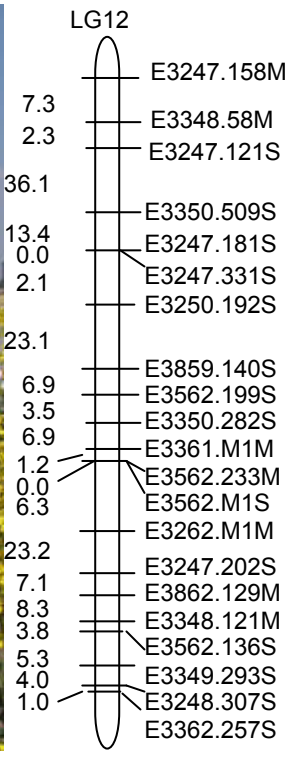
Berisso Kebede

Development of intervarietal substitution lines in *Brassica napus* L. using marker assisted selection and mapping of QTL for agronomically important traits



Development of intervarietal substitution lines in *Brassica napus* L. using marker assisted selection and mapping of QTL for agronomically important traits

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In memory of my father, late Mr. Kebede Demo Kemesi

AB-QTL	Advanced backcross quantitative trait loci
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BC	Backcross
bp	base pairs
C16:0	Palmitic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C22:1	Erucic acid
cM	centi Morgan
CoA	Coenzyme A
CORR	Correlation
^{o}C	degree Celsius
DF	Degree of freedom
DH	Double haploid
Diff.	Difference
DNA	Deoxyribonucleic acid
DSV	Deutsche Saatveredelung
dNTPs	mixture of deoxyribosenucleotide triphosphates
Eru6	Erucic acid gene on linkage group 6 on the map
Eru12	Erucic acid gene on linkage group 12 on the map
F	Filala
FAO	Food and Agriculture Organization
g	gram
GC	Gas chromatography
GLM	General Linear Model
gsl (GSL)	Glucosinolate
hr	hour
kg	Killogram
Km	Killometer
KWS	KWS SAAT AG
L	Length
LG	Linkage group
LSMEANS	Least square means
M	Mansholt
mA	milli Amper
MAS	Marker assisted selection
mg	milligram
Min.	Minute
mM	milli Molar
MS	Mean of squares
ng	nanogram
NILs	Near isogenic lines
NIRS	Near Infrared spectroscopy
nm	nanometer
No	Number
NPZ	Norddeutschen Pflanzenzucht HG Lembke KG

P	Probability value
PCR	Polymerase Chain Reaction
Pmoles	Pico moles
Pro	Protein
PROC	Procedure
QTL	Quantitative trait loci
RAPD	Random Amplified Polymorphism of DNA
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant inbreed lines
RL	Restriction ligation
Sec.	Second
SNP	Single Nucleotide Polymorphism
SS	Sum of squares
SSR	Single Sequence Repeat
Sta	Start
SW	SW seed GmbH
V	Volt
W	Watt
%	Percentage
µl	microlitre
µmol	micro mole
Plh	Plant height
BOF	Begin of flowering
EOF	End of flowering
DOF	Duration of flowering
WS	Winter survival

1. General introduction

Rapeseed belongs to the family *Brassicaceae* from which a total of 6 species play an important role in agriculture. These are *Brassica rapa*, *B. olreacea*, *B. napus*, *B. juncea*, *B. nigra* and *B. carinata*. After the release of canola quality cultivars which contain low erucic acid and low glucosinolate content, rapeseed became an important oil crop in temperate areas. In the last decades, the area coverage and amount of production are continuously increasing in Europe and China. In 2005, a total of 27 million hectare land was covered by rapeseed, an increase of 1.03% from the year before (Oil World 2007). The trend still shows an increase in rapeseed production worldwide. In Germany, rapeseed production is increasing each year due to the diversification of the use of the crop as biodiesel and a total of 1.5 million hectare land was covered in 2006, an increase of 0.15 million hectares from the year before (Oil World 2007).

In the last decades, breeding objectives of rapeseed were mainly concentrated on improving oil content and oil related traits. However, breeding new and better cultivars that can cope with existing environment and produce maximum yield are also attractive breeding aims. Additionally, rapeseed meal, which remains after the extraction of oil can be used in animal feeding and may require development of cultivars with high protein and high oil content to be used for multiple purposes. Development of better cultivars requires better knowledge of inheritance of the desired traits. Most agronomically important traits are controlled by many genes and unlike monogenic traits they don't follow Mendelian pattern of inheritance. Moreover, the level of each gene effect differs in which some genes contribute large effects while others have only small effects on the trait and are usually described as major and minor genes, respectively. These genes can interact with other genes and with the environment making it difficult to clearly define their effects. Classical quantitative genetics is limited to statistical approaches dealing with the average effects of genes and provides little information about the localization and number of QTL (Quantitative trait loci).

In the last decade's development of molecular markers like RFLP, RAPD, AFLP, SSR and SNP allowed the construction of dense maps for many plant species. These new methods opened a new approach of QTL mapping to study quantitative traits (Lander and Botstein 1989). QTL mapping identifies genome regions that harbour genes affecting a quantitative trait and can also estimate QTL effects. Knowledge of the number of QTL and their effects on phenotypic traits can help to understand the genetic architecture of the traits. For instance, oil content in rapeseed may be controlled by many genes with small effects, or by a few genes with large effects. QTL

mapping studies can also be used to identify candidate genes if the genome region contributing to a phenotypic trait is identified.

QTL mapping

QTL mapping involves selecting mapping population, genetic marker assays, evaluation of traits of interest and making inferences about QTL based on the association analysis between genetic markers and the trait (Collard et al. 2005). Several statistical methods such as t-test, analysis of variance, regression and generalized likelihood approach are usually used to detect QTL. A significant association between a trait and genetic markers may be an indication of a QTL residing near the markers (Collard et al. 2005). However, statistical significance does not always reveal a biological significance due to the multiple test problem associated with QTL mapping (Liu 1998). Generally, QTL mapping is affected by the heritability of a trait, the total number of QTL affecting the trait, the distribution of the QTL in the genome, interaction between genes, variation due to environment, type and size of the population used for mapping, genome size and resolution of markers (Liu 1998) and method of QTL mapping. A number of QTL mapping methods are currently available like simple interval mapping and composite interval mapping, relaying on statistical methods to find relationships between molecular markers and QTL in a segregating population.

Interval mapping (Lander and Botstein 1989) uses information of two linked markers to test for presence of a putative QTL in the intervening interval. Interval mapping is widely done using a software package MapMaker/QTL (Lincoln et al. 1992). However, interval mapping gives a rough estimate of QTL position. Depending on the population size used for mapping, variance analysed and the QTL effects, QTL localization could be in the range of confidence interval of 10's of cM (van Ooijen 1992, Darvasi et al. 1993). Interval mapping is influenced by closely linked QTL and may consider them as a single QTL. Additionally, the number of QTL cannot be resolved and the statistical power is relatively low (Liu 1998). Simulation results revealed that a "ghost QTL" might appear between two linked QTL in interval mapping, while the two real QTL are hidden by "ghost QTL" (Moreno-Gonzalez 1992).

Composite interval mapping (CIM) uses multiple markers as factors in the analysis of QTL and overcomes the problems of low testing power and "ghost QTL" of the interval mapping. Computer software packages like PLAB/QTL (Utz and Melchinger 1996) and MapManager QTX (Manly et al. 2001) are commonly used to map QTL in CIM. CIM method uses the combination of simple interval mapping and multiple linear regressions to test the presence of

QTL in the entire genome (Jansen, 1993, Zeng 1994). For composite interval mapping various algorithms such as multiple linear regressions (Jansen 1993) maximum likelihood function (Zeng 1994) and Markov Chain Monte Carlo (MCMC) approaches can be used. The CIM approach has advantages over simple interval mapping by increasing QTL resolution (Liu 1998).

