1C06-4

TCTGGCTTCAGGCTTGTGTTATATAATTTGCATGGAAGCTTCTTATTAGGGACTAAAGAAGTGATTTTGTGGATGGATGCA
GGTCGAGTAGATTTGCGAGGCTGCAGAGTGTTATGTGCACGCTCGCTGGGAAGAATGCTTATGTGAGGTTCAGTTGTAGTA
CTGGTGATGCTATGGGGATGAACATGGTATCCAAAGGTGTTCAGAATGTTCTTGAGTTTCTTACTGAAGATTTTCCCGATA
TGGATGTCATTGGAATCTCTGGTGAGTTCCTTGAAAGACTCTAGATTTATTGTTTGATGTTATGTTGAGAAGCTATGAAGC
GCTAAAGATTTGATTTTTTCTGTGTGTTTTATTTGTTTCAGGTAACTTTTGTTCGGACAAGAAGCCAGCTGCTGTGAACTG
GATCGAGGGACGTGGCAAATCAGTTGTGTGCGAGGCGGTGATCAGAGGAGAGACCGTGAACAAGGTGTTGAAAACGAGCGT
GGCTTCTTTGGTGGAGCTCAACATGCTCAAGAACCTCACGGGATCTGCTATTGCAGGGTCTCTAGGTGGATTCAACGCTCA
CGCCAGCAACATTGTTTCTGCTGTGTTCTTAGCGACTGGTCAGGATCCAGCTCAAAACGTGGAGAGCTCTCAGTGCATCAC
AATGATGGAAGCCATTAACGACGG

HMG1C02-1

ATCGTTCATCTCACGCGCCGACACCAACGACACCGATGCTAACGATTTCGAAGACCACCGCCTCGTCACGTGCCCACCCAG
GGGCGGACGTAGGTTGGATTTGGAAAGAGGTGTGGCACGTGCCTCTTTAAATAATTAATCTAAAATTTATTTATATATCCT
AATGTTTGTTTATATTACATTTTCCGCCCCATACTAAATTACTCTCTAGATCCGCCCCCACCACCGATCGTCTGCGTCGCC
AAATCACCGATTCCGGATCCCGCGCTCCCCGAGGAAGACGAGGAGATAGTGAAATCCGTAATCCACGGCGCGATCCCTTCC
TACTCTCTCGAGTCTCGTCTCGGAGATTGCAAGAGAGCGGCGACGATACGGCGCGAGGCGCTGCAGAGGATAACCGGGAGG
TCGAT

HMG2A10-1 (Sansibar)

TCATGGCATCCTTCATGAGAAAACTGAAAGCACCACCAGACAAGTGAATAGCCTTGCATCCTCTGTTCGTGCTTGCAACCA
AACACCCCTCCGTCGTCGCCATTGGCACCGAGTACTCTTTCCCGTCGAGCAACAGAGGTCCGGCGATCCCCACCGGAATCT
GAACGTACCCAACCGGCATCTCGCAGCACTGTCCCAAGATCGAATCGTAATCGAAACCGTCCAACGGAAGTCCGGTTAAGG
ATTTTCCGGTCATCCTCTGAACCGCTTCTCGTCTAATCGCCGCGGCTCGCTTGCAGTCTCCGAGCTTCGTCTCGAGCGAGT
GCGACGAGGTGGCTCCGTCGACCACGGACTTGACGATCGCTTCGTCTTCCTCGGAAGTAAGATCAGACTCAATCTTGACGA
GGCGATCGATCTCGTCGTCGTTGGGGATCCAAACGTCGTCGTCGTCGTCGGAGGATGATCGGAGGAAGACGGATTGAACGA
AGTCGACGCCGAGGAAGCCGATGAGGTAGATGCAGGAGGCGATGACTCCAATGAGTGAGAGGATCTCGGAGAGGTTGACGA
CGTGAAAAAGGCGTCAACCTCTCCGAGATCCTCTCACTCATTGGAGTCATCGCCTCCTGCATCTACCTCATCGGCTTCCTC
GGCGTCGACTTCGTTCAATCCGTCTTCCTCCGATCATCCTCCGACGACG

HMG2A10-2 (Sansibar)

GAACCAGTAAGGTTCTTGAGCACGTGAAGCTCGACAAGATCCTCCACGGTAGTCTTCAACACATTCCTCACAATCCCACCT
TTAATAAGGGCTTCACACACAACATGCTTCCCACGTCCTTGGATCCAGTTTATAGCCGAAGCTTTCTTGTCGGAACAGCAA
TTCCCTGAACACCAAATTAACACCACGAATCAAAAGACCTGACTTTAATCCAGAAACCAAGACAAGGAAAGAGTAGACTAC
TACAAACCTGAGATGCCAATCACAACCATGTCAGGGAACATTGTCTTCATAAACTCCAAGACATTCTGAACGCCTTTGGAG
ACCATGTTCATCCCCATAGCATCGCCAGTACCACACGCAAACCTCGGATAGAGATTCCTCCCAGAAATCGAGCAAGTGATA
CTCTGAAGCCAAGCAAATCTACTCGATCTACAAACAAACAAACACACACAATGGTCAATACAAAATCACACCATCCTAGCT
TAGAAGCTTAGACAAAGTAGTCAAAACTTTACTTGCTGAAAATAAGAGAGAGTCTCTCGAAATTAGCAGGATCCTGTAGAT
AAAACATGGCACGAGCAGCTCTTTTCACAGAAGGGAACTTAACAACAGGAGCTCTAGTCATGGCATCCTTCATGAGAAAAC
TGAAAGCACCACCAGACAAGTGAATAGCCTTGCATCCTCTGTTCGTGCTTGCAACCAAACACCCCTCCGTCGTCGCCATTG
GCACCGAGTACTCTTTCCCGTCGAGCAACAGAGGTCCGGCGATCCCCACCGGAATCTGAACGTACCCAAC

HMG2C07-1

ACGTCGTTAACCTCTCCGAGATCCTCTCACTCATTGGAGTCATCGCGTCGTGCATCTACCTCATCGGCTTCCTCGGCGTCG
ACTTCGTTCAATCAGTCTTCCTCCGATCATCCTCCGACGACGACGACGGTTGGATCCCAAACGACGACGAGATCGATCGCC
TCGTCAAGATTGAGTCTGATCTTACTTCCGAGGAAGACGAAGAGATCGTCAAGTCCGTGATCGACGGAGCCACCTCGTCGT
ACTCACTCGAGACGAAGCTCGGAGACTGCAAGCGAGCCGCGGCGATTAGACGAGAAGCGGTTCAGAGGATGACCGGAAAAT
CCTTAACTGGACTTCCGTTTGACGGTTTCGATTACGATTCGATCTTGGGACAGTGCTGCGAGATGCCGGTTGGGTACGTTC
AGATTCCGGTGGGGATCGCCGGACCTCTGTTGCTCGACGGGAAAGAGTACTCGGTGCCAATGGCGACTACGGAGGGTTGTT
TGGTTGCAAGCACTAACAGAGGATGCAAGGCTATTCACTTATCTGGTGGTGCTTTCAGTTTTCTCATGAAGGATGCGATG

HMG2C07-2

AGAGGATGCAAGGCTATTCACTTATCTGGTGGTGCTTTCAGTTTTCTCATGAAGGATGCGATGACTAGAGCTCCTGTTGTT
AAGTTCCCTTCTGTGAGAAGAGCTGCTCGTGCCATGTTTTATCTACAGAATCCTTCTAATTTCGAGAGACTCTCTCTGATT
TTCAGCAAGTAAAGTTTTGAACTTTGTCTAAGCTTCTAAGCTAGGATGGTGATTTTGTATTGACATTGTGTGTGTGTTTT
GTTTGTTTGTAGATCGAGTAGATTTGCTTGGCTTCAGAGTATCACATGCTCGATTTCTGGGAGGAATCTCTATCCGAGGTT
TGCGTGTGGTACTGGCGATGCTATGGGGATGAACATGGTCTCCAAAGGCGTTCAGAATGTCTTGGAGTTTATTAAGACAAA
GTTCCCTGACATGGTTGTTATAGGCATCTCAGGTTTGTAGTCTACTCTTTACTTGTCTTGGTTTCTGGATTAAAGTCAGGT
CTTTTGATTCGTGTTGTTAATTTGGTGTTCAGGGAATTGCTGTTCCGACAAGAAGGCTTCGGCTATAAACT

HMG2C07-3

CTTGCATTTGCATTAGGTCATATCTGTATGTATAATCATTATCAATTAGATCAATACAGTTTTGTAAAACGCATATGAAAT
GTCTGAAATTTTCTATTTTTTTCATTTTTTAAGTCAATAGATAAGAAATTTATTTTGAAAATTCCTTGCTTTTAATGGGAT
AATATTGTAAGAAAAAAACAATTGAAGAGGAAAACCTCACGTTATCTATCACTTTCAATAGTTTTTTGGTCTCCATTAAAA
AGGATAATTATTTTTAATATTTTAGGCTCAACAGAATTTTTTAAAATTCTATAGATTTAATTTTCAATTAATAAACAAACT
AAAAGATGCCTAAATCTGTAAACTTTTAAACTAAGGACAAAAGAATAAACTCAAAAAGGAATATTAATTAATTTCAACTAA
CAATTAAGAATTAATAGAAGTAGTTAGCAAGATAGAGACGAAGGCCAAATCATCTCCTCTCTCCCAAACAAACATTAAACT
CTTGAGACTTGACGTTGATTTCGATGTCCACCACTACCACCACCCCAATATATATATATATATATAAACCCCTCCAAAACC
TAAAGATTTCATAATCACATCTCCGTTTATAAAAAGAAAAAAAGACCGTCAATGGAAATCCATCGAAGATTACCGAGTAGA
GAACTCCCAACACCAAACGACGACTTTG

HMGSUNC-3

CTCCATCAATGATGCAGACTACTTTGTATTCCATTCTCCATACAACAAAGTAATCTCTTAACCCTCCCAACAACAGTTTTG
TTCTTTGCCTGAACCGTCTCTGACCAGTCATCTCTTCTTTGCTGTTGCAGCTTGTACAGAAAAGCTTTGCTCGTCTCTTGT
ACAACGACTTCTTGAGAAACGCAAGGTTCTTGCTCTGGATGAAAGATTCTTAAACTTGTTTATATTAATTGAGTTAAACAT
ATCTGTTTCCTCTATTTCAGCTCCATTGATGAGGCTGCCAAAGAGAAGTTCACTCCTTATTCGTCTTTGCCACTCGACGAG
AGTTACCAAAGCCGTGATCTTGAAAAGGTTTTCATCTTTTGTTTTTTTGTGATACTTGGAAAAAGCAAAACCAGAGTTATT
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ACCAAAAGAAGTTGGTAACATGTACACCGCTTCTCTCTACGCAGCTTTTGCTTCTCTCATCCACAACAAACACAGCGATTT
GGTAAACTAAAGAAAACAAACTTTTCTGAGTCTCTTGAAATTTATTATTTACATTTTTGTTTTTTTGTATAGGCGGGGAAG
AGGGTGGTTATGTTCTCATATGGAAGTGGTTCAACCGCAACGATGTTCTCATTGCGTCTCTGCGAAAACCAGTCTCCTTTC
AGCCTCTCAAACATTGCATCTGTGATGGACATCGGTGGTAA

HMGSUNC-5

ACGCTACTCTATCATATGAAAACTAAACGCAATGGCAAAGTTATATATAAACTTATAACTTTGGCATTGCTATAATAAATT
AAGGAAAATGGAAAGGGATAAATAATGAAATAAAAAAGAGAAGCACGAGGGAGGGAGGTAACTCACGCGGTCTGCGTTGTC
CAGATTCCGGAGGAAACAACTCCTGCCCCCCAACCTCTCTCTCTCTCACGCACACATACAAACACACTTTATCTTATTTTC
CATTTATTTTTCGACACATAAATCTTTATTTTTTTTAATATACAGATAAATCTTAATTGTATATTTCAGATAGCTATTTAT

TCTTTTTGACGTGTAATTGTCCCACACGGAGGAAATTTTTTTTTTAATCAATTAATATATAAGGACATATTAGTAATTAAT
TATCCAGAATTTTAATTTTTGAACACAAAAAAAAAAGATTTTAAAACACAAGTGTACAAGAGTATTGTGTTTGACCGAAAC
AAAACATACAACTAATTATTAAATTATTTAGGAAAATAAAAAGAGAAAAATCCACCTAATATCTCTCCTGGATTTGTTATT
AAAAAACAAATTAAGTTTCGTTTCTATTTTCAACCGATTCATATACTATTTCTTTTATAGAGCTTCAAATCTCTCTCTCTC
ATTCTCTTCTGCTAAGATCAAACCTT

HMGSA07-1

CAACAGTTTTGTTCTTTGCCTGAACCGTCTCTGACCAGTCATCTCTTCTTTGCTGTTGCAGCTTGTACAGAAAAGCTTTGC
TCGTCTCTTGTACAACGACTTCTTGAGAAACGCAAGGTTCTTGCTCTGGATTGAAAGTTTCCTAAACTTGTTTATATTAAT
TGAGTTAAACATATCTGTTTCCTCTATTTCAGCTCCATTGATGAGGCTGCCAAAGAGAAGTTCACTCCTTATTCGTCTTTG
TCACTCGACGAGAGTTACCAAAGCCGTGATCTTGAAAAGGTTTTTCATCTTTTTTTTTTTTTTTGATATTGGAAAAAGCAA
AACCAGAGTTATTATAGCTTTGTTGTCTTTTGCAGGTGTCACAACAAGTTGCAAAAACGTTTTATGATGCTAAAGTGCAAC
CAACGACTTTAGTACCAAAAGAAGTCGGTAACATGTACACTGCTTCTCTCTACGCAGCTTTTGCTTCTCTCATCCACAACA
AACACAGCGATTTGGTAAACTATAAAAATAACAAACAAACTTTTTCTTCTCACTTTCT

SMT1A03-2

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AATGTCTTAAAGTGCTGAAAACATATATTAGTAGGATTATATACCATGTCAGTGTAATTAGCTTTTCGCTCTTCCTCGTCT
CCTCCGTGGAATTCATGATATTGCTCATACCTGAATCGTCGTCGAATCACTAAAATTTAGTATAATGTCCAGTAAGAAAAT
CATCGTCTAGACGCATACATTTCTACATCTTCGAGAGAGATGAAACTAGAAGCAACCCAATATTTTTTTTTTGTTTTAAAA
TCTCAATCTTTTTAACCAAAAGTCGTTAATGGCCAAGAGAAAAAACAACCAGCCGGGCCCTCCAGAGCGTGACAACAGCGA
AATACACGTCAAGAACAGGGACCATCTCAGTATCCCTTCCTTTTTCTACTGTATCGAAAGCTCACTTCCAAAGAATATTCA
ATGCATTATTTTCCATTAATTGGCAAACAAACAAACAAAAAAATGGATAGAAATAGATTTTCGTAACGATATAGTGGATCC
ACAATATCAATATGCATGGTAAAGAAAACGAGTGTTGTTAAGTGAATCGGAGAACGAAACTACGTTTACGATCGAGGAAAC
AAACGTACTTCTCGACGGCGGTGAGAACATCGGATTTATCGATCTTGCCACCAAGGTTCGAGGC

SMT1C03-1

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GTTAAACCAGGACAAAAGGTTTGATAACTTTGGCTGAGCGCTCGAAAAGTGGAACCTTCACCATCAGTTGCTCATTTCATC
CCTTTATGTTTCTCAACAGGTACTGGATGTAGGGTGTGGAATTGGTGGACCACTGAGAGAAATTGCACGATTCAGGTAAAA
TGATCACACGTTATTATTTACATCCTCTCTACATTAGATTTAGAGTACATGTCATGGTTAGGAAAATATTTCAATAATATA
GATTCTGTTTGTGTTTGCTCTGTTACAGCAATTCATCTGTTACCGGGATCAACAATAATGAATACCAGATCACCAGGGGCA
AGGTAACATATCTATATTTGTGGAATATAATGCATTTTCTGTCATATAATTGGTTGCTGGAATTTCTTGCAAGTGTTATTT
CCTGTCTCTTTGAACTTGATTTTGATTGATTTCAGAATGTGGATTATATGACTATAAATTCCATGATTGATATCTGGTTTT
TTTTTATAATTCATATTGGCATCCCTCTTCCTCGCAGGAGCTAAACCGACTTGCAGGTGTCGACAAGACATGTAACTTTGT
CAAGGTCAGTTTAATTCAAGTTTCTTGTACTTACTACATAAGTTGTAAAATCCTGGGTTGATAAGAAAAAAACCTTGTAAC
GGGCAACGTGATGAATTTGTAGG