Mapping populations

Selection of appropriate parents is an important step in generation of a mapping population. Usually, parents with contrasting phenotypic characteristics are preferred to produce high polymorphisms in a cross, which can help to generate enough markers covering the genome. Commonly four types of populations are widely used in QTL mapping like F_2 population, backcross population (BC), double haploid (DH) population and recombinant inbreed lines (RIL)

F_2 population are derived from F_1 hybrids and can be produced in short time. The population produces a unique genotype from each F_1 hybrid therefore seed multiplication can not produce uniform lines; therefore, experimental design cannot be employed to control environmental effects (Hai 2006). The F_2 plants can be self-pollinated to produce a F_3 population, which contains all of the alleles that were present in the F_2 and can be used for QTL mapping. However, precision of the QTL mapping in F_3 population cannot be high due to higher heterogeneity. Moreover, simulation study showed that the number of QTL detected in F_3 population is relatively lower than in F_2 (Tanksley and Nelson 1996).

BC population: derived by crossing F_1 to one of the respective parents. A BC population has similar disadvantages and drawbacks as in F_2 population. BC_1 population is usually used for QTL mapping. However, BC_2, BC_3, and BC_4 can also be used for QTL mapping although simulation study showed that the power of QTL detection decreases as the backcrossing generation increases (Tanksley and Nelson 1996). BC populations are less informative as compared to F_2 because additive effects cannot be distinguished from dominance effects and some epistatic effects can be confounding. Another disadvantage of using F_2 and BC population is that marker data cannot be applied for repeated trials since plants from the next trials are not similar to lines analysed by markers due to allelic recombination.

DH population: Commonly DH populations are developed from F_1 plants (F_1DH) by using anther or microspore culture. The DH genotypes possess duplicated homologues chromosomes and large number of plants can be produced by self-pollination, which permits replicated field trials. Moreover, unlike in F_2 and BC population, marker information can be repeatedly used for

multiple field trials in the same population. Additionally, successful microspore culture depends on the genotypes making it difficult to develop DH lines from all F_1 plant in many species.

RIL populations can be developed by self-pollination and sib-mating of the F_2 plants repeatedly using single decent approach until all of the segregating loci become homozygous. RIL consist series of homozygous lines in which each line contains a unique combination of chromosomal segments from the parents. These lines can be selfed to produce 'true breeding' lines (Collard et al. 2005) that can be tested in multiple field trials. The main disadvantage of using RIL in QTL mapping is the same to that of DH population that dominance and related epistasis cannot be analysed (Zhao 2002). Additionally, development of RIL population requires more time than any of the above population due to the need of at least 7 to 8 generation of selfing to produce nearly homozygous lines. Generally, QTL mapping in segregating populations like F_2, BC, F_1DH and RIL populations revealed low power of QTL detection and poor precision of QTL localization (Eshed and Zamir 1994; 1995; Kearsey and Farquhar 1998; Melchinger et al. 1998).

In contrast to the use of the above mentioned mapping populations, QTL mapping can be done using substitution lines. **Substitution lines** contain complementary sets of donor segments in the genetic background of the recurrent parent in which the whole donor genome is represented in a well-defined way. Therefore, these lines differ only by overlapping recombinant segments. Eshed and Zamir (1994; 1995) have developed introgression lines (ILs) of tomato each containing a single homozygous chromosome segment of the donor parent of *Lycopersicon pennellii* in the genetic background of an elite cultivar of *L. esculentum*. Field trials of these ILs have revealed 104 QTL for 6 agronomic traits: total soluble solids content, fruit mass, plant weight, percentage green fruit weight, total yield and total solid yield. However, if a segregating population has been used the number of QTL identified could have been as low as 4 to 5 QTL per trait in tomato (Paterson et al. 1990).

For the term "substitution lines" as used in the current study, often another term "introgression lines" was used (Eshed and Zamir 1994; 1995, Paran and Zamir 2003). Other synonyms are also found in the literature for example, "Recombinant Chromosome Substitution Lines" (Matus et al. 2003), defined as an overlapping set of nearly isogenic lines in a common genetic background. Howell et al. (1996) called the substitution lines developed by them as material carrying small defined homozygous substitutions (i.e., near isogenic lines, NILs). The difference between the term "Substitution lines" and NILs is that the substitution lines should cover the whole donor genome while NILs do not necessarily cover the genome. Another difference is that

NILs could be used to introgress gene of interest into the recurrent parent from pre-selected parents to produce a better cultivar while in the case of substitution lines although all donor segments are well defined, they are not necessarily expected to contain favourable genes. Moreover, a method demonstrated by Tanksley and Nelson (1996), Advanced Backcross QTL (AB-QTL), can be used simultaneously to discover and transfer valuable QTL from unadapted germplasm into elite cultivars using a backcrossing program followed by marker assisted selection (MAS) of elite NILs for variety production. All these methods follow backcrossing of donor genotypes carrying a specific trait of interest to the recurrent parent with equally desirable or better attributes to improve cultivars or study complex traits.

NILs have been used to verify the effects of QTL (Tanksley et al. 1996), study QTL x genetic background, QTL x environment and QTL x QTL interactions (Monforte et al. 2001). NILs are also demonstrated as a method for introgression of new genetic variability from wild species to the elite germplasm (Tanksley and McCouch 1997; Zamir 2001)

In recent years many NILs were developed for QTL analysis in plant species including tomato (Eshed and Zamir 1994; Monforte and Tanksley 2000), cabbage (Ramsay et al. 1996), rapeseed (Howell et al. 1996), rice (Lin et al. 1998; Wan et al. 2004), sorghum (Tuinstra et al. 1998), lettuce (Jeuken and Lindhout 2004), barely (von Korff et al. 2004), lemon (Eduardo et al. 2005), wheat (Law and Worland 1996; Pestsova et al. 2001, 2006) and plant model organism, *Arabidopsis thaliana* (Keurentjes et al. 2007). In mice, which are used as animal model organism, substitution strains were also developed to study complex traits (Singer et al. 2004). As scientists are more interested to study complex traits and identify QTL precisely, the need to develop substitution line libraries, which can avoid background interference in the analysis, is also increasing despite high cost and labour efforts.

Objectives of the study

The first objective of the current study was to develop intervarietal substitution lines in *Brassica napus* L from two different crosses of rapeseed cultivars. The second objective was mapping of QTL for agronomically important traits in the substitution lines developed from a cross of 'Mansholts Hamburger Raps' and 'Samourai'.

Therefore, this study was proposed with the following specific objectives:

To develop two series of intervarietal substitution lines in *Brassica napus* L. using backcrossing and marker assisted selection

To map QTL for oil content and oil quality traits in intervarietal substitution lines developed from a cross of 'Mansholts Hamburger Raps' and 'Samourai'

To map QTL for protein and glucosinolate contents

To map QTL for flowering time, plant height and winter survival

To study the effect of erucic acid genes on individual phytosterol and sinapate esters contents

Thesis outline

This introductory part will be followed by chapter 2 describing the development of substitution lines in a cross of 'Express' and a resynthesized rapeseed, 'R239'. An attempt was made to depict methods and selection patterns followed through backcrossing and marker assisted selection approaches. Chapter 3 reveals development of the second set of substitution lines from the cross of 'Mansholts Hamburger Raps' and 'Samourai'. In chapter 4, mapping of QTL for oil and fatty acid contents were performed using the substitution lines developed from the cross of 'Mansholts Hamburger Raps' and 'Samourai'. Chapter 5 deals with QTL mapping for protein and glucosinolate content in the same population. Moreover, in chapter 5 an attempt was made to show relationships between protein and oil content. Chapter 6 deals with QTL mapping for flowering time, plant height and winter survival. Chapter 7 deals with the effect of erucic acid genes on sinapate esters and individual phytosterol content.

2. Development of intervarietal substitution lines in the cross of 'Express' with the resynthesized line 'R239' using marker assisted selection

2.1 Introduction

Most agronomically important traits such as oil content and fatty acid compositions have complex patterns of phenotypic variation and are controlled by many genes called quantitative trait loci (QTL). Moreover, they are influenced by environment and genotype by environment interactions. QTL studies could help to understand the effects of the genes, their mode of inheritance and indicate possible ways to manipulate the genetic variation in crop improvement programs. In the last decades, in many studies QTL have been mapped using segregating populations of F_2, double haploid lines (DH), recombinant inbreed lines (RIL) or backcross populations (BC) in which phenotypic variation and genetic marker alleles could be associated using statistical approaches (Lander and Botstein 1989; Haley and Knott 1992; Kearsey and Hyne 1994). However the precision of estimating the number and effects of the QTL using the above population and analytical methods is limited (Kearsey and Farquhar 1998; Melchinger et al. 1998).

According to simulation studies the QTL detected using the above populations underestimate the number of QTL affecting the trait. In some cases the effect of the few detected QTL are overestimated because the effect of small QTL is ignored or summed up to the larger QTL which can lead to misinformation of the true QTL effect. Larger populations of up to 1000 genotypes are needed to identify QTL with small effects (Soller et al. 1976; Schön et al. 2004). For practical reasons such as high costs required to collect and analyse the genotypic and phenotypic characteristics, QTL mapping is limited to populations of few hundred lines, which can lead to small number of QTL detection and a poor precision of QTL localization. Even a segregating population of several hundred lines can give misleading results when used for QTL analysis (Beaves et al. 1994; Hyne et al. 1995; Melchinger et al. 1998).

In contrast to the populations used and methods described above, QTL effects can be estimated using substitution lines having a low proportion of the donor genome. Developing of a set of lines with low proportion of the donor genome can efficiently estimate the QTL effects since the background effects are avoided by introgression of only a single donor segment to the recurrent parent. Substitution lines differ from the recurrent parent by only small, defined donor segments, therefore, phenotypic difference between a line and the recurrent parent can be associated with a QTL located in a single donor segment (Eshed and Zamir 1994; 95). Moreover, these lines can

be self-pollinated to produce uniform lines with sufficient seeds allowing multiple field trials to control environment and identify reproducible QTL effects harboured in the introgressed region of donor segments. The lines can also be used as addition of important character to elite cultivars to be directly used in crop improvement program because substitution lines comprise lower linkage drag except the introgressed donor segment compared to segregating populations.

The objective of the current study was to develop a substitution line library for *Brassica napus* L. The donor parent was a resynthesised rapeseed, 'R239', which is introgressed in to the genetic background of 'Express' through a backcrossing program.

2.2 Materials and methods

2.2.1 Plant materials and genetic map

In the development of substitution lines the donor parent was a resynthesised rapeseed line, R239, developed through interspecific hybridization of *Brassica rapa* (yellow sarson) and *Brassica olereacea* (cauliflower). An inbreed line, E617, from the variety 'Express' was used as a recurrent parent. Express is characterised as a short and high yielding variety of canola quality released by Norddeutschen Pflanzenzucht HG Lembke KG in 1993. After producing a F_1 from the two parents, they were backcrossed to E617 and BC_1 plants were produced. 90 BC_1 plants were genotyped to develop a genetic map of 1327.1 cM using 23 AFLP primer pairs producing 220 polymorphic markers distributed across 22 linkage groups (LG) (Ecke personal communication). This genetic map was used as a reference in the further backcrossing program and in the development of a substitution line library. A total of 10 genotypes with donor segments covering the whole genome of the donor parent were selected in BC_1 and used to begin with the current study. Fig. 2.1 depicts the scheme used to develop the substitution lines.

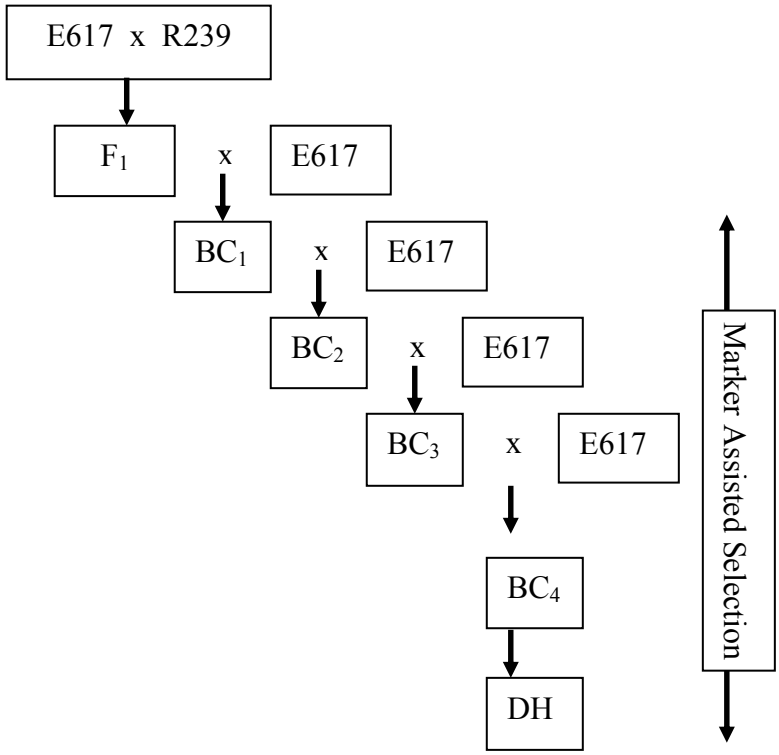


Fig. 2.1 Scheme for the development of substitution lines from the cross of 'E617' x 'R239'. Number after BC describes the backcross generation. All the BC generations were subjected to MAS.
E617: inbreed lines from variety 'Express'; R239: resynthesized rapeseed; BC: back crossing; DH: doubled haploid.

2.2.2 DNA extraction and AFLP markers

2.2.2.1 DNA extraction

Fresh leave material of 100 mg were taken from 15 to 21 day old plants and put in 1.5 ml reaction tubes (Eppendorf) and then immersed in liquid nitrogen (-191^{o}C) and finally stored at -20^{o}C in a refrigerator. DNA was extracted using Nucleon PhytoPure plant extraction kits from Amersham (Amersham^{TM} Biosciences, Freiburg, Germany, 1999) according to the manufacturers' instructions with the following modification: After DNA extraction 30 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) was added to the pellet and then incubated at 65^{o}C in a water bath for two hours to speed resuspension. Finally, the DNA solution was centrifuged and stored in the refrigerator at 4^{o}C. The concentration of DNA was measured using a Versa FluroTM flurometer (Bio-Rad laboratories, Hercules, USA) with the flurochrome dye Hoechst 33258. The fluroscence was measured using the excitation wavelength of 360 nm (optical filter excitation, Ex 360/40 – 340-380 nm) and emission wavelength of 460 nm (optical filter emission, Em 460/10 – 455-485).

The quality of the extracted DNA was verified on a 1.5% agarose gel (15 cm length) prepared with TAE buffer (40 mM Tris-Acetat, 1 mM EDTA, pH 8). Each sample used for the gel was prepared taking 2 µl from the DNA and 5 µl of a loading buffer. The loading buffer stock solution was composed of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose and water. Gels were run in TAE electrophoresis buffer at 200-volts for 2 hours. After this step, the gels were stained in an ethidium bromide solution (1.0 mg/l H_2O) for 20 min. followed by 20 min. incubation in water to remove excess of ethidium bromide. DNA was visualised under UV light (λ = 254 nm) and a photo was taken from the gel with a Polaroid MP-4 Land Camera (Polaroid film 667). For plants with a low amount or degraded DNA based on the gel image analysis, extraction of DNA was repeated. The DNA was diluted to a concentration of 50 ng per µl with TE buffer and stored at 4 ^{o}C in the refrigerator.

2.2.2.2 AFLP markers

Restriction: A total of 250 ng DNA was incubated at 37^{o}C for 1 h and 30 min. in a total volume of 30 µl containing 1x restriction-ligation (RL) buffer (10 mM Tris HAc, 10 mM MgAc, 50 mM KAc, 5 mM DTT (Dithiothreitol ($C_4H_{10}O_2S_s$)), pH 7.5), 4 units of each of EcoRI and MseI enzymes.