SMT1UNC-1

CAATGTTTCGCTGCCTATGAGTGGTGCATGACTGATGCATTTGACCCTGCTAACGCCCAGCATCAGAAAATAAAGGTCTAG
TGTGGTCTCTTGCATTATATATCCATATTATCTTTGCATCTATTGTAATACAAACAAATACTCTGTTGTGCAGGCAGAGAT
AGAGATTGGAGATGGTCTCCCTGACATTAGGCTGACTTCAAAATGCCTCGAAGCCCTGAAACAGGCCGGTTTTGAAGTAAG
TATCTGACTACAGATCCCTCTCTATTTCCAAACGTAAAACATTTTAACTATAATTAGTACTATTATCATCGGCTTTGGATA
TAAGGTGATATGGGAAAAGGATCTGGCGGAGGACTCGCCAGTCCCATGGTACTTACCTCTGGACAAAAACCACTTCTCGCT
CAGTAGCTTCCGCCTAACAGCTGTTGGACGGTTCATAACCAAAAACATGGTAACCACCAAAAACTTGCTCTGTCTCATCTG
CCTAATTTCATTCATTAACTACTGATTGTTCATACATCTGAACTTGCCATTGTTATTTATATAGGTGAAGGTCCTTGAGTA

CATAAGACTTGCACCTAAAGGAAGCCAAAGGGTGTCAGATTTTCTGGAGAAGGCCGCAGAAGGATTAGTCGACGGTGGAAG
GTGAGAGGATCCCAGCTTTATATATATATATTCAAACTAATTTTGATATACTAAATTAATTACTAGTGTCAACTTCTAAAT
CTATGTGTGTTAACAGGAGGGAGATTTTCACGCCAATGTATTTCTT

SMT2A06-1

TCTCCTTCCATCCGAGGCAAATCCCACCGTGACGCCACGCGCCTCCACGAAGAGATGGCCGTAGATCTGATCCAAGTGAAA
CCGGGTCAAAAGATCCTCGATGTCGGATGCGGCGTGGGAGGTCCGATGCGCGCGATTGCATCCCACTCGCGAGCCAACGTG
GTGGGGATCACGATCAACGAGTACCAGGTGAAGAGAGCGCGTGACCACAACAGGAAAGCAGGACTCGACGCGCTCTGCGAG
GTCGTGTGTGGTAACTTCCTCCAGATGCCCTTCGATGATAATACCTTCGACGGCGCGTACTCTATCGAAGCGACGTGTCAC
GCGCCGAACCTGGAAGAAGTCTACGCCGAGATCTACAGGGTGTTGAAACCCGGATCTTTGTATGTATCGTACGAGTGGGTC
ACCACTGATAAGTTCAACGCCCAGGATGAGGAACACGTGGAGGTCATCCAAGGGATCGAGAGGGGTGACGCGCTTCCTGGC
CTTAGGGCTTACTCCGATATAGCCCAGGCCGCCAAGAAAGTTGGGTTCCAAATTGTCAAGGAGAAGGATCTTGCCGCTCCA
CCGGCTCAGCCGTGGTGGACTAGGCTTAAGATGGGTCGCCTCGCTTATTGGAGGAACCACGTTGTCGTTCAGATCTTGTCT
GCCGTTGGA

SMT2A06-2

ACTCTTCTTCACTGGAGCTCTCGTAGCCGGCGGAATCTACTGGTTCCTGTGCGTCCTGGGACCAGCGGAGCGTAAAGGCAA
ACGAGCGTTGGATCTATCGGGCGGGTCCATCTCGGCGGAGAAAGTTCAAGACAAGTACAAACAGTACTGGTCGTTCTTCCG
CCGTCCGAAAGAGATCGAAACCGCCGAGAAAGTCCCGGACTTCGTGGACACGTTCTACAACCTCGTCACCGACATCTACGA
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GATGGCCGTAGATCTGATCCAAGTGAAACCGGGTCAAAAGATCCTCGATGTCGGATGCGGCGTGGGAGGTCCGATGCGCGC
GATTGCATCCCACTCGCGAGCCAACGTGGTGGGGATCACGATCAACGAGTACCAGGTGAAGAGAGCGCGTGACCACAACAG
GAAAGCAGGACTCGACGCGCTCTGCGAGGTCGTGTGTGGTAACTTCCTCCAGATGCCCTTCGATGATAATACCTTCGACGG
CGCGTACTCTATCGAAGCGACGTGTCACGCGCCGAACCTGGAAGAAGTCTACGCCGAGATCTACAGGGTGTTGAAACCCGG
ATCTTTGTATGTATCGTACG

SMT2C05-1

CCAGATGCCCTTCGATGATAACACCTTCGACGGCGCGTACTCTATCGAAGCGACGTGTCACGCGCCGAAGCTGGAAGAAGT
CTACGCCGAGATCTACAGGGTGTTGAAACCCGGATCTTTGTACGTATCGTACGAGTGGGTCACCACTGATAAGTTCAACGC
CGAGGATGAGGAACACGTGGAGGTCATCCAAGGCATCGAGAGGGGTGACGCGCTTCCTGGGCTTAGGGCTTACTCGGATAT
AGCCGAGGCCGCCAAGAAAGTTGGCTTCCAAGTTGTCAAGGAGAAGGATCTAGCGGCTCCACCGGCTGAGCCGTGGTGGAC
TAGGCTTAAGATGGGTCGGCTCGCTTATTGGAGGAACCACATTGTGGTTCAGATTCTGTCTGCTGTTGGAGTTGCGCCTAA
GGGAACCGTCGATGTTCACGAGATGTTGTTTAAGACTGCTGAT

SMT2C05-2

CTTCTTCACCGGAGCTCTCGTAGCCGGCGGAATCTACTGGTTCCTGTGCGTCCTGGGACCAGCGGAGCGAAAAGGAAAACG
AGCGTTGGATCTATCGGGGGGGTCCATCTCGGCCGAGAAAGTTCAAGACAAGTACAAACAGTACTGGTCGTTCTTCCGCCG
TCCGAAAGAGATCGAAACCGCCGAGAAAGTCCCGGACTTCGTCGACACGTTCTACAACCTCGTCACCGACATCTACGAGTG
GGGATGGGGACAGTCCTTCCACTTCTCTCCTTCCATCCGAGGCAAATCCCACCGCGACGCCACGCGCCTCCACGAAGAGAT
GGCCGTAGATCTGATCCAAGTGAAACCGGGTCAAAAGATCCTCGATGTCGGATGCGGCGTCGGAGGTCCGATGCGCGCGAT
TGCATCCCACTCGCGAGCCAACGTGGTGGGGATCACGATCAACGAGTACCAGGTGAAGAGAGCGCGTGACCACAACAGAAA
AGCAGGACTCGACGCGCTCTGCGAGGTCGTGTGTGGTAACTTCCTCCAGATGCCCTTCGATGATAACACCTTCGACGGCGC
GTACTCTATCGAAGCGACGTGTCACGCGCCGAAGCTGGAAGAAGTCTACGCCGAGATCTACAGGGTGTTGAAACCCGGATC
TT

SMT2C05-3

GTTGATCATTATCCTCCCTCCAATCAACATCAAAATTAGGATCATGTATGCTTGATTTACGTACGAAGTAATGTAGTGTCA
CAGTTGCTGCCACGAGCTTTCTTGTTGTTCCAATGTCGTACCTCATTTTATTTTGAGTACATTTCCATTTCCAAATATCAA
ATGTTCTTTCAATGACACATCGTAGTGGAGAATGACAACGATTAAATTTTACAAATTACTCTGGATATAAATTTTTTGTTA
AGTCTTGATCGCTTTAGATTTAAATTTAGATTTAGTGTTGTGAATATAAATGTTGTTGTGTAGAGTTCCCACAGCCGTTAC
TAATCATTTTCATAGAGATTCTTTCGAAAATGCTCTGAACAGTACATATTAGAGGTTTGTTTCTTTAGATTAATTACTAAA
CTGTAATTACACTGAATGATCTTTTGTTTCTTTAGATTAATTACTAGCAGTAAATTTTCATTTGACTAAGCGTTTTTTTTT
TCTTCTTGGATATATACTTGTTCACTGAAAAAAAAAATCTTTTTAAATTGTTCAAGTCAAAGGCCAAATCAGGCTTCTT

A.8 Linkage map of SODH population

Linkage map of SODH population. The full map comprises of high fidelity markers (highlighted in grey), framework markers (indicated in bold and highlighted in grey), and placed markers (indicated in normal font). High fidelity markers are a subset of markers in which all marker orders are supported by a minimal log-likelihood difference of 3.0 with a maximal distance to the previous marker of 30 cM. Framework markers are selected from high-fidelity markers for QTL mapping. Placed markers are markers placed individually relative to the high-fidelity markers at their most likely position which are supported at a LOD score of less than three.