Ligation: A total of 10 µl ligation mix containing 5 pmoles EcoRI adaptors (5'-CTC GTA GAC TGC GTA CC-3', 3'-CTG ACG CAT GGT TAA-5'), 50 pmoles MseI adaptors (5'-GAC GAT GAG TCC TGA G-3', 5'-TAC TCA GGA CTC AT-3'), 1 unit T4 ligase (Promega GmbH, Germany), 1x RL buffer, 1 mM ATP, was added to 30 µl of the restriction product giving a final reaction volume of 40 µl. Ligation was carried out at 37^{o}C for 3 hrs and 10 min., followed by 33.5^{o}C for 3 min., 30^{o}C for 3 min., 26^{o}C for 4 min., and 22^{o}C for 15 min. Reaction temperature was designed in order to maintain optimum activity of EcoRI and MseI for the first 3 hrs and 10 min. to restrict fragment to fragment ligation. The digested ligation product was diluted 1:5 with TE buffer pH 8.

Preamplification: preamplifications were carried out with two selective primers, E01 (5'-CTG CGT ACC AAT TCA-3') and M02 (5'-GAT GAG TCC TGA GTA AC-3'). A total of 20 µl preamplification mix contained 8 µl of the diluted restriction-ligation product, 0.3 mM dNTPs, 1.5 units Taq-DNA-Poymerae (Amersham Biosciences, Germany), 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8), 4 mM $MgCl_2$, 10 pmoles E01 and 8.7 pmoles M02. PCR cycling condition was an initial DNA denaturation at 94^{o}C for 30 sec., followed by 20 cycles of 94^{o}C for 30 sec. (denaturation), 56^{o}C for 30 sec. (annealing) and 72^{o}C for 1 min. (extension). After the

final cycle the 72°C extension step was extended to 5 min. Finally, the preamplification product was diluted 1:10 with TE buffer pH 8.

Amplification: The amplification reaction was carried out using Eco and Mse primers with three selective nucleotides at their 3' ends. In sum 23 primer combinations were used (Table 2.1). A total of 20 µl of amplification mix contained 6 µl of the diluted preamplification product, 0.24 mM dNTPs, 2 pmoles Eco primer, 7 pmoles Mse primer, 0.6 units Taq-DNA-polymerase, 1 x PCR buffer and 4 mM $MgCl_2$. PCR condition was an initial denaturation at 94°C for 30 sec., one cycle of denaturation at 94°C for 30 sec., annealing at 65°C for 30 sec. and extension at 72°C for 1 min., and then 11 cycles of a touch down protocol where the annealing temperature was lowered by - 0.7°C in each cycle to give optimum conditions for all primer pairs. This was followed by a further 24 cycles at 94°C for 30 sec., 56°C for 30 sec. and 72°C for 60 sec. of denaturation, annealing and extension, respectively. After the final cycle a 72°C extension step was extended for 5 min.

Gel electrophoresis: Detection of AFLP fragments was carried out on an automated DNA sequencer (LI-COR 4200 IR2, LI-COR Inc. Nebraska, USA). To 20 µl of the amplification product 10 µl of loading dye (98% (v/v) formamide, 10 mM EDTA, 4 mM NaOH, and 0.025% (w/v) rhodamin B ($C_{28}H_{31}ClN_2O_3$)) was added. Then the DNA was denaturated at 94°C for 4 min. The AFLP fragments were resolved using a 25 cm gel apparatus with 0.2 mm spacers on 6% polyacrylamide gels (Long ranger, Bio Whittaker Molecular Applications ApS, Denmark) which contained 7.8 M urea (NF-urea Rotiphore®), 1 x long run TBE buffer (1.34 M Tris-HCl, 0.45 M boric acid, 25 mM EDTA, pH 9.2), 10% (w/v) ammonium persulfate and 0.01% (v/v) TEMED. Before loading the samples, a 15 min prerun was carried out with the following settings: Voltage 1000 V, Temperature 45°C, Current 37 mA, Power 40 W. A 64 shark teeth comb was used to make slots and 1 µl of each DNA sample was loaded. Fragment mobility was measured by real-time laser fluorescence at 800 nm and was converted to a TIFF-image of the gel. Image data were collected for 5 h with the same settings of voltage, temperature, current and power as in the prerun.

2.2.3 Data scoring and analysis

DNA fragment sizes were determined using a 50 to 700 bp sizing standard (LI-COR® Biotechnology, USA). The gel image was electronically stored and opened in Adobe Photoshop for the scoring. The two parents, R239 (R) and E617 (E), were used as a reference to determine polymorphic bands. Dominant marker alleles of the donor parent were scored from the TIFF-image of the gel based on the presence or absence of 'R239' bands in the offsprings. The

information from the TIFF-image were transferred to an Excel sheet containing the following information: gel identification number, primer pairs used, identification number of the offspring plants, estimated size of a polymorphic band in bp, presence of the polymorphic donor parent band scored as R and absence of the band scored as E. Finally, the information was integrated into another Excel data base of marker allele positions of the mapped genome from the genetic map developed in BC_1.

Selection strategy of plants with the target donor segment in the backcrossing program followed the following criteria: The selected line should have uninterrupted target donor segments (no recombination within the target donor segment) and as long a segment of the target donor chromosome as possible. The line should have no or few non-target donor segments, contain no double crossing over and no missing values with respect to marker information.

The minimum donor segment length (in centi Morgan) on the mapped genome was determined by subtracting the position of the last marker scored as a donor allele from the position of the first marker scored as the donor allele. If the donor segments in the selected lines contained only one marker with a donor allele in which the flanking markers on both sides showed the recurrent parent genotype, the length of the donor segment was assigned a length of 0 cM although in realty the length of the donor segment is greater than 0 cM. If this segment would be of an interest after phenotypic analysis, screening of more markers could be done to place more markers in the region of the target donor segment.

2.2.4 Microspore culture

Selected BC_4 families were sown in 40 cm^2 pots filled with compost soil. After the plants were grown for 3 to 4 weeks in the green house at 22^oC for 16 h day length, they were transferred to a vernalization chamber (4^oC) for 8 weeks of artificial cold treatment to initiate flowering. After 8 weeks plants were taken out from the vernalization chamber and transferred to 13 x 13 cm^2 pots filled with compost soil. The plants were kept in the greenhouse until flower bud initiation. When flowering started, selected BC_4 plants were transferred to a growth chamber. Microspore culture followed the procedure of Lichter (1982) and Möllers et al. (1994) with the following modification: Temperature in the growth chamber was kept at 12^o C for 16 h day length, at 6^oC for 8 h dark and another growth chamber kept at 16^o C for 16 h day length, at 10^oC for 8 h dark. In the microspore culture 0.3% colchcine treatment was used to double the chromosome number. For matured haploid plants, the roots were immersed in 0.3% colchcine overnight. For older

plants that had not responded to repeated colchcine treatment, the side branches of the plants were cut and immersed in 0.3% colchcine over night and then the cut ends were dipped in rhizopon powder (0.1%) containing 1-naphthaleneacetic acid (Rhizopon B. V, Rijndik, the Netherlands) to initiate root development.