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
A01				
1	1	-1.3	3116018 | F | 0	SilicoDArT
2	**2**	**0.0**	**3091853 | F | 0**	**SilicoDArT**
3	3	5.3	3143480 | F | 0	SilicoDArT
4	4	5.5	3129410 | F | 0	SilicoDArT
5	5	5.7	3085018 | F | 0	SilicoDArT
6	6	5.7	3095806 | F | 0	SilicoDArT
7	7	6.2	100000505 | F | 0	SilicoDArT
8	**8**	**6.8**	**3083750 | F | 0**	**SilicoDArT**
9	9	8.4	ra00486s01	SNP
11	**11**	**10.0**	**CB10097b**	**SSR**
10	10	10.0	CB10097a	SSR
12	12	11.8	3101886 | F | 0	SilicoDArT
13	13	11.8	3083038 | F | 0	SilicoDArT
14	14	13.5	100002928 | F | 0	SilicoDArT
15	**15**	**16.2**	**3091606 | F | 0**	**SilicoDArT**
16	16	16.7	100002918 | F | 0	SilicoDArT
17	17	18.3	3083677 | F | 0	SilicoDArT
18	**18**	**20.1**	**3094873 | F | 0**	**SilicoDArT**
19	19	23.9	ra00268s01	SNP
20	20	24.0	3124008 | F | 0	SilicoDArT
21	**21**	**32.4**	**CB10099**	**SSR**
22	**22**	**34.7**	**E39M48-194**	**AFLP**
23	23	40.0	3082222 | F | 0	SilicoDArT
24	24	44.3	3111529 | F | 0	SilicoDArT
25	25	44.3	3109073 | F | 0	SilicoDArT
26	**26**	**45.3**	**3115206 | F | 0**	**SilicoDArT**
27	27	47.9	3154845 | F | 0	SilicoDArT
28	28	50.0	3184971 | F | 0	SilicoDArT
29	29	50.3	brPb-660511	DArT
30	30	52.2	BNKS003001	KASP
31	31	52.7	100002260 | F | 0	SilicoDArT
32	**32**	**53.8**	**100000853 | F | 0**	**SilicoDArT**
33	33	56.5	3144953 | F | 0	SilicoDArT
34	34	56.5	3101303 | F | 0	SilicoDArT
35	35	57.0	3075518 | F | 0	SilicoDArT
36	**36**	**57.5**	**E38M59-285**	**AFLP**
37	37	64.2	100000242 | F | 0	SilicoDArT
38	38	64.2	3109845 | F | 0	SilicoDArT
39	39	64.2	3106173 | F | 0	SilicoDArT
40	**40**	**64.8**	**3094588 | F | 0**	**SilicoDArT**
41	41	66.4	3150404 | F | 0	SilicoDArT
42	**42**	**68.0**	**3133092 | F | 0**	**SilicoDArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
43	43	69.1	3095557 | F | 0	SilicoDArT
44	44	69.3	ra00193s01	SNP
45	45	69.3	100006185 | F | 0	SilicoDArT
46	46	69.4	3173412 | F | 0	SilicoDArT
47	47	70.0	3136802 | F | 0	SilicoDArT
48	48	70.1	ra00519s01	SNP
49	49	71.7	100004900 | F | 0	SilicoDArT
50	**50**	**73.3**	**brPb-661536**	**DArT**
51	51	73.7	brPb-661298	DArT
52	52	73.7	brPb-662426	DArT
53	53	74.0	100001595 | F | 0	SilicoDArT
54	54	74.0	3110311 | F | 0	SilicoDArT
55	55	74.1	3128249 | F | 0	SilicoDArT
56	56	77.1	3085239 | F | 0	SilicoDArT
57	57	77.6	3101219 | F | 0	SilicoDArT
58	58	77.7	3107527 | F | 0	SilicoDArT
59	**59**	**78.9**	**3195628 | F | 0**	**SilicoDArT**
60	60	81.0	3093342 | F | 0	SilicoDArT
61	61	86.7	100001355 | F | 0	SilicoDArT
62	62	87.0	100003913 | F | 0	SilicoDArT
63	63	87.1	3083685 | F | 0	SilicoDArT
64	64	87.4	3157600 | F | 0	SilicoDArT
65	65	87.5	3215074 | F | 0	SilicoDArT
66	**66**	**88.5**	**3114358 | F | 0**	**SilicoDArT**
67	67	89.9	100000381 | F | 0	SilicoDArT
68	68	90.6	100000607 | F | 0	SilicoDArT
69	69	90.9	3168233 | F | 0	SilicoDArT
70	70	91.0	brPb-657961	DArT
71	71	91.0	3124933 | F | 0	SilicoDArT
72	**72**	**92.2**	**brPb-839886**	**DArT**
73	73	93.3	3111870 | F | 0	SilicoDArT
74	74	93.7	3128859 | F | 0	SilicoDArT
75	**75**	**96.7**	**CB10572**	**SSR**
A02				
76	**1**	**0.0**	**ra00578s01**	**SNP**
77	2	2.8	ra00241s01	SNP
78	3	7.0	3143354 | F | 0	SilicoDArT
79	4	7.3	3123526 | F | 0	SilicoDArT
80	**5**	**8.7**	**brPb-809917**	**DArT**
81	6	8.7	3144140 | F | 0	SilicoDArT
82	7	9.3	3103750 | F | 0	SilicoDArT
83	8	9.9	100000348 | F | 0	SilicoDArT
84	9	10.4	3102285 | F | 0	SilicoDArT
85	10	10.7	3159697 | F | 0	SilicoDArT
86	**11**	**11.7**	**100005036 | F | 0**	**SilicoDArT**
87	12	14.6	3091433 | F | 0	SilicoDArT
88	13	15.3	100001830 | F | 0	SilicoDArT
89	14	15.9	3110832 | F | 0	SilicoDArT
90	15	16.6	100001738 | F | 0	SilicoDArT
91	16	17.8	E40M50-120	AFLP
92	17	18.3	ra00496s01	SNP
93	**18**	**21.4**	**E38M51-157**	**AFLP**
94	19	22.3	E38M51-159	AFLP
95	20	23.6	3080289 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
96	21	23.6	100002458 | F | 0	SilicoDArT
97	22	24.1	brPb-660621	DArT
98	23	24.3	3127843 | F | 0	SilicoDArT
99	24	24.5	3085028 | F | 0	SilicoDArT
101	**26**	**24.9**	**brPb-670777**	**DArT**
100	25	24.9	brPb-659698	DArT
102	27	24.9	3117488 | F | 0	SilicoDArT
103	28	25.2	100003456 | F | 0	SilicoDArT
104	29	25.4	3168423 | F | 0	SilicoDArT
105	30	25.9	3111880 | F | 0	SilicoDArT
106	31	26.4	3128181 | F | 0	SilicoDArT
107	32	27.0	100001659 | F | 0	SilicoDArT
108	**33**	**27.9**	**3081349 | F | 0**	**SilicoDArT**
109	34	30.0	3133746 | F | 0	SilicoDArT
110	35	30.5	3084341 | F | 0	SilicoDArT
111	36	31.7	3094351 | F | 0	SilicoDArT
112	**37**	**40.7**	**brPb-839048**	**DArT**
A03				
113	**1**	**0.0**	**3131550 | F | 0**	**SilicoDArT**
114	2	11.8	3151622 | F | 0	SilicoDArT
115	3	13.6	3210571 | F | 0	SilicoDArT
116	4	14.6	3085579 | F | 0	SilicoDArT
117	5	15.0	3079212 | F | 0	SilicoDArT
118	6	15.1	3095947 | F | 0	SilicoDArT
119	7	15.2	3101888 | F | 0	SilicoDArT
120	8	15.3	3157144 | F | 0	SilicoDArT
121	9	16.0	3160113 | F | 0	SilicoDArT
122	10	16.1	100000304 | F | 0	SilicoDArT
123	11	16.2	100002646 | F | 0	SilicoDArT
124	12	16.6	3107540 | F | 0	SilicoDArT
125	13	16.8	3095727 | F | 0	SilicoDArT
126	**14**	**20.1**	**E32M48-354**	**AFLP**
127	15	21.4	brPb-670404	DArT
128	16	21.4	100000481 | F | 0	SilicoDArT
129	17	21.8	3191853 | F | 0	SilicoDArT
130	18	22.1	3190259 | F | 0	SilicoDArT
131	19	22.2	3168849 | F | 0	SilicoDArT
132	20	22.6	3190375 | F | 0	SilicoDArT
133	21	22.6	3113490 | F | 0	SilicoDArT
134	22	23.6	brPb-657959	DArT
135	**23**	**24.0**	**brPb-842179**	**DArT**
136	24	24.5	brPb-660232	DArT
137	25	26.3	brPb-661557	DArT
138	**26**	**30.7**	**ra02449s01**	**SNP**
139	**27**	**34.4**	**ra02025s01**	**SNP**
140	**28**	**42.0**	**ra00558s01**	**SNP**
141	29	46.9	BNKS003004	KASP
142	30	53.6	3092169 | F | 0	SilicoDArT
143	31	54.7	ra04368s01	SNP
144	32	61.3	3171705 | F | 0	SilicoDArT
145	33	62.3	3214361 | F | 0	SilicoDArT
146	34	62.3	3122883 | F | 0	SilicoDArT
147	35	62.4	3099613 | F | 0	SilicoDArT
148	36	62.9	3083027 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
149	**37**	**63.5**	**brPb-671217**	**DArT**
150	38	63.5	3192350 | F | 0	SilicoDArT
151	39	64.5	3148990 | F | 0	SilicoDArT
152	40	66.7	3082832 | F | 0	SilicoDArT
153	41	67.9	3126915 | F | 0	SilicoDArT
154	42	71.0	ra00527s01	SNP
155	43	72.1	100003668 | F | 0	SilicoDArT
156	44	72.2	3100448 | F | 0	SilicoDArT
157	45	74.1	100000670 | F | 0	SilicoDArT
158	46	76.1	3122968 | F | 0	SilicoDArT
159	47	76.1	3104408 | F | 0	SilicoDArT
160	48	76.5	3133727 | F | 0	SilicoDArT
163	**51**	**77.1**	**brPb-838885**	**DArT**
161	49	77.1	brPb-658964	DArT
162	50	77.1	brPb-807766	DArT
164	52	77.6	100000743 | F | 0	SilicoDArT
165	**53**	**82.0**	**ra02551s01**	**SNP**
166	54	82.3	ra02552s01	SNP
167	55	83.4	ra00721s01	SNP
168	56	89.6	3103433 | F | 0	SilicoDArT
169	57	90.9	BNKS003003	KASP
170	58	91.0	3110228 | F | 0	SilicoDArT
171	59	97.4	3218631 | F | 0	SilicoDArT
172	60	100.2	3107225 | F | 0	SilicoDArT
173	61	100.6	100001790 | F | 0	SilicoDArT
174	62	101.3	3107173 | F | 0	SilicoDArT
175	63	101.4	3086942 | F | 0	SilicoDArT
176	64	101.8	3102441 | F | 0	SilicoDArT
177	65	101.9	3134197 | F | 0	SilicoDArT
178	66	102.0	3216516 | F | 0	SilicoDArT
179	67	102.0	3081514 | F | 0	SilicoDArT
180	68	102.0	100000697 | F | 0	SilicoDArT
181	69	102.1	3204743 | F | 0	SilicoDArT
182	70	102.4	3079868 | F | 0	SilicoDArT
183	71	102.5	3141063 | F | 0	SilicoDArT
184	72	102.8	brPb-808787	DArT
185	73	102.9	brPb-660346	DArT
186	74	103.1	brPb-662347	DArT
187	75	103.1	brPb-809799	DArT
188	76	103.6	BNKS00092	KASP
189	**77**	**108.2**	**ra02122s01**	**SNP**
190	78	111.0	ra00584s01	SNP
191	79	111.0	ra01317s01	SNP
192	80	115.8	ra00513s01	SNP
193	**81**	**117.3**	**ra03222s01**	**SNP**
194	82	121.7	3118779 | F | 0	SilicoDArT
195	83	123.3	3104943 | F | 0	SilicoDArT
196	84	123.7	3115329 | F | 0	SilicoDArT
197	85	132.4	3129789 | F | 0	SilicoDArT
198	86	133.0	3080926 | F | 0	SilicoDArT
199	**87**	**143.6**	**ra00720s01**	**SNP**
200	88	148.3	E37M50-393	AFLP
201	89	148.6	3144045 | F | 0	SilicoDArT
202	90	148.7	3105670 | F | 0	SilicoDArT
203	91	148.8	100001823 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
204	92	149.2	100020227 | F | 0	SilicoDArT
205	93	150.4	100004251 | F | 0	SilicoDArT
206	**94**	**156.2**	**ra00109s01**	**SNP**
207	**95**	**159.9**	**E37M50-87**	**AFLP**
208	96	162.8	3083532 | F | 0	SilicoDArT
209	97	162.8	3145944 | F | 0	SilicoDArT
210	98	163.0	brPb-660733	DArT
211	99	163.1	brPb-661038	DArT
212	100	163.1	brPb-661655	DArT
213	101	163.1	100008069 | F | 0	SilicoDArT
214	102	163.1	3101876 | F | 0	SilicoDArT
215	103	163.1	3164474 | F | 0	SilicoDArT
216	104	163.2	brPb-661458	DArT
217	105	163.2	3199200 | F | 0	SilicoDArT
218	106	163.2	100000292 | F | 0	SilicoDArT
219	107	163.3	3084401 | F | 0	SilicoDArT
220	108	163.4	brPb-658102	DArT
221	109	163.5	3092220 | F | 0	SilicoDArT
222	110	163.6	100002001 | F | 0	SilicoDArT
223	111	163.9	3101206 | F | 0	SilicoDArT
224	112	166.4	3104803 | F | 0	SilicoDArT
225	113	166.5	3128719 | F | 0	SilicoDArT
226	114	166.5	3205802 | F | 0	SilicoDArT
227	115	166.7	3155103 | F | 0	SilicoDArT
228	116	166.8	3103559 | F | 0	SilicoDArT
229	117	166.8	3099386 | F | 0	SilicoDArT
230	118	167.3	3098582 | F | 0	SilicoDArT
231	119	167.4	100002293 | F | 0	SilicoDArT
232	120	167.8	3097691 | F | 0	SilicoDArT
233	121	168.2	3086380 | F | 0	SilicoDArT
234	**122**	**172.2**	**E44M51-62**	**AFLP**
235	123	174.5	3082651 | F | 0	SilicoDArT
236	124	174.5	E39M48-253	AFLP
237	125	174.7	3150452 | F | 0	SilicoDArT
238	126	175.0	CB10425b	SSR
239	127	175.2	100000301 | F | 0	SilicoDArT
240	128	175.4	100002192 | F | 0	SilicoDArT
241	129	175.5	3086708 | F | 0	SilicoDArT
242	130	175.8	3103603 | F | 0	SilicoDArT
243	131	175.9	100004378 | F | 0	SilicoDArT
244	132	176.1	100000887 | F | 0	SilicoDArT
245	133	176.2	3096558 | F | 0	SilicoDArT
246	134	176.2	3081220 | F | 0	SilicoDArT
247	135	176.4	3102376 | F | 0	SilicoDArT
248	136	176.5	100001051 | F | 0	SilicoDArT
249	137	176.9	3085779 | F | 0	SilicoDArT
250	138	177.0	3127765 | F | 0	SilicoDArT
251	139	177.2	3207061 | F | 0	SilicoDArT
252	140	177.2	3105467 | F | 0	SilicoDArT
253	141	178.9	3165152 | F | 0	SilicoDArT
254	142	179.2	3079470 | F | 0	SilicoDArT
255	**143**	**196.3**	**3093734 | F | 0**	**SilicoDArT**
256	144	196.8	3109183 | F | 0	SilicoDArT
257	145	198.1	3090226 | F | 0	SilicoDArT
258	**146**	**203.5**	**brPb-840964**	**DArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
259	147	203.5	3082447 | F | 0	SilicoDArT
260	148	204.6	3094830 | F | 0	SilicoDArT
261	149	204.9	3097684 | F | 0	SilicoDArT
262	150	205.2	3092016 | F | 0	SilicoDArT
263	**151**	**220.6**	**E36M51-388**	**AFLP**
264	152	224.1	E36M51-386	AFLP
A04				
265	**1**	**0.0**	**3115752 | F | 0**	**SilicoDArT**
266	2	3.2	brPb-657581	DArT
267	3	3.2	brPb-660920	DArT
268	4	3.2	brPb-661134	DArT
269	5	3.2	brPb-671364	DArT
270	6	3.2	brPb-808993	DArT
271	7	3.2	brPb-839577	DArT
272	8	3.2	brPb-841534	DArT
273	9	3.2	3078419 | F | 0	SilicoDArT
274	10	3.5	100000025 | F | 0	SilicoDArT
275	**11**	**4.1**	**3097895 | F | 0**	**SilicoDArT**
276	12	4.7	100013967 | F | 0	SilicoDArT
277	13	5.6	3104210 | F | 0	SilicoDArT
278	**14**	**12.5**	**100002422 | F | 0**	**SilicoDArT**
279	15	24.2	3082753 | F | 0	SilicoDArT
280	**16**	**27.9**	**3115220 | F | 0**	**SilicoDArT**
281	17	28.5	3121695 | F | 0	SilicoDArT
282	18	30.9	100020245 | F | 0	SilicoDArT
283	19	47.9	3106537 | F | 0	SilicoDArT
284	20	48.0	3080120 | F | 0	SilicoDArT
285	21	48.5	3108985 | F | 0	SilicoDArT
286	22	48.9	3089872 | F | 0	SilicoDArT
287	**23**	**50.8**	**3100326 | F | 0**	**SilicoDArT**
288	24	52.6	3120203 | F | 0	SilicoDArT
289	25	65.3	3112387 | F | 0	SilicoDArT
290	**26**	**67.8**	**3096579 | F | 0**	**SilicoDArT**
291	27	68.6	100017579 | F | 0	SilicoDArT
292	28	70.8	3131875 | F | 0	SilicoDArT
293	29	71.6	100007020 | F | 0	SilicoDArT
294	30	71.9	3081208 | F | 0	SilicoDArT
295	**31**	**74.4**	**3076080 | F | 0**	**SilicoDArT**
296	32	77.1	3079322 | F | 0	SilicoDArT
297	33	79.0	3089077 | F | 0	SilicoDArT
298	34	79.1	100000922 | F | 0	SilicoDArT
299	35	79.3	100000689 | F | 0	SilicoDArT
300	36	79.5	3202105 | F | 0	SilicoDArT
301	37	80.6	100000367 | F | 0	SilicoDArT
302	**38**	**82.0**	**100000708 | F | 0**	**SilicoDArT**
303	39	83.4	3138203 | F | 0	SilicoDArT
304	40	84.0	3112776 | F | 0	SilicoDArT
305	41	84.0	3078456 | F | 0	SilicoDArT
306	42	86.3	ra01618s01	SNP
307	43	86.5	ra01619s01	SNP
308	**44**	**88.0**	**ra00529s01**	**SNP**
309	45	92.4	100000958 | F | 0	SilicoDArT
310	46	92.8	3119670 | F | 0	SilicoDArT
311	47	93.2	3106425 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
312	48	93.7	3085072 | F | 0	SilicoDArT
313	49	94.3	3122899 | F | 0	SilicoDArT
314	50	94.5	3152827 | F | 0	SilicoDArT
315	51	94.5	3089820 | F | 0	SilicoDArT
316	52	94.7	3152868 | F | 0	SilicoDArT
317	53	95.3	3217357 | F | 0	SilicoDArT
318	54	97.1	3096433 | F | 0	SilicoDArT
319	55	97.3	3204804 | F | 0	SilicoDArT
320	56	97.7	100004692 | F | 0	SilicoDArT
321	57	97.8	3142478 | F | 0	SilicoDArT
322	58	98.0	3165412 | F | 0	SilicoDArT
323	59	98.5	3085886 | F | 0	SilicoDArT
324	**60**	**98.9**	**brPb-661999**	**DArT**
325	61	98.9	3130212 | F | 0	SilicoDArT
326	62	99.6	3112445 | F | 0	SilicoDArT
327	63	100.9	100004210 | F | 0	SilicoDArT
328	64	102.9	3080399 | F | 0	SilicoDArT
329	65	103.8	brPb-808367	DArT
330	66	103.9	brPb-808366	DArT
331	**67**	**104.2**	**brPb-841775**	**DArT**
332	68	105.5	E39M48-188	AFLP
333	69	106.7	3075129 | F | 0	SilicoDArT
334	70	107.9	3140189 | F | 0	SilicoDArT
335	71	109.4	3123541 | F | 0	SilicoDArT
336	**72**	**110.4**	**3102738 | F | 0**	**SilicoDArT**
337	73	111.2	100001515 | F | 0	SilicoDArT
338	74	113.0	100002717 | F | 0	SilicoDArT
339	75	113.1	100001017 | F | 0	SilicoDArT
340	76	113.3	3129057 | F | 0	SilicoDArT
341	77	113.3	3079617 | F | 0	SilicoDArT
342	78	113.3	100000999 | F | 0	SilicoDArT
343	79	113.6	3103799 | F | 0	SilicoDArT
344	80	114.1	3107915 | F | 0	SilicoDArT
345	81	116.6	3137305 | F | 0	SilicoDArT
346	**82**	**117.2**	**brPb-660337**	**DArT**
347	83	117.2	3123638 | F | 0	SilicoDArT
348	84	119.4	3083857 | F | 0	SilicoDArT
349	85	120.5	3098597 | F | 0	SilicoDArT
350	86	122.2	3125501 | F | 0	SilicoDArT
351	87	123.0	3153528 | F | 0	SilicoDArT
352	88	123.3	3115002 | F | 0	SilicoDArT
353	89	123.4	3095207 | F | 0	SilicoDArT
354	90	123.4	3087893 | F | 0	SilicoDArT
355	91	123.5	3092191 | F | 0	SilicoDArT
356	92	123.6	3091626 | F | 0	SilicoDArT
357	93	123.9	3075971 | F | 0	SilicoDArT
358	**94**	**124.5**	**brPb-658275**	**DArT**
359	95	124.8	brPb-841544	DArT
360	96	124.9	3082820 | F | 0	SilicoDArT
361	97	125.0	3134624 | F | 0	SilicoDArT
362	98	125.4	100002172 | F | 0	SilicoDArT
363	99	125.4	3138448 | F | 0	SilicoDArT
364	100	125.4	100020167 | F | 0	SilicoDArT
365	101	127.0	CB10493b	SSR
366	102	128.0	CB10493a	SSR