2.3 Results

2.3.1 AFLP markers

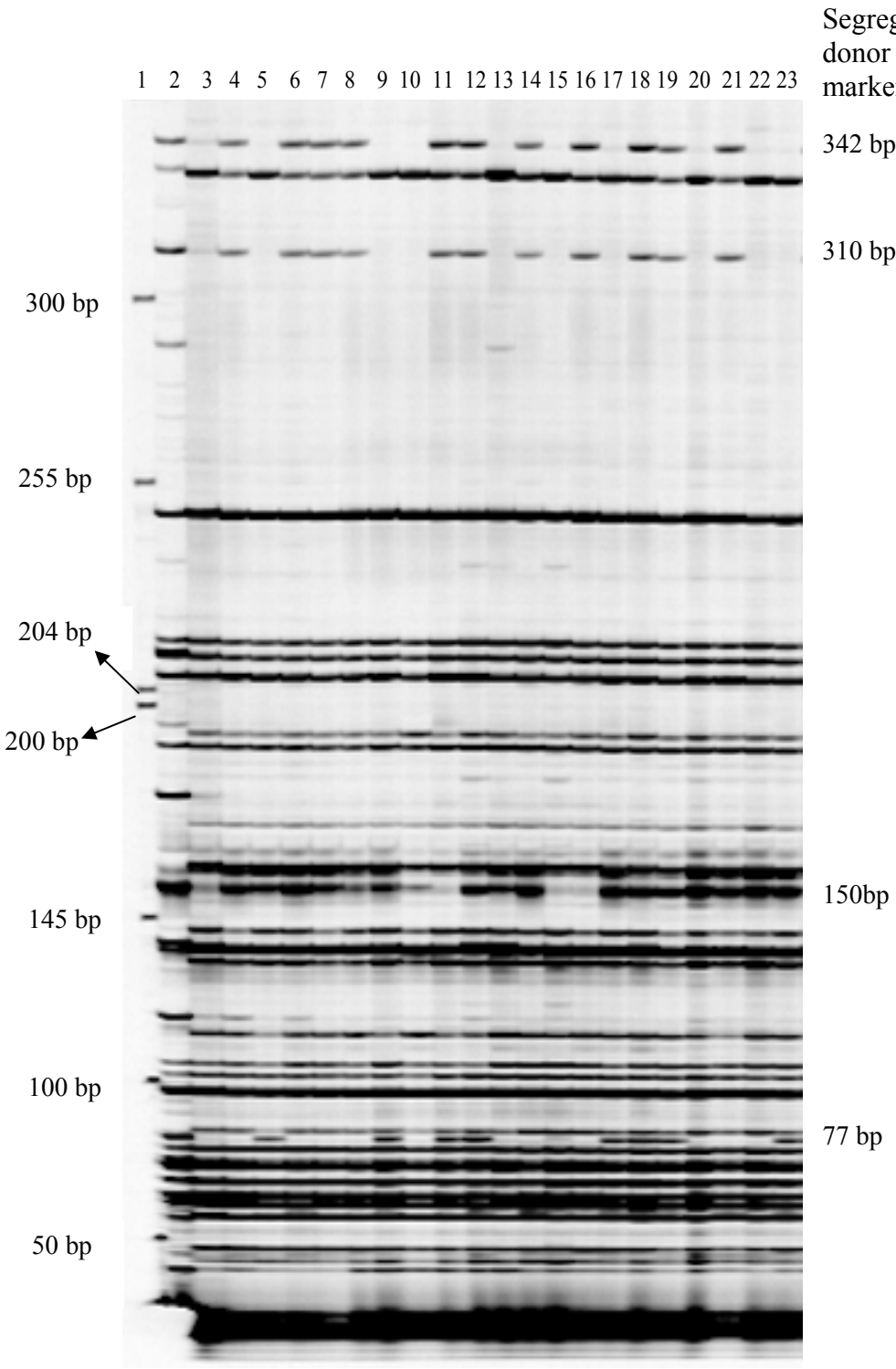
The electrophoresis image presented in Figure 2.2 shows (1) a molecular weight standard in the first lane of the gel used to facilitate sizing of the markers, (2) the AFLP banding patterns of the parents, R239 and E617, at positions 2 and 3 in the gel from the left, respectively, (3) monomorphic bands and (4) polymorphic dominant markers from the donor parent R239.

2.3.2 Selection in BC_2

A total of 268 BC_2 plants were analysed using 23 primer combinations. Figure 2.3 shows the distribution of percentage of donor segment coverage in the plants of the different backcross generation. The mean coverage by donor segments in the BC_2 population was 299.6 cM ranging from 2.3 to 572.5 cM (Table 2.2). Figure 2.4 depicts the distribution of the number of donor segments in the plants of the different backcross generations. The mean number of donor fragments in BC_2 was 14 ranging from 3 to 24 fragments. In BC_2, 20 genotypes were selected with the donor coverage ranging from 161.5 to 370.8 cM with a mean of 259 cM. The selected lines have coverage of at least one full linkage group of a donor parent and additional donor segments from other linkage groups. The number of donor fragments in the selected BC_2 plants ranged from 6 to 14 (mean 10) (Fig. 2.5).

2.3.3 Selection in BC_3

From each of the 20 selected BC_2 genotypes 20 offsprings were sown and a total of 400 plants were analysed for donor specific AFLP markers at all loci that had been heterozygous in the respective parental BC_2 plant. The mean coverage of donor segments in the BC_3 population was 121.2 cM ranging from 5.2 to 276.5 cM. The number of donor fragments ranged from 2 to 26 (mean 9.2). In the BC_3 generation 18 genotypes that contained ≤ 4 donor segments were selected. In sum all donor segments of the selected plants covered 60% of the mapped rapeseed genome. The donor segment coverage of the selected genotypes ranged from 32.5 to 133.9 cM with a mean of 86.9 cM, which is lower than the population mean due to intensive MAS. In



Lanes
1: Molecular weight standard, 50 – 700 bp,
2: Donor parent, R239,
3: Recurrent parent, E617,
4 -23: Individual plants from the BC_4 family 2.2

Fig. 2.2 Part of an electrophoresis image adjusted by Adobe Photoshop showing AFLP banding patterns of 25 the BC_4 family 2.2 progenies and their parents for primer combination E38M51. bp: base pairs

the selected plants the number of donor fragments ranged from 2 to 4 segments with a mean of 3.6.

2.3.4 Selection in BC_4

From each of the 18 selected BC_3 plants, 14 to 20 seeds were sown and a total of 349 plants were again analysed using AFLP markers at all loci that had been heterozygous in the respective parental BC_3 plant. The mean coverage of donor segments in the BC_4 population was 37.9 cM ranging from 0 to 133.9 cM (Table 2.2). The number of donor fragments ranged from 0 to 8 with a mean of 3.2. A total of 27 genotypes containing ≤ 2 and one with 3 donor segments were selected for the development of DH substitution lines. The donor segment coverage in the selected plants ranged from 2.3 to 89.2 cM with a mean of 39.3 cM. The donor segment fragments ranged from 1 to 4 with a mean of 2. The selected BC_4 plants were used for the development of DH lines using microspore culture in order to produce genotypes homozygous for the selected donor segments.

The largest coverage by donor segments in the selected BC_4 plants was 86.9 cM of which 69.9 cM was located on linkage group 10 and 17 cM on linkage group 5, in sum both of the donor segments covered 6.4% of the donor genome. Based on the expectation of classical backcrossing in which the percentage of donor genome is halved per generation of backcrossing to the recurrent parent, the selected plants with the largest donor segment coverage in the BC_4 generation have slightly more donor genome than the expected 6.25%. The smallest donor segments in the selected lines contained only one marker with a donor allele and assigned a length of 0 cM, because the flanking markers on both sides showed the recurrent parent genotype. Table 2.2 depicts the overview of donor segments coverage in the mapped rapeseed genome in each generations of the backcrossing program and in the plants selected by MAS.