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
367	**103**	**132.1**	**ra00443s01**	**SNP**
368	**104**	**137.1**	**ra02159s01**	**SNP**
369	**105**	**143.2**	**100001117 | F | 0**	**SilicoDArT**
370	**106**	**167.4**	**E44M50-368**	**AFLP**
371	107	194.2	3094806 | F | 0	SilicoDArT
A05				
372	**1**	**0.0**	**E37M59-119**	**AFLP**
374	**3**	**21.9**	**CB10545b**	**SSR**
373	2	21.9	CB10545a	SSR
375	4	24.9	3147876 | F | 0	SilicoDArT
376	5	25.0	brPb-809749	DArT
377	6	25.4	brPb-662183	DArT
378	7	26.2	3110316 | F | 0	SilicoDArT
379	8	26.4	3140823 | F | 0	SilicoDArT
380	**9**	**26.9**	**brPb-658634**	**DArT**
381	10	27.5	3090721 | F | 0	SilicoDArT
382	11	27.5	3080546 | F | 0	SilicoDArT
383	**12**	**28.8**	**E38M50-57**	**AFLP**
384	13	32.5	100000939 | F | 0	SilicoDArT
385	14	32.7	3150271 | F | 0	SilicoDArT
386	15	32.8	3115448 | F | 0	SilicoDArT
387	16	32.8	3092074 | F | 0	SilicoDArT
388	17	32.9	100002239 | F | 0	SilicoDArT
389	18	34.4	3134509 | F | 0	SilicoDArT
390	19	35.3	3161216 | F | 0	SilicoDArT
391	20	39.9	ra00631s01	SNP
392	**21**	**42.8**	**ra03312s01**	**SNP**
393	**22**	**46.3**	**ra03757s01**	**SNP**
394	23	50.0	3203233 | F | 0	SilicoDArT
395	24	50.2	3104246 | F | 0	SilicoDArT
396	**25**	**51.4**	**3101879 | F | 0**	**SilicoDArT**
397	**26**	**56.2**	**100001954 | F | 0**	**SilicoDArT**
398	27	58.6	100001325 | F | 0	SilicoDArT
399	28	59.3	3101878 | F | 0	SilicoDArT
400	**29**	**60.5**	**3159067 | F | 0**	**SilicoDArT**
401	30	66.6	3132264 | F | 0	SilicoDArT
402	**31**	**69.1**	**3080783 | F | 0**	**SilicoDArT**
403	32	70.6	100000411 | F | 0	SilicoDArT
404	33	71.0	3123450 | F | 0	SilicoDArT
405	**34**	**74.3**	**100002174 | F | 0**	**SilicoDArT**
406	35	76.5	3178758 | F | 0	SilicoDArT
407	36	76.6	3080809 | F | 0	SilicoDArT
408	**37**	**79.6**	**100006261 | F | 0**	**SilicoDArT**
409	38	95.6	ra00630s01	SNP
410	39	96.3	3140870 | F | 0	SilicoDArT
411	40	96.6	ra01288s01	SNP
412	41	96.7	3158186 | F | 0	SilicoDArT
413	42	96.8	3096094 | F | 0	SilicoDArT
414	43	96.8	3079865 | F | 0	SilicoDArT
415	44	96.9	3220238 | F | 0	SilicoDArT
416	45	97.1	3081506 | F | 0	SilicoDArT
417	46	97.1	3092837 | F | 0	SilicoDArT
418	47	97.3	CB10080	SSR
419	48	97.9	3133251 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
420	49	98.4	3119024 | F | 0	SilicoDArT
421	50	98.5	3155149 | F | 0	SilicoDArT
422	51	98.6	brPb-662228	DArT
423	52	100.2	3082071 | F | 0	SilicoDArT
424	53	100.4	3149242 | F | 0	SilicoDArT
425	54	100.7	3123068 | F | 0	SilicoDArT
426	55	100.7	100000782 | F | 0	SilicoDArT
427	56	100.8	3125380 | F | 0	SilicoDArT
428	57	100.9	100003440 | F | 0	SilicoDArT
429	58	100.9	3149178 | F | 0	SilicoDArT
430	**59**	**101.1**	**100001204 | F | 0**	**SilicoDArT**
431	60	101.6	brPb-671027	DArT
432	61	103.0	100000641 | F | 0	SilicoDArT
433	62	103.6	100005373 | F | 0	SilicoDArT
434	63	103.7	3178683 | F | 0	SilicoDArT
435	64	105.2	100000931 | F | 0	SilicoDArT
436	65	105.7	brPb-841413	DArT
437	66	105.7	3187748 | F | 0	SilicoDArT
438	67	105.8	3127498 | F | 0	SilicoDArT
439	**68**	**105.9**	**3086981 | F | 0**	**SilicoDArT**
440	69	108.4	3214359 | F | 0	SilicoDArT
441	**70**	**112.1**	**brPb-838478**	**DArT**
442	71	113.2	3117146 | F | 0	SilicoDArT
443	72	113.3	3120075 | F | 0	SilicoDArT
444	73	114.3	3107878 | F | 0	SilicoDArT
445	**74**	**116.5**	**3124967 | F | 0**	**SilicoDArT**
446	75	118.2	3082516 | F | 0	SilicoDArT
447	76	118.7	BNKS00151	KASP
448	77	119.3	100017601 | F | 0	SilicoDArT
449	**78**	**121.1**	**3159039 | F | 0**	**SilicoDArT**
450	79	122.7	100004695 | F | 0	SilicoDArT
451	**80**	**135.4**	**3130915 | F | 0**	**SilicoDArT**
452	**81**	**160.5**	**3085630 | F | 0**	**SilicoDArT**
453	82	163.8	BNKS003005	KASP
A06				
454	**1**	**0.0**	**ra00669s01**	**SNP**
455	2	4.5	3165997 | F | 0	SilicoDArT
456	3	5.9	3127612 | F | 0	SilicoDArT
457	4	6.2	3095104 | F | 0	SilicoDArT
459	**6**	**6.7**	**brPb-841736**	**DArT**
458	5	6.7	brPb-661213	DArT
460	7	6.7	3077210 | F | 0	SilicoDArT
461	8	8.6	100001454 | F | 0	SilicoDArT
462	**9**	**11.2**	**3085327 | F | 0**	**SilicoDArT**
463	10	13.0	3104397 | F | 0	SilicoDArT
464	11	14.9	100001528 | F | 0	SilicoDArT
465	**12**	**22.2**	**3097489 | F | 0**	**SilicoDArT**
466	13	28.0	BNKS003006	KASP
467	**14**	**30.3**	**brPb-808844**	**DArT**
468	15	31.2	brPb-660024	DArT
469	16	31.2	brPb-660405	DArT
470	17	31.2	brPb-662153	DArT
471	18	31.2	brPb-671060	DArT
472	19	31.2	brPb-809719	DArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
473	20	31.2	brPb-840355	DArT
474	21	31.2	brPb-840896	DArT
475	22	34.2	3144593 | F | 0	SilicoDArT
476	23	35.8	3152360 | F | 0	SilicoDArT
477	24	36.1	3079299 | F | 0	SilicoDArT
478	25	36.6	100000418 | F | 0	SilicoDArT
479	26	37.2	3122471 | F | 0	SilicoDArT
480	**27**	**37.8**	**3077601 | F | 0**	**SilicoDArT**
481	28	39.7	ra02880s01	SNP
482	29	41.8	100001400 | F | 0	SilicoDArT
483	30	42.7	3146960 | F | 0	SilicoDArT
484	31	43.2	100000898 | F | 0	SilicoDArT
485	**32**	**45.0**	**3080133 | F | 0**	**SilicoDArT**
486	33	45.7	3086008 | F | 0	SilicoDArT
487	34	49.2	3166957 | F | 0	SilicoDArT
488	35	49.3	3158598 | F | 0	SilicoDArT
489	36	49.5	3117776 | F | 0	SilicoDArT
490	**37**	**50.1**	**3156714 | F | 0**	**SilicoDArT**
491	38	53.6	3110950 | F | 0	SilicoDArT
492	39	55.3	3143447 | F | 0	SilicoDArT
493	40	57.4	3154085 | F | 0	SilicoDArT
494	**41**	**58.2**	**3111072 | F | 0**	**SilicoDArT**
495	42	58.7	3169284 | F | 0	SilicoDArT
496	43	61.2	3103251 | F | 0	SilicoDArT
497	44	61.8	brPb-808961	DArT
498	45	61.8	brPb-839172	DArT
499	46	61.8	3102015 | F | 0	SilicoDArT
500	47	62.5	3161827 | F | 0	SilicoDArT
501	48	62.8	3090481 | F | 0	SilicoDArT
502	49	63.6	ra00695s01	SNP
503	50	63.7	ra00098s01	SNP
504	51	63.9	BNKS003007	KASP
505	52	64.3	3155791 | F | 0	SilicoDArT
506	53	64.4	3155856 | F | 0	SilicoDArT
507	54	64.7	E37M51-262	AFLP
508	55	64.8	3111802 | F | 0	SilicoDArT
509	56	65.0	3164979 | F | 0	SilicoDArT
510	**57**	**65.8**	**brPb-662354**	**DArT**
511	58	66.3	3091828 | F | 0	SilicoDArT
512	59	67.8	3095218 | F | 0	SilicoDArT
513	60	67.8	ra00122s01	SNP
514	61	68.1	3101890 | F | 0	SilicoDArT
515	62	68.3	100002746 | F | 0	SilicoDArT
516	63	68.4	3168069 | F | 0	SilicoDArT
517	64	68.5	3128731 | F | 0	SilicoDArT
518	65	68.6	3097844 | F | 0	SilicoDArT
519	66	68.6	3082335 | F | 0	SilicoDArT
520	67	69.0	3157918 | F | 0	SilicoDArT
521	68	69.5	3092874 | F | 0	SilicoDArT
522	**69**	**70.6**	**3114544 | F | 0**	**SilicoDArT**
523	70	71.1	3111903 | F | 0	SilicoDArT
524	71	71.2	3174755 | F | 0	SilicoDArT
525	72	71.4	3103617 | F | 0	SilicoDArT
526	73	71.7	3083075 | F | 0	SilicoDArT
527	74	72.0	3096722 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
528	75	72.0	3118455 | F | 0	SilicoDArT
529	76	72.2	3146385 | F | 0	SilicoDArT
530	77	72.7	3078562 | F | 0	SilicoDArT
531	78	73.3	3106588 | F | 0	SilicoDArT
532	79	73.8	brPb-839202	DArT
533	80	74.1	3145968 | F | 0	SilicoDArT
534	81	74.5	3087382 | F | 0	SilicoDArT
535	82	74.5	brPb-657724	DArT
536	83	74.6	100001663 | F | 0	SilicoDArT
537	84	74.7	3178905 | F | 0	SilicoDArT
538	85	75.0	100002574 | F | 0	SilicoDArT
539	**86**	**75.6**	**brPb-659119**	**DArT**
540	87	75.6	3154803 | F | 0	SilicoDArT
541	88	75.9	3120800 | F | 0	SilicoDArT
542	89	76.6	3099241 | F | 0	SilicoDArT
543	90	77.2	100000571 | F | 0	SilicoDArT
544	91	77.6	3081483 | F | 0	SilicoDArT
545	92	77.6	3093423 | F | 0	SilicoDArT
546	93	79.8	100002078 | F | 0	SilicoDArT
547	94	80.1	3104196 | F | 0	SilicoDArT
548	95	80.4	3118041 | F | 0	SilicoDArT
549	**96**	**80.9**	**100003367 | F | 0**	**SilicoDArT**
550	97	83.3	3159585 | F | 0	SilicoDArT
551	**98**	**84.1**	**3091100 | F | 0**	**SilicoDArT**
552	99	85.3	3085567 | F | 0	SilicoDArT
553	**100**	**90.6**	**3129180 | F | 0**	**SilicoDArT**
554	**101**	**94.4**	**brPb-838969**	**DArT**
555	102	95.8	3081203 | F | 0	SilicoDArT
556	103	96.5	E45M48-103	AFLP
557	104	98.0	3082035 | F | 0	SilicoDArT
558	105	100.7	3084028 | F | 0	SilicoDArT
559	106	101.1	3095659 | F | 0	SilicoDArT
560	**107**	**101.6**	**brPb-658875**	**DArT**
561	108	102.7	3094015 | F | 0	SilicoDArT
562	109	103.0	3140972 | F | 0	SilicoDArT
563	110	103.0	3136242 | F | 0	SilicoDArT
564	111	103.2	E44M48-278	AFLP
565	112	103.2	E44M50-238	AFLP
566	113	103.8	3079522 | F | 0	SilicoDArT
567	114	104.4	100003267 | F | 0	SilicoDArT
568	**115**	**105.5**	**3075341 | F | 0**	**SilicoDArT**
569	116	106.0	3117463 | F | 0	SilicoDArT
570	117	106.8	3221610 | F | 0	SilicoDArT
571	118	109.1	3086832 | F | 0	SilicoDArT
572	**119**	**114.1**	**3112388 | F | 0**	**SilicoDArT**
573	120	115.0	3098112 | F | 0	SilicoDArT
574	121	115.1	3147893 | F | 0	SilicoDArT
575	**122**	**120.1**	**3096863 | F | 0**	**SilicoDArT**
576	123	120.8	100001404 | F | 0	SilicoDArT
577	**124**	**128.0**	**3216051 | F | 0**	**SilicoDArT**
A07				
578	**1**	**0.0**	**HMG2A10-2**	**Candidate gene - Bra002053**
579	2	11.5	D120E-3	Candidate gene - Bra039003
580	**3**	**18.0**	**3110517 | F | 0**	**SilicoDArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
581	4	20.8	3102883 | F | 0	SilicoDArT
582	5	22.0	100004241 | F | 0	SilicoDArT
583	6	22.6	100020171 | F | 0	SilicoDArT
584	7	23.3	100000991 | F | 0	SilicoDArT
585	8	25.0	100004676 | F | 0	SilicoDArT
586	9	27.4	3125506 | F | 0	SilicoDArT
587	10	28.4	3168208 | F | 0	SilicoDArT
588	11	28.7	100011842 | F | 0	SilicoDArT
589	12	29.0	3215155 | F | 0	SilicoDArT
590	13	29.0	3109557 | F | 0	SilicoDArT
591	**14**	**32.8**	**3126880 | F | 0**	**SilicoDArT**
592	15	34.3	3144490 | F | 0	SilicoDArT
593	16	34.7	3100356 | F | 0	SilicoDArT
594	17	35.1	3199991 | F | 0	SilicoDArT
595	18	35.9	3093071 | F | 0	SilicoDArT
596	19	35.9	3084921 | F | 0	SilicoDArT
597	20	35.9	100020114 | F | 0	SilicoDArT
598	21	36.2	brPb-839636	DArT
599	22	36.2	3107557 | F | 0	SilicoDArT
600	23	36.4	3082243 | F | 0	SilicoDArT
601	24	36.7	3077078 | F | 0	SilicoDArT
602	25	37.0	100003906 | F | 0	SilicoDArT
603	26	37.2	3161729 | F | 0	SilicoDArT
604	**27**	**37.7**	**3201696 | F | 0**	**SilicoDArT**
605	28	38.9	3117058 | F | 0	SilicoDArT
606	29	40.1	3170325 | F | 0	SilicoDArT
607	30	42.8	3147391 | F | 0	SilicoDArT
608	31	42.9	3158086 | F | 0	SilicoDArT
609	32	43.2	3091032 | F | 0	SilicoDArT
610	33	43.4	3146730 | F | 0	SilicoDArT
611	34	43.4	3101139 | F | 0	SilicoDArT
612	35	43.8	3150019 | F | 0	SilicoDArT
613	36	43.9	3119858 | F | 0	SilicoDArT
614	37	43.9	3104689 | F | 0	SilicoDArT
615	**38**	**44.3**	**brPb-671083**	**DArT**
616	39	44.3	3138917 | F | 0	SilicoDArT
617	40	45.0	3082028 | F | 0	SilicoDArT
618	41	45.4	BNKS003013	KASP
619	42	46.8	ra00662s01	SNP
620	43	47.3	ra03823s01	SNP
621	**44**	**49.3**	**ra00106s01**	**SNP**
622	45	51.6	ra00124s01	SNP
623	**46**	**55.4**	**ra00123s01**	**SNP**
624	**47**	**61.8**	**ra00476s01**	**SNP**
625	48	63.9	3079664 | F | 0	SilicoDArT
626	49	63.9	100006288 | F | 0	SilicoDArT
627	50	63.9	3104913 | F | 0	SilicoDArT
628	51	64.0	100003344 | F | 0	SilicoDArT
629	52	64.1	3146009 | F | 0	SilicoDArT
630	53	64.2	brPb-671004	DArT
631	54	64.2	brPb-839259	DArT
632	**55**	**65.5**	**ra00366s01**	**SNP**
633	56	66.2	brPb-661422	DArT
634	57	66.9	ra03975s01	SNP
635	**58**	**68.3**	**ra00069s01**	**SNP**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
636	59	70.8	3091635 | F | 0	SilicoDArT
637	60	71.2	3095296 | F | 0	SilicoDArT
638	61	71.3	3081366 | F | 0	SilicoDArT
639	62	71.7	3087873 | F | 0	SilicoDArT
640	63	72.2	100000677 | F | 0	SilicoDArT
641	64	72.5	3118053 | F | 0	SilicoDArT
642	65	72.5	3084997 | F | 0	SilicoDArT
643	66	72.6	3098875 | F | 0	SilicoDArT
644	67	72.7	3103317 | F | 0	SilicoDArT
645	68	73.0	3130692 | F | 0	SilicoDArT
646	69	73.5	3133446 | F | 0	SilicoDArT
647	70	73.6	3152181 | F | 0	SilicoDArT
648	71	74.2	100002833 | F | 0	SilicoDArT
649	72	74.3	brPb-808556	DArT
650	73	75.4	100000104 | F | 0	SilicoDArT
651	74	75.6	3155824 | F | 0	SilicoDArT
652	75	76.4	3081825 | F | 0	SilicoDArT
653	76	78.4	3096398 | F | 0	SilicoDArT
654	77	79.9	3104105 | F | 0	SilicoDArT
655	78	81.2	3134422 | F | 0	SilicoDArT
656	79	81.3	3085294 | F | 0	SilicoDArT
657	80	81.3	3099641 | F | 0	SilicoDArT
658	81	81.4	100001652 | F | 0	SilicoDArT
659	82	81.5	100001122 | F | 0	SilicoDArT
660	83	81.6	3105682 | F | 0	SilicoDArT
661	84	81.6	brPb-657950	DArT
662	85	81.6	3105562 | F | 0	SilicoDArT
663	86	81.7	3113721 | F | 0	SilicoDArT
664	87	81.9	3165473 | F | 0	SilicoDArT
665	88	82.0	3107917 | F | 0	SilicoDArT
666	89	82.1	100001576 | F | 0	SilicoDArT
667	90	82.2	3213750 | F | 0	SilicoDArT
668	91	82.4	3094803 | F | 0	SilicoDArT
669	**92**	**87.4**	**ra04226s01**	**SNP**
670	93	89.7	ra02893s01	SNP
671	94	91.6	3221850 | F | 0	SilicoDArT
672	95	92.0	3089394 | F | 0	SilicoDArT
673	96	92.0	3148683 | F | 0	SilicoDArT
674	97	92.2	3085559 | F | 0	SilicoDArT
675	98	92.3	3092466 | F | 0	SilicoDArT
676	99	92.5	3155716 | F | 0	SilicoDArT
677	100	92.6	3169755 | F | 0	SilicoDArT
678	101	92.7	100001438 | F | 0	SilicoDArT
679	102	92.7	3210809 | F | 0	SilicoDArT
680	103	93.0	3138064 | F | 0	SilicoDArT
681	104	93.1	3127470 | F | 0	SilicoDArT
682	105	93.1	100000366 | F | 0	SilicoDArT
683	106	93.2	3158614 | F | 0	SilicoDArT
684	107	93.3	3101529 | F | 0	SilicoDArT
685	108	93.5	3116838 | F | 0	SilicoDArT
686	109	93.5	3096443 | F | 0	SilicoDArT
687	110	93.6	3182734 | F | 0	SilicoDArT
688	111	93.8	3155006 | F | 0	SilicoDArT
689	112	94.0	3093208 | F | 0	SilicoDArT
690	113	94.2	3078747 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
691	114	94.4	3144459 | F | 0	SilicoDArT
692	115	94.8	3077429 | F | 0	SilicoDArT
693	116	94.9	3102141 | F | 0	SilicoDArT
694	117	95.0	3122069 | F | 0	SilicoDArT
695	118	95.1	3087787 | F | 0	SilicoDArT
696	119	95.1	3110307 | F | 0	SilicoDArT
697	120	95.2	100016758 | F | 0	SilicoDArT
698	121	96.1	100014492 | F | 0	SilicoDArT
699	**122**	**101.0**	**ra00688s01**	**SNP**
700	123	104.0	3080964 | F | 0	SilicoDArT
701	124	104.7	3105846 | F | 0	SilicoDArT
702	125	106.4	3086581 | F | 0	SilicoDArT
703	126	106.8	ra00502s01	SNP
704	**127**	**109.5**	**E40M50-263**	**AFLP**
705	128	113.5	3087651 | F | 0	SilicoDArT
706	129	113.5	100009756 | F | 0	SilicoDArT
707	130	113.6	3095820 | F | 0	SilicoDArT
708	131	113.8	100000635 | F | 0	SilicoDArT
709	132	113.9	3108912 | F | 0	SilicoDArT
710	133	114.0	100000192 | F | 0	SilicoDArT
711	134	114.1	3081643 | F | 0	SilicoDArT
712	135	114.4	ra00696s01	SNP
713	136	114.5	ra04111s01	SNP
714	137	115.5	3163256 | F | 0	SilicoDArT
715	138	115.6	3079046 | F | 0	SilicoDArT
716	139	116.9	3110657 | F | 0	SilicoDArT
717	140	116.9	3094109 | F | 0	SilicoDArT
718	141	117.0	3102071 | F | 0	SilicoDArT
719	**142**	**117.5**	**3122992 | F | 0**	**SilicoDArT**
720	143	118.7	3077979 | F | 0	SilicoDArT
721	144	119.0	100001444 | F | 0	SilicoDArT
722	145	119.4	brPb-657606	DArT
723	146	119.4	brPb-657676	DArT
724	147	120.0	BNKS003008	KASP
725	148	120.1	100003359 | F | 0	SilicoDArT
726	149	120.1	HMG1A07	Candidate gene - Bra015739
727	150	120.7	100020173 | F | 0	SilicoDArT
728	151	121.8	3100601 | F | 0	SilicoDArT
729	152	123.2	3092395 | F | 0	SilicoDArT
730	**153**	**123.6**	**3124997 | F | 0**	**SilicoDArT**
731	154	124.8	3083394 | F | 0	SilicoDArT
732	155	124.8	3188698 | F | 0	SilicoDArT
733	156	125.0	100004780 | F | 0	SilicoDArT
734	157	125.9	3135423 | F | 0	SilicoDArT
735	158	126.3	3162490 | F | 0	SilicoDArT
736	159	126.6	3085420 | F | 0	SilicoDArT
737	**160**	**127.2**	**3174697 | F | 0**	**SilicoDArT**
738	161	128.3	100007132 | F | 0	SilicoDArT
739	162	128.4	3087003 | F | 0	SilicoDArT
740	163	128.5	3081222 | F | 0	SilicoDArT
741	**164**	**133.7**	**3100149 | F | 0**	**SilicoDArT**
A08				
742	**1**	**0.0**	**ra00574s01**	**SNP**
743	**2**	**9.4**	**ra01507s01**	**SNP**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
744	3	13.7	brPb-840706	DArT
745	4	13.7	brPb-841145	DArT
746	5	13.8	3108185 | F | 0	SilicoDArT
747	6	13.9	3088451 | F | 0	SilicoDArT
748	7	13.9	100000779 | F | 0	SilicoDArT
749	8	13.9	3080518 | F | 0	SilicoDArT
750	9	14.5	3078154 | F | 0	SilicoDArT
751	10	15.5	E37M59-302	AFLP
752	11	16.5	3096017 | F | 0	SilicoDArT
753	12	16.5	BNKS00264	KASP
754	**13**	**18.0**	**E44M48-412**	**AFLP**
755	14	19.3	3101417 | F | 0	SilicoDArT
756	15	20.0	3191113 | F | 0	SilicoDArT
757	16	21.3	3079969 | F | 0	SilicoDArT
758	17	21.9	brPb-658031	DArT
759	18	21.9	brPb-808147	DArT
760	19	21.9	brPb-809516	DArT
761	20	21.9	3214939 | F | 0	SilicoDArT
762	21	22.2	3216686 | F | 0	SilicoDArT
763	22	22.2	3086154 | F | 0	SilicoDArT
764	23	22.2	100002170 | F | 0	SilicoDArT
765	24	22.4	brPb-808461	DArT
766	25	22.4	brPb-841039	DArT
767	**26**	**22.8**	**brPb-660348**	**DArT**
768	27	22.8	brPb-838822	DArT
769	28	22.8	3075762 | F | 0	SilicoDArT
770	29	24.8	3101278 | F | 0	SilicoDArT
771	30	25.4	100001202 | F | 0	SilicoDArT
772	31	26.1	100000347 | F | 0	SilicoDArT
773	32	26.7	100000420 | F | 0	SilicoDArT
774	33	27.1	3101673 | F | 0	SilicoDArT
775	34	27.4	100001705 | F | 0	SilicoDArT
776	35	28.1	3082230 | F | 0	SilicoDArT
777	36	28.1	3084249 | F | 0	SilicoDArT
778	37	28.2	3142601 | F | 0	SilicoDArT
779	38	28.4	3077168 | F | 0	SilicoDArT
780	**39**	**29.3**	**100005340 | F | 0**	**SilicoDArT**
781	40	29.8	100002894 | F | 0	SilicoDArT
782	41	30.0	3105911 | F | 0	SilicoDArT
783	42	30.4	100000754 | F | 0	SilicoDArT
784	**43**	**32.3**	**3107840 | F | 0**	**SilicoDArT**
785	44	33.6	3085908 | F | 0	SilicoDArT
786	45	37.8	3134824 | F | 0	SilicoDArT
A08-II				
787	**1**	**0.0**	**3183479 | F | 0**	**SilicoDArT**
788	2	3.4	brPb-657962	DArT
789	3	3.4	3086525 | F | 0	SilicoDArT
790	**4**	**5.1**	**3107179 | F | 0**	**SilicoDArT**
791	5	6.8	3108568 | F | 0	SilicoDArT
792	6	8.1	3104723 | F | 0	SilicoDArT
793	**7**	**9.7**	**3130947 | F | 0**	**SilicoDArT**
A09				
794	1	-10.2	3117526 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
795	2	-9.5	CB10103	SSR
796	3	-9.2	BNKS003012	KASP
797	4	-8.0	3086052 | F | 0	SilicoDArT
798	5	-7.4	100001262 | F | 0	SilicoDArT
799	6	-7.4	3077115 | F | 0	SilicoDArT
800	7	-6.3	3167079 | F | 0	SilicoDArT
801	8	-6.3	3160393 | F | 0	SilicoDArT
802	9	-5.7	100002348 | F | 0	SilicoDArT
803	10	-3.7	3163543 | F | 0	SilicoDArT
804	**11**	**0.0**	**100001587 | F | 0**	**SilicoDArT**
805	12	3.1	brPb-658420	DArT
806	13	3.1	brPb-659767	DArT
807	14	3.1	3105182 | F | 0	SilicoDArT
808	15	3.9	3090195 | F | 0	SilicoDArT
809	16	4.0	3076259 | F | 0	SilicoDArT
810	17	4.2	E44M51-274	AFLP
811	18	4.5	3076863 | F | 0	SilicoDArT
812	19	4.7	E45M48-277	AFLP
813	20	4.9	3088326 | F | 0	SilicoDArT
814	21	5.3	3120227 | F | 0	SilicoDArT
815	**22**	**24.6**	**3128791 | F | 0**	**SilicoDArT**
816	**23**	**30.7**	**3123759 | F | 0**	**SilicoDArT**
817	**24**	**36.4**	**100000807 | F | 0**	**SilicoDArT**
818	25	37.5	3161096 | F | 0	SilicoDArT
819	26	39.8	3129190 | F | 0	SilicoDArT
820	**27**	**53.0**	**100001207 | F | 0**	**SilicoDArT**
821	28	55.4	3120132 | F | 0	SilicoDArT
822	29	56.1	100007236 | F | 0	SilicoDArT
823	30	58.7	100013073 | F | 0	SilicoDArT
824	**31**	**59.8**	**3129027 | F | 0**	**SilicoDArT**
825	32	60.5	3094134 | F | 0	SilicoDArT
826	33	60.6	100001544 | F | 0	SilicoDArT
827	34	60.6	3104685 | F | 0	SilicoDArT
828	35	61.5	100002145 | F | 0	SilicoDArT
829	**36**	**63.8**	**100010105 | F | 0**	**SilicoDArT**
830	37	64.3	100001796 | F | 0	SilicoDArT
831	38	64.3	100001200 | F | 0	SilicoDArT
832	39	65.7	100003604 | F | 0	SilicoDArT
833	40	73.0	ra00232s01	SNP
834	41	74.5	E44M59-288	AFLP
835	42	75.0	3079866 | F | 0	SilicoDArT
836	**43**	**76.3**	**100001522 | F | 0**	**SilicoDArT**
837	**44**	**78.9**	**3086664 | F | 0**	**SilicoDArT**
838	45	82.4	3112150 | F | 0	SilicoDArT
839	46	83.9	ra00324s01	SNP
840	47	96.6	3160715 | F | 0	SilicoDArT
841	**48**	**99.3**	**brPb-840102**	**DArT**
842	49	99.3	100000398 | F | 0	SilicoDArT
843	50	100.6	brPb-661799	DArT
844	51	100.6	100019446 | F | 0	SilicoDArT
845	52	101.1	100002870 | F | 0	SilicoDArT
846	53	101.8	3167528 | F | 0	SilicoDArT
847	54	103.6	3084822 | F | 0	SilicoDArT
848	55	104.2	3153877 | F | 0	SilicoDArT
850	**57**	**107.8**	**brPb-661741**	**DArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
849	56	107.8	brPb-657939	DArT
851	58	107.8	3078875 | F | 0	SilicoDArT
852	59	108.2	brPb-658630	DArT
853	60	108.2	brPb-661730	DArT
854	61	109.7	BNKS00281	KASP
855	62	110.6	100004872 | F | 0	SilicoDArT
856	**63**	**115.6**	**3078836 | F | 0**	**SilicoDArT**
857	64	116.9	3092922 | F | 0	SilicoDArT
858	**65**	**120.0**	**3106147 | F | 0**	**SilicoDArT**
A10				
859	**1**	**0.0**	**E40M50-486**	**AFLP**
860	2	13.0	3082551 | F | 0	SilicoDArT
861	3	13.3	100003139 | F | 0	SilicoDArT
862	4	13.5	3122317 | F | 0	SilicoDArT
863	5	13.7	3105477 | F | 0	SilicoDArT
864	6	14.0	100020237 | F | 0	SilicoDArT
865	7	14.1	3171611 | F | 0	SilicoDArT
866	8	14.1	ra00309s01	SNP
867	9	14.2	ra00722s01	SNP
868	10	14.3	100005647 | F | 0	SilicoDArT
869	11	14.5	100001087 | F | 0	SilicoDArT
870	12	14.6	3078744 | F | 0	SilicoDArT
871	13	14.6	3113208 | F | 0	SilicoDArT
872	14	14.7	3155415 | F | 0	SilicoDArT
873	15	14.9	E40M50-325	AFLP
874	16	14.9	3077374 | F | 0	SilicoDArT
875	17	15.1	3124471 | F | 0	SilicoDArT
876	18	15.3	100007238 | F | 0	SilicoDArT
877	19	15.3	100002494 | F | 0	SilicoDArT
878	20	15.6	3206921 | F | 0	SilicoDArT
879	21	15.6	E32M48-191	AFLP
880	22	15.9	3124821 | F | 0	SilicoDArT
881	23	16.4	100000387 | F | 0	SilicoDArT
882	24	16.5	3151448 | F | 0	SilicoDArT
883	25	16.7	100001142 | F | 0	SilicoDArT
884	26	16.9	E37M50-350	AFLP
885	27	17.5	3117316 | F | 0	SilicoDArT
886	**28**	**18.7**	**E37M50-346**	**AFLP**
887	29	19.6	100001764 | F | 0	SilicoDArT
888	30	19.6	3082738 | F | 0	SilicoDArT
889	31	19.8	100000424 | F | 0	SilicoDArT
890	32	19.8	3160334 | F | 0	SilicoDArT
891	33	19.8	3092718 | F | 0	SilicoDArT
892	34	19.8	E44M48-61	AFLP
893	35	20.1	100002245 | F | 0	SilicoDArT
894	36	20.1	3148980 | F | 0	SilicoDArT
895	37	20.3	3075752 | F | 0	SilicoDArT
896	38	20.3	brPb-658597	DArT
897	39	20.4	3192191 | F | 0	SilicoDArT
898	40	20.4	3139183 | F | 0	SilicoDArT
899	41	20.5	3074354 | F | 0	SilicoDArT
900	42	20.6	3170450 | F | 0	SilicoDArT
901	43	20.7	3169562 | F | 0	SilicoDArT
902	44	21.1	brPb-658977	DArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
903	45	21.1	3118361 | F | 0	SilicoDArT
904	46	22.9	brPb-671233	DArT
905	47	23.1	3140449 | F | 0	SilicoDArT
906	48	23.1	3104190 | F | 0	SilicoDArT
907	49	23.1	3081100 | F | 0	SilicoDArT
908	50	23.1	3084063 | F | 0	SilicoDArT
909	51	23.1	100003731 | F | 0	SilicoDArT
910	52	23.3	E44M59-142	AFLP
911	53	23.3	3183448 | F | 0	SilicoDArT
912	54	23.4	brPb-838630	DArT
913	55	23.5	100005383 | F | 0	SilicoDArT
914	56	23.5	3115940 | F | 0	SilicoDArT
915	57	23.5	3112941 | F | 0	SilicoDArT
916	58	23.5	3101763 | F | 0	SilicoDArT
917	59	23.5	3155296 | F | 0	SilicoDArT
918	**60**	**23.7**	**brPb-663458**	**DArT**
919	61	24.0	3125308 | F | 0	SilicoDArT
920	62	24.1	3123350 | F | 0	SilicoDArT
921	63	24.1	3091465 | F | 0	SilicoDArT
922	64	24.1	3079116 | F | 0	SilicoDArT
923	65	24.1	3108659 | F | 0	SilicoDArT
924	66	24.1	3103408 | F | 0	SilicoDArT
925	67	24.1	3106273 | F | 0	SilicoDArT
926	68	24.1	3106844 | F | 0	SilicoDArT
927	69	24.2	3176982 | F | 0	SilicoDArT
928	70	24.2	3186758 | F | 0	SilicoDArT
929	71	24.3	100002471 | F | 0	SilicoDArT
930	72	24.8	100020270 | F | 0	SilicoDArT
931	73	25.4	3209880 | F | 0	SilicoDArT
932	74	25.4	3098925 | F | 0	SilicoDArT
933	75	25.4	3098474 | F | 0	SilicoDArT
934	76	25.9	brPb-657811	DArT
935	77	26.2	3090321 | F | 0	SilicoDArT
936	78	26.4	3139676 | F | 0	SilicoDArT
937	**79**	**27.0**	**3142772 | F | 0**	**SilicoDArT**
938	80	28.2	3199708 | F | 0	SilicoDArT
939	81	28.3	100005196 | F | 0	SilicoDArT
940	82	28.4	3171065 | F | 0	SilicoDArT
941	83	29.3	100000526 | F | 0	SilicoDArT
942	84	30.5	3197727 | F | 0	SilicoDArT
943	85	31.0	100004037 | F | 0	SilicoDArT
944	86	31.9	3130241 | F | 0	SilicoDArT
945	87	32.2	3108620 | F | 0	SilicoDArT
946	**88**	**33.4**	**3087218 | F | 0**	**SilicoDArT**
947	89	34.7	100012313 | F | 0	SilicoDArT
948	90	35.5	100001882 | F | 0	SilicoDArT
949	91	36.0	100005354 | F | 0	SilicoDArT
950	92	36.5	3102759 | F | 0	SilicoDArT
951	**93**	**37.0**	**brPb-839046**	**DArT**
952	94	37.7	3151538 | F | 0	SilicoDArT
953	95	39.6	3082509 | F | 0	SilicoDArT
954	96	40.1	3111468 | F | 0	SilicoDArT
955	97	41.2	3090635 | F | 0	SilicoDArT
956	**98**	**41.9**	**brPb-809526**	**DArT**
957	99	44.0	3100364 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
958	100	45.8	brPb-658429	DArT
959	101	46.9	3141120 | F | 0	SilicoDArT
960	102	47.2	3173168 | F | 0	SilicoDArT
961	**103**	**48.5**	**E38M51-138**	**AFLP**
962	104	48.9	3147607 | F | 0	SilicoDArT
963	105	49.1	3080720 | F | 0	SilicoDArT
964	106	49.4	3141115 | F | 0	SilicoDArT
965	107	50.8	3110112 | F | 0	SilicoDArT
966	108	52.3	3113938 | F | 0	SilicoDArT
968	**110**	**53.7**	**brPb-670984**	**DArT**
967	109	53.7	brPb-661312	DArT
969	111	57.3	100001299 | F | 0	SilicoDArT
970	112	57.3	3092784 | F | 0	SilicoDArT
971	113	58.3	3134528 | F | 0	SilicoDArT
972	114	58.4	3139478 | F | 0	SilicoDArT
973	**115**	**60.9**	**100003622 | F | 0**	**SilicoDArT**
974	116	62.2	3127574 | F | 0	SilicoDArT
975	**117**	**64.8**	**3121146 | F | 0**	**SilicoDArT**
976	118	65.4	3132451 | F | 0	SilicoDArT
977	119	68.0	ra00262s01	SNP
978	120	76.0	3113923 | F | 0	SilicoDArT
979	121	76.5	100005645 | F | 0	SilicoDArT
981	**123**	**77.0**	**3162075 | F | 0**	**SilicoDArT**
980	122	77.0	3143860 | F | 0	SilicoDArT
982	124	78.7	3114709 | F | 0	SilicoDArT
983	125	79.8	3088349 | F | 0	SilicoDArT
984	126	79.8	3078994 | F | 0	SilicoDArT
985	**127**	**83.9**	**3121519 | F | 0**	**SilicoDArT**
986	**128**	**93.4**	**3080489 | F | 0**	**SilicoDArT**
987	129	94.1	3090919 | F | 0	SilicoDArT
C01				
988	**1**	**0.0**	**3089602 | F | 0**	**SilicoDArT**
989	2	2.3	3125697 | F | 0	SilicoDArT
990	**3**	**5.6**	**3218184 | F | 0**	**SilicoDArT**
991	4	6.4	3075934 | F | 0	SilicoDArT
992	5	7.2	3117027 | F | 0	SilicoDArT
993	6	7.9	3099172 | F | 0	SilicoDArT
994	7	8.3	100000264 | F | 0	SilicoDArT
995	**8**	**10.0**	**3105213 | F | 0**	**SilicoDArT**
996	9	11.8	ra00575s01	SNP
997	**10**	**15.8**	**100003372 | F | 0**	**SilicoDArT**
998	11	16.3	3195275 | F | 0	SilicoDArT
999	12	16.7	3078343 | F | 0	SilicoDArT
1000	13	22.6	3186788 | F | 0	SilicoDArT
1001	**14**	**23.3**	**brPb-841618**	**DArT**
1002	15	33.2	CB10208a	SSR
1003	16	34.3	ra00586s01	SNP
1004	17	34.4	CB10208b	SSR
1005	**18**	**38.9**	**100001436 | F | 0**	**SilicoDArT**
1006	19	40.7	3134706 | F | 0	SilicoDArT
1007	**20**	**41.6**	**100000250 | F | 0**	**SilicoDArT**
1008	21	42.2	brPb-670827	DArT
1009	22	42.2	100000478 | F | 0	SilicoDArT
1010	23	42.2	100000654 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1011	24	42.3	100003200 | F | 0	SilicoDArT
1012	25	48.5	ra00057s01	SNP
1013	26	55.2	100002286 | F | 0	SilicoDArT
1014	**27**	**59.9**	**100001283 | F | 0**	**SilicoDArT**
1015	28	68.6	3102163 | F | 0	SilicoDArT
1016	29	70.1	100003708 | F | 0	SilicoDArT
1018	**31**	**71.5**	**brPb-661581**	**DArT**
1017	30	71.5	brPb-658209	DArT
1019	32	71.5	100001362 | F | 0	SilicoDArT
1020	33	72.1	3217953 | F | 0	SilicoDArT
1021	**34**	**77.5**	**ra02156s01**	**SNP**
C02				
1022	**1**	**0.0**	**3181853 | F | 0**	**SilicoDArT**
1023	2	1.8	brPb-838939	DArT
1024	3	1.8	3100433 | F | 0	SilicoDArT
1025	4	4.7	3081013 | F | 0	SilicoDArT
1026	5	5.7	100005406 | F | 0	SilicoDArT
1027	**6**	**6.2**	**3098215 | F | 0**	**SilicoDArT**
1028	7	7.8	3079210 | F | 0	SilicoDArT
1029	**8**	**10.1**	**100003257 | F | 0**	**SilicoDArT**
1030	9	14.0		KASP
1031	**10**	**16.8**	**3077481 | F | 0**	**SilicoDArT**
1032	11	34.7	3078746 | F | 0	SilicoDArT
1033	12	34.7	3135517 | F | 0	SilicoDArT
1034	**13**	**39.9**	**ra00403s01**	**SNP**
1035	14	48.2	100003330 | F | 0	SilicoDArT
C02-II				
1036	**1**	**0.0**	**MD38**	**SSR**
1037	**2**	**5.8**	**3130831 | F | 0**	**SilicoDArT**
1038	**3**	**28.5**	**ra00265s01**	**SNP**
1039	4	31.5	3144088 | F | 0	SilicoDArT
1040	**5**	**32.6**	**3114214 | F | 0**	**SilicoDArT**
1041	6	34.4	3112452 | F | 0	SilicoDArT
1042	7	34.9	3116358 | F | 0	SilicoDArT
1043	**8**	**36.1**	**3101131 | F | 0**	**SilicoDArT**
1044	9	37.3	100000299 | F | 0	SilicoDArT
1045	10	37.6	3076891 | F | 0	SilicoDArT
1046	11	37.7	100003435 | F | 0	SilicoDArT
1047	**12**	**40.3**	**E38M59-241**	**AFLP**
1048	13	45.9	3130662 | F | 0	SilicoDArT
1049	**14**	**66.3**	**3134297 | F | 0**	**SilicoDArT**
1050	**15**	**90.1**	**3110160 | F | 0**	**SilicoDArT**
1051	16	92.9	3075380 | F | 0	SilicoDArT
1052	17	93.3	3097235 | F | 0	SilicoDArT
1053	18	97.1	brPb-657639	DArT
1054	19	97.1	100002713 | F | 0	SilicoDArT
1055	20	97.9	3102949 | F | 0	SilicoDArT
C03				
1056	**1**	**0.0**		**KASP**
1057	2	3.7	100001909 | F | 0	SilicoDArT
1058	3	4.1	3172996 | F | 0	SilicoDArT
1059	4	4.1	brPb-661033	DArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1060	5	4.3	3075957 | F | 0	SilicoDArT
1061	6	4.3	100004366 | F | 0	SilicoDArT
1062	7	4.3	100001039 | F | 0	SilicoDArT
1063	8	4.3	brPb-841314	DArT
1064	9	4.4	100008582 | F | 0	SilicoDArT
1065	10	4.5	100001513 | F | 0	SilicoDArT
1066	11	4.6	100003174 | F | 0	SilicoDArT
1067	**12**	**5.8**	**3141420 | F | 0**	**SilicoDArT**
1068	13	7.2	100001744 | F | 0	SilicoDArT
1069	14	7.7	3130599 | F | 0	SilicoDArT
1070	15	9.2	100011291 | F | 0	SilicoDArT
1071	16	9.3	3163034 | F | 0	SilicoDArT
1072	17	10.5	3094659 | F | 0	SilicoDArT
1073	18	11.4	3107451 | F | 0	SilicoDArT
1074	19	12.3	3082352 | F | 0	SilicoDArT
1075	**20**	**13.5**	**3079122 | F | 0**	**SilicoDArT**
1076	21	13.9	3106642 | F | 0	SilicoDArT
1077	22	14.1	3101243 | F | 0	SilicoDArT
1078	23	14.1	3088817 | F | 0	SilicoDArT
1079	24	15.2	3094325 | F | 0	SilicoDArT
1080	25	17.2	3161741 | F | 0	SilicoDArT
1081	26	17.5	E37M59-318	AFLP
1082	27	17.8	3123732 | F | 0	SilicoDArT
1083	28	17.8	3080728 | F | 0	SilicoDArT
1084	29	17.9	3169487 | F | 0	SilicoDArT
1085	30	17.9	3134018 | F | 0	SilicoDArT
1086	31	17.9	3135058 | F | 0	SilicoDArT
1087	32	18.0	ra00706s02	SNP
1088	**33**	**23.8**	**3124978 | F | 0**	**SilicoDArT**
1089	34	24.5	3154171 | F | 0	SilicoDArT
1090	35	25.6	3151124 | F | 0	SilicoDArT
1091	36	28.4	3199342 | F | 0	SilicoDArT
1092	37	28.5	E40M50-293	AFLP
1093	38	29.2	3158428 | F | 0	SilicoDArT
1094	**39**	**29.7**	**brPb-839425**	**DArT**
1095	40	30.2	100005393 | F | 0	SilicoDArT
1096	41	30.9	3144775 | F | 0	SilicoDArT
1097	42	32.2	ra00652s01	SNP
1098	**43**	**39.1**		**KASP**
1099	44	39.8	100000706 | F | 0	SilicoDArT
1100	45	42.3	100001728 | F | 0	SilicoDArT
1101	46	42.3	3082293 | F | 0	SilicoDArT
1102	47	42.4	3078479 | F | 0	SilicoDArT
1103	**48**	**44.4**	**brPb-663595**	**DArT**
1104	49	45.6	3117172 | F | 0	SilicoDArT
1105	50	45.8	3079795 | F | 0	SilicoDArT
1106	51	46.0	3138311 | F | 0	SilicoDArT
1107	52	46.1	100003626 | F | 0	SilicoDArT
1108	53	46.7	3098806 | F | 0	SilicoDArT
1109	54	47.5	brPb-661171	DArT
1110	55	47.5	3106682 | F | 0	SilicoDArT
1111	56	48.6	3096432 | F | 0	SilicoDArT
1112	57	49.7	3080238 | F | 0	SilicoDArT
1113	58	50.0	3145549 | F | 0	SilicoDArT
1115	**60**	**50.3**	**3099333 | F | 0**	**SilicoDArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1114	59	50.3	3110585 | F | 0	SilicoDArT
1116	61	50.9	3151559 | F | 0	SilicoDArT
1117	62	52.9	3151444 | F | 0	SilicoDArT
1118	**63**	**54.3**	**3111584 | F | 0**	**SilicoDArT**
1119	64	55.4	100001058 | F | 0	SilicoDArT
1120	**65**	**61.6**	**3082377 | F | 0**	**SilicoDArT**
1121	66	63.2	3093258 | F | 0	SilicoDArT
1122	**67**	**70.7**	**100001264 | F | 0**	**SilicoDArT**
1123	68	79.7	3093577 | F | 0	SilicoDArT
1124	**69**	**83.2**	**3093435 | F | 0**	**SilicoDArT**
1125	70	85.2	3137975 | F | 0	SilicoDArT
1126	71	86.0	3099734 | F | 0	SilicoDArT
1127	72	89.7	3155403 | F | 0	SilicoDArT
1128	73	90.5	3095558 | F | 0	SilicoDArT
1129	74	90.7	3098753 | F | 0	SilicoDArT
1130	75	91.0	100004401 | F | 0	SilicoDArT
1131	76	91.2	3081663 | F | 0	SilicoDArT
1132	**77**	**92.0**	**3126608 | F | 0**	**SilicoDArT**
1133	78	94.0	100014252 | F | 0	SilicoDArT
1134	79	94.5	3082170 | F | 0	SilicoDArT
1135	80	99.3	3099517 | F | 0	SilicoDArT
1136	**81**	**100.3**	**3113836 | F | 0**	**SilicoDArT**
1137	82	101.5	3082224 | F | 0	SilicoDArT
1138	**83**	**103.9**	**3093598 | F | 0**	**SilicoDArT**
1139	**84**	**111.3**	**ra00285s01**	**SNP**
C03-II				
1140	**1**	**0.0**	**E40M50-355**	**AFLP**
1141	2	19.4	3159610 | F | 0	SilicoDArT
1142	**3**	**27.4**	**ra02841s01**	**SNP**
1143	4	32.0	3107287 | F | 0	SilicoDArT
1144	5	32.3	3079902 | F | 0	SilicoDArT
1145	6	32.4	3098341 | F | 0	SilicoDArT
1146	7	32.4	3097760 | F | 0	SilicoDArT
1147	**8**	**32.9**	**brPb-659400**	**DArT**
1148	9	35.3	3090391 | F | 0	SilicoDArT
1149	10	35.6	3105494 | F | 0	SilicoDArT
1150	11	36.0	3089578 | F | 0	SilicoDArT
1151	12	37.7	100000694 | F | 0	SilicoDArT
1152	**13**	**38.4**	**3087789 | F | 0**	**SilicoDArT**
1153	14	39.1	3113122 | F | 0	SilicoDArT
1154	15	40.2	3156183 | F | 0	SilicoDArT
1155	16	40.6	3089940 | F | 0	SilicoDArT
1156	17	41.0	brPb-663367	DArT
1157	18	41.0	brPb-839114	DArT
1158	19	41.1	3126246 | F | 0	SilicoDArT
1159	**20**	**41.8**	**3151023 | F | 0**	**SilicoDArT**
1160	21	43.6	100004699 | F | 0	SilicoDArT
1161	22	44.0	brPb-663065	DArT
1162	23	45.2		KASP
1163	24	60.6	CB10036	SSR
1164	**25**	**66.3**	**3132883 | F | 0**	**SilicoDArT**
1165	**26**	**74.2**	**3101542 | F | 0**	**SilicoDArT**
1166	27	75.8	3088878 | F | 0	SilicoDArT
1167	28	76.5	100003886 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1168	29	77.6	3157873 | F | 0	SilicoDArT
1169	30	79.3	3160778 | F | 0	SilicoDArT
1170	31	80.0	3121037 | F | 0	SilicoDArT
1171	32	80.1	3091189 | F | 0	SilicoDArT
1172	33	80.5	3158067 | F | 0	SilicoDArT
1173	34	80.5	3168074 | F | 0	SilicoDArT
1174	35	81.3	100001306 | F | 0	SilicoDArT
1175	36	81.3	3170572 | F | 0	SilicoDArT
1176	37	81.3	E36M51-151	AFLP
1177	38	81.4	3088309 | F | 0	SilicoDArT
1178	39	81.4	E38M50-53	AFLP
1179	40	81.6	3106690 | F | 0	SilicoDArT
1180	41	81.6	3109194 | F | 0	SilicoDArT
1181	**42**	**82.2**	**brPb-670779**	**DArT**
1182	43	82.2	3081727 | F | 0	SilicoDArT
1183	44	82.8	3109773 | F | 0	SilicoDArT
1184	45	83.4	3099182 | F | 0	SilicoDArT
1185	46	83.6	ra00466s01	SNP
1186	47	83.7	ra03820s01	SNP
1187	48	83.8	3111330 | F | 0	SilicoDArT
1188	49	83.8	3120837 | F | 0	SilicoDArT
1189	50	84.7	3128244 | F | 0	SilicoDArT
1190	51	88.5	3138709 | F | 0	SilicoDArT
1191	**52**	**88.9**	**3115435 | F | 0**	**SilicoDArT**
1192	53	92.4	100000573 | F | 0	SilicoDArT
1193	**54**	**94.3**	**3205118 | F | 0**	**SilicoDArT**
1194	**55**	**99.0**	**ra00281s01**	**SNP**
1195	**56**	**102.3**	**ra00115s01**	**SNP**
1196	57	102.7	ra02505s01	SNP
1197	58	106.1	100001721 | F | 0	SilicoDArT
1198	**59**	**107.6**	**3083936 | F | 0**	**SilicoDArT**
1199	60	111.2	100001718 | F | 0	SilicoDArT
1200	**61**	**134.8**	**3208561 | F | 0**	**SilicoDArT**
C04				
1201	1	-6.3	3081759 | F | 0	SilicoDArT
1202	**2**	**0.0**	**100001866 | F | 0**	**SilicoDArT**
1203	3	2.9	100000930 | F | 0	SilicoDArT
1204	4	3.5	3121308 | F | 0	SilicoDArT
1205	**5**	**4.0**	**brPb-839864**	**DArT**
1206	6	4.0	100002964 | F | 0	SilicoDArT
1207	7	4.6	3127821 | F | 0	SilicoDArT
1208	8	4.9	100001230 | F | 0	SilicoDArT
1209	9	5.1	100000822 | F | 0	SilicoDArT
1210	10	5.9	3147703 | F | 0	SilicoDArT
1211	11	6.6	CB10493c	SSR
1212	12	6.9	MR155a	SSR
1213	**13**	**10.0**	**ra00567s01**	**SNP**
1214	14	17.7	3092630 | F | 0	SilicoDArT
1215	15	18.4	100000611 | F | 0	SilicoDArT
1216	16	19.6	3149385 | F | 0	SilicoDArT
1217	**17**	**26.3**	**ra03588s01**	**SNP**
1218	**18**	**30.6**	**3098987 | F | 0**	**SilicoDArT**
1219	**19**	**34.2**	**3146544 | F | 0**	**SilicoDArT**
1220	20	35.1	3082001 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1221	21	36.5	100001583 | F | 0	SilicoDArT
1222	**22**	**42.5**	**3138243 | F | 0**	**SilicoDArT**
1223	23	43.8	3167909 | F | 0	SilicoDArT
1224	24	73.2	3214175 | F | 0	SilicoDArT
C04-II				
1225	**1**	**0.0**	**E36M59-382**	**AFLP**
1226	2	13.3	E36M59-181	AFLP
1227	3	14.9	ra00066s01	SNP
1228	**4**	**18.6**	**E44M59-383**	**AFLP**
1229	5	20.6	E39M48-104	AFLP
1230	6	21.3	ra02538s01	SNP
1231	7	21.3	ra04165s01	SNP
1232	**8**	**22.0**	**E45M48-139**	**AFLP**
1233	9	22.9	ra02651s01	SNP
1234	10	22.9	ra02073s01	SNP
1235	11	23.3	100020196 | F | 0	SilicoDArT
1236	12	23.5	3150078 | F | 0	SilicoDArT
1237	13	24.3	3090408 | F | 0	SilicoDArT
1238	14	24.7	3106150 | F | 0	SilicoDArT
1239	15	24.7	3180316 | F | 0	SilicoDArT
1240	16	24.7	100002196 | F | 0	SilicoDArT
1241	17	24.7	3210112 | F | 0	SilicoDArT
1242	18	24.9	3129137 | F | 0	SilicoDArT
1243	19	24.9	100001286 | F | 0	SilicoDArT
1244	20	24.9	3119350 | F | 0	SilicoDArT
1245	21	25.0	3122115 | F | 0	SilicoDArT
1246	22	25.1	100002244 | F | 0	SilicoDArT
1247	23	25.2	3080821 | F | 0	SilicoDArT
1248	24	25.2	3126142 | F | 0	SilicoDArT
1249	25	25.3	3085780 | F | 0	SilicoDArT
1250	26	25.4	3103855 | F | 0	SilicoDArT
1251	**27**	**27.3**	**3096503 | F | 0**	**SilicoDArT**
1252	**28**	**31.1**	**3164705 | F | 0**	**SilicoDArT**
1253	29	36.2	3129822 | F | 0	SilicoDArT
1254	30	36.7	3098822 | F | 0	SilicoDArT
1255	31	36.7	3083242 | F | 0	SilicoDArT
1256	32	36.8	3078133 | F | 0	SilicoDArT
1257	33	36.9	brPb-840880	DArT
1258	34	37.1	3085231 | F | 0	SilicoDArT
1259	35	37.1	3097845 | F | 0	SilicoDArT
1260	36	37.3	3153513 | F | 0	SilicoDArT
1261	37	37.4	3074933 | F | 0	SilicoDArT
1262	38	37.4	100017801 | F | 0	SilicoDArT
1263	39	37.4	3098880 | F | 0	SilicoDArT
1264	40	37.8	3085199 | F | 0	SilicoDArT
1265	41	37.8	3192882 | F | 0	SilicoDArT
1266	42	37.9	3089644 | F | 0	SilicoDArT
1267	43	37.9	100000616 | F | 0	SilicoDArT
1268	44	38.0	100003082 | F | 0	SilicoDArT
1269	45	38.0	3119539 | F | 0	SilicoDArT
1270	46	38.1	3122916 | F | 0	SilicoDArT
1271	47	38.1	3080189 | F | 0	SilicoDArT
1272	48	38.2	100003987 | F | 0	SilicoDArT
1273	49	38.2	3181679 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1274	50	38.2	3108582 | F | 0	SilicoDArT
1275	51	38.5		KASP
1276	52	38.8	3105936 | F | 0	SilicoDArT
1277	53	38.8	3086475 | F | 0	SilicoDArT
1278	54	38.9	3120311 | F | 0	SilicoDArT
1279	55	39.2	3131174 | F | 0	SilicoDArT
1280	56	41.8		KASP
1281	57	42.8	3096418 | F | 0	SilicoDArT
1282	58	43.0	100002097 | F | 0	SilicoDArT
1283	59	43.6	3074798 | F | 0	SilicoDArT
1284	60	43.7	brPb-660096	DArT
1285	61	43.8	brPb-841817	DArT
1286	62	44.3	3210998 | F | 0	SilicoDArT
1287	63	46.2	3094877 | F | 0	SilicoDArT
1288	64	49.5	ra04231s01	SNP
1289	65	51.4	3127188 | F | 0	SilicoDArT
1290	**66**	**55.4**	**3084493 | F | 0**	**SilicoDArT**
1291	67	57.3	100003606 | F | 0	SilicoDArT
1292	68	58.4	3089393 | F | 0	SilicoDArT
1293	69	59.5	3151340 | F | 0	SilicoDArT
1294	70	59.6	3130008 | F | 0	SilicoDArT
1295	71	59.7	3117265 | F | 0	SilicoDArT
1296	72	59.8	3137785 | F | 0	SilicoDArT
1297	73	60.2	3132945 | F | 0	SilicoDArT
1298	**74**	**60.7**	**100008181 | F | 0**	**SilicoDArT**
1299	75	62.2	3098394 | F | 0	SilicoDArT
1300	76	64.2		KASP
1301	77	66.5	3130203 | F | 0	SilicoDArT
1302	**78**	**67.1**	**3079359 | F | 0**	**SilicoDArT**
1303	79	69.9	3203812 | F | 0	SilicoDArT
1304	80	71.2	3085903 | F | 0	SilicoDArT
1305	81	71.9	3215423 | F | 0	SilicoDArT
1306	82	72.5	100000603 | F | 0	SilicoDArT
1307	83	73.0	3160534 | F | 0	SilicoDArT
1308	**84**	**74.9**	**3077089 | F | 0**	**SilicoDArT**
1309	85	91.6	100000363 | F | 0	SilicoDArT
1310	86	92.4	3089635 | F | 0	SilicoDArT
1311	**87**	**94.2**		**KASP**
1312	88	97.9	3169070 | F | 0	SilicoDArT
1313	89	100.0	3099782 | F | 0	SilicoDArT
1314	90	100.8	100004246 | F | 0	SilicoDArT
1315	91	100.9	3092725 | F | 0	SilicoDArT
1316	92	101.0	3089038 | F | 0	SilicoDArT
1317	93	101.0	3087411 | F | 0	SilicoDArT
C05				
1318	**1**	**0.0**	**100001020 | F | 0**	**SilicoDArT**
1319	**2**	**4.1**	**100001059 | F | 0**	**SilicoDArT**
1320	3	10.3	BNKS003011	KASP
1321	4	11.7	3135471 | F | 0	SilicoDArT
1322	5	12.7	3078305 | F | 0	SilicoDArT
1323	**6**	**13.7**	**3187836 | F | 0**	**SilicoDArT**
1324	7	15.3	3148284 | F | 0	SilicoDArT
1325	8	15.4	3158912 | F | 0	SilicoDArT
1326	9	16.0	3077295 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1327	10	16.7	E45M48-186	AFLP
1328	**11**	**23.2**	**ra00447s01**	**SNP**
1329	12	30.5	3107673 | F | 0	SilicoDArT
1330	13	30.5	3086141 | F | 0	SilicoDArT
1331	14	31.3	3113517 | F | 0	SilicoDArT
1332	15	31.3	3099529 | F | 0	SilicoDArT
1333	16	31.6	3085952 | F | 0	SilicoDArT
1334	17	31.6	3215116 | F | 0	SilicoDArT
1335	18	31.8	3134611 | F | 0	SilicoDArT
1336	19	32.5	3107756 | F | 0	SilicoDArT
1337	20	32.9	3186413 | F | 0	SilicoDArT
1338	**21**	**38.8**	**ra00237s01**	**SNP**
1339	22	42.2	100001580 | F | 0	SilicoDArT
1340	23	42.3	brPb-839592	DArT
1341	24	42.4	3084391 | F | 0	SilicoDArT
1342	25	42.4	3142945 | F | 0	SilicoDArT
1343	26	42.6	3119110 | F | 0	SilicoDArT
1344	27	42.8	3121010 | F | 0	SilicoDArT
1345	28	43.3	E40M50-341	AFLP
1346	29	43.5	3126131 | F | 0	SilicoDArT
1347	30	44.1	3121351 | F | 0	SilicoDArT
1348	31	44.3	3176872 | F | 0	SilicoDArT
1349	32	45.9	100001511 | F | 0	SilicoDArT
1350	33	45.9	3089519 | F | 0	SilicoDArT
1351	34	49.9	3103176 | F | 0	SilicoDArT
1352	35	51.8	3130525 | F | 0	SilicoDArT
1353	36	53.0	100000496 | F | 0	SilicoDArT
1354	37	53.4	100000289 | F | 0	SilicoDArT
1355	38	53.5	100002575 | F | 0	SilicoDArT
1356	39	54.3	3081126 | F | 0	SilicoDArT
1357	**40**	**55.3**	**3084373 | F | 0**	**SilicoDArT**
1358	41	55.8	3090482 | F | 0	SilicoDArT
1359	42	56.9	3075876 | F | 0	SilicoDArT
1360	43	58.1	3088390 | F | 0	SilicoDArT
1361	**44**	**63.5**	**3091164 | F | 0**	**SilicoDArT**
1362	45	66.0	100001238 | F | 0	SilicoDArT
1363	46	66.2	100002711 | F | 0	SilicoDArT
1364	47	66.3	100002141 | F | 0	SilicoDArT
1365	48	67.9	ra00544s01	SNP
1366	**49**	**69.4**	**ra00678s01**	**SNP**
1367	**50**	**86.2**	**100001393 | F | 0**	**SilicoDArT**
1368	51	88.5	3112905 | F | 0	SilicoDArT
1369	52	89.3	3085758 | F | 0	SilicoDArT
1370	**53**	**92.1**	**100002303 | F | 0**	**SilicoDArT**
C06				
1371	**1**	**0.0**	**3193324 | F | 0**	**SilicoDArT**
1372	**2**	**22.1**	**3136669 | F | 0**	**SilicoDArT**
1373	3	25.1	3136866 | F | 0	SilicoDArT
1374	4	25.1	100015824 | F | 0	SilicoDArT
1375	5	25.3	3212698 | F | 0	SilicoDArT
1376	6	25.3	3136795 | F | 0	SilicoDArT
1377	7	25.4	3171233 | F | 0	SilicoDArT
1378	8	25.6	100002982 | F | 0	SilicoDArT
1379	9	25.7	brPb-660868	DArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1380	**10**	**26.5**	**brPb-841049**	**DArT**
1381	11	26.5	3085522 | F | 0	SilicoDArT
1382	12	28.1		KASP
1383	13	28.6	3112397 | F | 0	SilicoDArT
1384	14	28.9	3076567 | F | 0	SilicoDArT
1385	15	28.9	ra00623s01	SNP
1386	16	29.0	ra04332s01	SNP
1387	17	29.7	3100211 | F | 0	SilicoDArT
1388	18	48.2	3081948 | F | 0	SilicoDArT
1389	19	49.5	100003870 | F | 0	SilicoDArT
1390	**20**	**49.8**	**brPb-659518**	**DArT**
1391	21	50.4	3096578 | F | 0	SilicoDArT
1392	22	50.9	3179814 | F | 0	SilicoDArT
1393	23	51.0	3155927 | F | 0	SilicoDArT
1394	24	51.1	E44M51-332	AFLP
1395	25	51.9	100002241 | F | 0	SilicoDArT
1396	26	52.8	3081113 | F | 0	SilicoDArT
1397	27	53.0	E44M48-114	AFLP
1398	28	53.0	3091775 | F | 0	SilicoDArT
1399	29	53.2	3156311 | F | 0	SilicoDArT
1400	30	53.3	3116862 | F | 0	SilicoDArT
1402	**32**	**54.2**	**brPb-841792**	**DArT**
1401	31	54.2	brPb-663544	DArT
1403	33	54.8	3150052 | F | 0	SilicoDArT
1404	34	54.9	3207235 | F | 0	SilicoDArT
1405	35	55.1	3179786 | F | 0	SilicoDArT
1406	36	55.3	3161037 | F | 0	SilicoDArT
1407	37	55.3	3097864 | F | 0	SilicoDArT
1408	38	56.5	3127890 | F | 0	SilicoDArT
1409	**39**	**61.2**	**3079275 | F | 0**	**SilicoDArT**
1410	40	64.3	3095882 | F | 0	SilicoDArT
1411	41	65.5	3082021 | F | 0	SilicoDArT
1412	42	66.7	3156678 | F | 0	SilicoDArT
1413	**43**	**69.3**	**3079253 | F | 0**	**SilicoDArT**
1414	44	71.2	ra00231s01	SNP
1415	45	71.7	3078351 | F | 0	SilicoDArT
1416	46	72.4	ra00276s01	SNP
1417	47	72.8	3103187 | F | 0	SilicoDArT
1418	48	74.7	100000804 | F | 0	SilicoDArT
1419	49	75.8	3077473 | F | 0	SilicoDArT
1420	50	76.2	3101739 | F | 0	SilicoDArT
1421	**51**	**76.9**	**3082596 | F | 0**	**SilicoDArT**
1422	52	77.4	3078615 | F | 0	SilicoDArT
1423	53	77.8	3085425 | F | 0	SilicoDArT
1424	54	78.7	3105052 | F | 0	SilicoDArT
1425	55	78.8	3110809 | F | 0	SilicoDArT
1426	56	79.5	3160180 | F | 0	SilicoDArT
1427	57	83.9	3082908 | F | 0	SilicoDArT
1428	**58**	**85.8**	**3083438 | F | 0**	**SilicoDArT**
1429	**59**	**89.9**	**100002535 | F | 0**	**SilicoDArT**
1430	60	90.8	3148087 | F | 0	SilicoDArT
1431	61	91.3	3084835 | F | 0	SilicoDArT
1432	**62**	**94.9**	**ra01830s01**	**SNP**
1433	63	94.9	ra01831s01	SNP
1434	64	95.2	ra00642s01	SNP