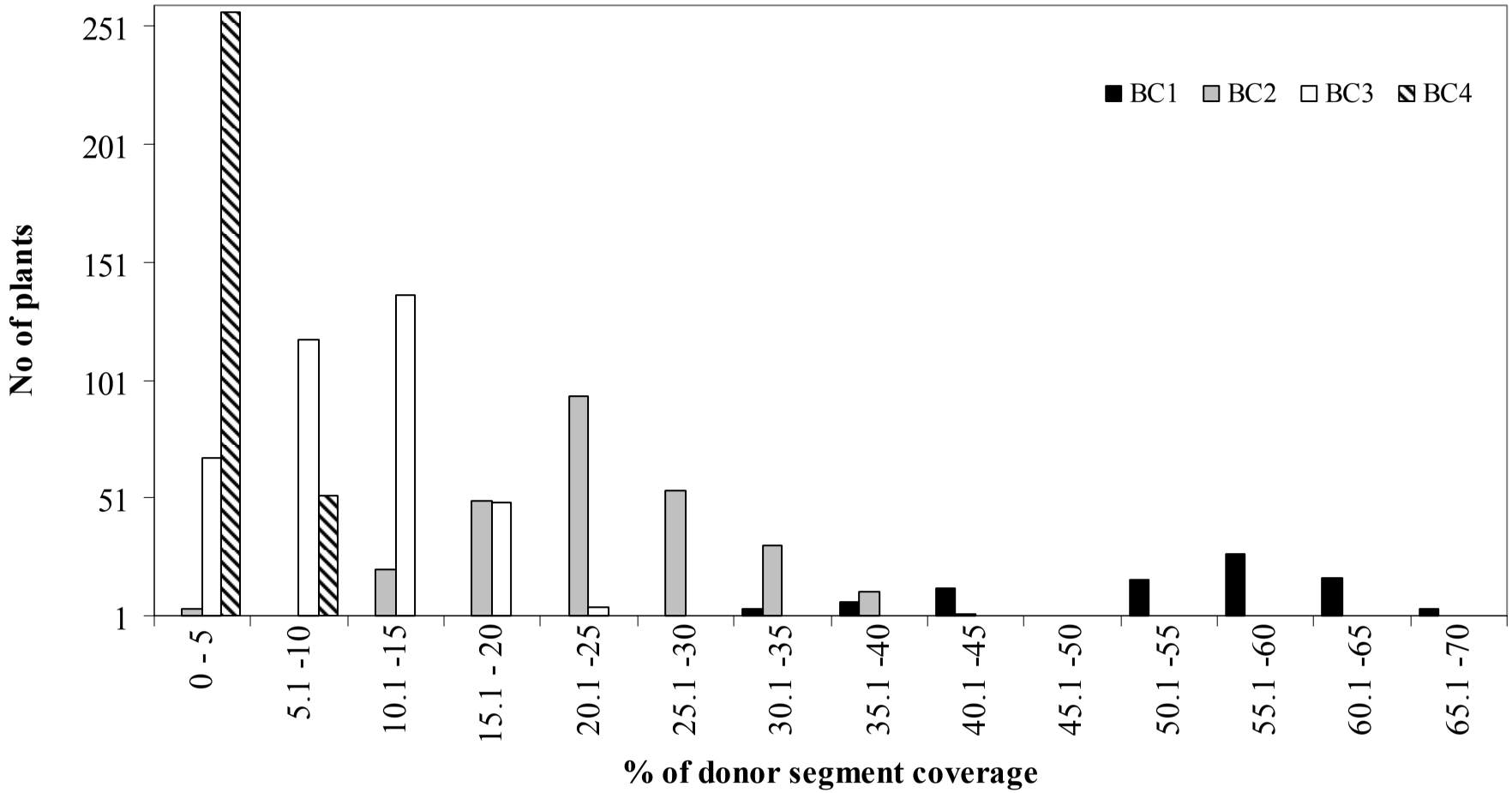


Fig 2.3 Distribution of percentage of donor segment coverage in the plants of the different backcross generation

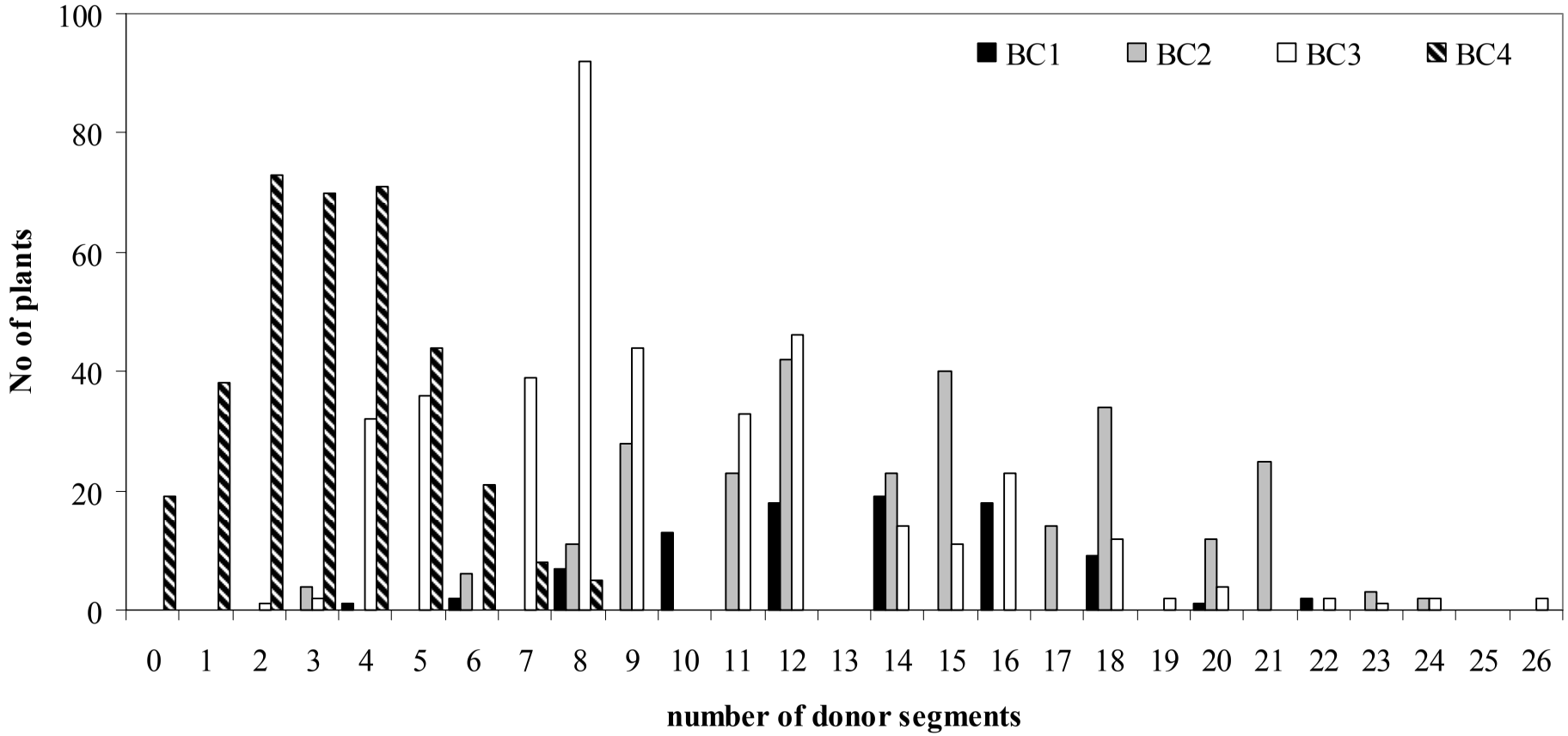


Fig. 2.4 Distribution of the number of donor segments in the plants of the different backcross generations

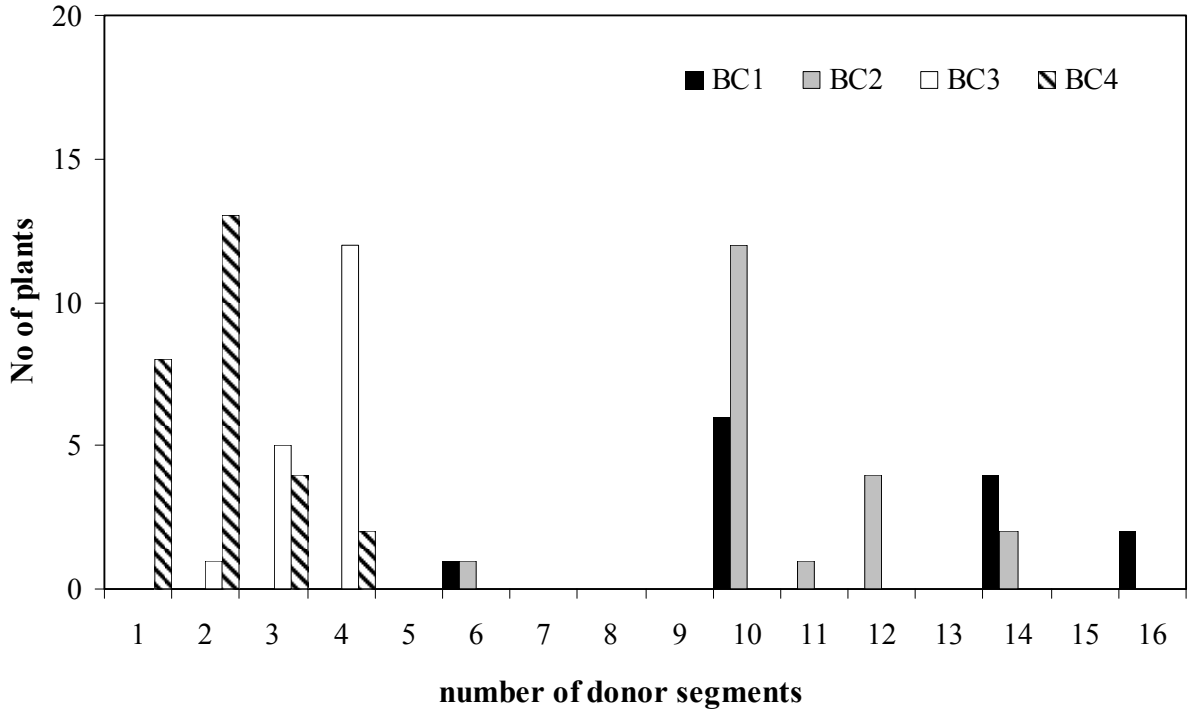


Fig. 2.5 Distribution of the number of donor segments in the plants selected by marker assisted selection in the different backcross generations