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
C07				
1435	**1**	**0.0**	**E36M51-214**	**AFLP**
1436	2	21.4	E36M51-183	AFLP
1437	**3**	**22.7**	**E36M51-217**	**AFLP**
1438	4	30.0	3101952 | F | 0	SilicoDArT
1439	5	30.1	3078289 | F | 0	SilicoDArT
1440	6	30.1	3081171 | F | 0	SilicoDArT
1441	7	30.7	3097143 | F | 0	SilicoDArT
1442	8	33.5	brPb-809585	DArT
1443	9	33.9	3085661 | F | 0	SilicoDArT
1444	**10**	**33.9**	**brPb-657886**	**DArT**
1445	11	35.0	3149733 | F | 0	SilicoDArT
1446	12	35.9	3094933 | F | 0	SilicoDArT
1447	13	35.9	100002384 | F | 0	SilicoDArT
1448	14	36.5	3081298 | F | 0	SilicoDArT
1449	15	37.2	CB10534	SSR
1450	**16**	**40.1**	**E37M59-134**	**AFLP**
1451	17	41.2	3158051 | F | 0	SilicoDArT
1452	18	41.6	3079508 | F | 0	SilicoDArT
1453	19	41.6	3143136 | F | 0	SilicoDArT
1454	20	41.6	3084934 | F | 0	SilicoDArT
1455	21	41.7	3136495 | F | 0	SilicoDArT
1456	22	41.7	100005225 | F | 0	SilicoDArT
1457	23	41.9	3089139 | F | 0	SilicoDArT
1458	24	41.9	3094601 | F | 0	SilicoDArT
1459	25	41.9	3081012 | F | 0	SilicoDArT
1460	26	42.0	3165317 | F | 0	SilicoDArT
1461	27	43.5	100001120 | F | 0	SilicoDArT
1462	28	45.6	brPb-838855	DArT
1463	**29**	**49.0**	**3083814 | F | 0**	**SilicoDArT**
1464	**30**	**55.0**	**100001027 | F | 0**	**SilicoDArT**
1465	31	64.2	ra00058s01	SNP
1466	**32**	**66.9**	**100001167 | F | 0**	**SilicoDArT**
1467	33	70.3	ra00451s01	SNP
1468	34	70.8	3160238 | F | 0	SilicoDArT
1469	35	73.8	3161835 | F | 0	SilicoDArT
1470	**36**	**74.9**	**brPb-808675**	**DArT**
1471	37	76.4		KASP
1472	38	79.1	3081175 | F | 0	SilicoDArT
1473	39	80.6	BRA014	SSR
1474	**40**	**82.1**	**CB10014**	**SSR**
1475	41	84.4	3091341 | F | 0	SilicoDArT
1476	42	84.6	ra04036s01	SNP
1477	43	85.5	100000962 | F | 0	SilicoDArT
1478	44	86.7	100001740 | F | 0	SilicoDArT
1479	45	90.5	3146788 | F | 0	SilicoDArT
1480	46	92.2	100003088 | F | 0	SilicoDArT
1481	**47**	**93.0**	**brPb-670555**	**DArT**
1482	48	94.2	100001361 | F | 0	SilicoDArT
1483	49	94.4	ra02098s01	SNP
1484	50	94.5	3140098 | F | 0	SilicoDArT
1485	51	94.5	3079995 | F | 0	SilicoDArT
1486	52	94.8	100001220 | F | 0	SilicoDArT
1487	53	94.8	3219799 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1488	54	95.1	3084983 | F | 0	SilicoDArT
1489	55	95.7	100001320 | F | 0	SilicoDArT
1490	56	96.1	3090436 | F | 0	SilicoDArT
1491	57	96.8	3085711 | F | 0	SilicoDArT
1492	58	97.8	3097288 | F | 0	SilicoDArT
1493	59	98.8	3164862 | F | 0	SilicoDArT
1494	**60**	**100.0**	**3113313 | F | 0**	**SilicoDArT**
1495	61	100.7	ra00178s01	SNP
1496	62	100.8	3211282 | F | 0	SilicoDArT
1497	63	101.2	3098040 | F | 0	SilicoDArT
1498	64	101.6	3166059 | F | 0	SilicoDArT
1499	65	102.1	3141542 | F | 0	SilicoDArT
1500	66	103.0	3137590 | F | 0	SilicoDArT
1501	67	109.1	3105141 | F | 0	SilicoDArT
1502	68	109.2	3126628 | F | 0	SilicoDArT
1503	69	109.4	3103394 | F | 0	SilicoDArT
1504	70	109.6	3140873 | F | 0	SilicoDArT
1505	71	109.6	3086239 | F | 0	SilicoDArT
1506	72	109.9	brPb-659287	DArT
1507	73	109.9	3096647 | F | 0	SilicoDArT
1508	74	110.1	100020246 | F | 0	SilicoDArT
1509	75	110.5	3085658 | F | 0	SilicoDArT
1510	76	110.6	3164606 | F | 0	SilicoDArT
1511	77	110.6	3140984 | F | 0	SilicoDArT
1512	78	111.4	3090573 | F | 0	SilicoDArT
1513	**79**	**113.1**	**E38M50-142**	**AFLP**
1514	80	114.5	CB10431	SSR
1515	81	114.8	E44M48-146	AFLP
1516	82	115.2	3119631 | F | 0	SilicoDArT
1517	83	117.0	E39M48-416	AFLP
1518	84	117.3	E37M51-114	AFLP
1519	85	118.5	ra00740s01	SNP
1520	86	118.5		KASP
1521	87	118.8	3097580 | F | 0	SilicoDArT
1522	88	119.0	3097182 | F | 0	SilicoDArT
1523	89	119.0	100000655 | F | 0	SilicoDArT
1524	90	119.1	3154828 | F | 0	SilicoDArT
1525	91	119.2	3090464 | F | 0	SilicoDArT
1526	92	119.9	brPb-669913	DArT
1527	93	119.9	brPb-838990	DArT
1528	**94**	**119.9**	**3122303 | F | 0**	**SilicoDArT**
1529	95	120.3	3108998 | F | 0	SilicoDArT
1530	96	120.4	3083022 | F | 0	SilicoDArT
1531	97	120.5	3143308 | F | 0	SilicoDArT
1532	98	120.7	100002401 | F | 0	SilicoDArT
1533	99	120.9	3080897 | F | 0	SilicoDArT
1534	100	121.0	3113940 | F | 0	SilicoDArT
1535	101	121.1	3085352 | F | 0	SilicoDArT
1536	102	121.1	3092557 | F | 0	SilicoDArT
1537	103	121.5	3155125 | F | 0	SilicoDArT
1538	104	121.6	100006260 | F | 0	SilicoDArT
1539	105	121.7	3150881 | F | 0	SilicoDArT
1540	106	121.8	3099748 | F | 0	SilicoDArT
1541	107	125.0	3146405 | F | 0	SilicoDArT
1542	**108**	**127.2**	**3119656 | F | 0**	**SilicoDArT**

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1543	109	127.7	3130887 | F | 0	SilicoDArT
1544	110	128.2	100005316 | F | 0	SilicoDArT
1545	111	129.2	3142015 | F | 0	SilicoDArT
1546	112	129.7	3076082 | F | 0	SilicoDArT
1547	113	130.2	3078096 | F | 0	SilicoDArT
1548	114	130.5	3112639 | F | 0	SilicoDArT
1549	115	130.6	3114986 | F | 0	SilicoDArT
1550	116	131.0	3165595 | F | 0	SilicoDArT
1551	117	131.0	3095662 | F | 0	SilicoDArT
1552	118	131.0	3162387 | F | 0	SilicoDArT
1553	**119**	**132.2**	**CB10425a**	**SSR**
1554	120	138.1	3151222 | F | 0	SilicoDArT
1555	**121**	**142.4**	**ra03178s01**	**SNP**
C08				
1556	1	-10.9	3114892 | F | 0	SilicoDArT
1557	2	-9.9	3114506 | F | 0	SilicoDArT
1558	**3**	**0.0**	**3133904 | F | 0**	**SilicoDArT**
1559	4	1.9	3080989 | F | 0	SilicoDArT
1560	5	7.4	ra02289s01	SNP
1561	**6**	**10.8**	**ra03760s01**	**SNP**
1562	7	13.8	3186200 | F | 0	SilicoDArT
1563	8	15.6	3101240 | F | 0	SilicoDArT
1564	9	17.1	E45M48-283	AFLP
1565	10	17.2	3216199 | F | 0	SilicoDArT
1566	11	17.4	3139695 | F | 0	SilicoDArT
1567	12	17.4	3124093 | F | 0	SilicoDArT
1568	13	17.4	100005728 | F | 0	SilicoDArT
1569	14	17.5	3091815 | F | 0	SilicoDArT
1570	15	17.5	3089494 | F | 0	SilicoDArT
1571	16	17.6	3127582 | F | 0	SilicoDArT
1572	17	17.8	3101502 | F | 0	SilicoDArT
1573	18	18.0	3088795 | F | 0	SilicoDArT
1574	19	18.0	3103333 | F | 0	SilicoDArT
1575	20	18.2	3098256 | F | 0	SilicoDArT
1577	**22**	**18.6**	**E38M51-65**	**AFLP**
1576	21	18.6	3091900 | F | 0	SilicoDArT
1578	23	19.1	100000412 | F | 0	SilicoDArT
1579	24	19.4	100000306 | F | 0	SilicoDArT
1580	25	19.5	3119229 | F | 0	SilicoDArT
1581	26	19.6	3097375 | F | 0	SilicoDArT
1582	27	19.6	E38M51-63	AFLP
1583	28	19.9	3097554 | F | 0	SilicoDArT
1584	**29**	**21.3**	**3159566 | F | 0**	**SilicoDArT**
1585	30	25.4	3189138 | F | 0	SilicoDArT
1586	31	25.7	100005461 | F | 0	SilicoDArT
1587	32	25.9	3095147 | F | 0	SilicoDArT
1588	**33**	**41.6**	**E32M48-253**	**AFLP**
C09				
1589	**1**	**0.0**	**Dx-3**	**Candidate gene - Bol029796**
1590	**2**	**16.2**	**3074850 | F | 0**	**SilicoDArT**
1591	3	22.4	3103440 | F | 0	SilicoDArT
1592	4	23.4	3077053 | F | 0	SilicoDArT
1593	5	23.6	3158618 | F | 0	SilicoDArT