Table 2.1 primer pairs used in the marker analysis

E32M48	E32M62	E35M48	E38M50	E40M50
E32M49	E33M48	E35M60	E38M51	E40M51
E32M51	E33M49	E35M62	E38M59	E40M62
E32M60	E33M59	E38M47	E38M60	
E32M61	E33M60	E38M49	E38M61	

† see appendix 2.1 for primer sequences

Table 2.2 Donor segment coverage of the mapped rapeseed genome in each backcross generations and in the plants selected by marker assisted selection

Generation	Population					Selected plants		
	No. of plants	Donor genome coverage (cM)				No. of plants	Donor genome coverage (cM)	
		Min	Max	Mean	SD		Mean	SD
BC_1	90	256.7	1032.4	647.7	144.0	10	628.8	100.3
BC_2	268	2.3	572.5	299.6	97.2	20	259.0	49.6
BC_3	400	5.2	276.5	121.2	53.1	18	86.9	26.7
BC_4	349	0.0	133.9	37.9	26.4	27	39.3	20.2

2.4 Discussion

2.4.1 Degree of polymorphisms

Marschalek (2003) started the development of the substitution lines from the cross 'Mansholt' x 'Samourai' using 164 AFLP markers covering 1325 cM of the genetic map. Half of these markers came from the recurrent parent, which should be scored co-dominantly because both genotypes, heterozygous and homozygous, show the marker band and are therefore undistinguishable by manual scoring. Hence, markers coming from the recurrent parent were scored using software, AFLP-Quantar^{TM} pro 1.0 (Keygene Products BV 2000), which was a difficult task in the backcross program due to the inefficiency of the software to perform the analysis. Therefore, these markers could only be properly detected and scored in the final stage of the marker analysis in the double haploid substitution lines. Generally, a low degree of polymorphism was observed in the cross of 'Mansholt' x 'Samouria'. In contrast, the cross used in the current study possessed a high degree of polymorphism providing more markers and more resolution of the genetic map and information on the donor segments through the backcross generations. This resulted in a higher density of the genetic map. The reason for a high degree of polymorphisms in the current study was due to the use of the cross between a resynthesized rapeseed and a cultivar in the development of the substitution lines. A high degree of polymorphisms in the cross could be because of diverse characteristics of the resynthesized genotypes compared to conventional cultivars. Since *Brassica napus* does not have wild relatives which limited the diversity of the crop, the high polymorphisms observed in this study might reveal a potential gene pool in the resynthesized rapeseed that can be used as addition to the existing cultivars to improve qualitative and quantitative traits (Becker et al. 1995, Girke et al. 1999, Girke 2002**)** in addition to landraces and old cultivars to complement the rapeseed breeding programmes.

Howell et al. (1996) also developed substitution lines from the cross of *Brassica napus* L. cultivars 'Victor' x 'Tapidor' using 158 RFLP marker loci that defined a genetic map of 1204 cM. They observed considerably less polymorphism in their population than a population developed from a cross of a conventional cultivar with a resynthesized rapeseed genotype (Parkin et al. 1995) in which 399 RFLP markers were generated covering 1656 cM of the rapeseed genome. Based on the analysis of the cross used by Marschalek (2003), low polymorphism observed by Howell et al. (1996) could be due to the use of the cross of conventional cultivars in the development of the substitution lines.

2.4.2 BC_2, BC_3 and BC_4 genome composition

Due to MAS the genetic background of the recurrent parent is recovered faster compared to the expectation of classical breeding without MAS. For instance, in BC_2, the average donor genome coverage in the selected lines was 19.5%. The expected average donor genome coverage without MAS would have been 25%. Therefore, the MAS helped to achieve both, on the one hand the introgression of the target donor segments and on the other hand a faster recovering of the recurrent genome. The genetic background of the recurrent parent has gained approximately 6% due to intensive selection despite the simultaneous selections for a complementary set of donor segments covering as much of the donor genome as possible. However, here it should be clear that the estimated length of donor segment in the current study was the minimum length between two flanking marker scored as donor allele.

In the selected BC_3 and BC_4 plants, recovering of the recurrent parent genome was 6 and 3.2%, respectively, compared with the expectation without MAS. MAS facilitated the recovering of the recurrent parent genome without omitting the target donor segments from BC_1 to BC_3 generations. The average genome coverage by donor segments among BC_4 selected plants and the BC_4 population was 3% and 2.9%, respectively, revealing that the selected plants contained a higher percentage of donor segments than the population. This is because of the reason that in the final generation most lines contained small segment of the donor genome. Since the main objective was to recover as large as possible complementary donor segments, selection of plants with larger donor segments to cover the donor genome was inevitable, resulting in a higher percentage of donor genome in the selected plants than the population mean.

Marschalek (2003) selected lines with only 7.7 and 3.8% donor genome coverage in BC_2 and BC_3 generations, respectively. Compared to the present study in which lines with average donor genome coverage of 19.5 and 6.6% were selected in BC_2 and BC_3, respectively, Marschalek (2003) has made an effort of early selection against the donor genome, resulting in small donor segments in the late backcrossing generations. This will risk the omission of large parts of the donor genome. Accordingly, selected genotypes with less or equal to 4 donor segments in BC_3 covered only 48% of the donor genome in the population used by Marschalek while in the present study 60% of the donor genome was covered in the same generation, although both studies used the BC_4 plants for the development of DH substitution lines.

The average number of donor segment fragments is also an important criterion for selection.

Cermakova et al (1999) found 5 to 10 and 1 to 6 fragments of donor segment in BC_2 and BC_3 population, respectively, while Marschalek (2003) found 5 to 19 and 2 to 17 fragments of donor in BC_2 and BC_3 population, respectively. In the present study 3 to 24 and 2 to 26 fragments of donor segment were found in BC_2 and BC_3 generation, respectively. The difference in the number of fragments of donor segment could be a result of the size, resolution and the number of informative markers on the genetic map. Cermakova et al. (1999) used only 77 RFLP markers covering 450 cM of the rapeseed genome (Sharpe and Lydiate 2003) while Marschalek (2003) used 164 AFLP markers covering 1325 cM; in contrast the current study used 220 AFLP markers distributed on 1327 cM of the genome.

Howell et al. (1996) reported that lines with only one donor segment could be found in BC_3, which can be used for the development of substitution lines at an earlier stage. Compared to the present study genotypes with only one segment were found only in BC_4. This might be due to selection of smaller regions of the donor genome at an early stage as BC_1 by omitting some donor genome regions, which was the case in the study by Howell et al. (1996) in which selection in BC_2 was made by excluding linkage groups 9 and 12 fully; and parts of linkage groups 4, 5 and 15. More over, they selected 19 BC_3 lines with one or two segments to develop substitution lines that didn't represent a total of 7 entire linkage groups of the mapped donor genome. By omitting entire linkage group of the donor genome in an early generation, the present study could also have produced the NILs earlier than BC_4 generation.

Ramsay et al. (1996) developed substitution lines in *Brassica olarecea* L. in which plants with only one segment were selected in BC_2S_1 population, However, the selected lines do not contained all set of complementary segments from the donor parent. Moreover, the selected lines did not cover the entire region of linkage group 6. Since their genetic map of *B. olreacea* has a length of 747 cM (approximately half of the *B. napus* genome) and in selected lines if only part of the donor genome is to be represented, one can expect that developing of genotypes with only one donor segment is more possible in an earlier generation of backcrossing. However with the aim of developing a full set of substitution lines covering all of the donor genome we have to still pursue the backcrossing program to BC_5 generation to get back all the donor segments that were not included in BC_4 selection.