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Appendix A.8: *continued from previous page*

No. of marker	No. of marker per LG	Pos. (cM)	Marker name	Marker type
1594	6	23.7	3160758 | F | 0	SilicoDArT
1595	**7**	**24.2**	**3080644 | F | 0**	**SilicoDArT**
1596	8	25.0	3153498 | F | 0	SilicoDArT
1597	9	25.8	3087600 | F | 0	SilicoDArT
1598	10	25.9	3082014 | F | 0	SilicoDArT
1599	11	26.6	3093558 | F | 0	SilicoDArT
1600	12	27.0	3082688 | F | 0	SilicoDArT
1601	13	28.3	100018988 | F | 0	SilicoDArT
1602	14	30.1	100000777 | F | 0	SilicoDArT
1603	**15**	**30.9**	**3092949 | F | 0**	**SilicoDArT**
1604	16	31.4	3094779 | F | 0	SilicoDArT
1605	17	31.6	brPb-660506	DArT
1606	18	31.6	brPb-840166	DArT
1607	19	32.2	3162543 | F | 0	SilicoDArT
1608	20	32.5	3077953 | F | 0	SilicoDArT
1609	21	32.6	3085566 | F | 0	SilicoDArT
1610	22	32.7	3081512 | F | 0	SilicoDArT
1611	23	32.7	3171107 | F | 0	SilicoDArT
1612	24	32.7	3171910 | F | 0	SilicoDArT
1613	25	33.3	3113558 | F | 0	SilicoDArT
1614	26	33.7	3099816 | F | 0	SilicoDArT
1615	27	33.9	3080113 | F | 0	SilicoDArT
1616	28	34.2	brPb-839138	DArT
1617	29	34.4	3142064 | F | 0	SilicoDArT
1618	30	34.4	100000972 | F | 0	SilicoDArT
1619	**31**	**35.0**	**3104273 | F | 0**	**SilicoDArT**
1620	32	36.1	100020310 | F | 0	SilicoDArT
1621	33	36.9	3115278 | F | 0	SilicoDArT
1622	34	37.7	100004306 | F | 0	SilicoDArT
1623	35	38.1	3076981 | F | 0	SilicoDArT
1624	36	38.4	3084037 | F | 0	SilicoDArT
1625	37	38.4	3197712 | F | 0	SilicoDArT
1626	38	38.5	3091232 | F | 0	SilicoDArT
1627	39	38.5	100003671 | F | 0	SilicoDArT
1628	40	39.4	3171616 | F | 0	SilicoDArT
1629	41	40.2	3103755 | F | 0	SilicoDArT
1630	**42**	**40.5**	**3163621 | F | 0**	**SilicoDArT**
1631	43	41.3	3199932 | F | 0	SilicoDArT
1632	44	41.6	3179809 | F | 0	SilicoDArT
1633	45	42.2	3077879 | F | 0	SilicoDArT
1634	46	42.7	3081845 | F | 0	SilicoDArT
1635	**47**	**44.5**	**100001132 | F | 0**	**SilicoDArT**
1636	48	45.1	3165968 | F | 0	SilicoDArT
1637	49	48.9	CB10109	SSR
1638	50	49.7	ra02422s01	SNP
1639	51	50.2	E39M48-153	AFLP
1640	52	51.8	ra01544s01	SNP
1641	53	53.3	ra00571s01	SNP
1642	**54**	**63.5**	**ra02032s01**	**SNP**

A.9 List of cultivars

List of winter oilseed rape cultivars (n = 81) used in association mapping

No.	Variety	Breeder	No.	Variety	Breeder
1	Jessica	-	41	Aragon	NPZ
2	Pollen	Adrien Momont	42	Aurum	NPZ
3	Bristol	DSV	43	Baros	NPZ
4	Capitol	DSV	44	Campari	NPZ
5	Contact	DSV	45	Caramba	NPZ
6	Idol	DSV	46	Express 617	NPZ
7	Licapo	DSV	47	Gefion	NPZ
8	Lion	DSV	48	HSL 1032	NPZ
9	Lipid	DSV	49	Lorenz	NPZ
10	Lipton	DSV	50	LSF 519	NPZ
11	Lirajet	DSV	51	Nugget	NPZ
12	Lisabeth	DSV	52	Prince	NPZ
13	Lisek	DSV	53	Rasmus	NPZ
14	Oase	DSV	54	SLM 413	NPZ
15	Vivol	DSV	55	SLM 512	NPZ
16	Adder	KWS	56	Viking	NPZ
17	Agalon	KWS	57	Wotan	NPZ
18	Alaska	KWS	58	Zephir	NPZ
19	Alesi	KWS	59	Amor	Petersen/Raps Gbr
20	Allure	KWS	60	Duell	Raps
21	KW1519	KWS	61	Orlando	Saaten Union
22	KW3077	KWS	62	Ascona	SW seed
23	Lord	KWS	63	Aviso	SW seed
24	Milena	KWS	64	Expert	SW seed
25	Picasso	KWS	65	Falstaff	SW seed
26	Pirola	KWS	66	Kvintett	SW seed
27	Remy	KWS	67	Musette	SW seed
28	Robust	KWS	68	Sansibar	SW seed
29	Rodeo	KWS	69	SW Gospel	SW seed
30	Atlantic	Limagrain-Nickerson	70	SW Sinatra	SW seed
31	Boston	Limagrain-Nickerson	71	Tenor	SW seed
32	Cooper	Limagrain-Nickerson	72	Verona	SW seed
33	Escort	Limagrain-Nickerson	73	Apex	Syngenta
34	Ladoga	Limagrain-Nickerson	74	Fortis	Syngenta
35	Manitoba	Limagrain-Nickerson	75	Laser	Syngenta
36	Missouri	Limagrain-Nickerson	76	Madrigal	Syngenta
37	Montego	Limagrain-Nickerson	77	Magnum	Syngenta
38	Pacific	Limagrain-Nickerson	78	NK Bravour	Syngenta
39	Rapid	Limagrain-Nickerson	79	Recital	Syngenta
40	Savannah	Limagrain-Nickerson	80	Roxet	Syngenta
			81	Smart	Syngenta

KWS KWS SAAT AG; *DSV* Deutsche Saatveredelung AG;*NPZ* Norddeutsche Pflanzenzucht Hans-Georg Lembke KG

Acknowledgement

I owe an enormous amount to my supervisor, Dr. Christian Möllers, whose supervision, encouragement, generosity and patience throughout my PhD journey has made this thesis become a reality. I would like to thank Dr. Wolfgang Ecke for his help in map construction and valuable advice throughout this research work, and Prof. Wolfgang Link, for his time in statistical assistance. I am grateful to Prof. Dr. Heiko Becker for leading the team and giving me the opportunity to join Plant Breeding Division.

I would like to express my words of gratitude to Prof. Dr. Thomas Debener and Prof. Dr. Elke Pawelzik for kindly consenting to be my examiners.

This study was funded by the Deutsche Forschungsgemeinschaft (DFG). I would like to thank the breeding companies and institute for carrying out field trials: KWS Saat AG and Deutsche Saatveredelung GmbH, Germany; Lantmännen SW Seed, Sweden; and Academy of Agricultural Sciences (Prof Jianyi Zhao), Hangzhou, China.

My sincere gratitudes go to Uwe Ammermann, Rosi Clemens, Gunda Aselmeyer, and Carmen Mensch for their excellent technical support. A HUGE thank you to a wonderful colleague and friend, Nina Behnke, who has helped me a lot during my stay here in Germany. Special thanks to Ariana Istar Valdés-Velasquez, Anthimos Kampouridis, Antje Sicherholt, Christopher Hoppe, Edy Suprianto, Gregor Welna, Haiko Brandes, Julia Rudloff, Mareile Stever, Sansanti Widiarsih, Siyang Liu, and Sebastian Miersch, for their kindness and generosity that have contributed to an enjoyable working environment.

I would also like to take this opportunity to thank two of my dearest friends, Daniela Cadamuro and Nikel Yamonto, for giving me the support I have always needed and their invaluable friendship. Lastly, I am forever grateful to my parents, Janis and Poh Huat, whose unconditional love have made all of this possible.

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