2.4.3 Time required for developing of the substitution lines

The development of substitution lines has been obtained by combining the backcrossing and selfing method. Eshed and Zamir (1994; 1995) needed three backcrossing and up to eight

generation of selfing for the development of inbreed lines in tomato while Pestsova et al. (2001; 2006) needed two backcrossing and up to three generation of selfing in wheat. Differences in the time of generating the substitution lines depends mostly on the factors such as pre-existing information on the markers, available resources such as number of markers analysed per generation and size of the genetic map (Eduardo et al. 2005). Additionally, factors such as the number of linkage groups, number of plants analysed per generation, the robustness of markers and technological advancement in marker technology, characteristic of the species studied such as easiness of selfing and crossing and technical advances in tissue culture to develop DH lines can also affect the time to generate the substitution lines. In the current study four generation of backcrossing and one generation of developing double haploids through microspore culture were required to develop the first set of substitution lines. Microspore culture can help to fasten the development of the substitution lines with homozygous donor segments on both chromosome pair. In addition, problems of possible segregation of the donor segments (Ramsay et al. 1996) could be avoided through the use of microspore culture in which first haploids are developed from microspores of the selected plant and then the chromosome number is doubled using colchicine treatment.

2.4.4 Precision of substitution lines library

Substitution lines that contained isolated segments of a donor genotype in the genetic background of an elite cultivar allow the detection of beneficial QTL. The quality of the substitution lines library depends on the development of a set of lines with complementary donor segments fully covering the donor genome. More important is that the number of donor segments in the line should be as low as possible, preferably only one or two in order to control background effect with the recurrent parent genome. Sets of substitution lines with many donor segments per line can result in QTL to QTL interactions. In addition, in lines with many donor segments, QTL for the same trait but opposite effect can neutralize one another and hinder the detection of the QTL. Furthermore, if substitution lines with many donor segments were used for QTL mapping, the same drawbacks of the classical QTL mapping in populations of F_2, BC, and RIL can be observed such as low power of QTL detection, poor precision of QTL localization and over estimation of the QTL effects. In the final stage of developing the substitution lines plants possessing more than four segments are not worth selecting because of the complexity to identify the QTL. However, substitution lines with two or three donor segments could be used to study QTL to QTL interactions more accurately than the classical approach. This is because lines with less than three donor segments can show constant genetic background except in those regions of introgression. Therefore, the interaction of QTL obtained from such lines with two to

three donor segments could be verified more accurately by comparing the result with the lines possessing only a single donor segment of each of those donor segments.

MAS over successive generations of backcrossing are a universally applicable method for the development of substitution lines (Howell et al. 1996). The microspore derived plants from selected candidate plants in the current study are now growing in the greenhouse for production of seeds. For the double haploid plants with seed sets, seed multiplication is undergoing in the greenhouse to produce enough seeds for field experiments in the coming years. The multiplied lines will be assessed in multiple field trials to be characterized for all important agronomic traits.

A complete set of the substitution lines will allow us to detect QTL across the whole donor genome. As a limitation of the current study only 60% of the resynthesized rapeseed genome was covered in BC_3 based on donor segment detected in these candidate plants. The remaining 40% of the uncovered target donor genome could not be included in the current development of the substitution lines because the respective segments were only present in the plants with more than 4 donor segments which prevented their inclusion in the current selection. This limitation could be solved after one more backcrossing in order to clean up those non target segments from the candidate lines possessing the remaining 40% target donor segments to cover the whole genome of resynthesized rapeseed. The development of substitution line library is costly and laborious; however, the wider applicability of the strategy such as its higher quality to be used for candidate gene studies, fine mapping and accurate analysis of complex traits will stimulate more scientists to develop substitution lines libraries in many other plant species.

3. Development of intervarietal substitution lines in the cross of 'Mansholts Hamburger Raps' x 'Samourai' using marker assisted selection

3.1 Introduction

Rapeseed is the third leading oilseed crop produced worldwide (FAO 2004). The successful development of double quality rapeseed (Canola) has opened a great opportunity to use the crop in human nutrition and animal feeding. The oil content in the seed determines the value of the crop and rapeseed growers are paid higher prices for seed lots with higher oil content. Therefore, in the last decades, improving oil content and oil quality traits became one of the most important breeding criteria. However, oil content in rapeseed is a complex quantitative trait and is characterized by a continuous variation in segregating populations. In addition, the oil is composed of individual fatty acids, which are synthesized concurrently due to common precursors, which result in a complicated genetic control of the trait. Classical genetics and conventional quantitative genetics could not provide a clear understanding of quantitative traits. Therefore, in the last decades, QTL mapping became a more powerful and preferred approach to study complex traits. In many studies QTL were mapped using segregating populations like F_2, RIL, BC, and F_1DH populations. However, mapping analysis using the above populations has many drawbacks such as masking effects of major QTL and epistatic interactions of multiple QTL. Also, simulation studies showed that QTL mapping using a segregating population usually give biased estimations of the number, size and localization of the QTL (Beavis et al. 1994; Melchinger et al. 1998).

Alternative to a segregating population, QTL can be mapped more precisely using a set of substitution lines which contain well defined segments of the donor parent in the genetic background of the recurrent parent. Developing a set of lines with low proportion of the donor genome can efficiently estimate the QTL effects since background effects are avoided by introgression of only a single donor segment in to the genome of the recurrent parent. QTL to QTL interactions can be avoided due to a constant genetic background in the set of lines except for the introgressed donor segment (Eshed and Zamir 1994; 1995). Substitution line libraries contain complementary sets of lines with defined segments of the donor parent in the genetic background of the recurrent parent. In the study of complex traits substitution lines were suggested as efficient materials to estimate QTL effects more precisely due to lower genetic background effects (Eshed and Zamir 1994; 1995; Howell et al. 1996). Moreover, using lines with overlapping complementary donor segments and their respective phenotypic value, QTL

position can be narrowed down to a few centi Morgan allowing higher precision of QTL localization. Higher precision of QTL localization is a prerequisite for studies of candidate genes and cloning. Therefore, these lines facilitate fine mapping of valuable donor segments/QTL and might also lead towards gene discovery by introgressing traits that exist in wild species (Zamir 2001). The main disadvantage of development of substitution lines is the length of time and laborious work required to develop a set of lines with full coverage of the donor genome.

Eshed and Zamir (1994; 1995) developed 50 introgression lines (ILs) of tomato containing a single homozygous chromosome segment of the donor parent *Lycopersicon pennellii* in the genetic background of an elite cultivar of *L. esculentum*. Field trials of these ILs identified a total of 104 QTL for total soluble solids, fruit mass, plant weight, percentage green fruit weight, total yield and total solid yield. Besides, they identified that the total soluble solids content in the modern cultivar could be improved by the introgression of genome segments from the wild species into the elite cultivar. The main advantage of this strategy is the applicability of the method in practical marker assisted breeding, for example lines with small donor segments that contained beneficial QTL from the wild species could be used as addition to elite cultivars to improve traits of interest since the background effect became free from the linkage drags of the wild genome after repeated backcrossing.

In barely, von Korff et al. (2004) developed candidate introgression lines using AB-QTL (Tanksley and Nelson 1996) from a cross of an exotic barely accession *Hordeum vulgare ssp. spontaneum* which was used as a donor parent into the genetic background of two different spring cultivars, 'Scarlett' and 'Thuringia'. The candidate lines were tested in multiple field trials and several favourable exotic QTL were identified improving the elite cultivars for important agronomical traits (von Korff et al. 2006). In addition, several QTL were also detected for resistance against powdery mildew, leaf rust and scald on introgressed segments from the wild barely (von Korff et al. 2005).

The objective of the current study was the development of a substitution line library for *Brassica napus* L. The donor parent was 'Mansholts Hamburger Raps', which was introgressed in to the genetic background of 'Samourai' through a marker assisted backcrossing program.

3.2 Materials and methods

3.2.1 Plant material and genome coverage

In the backcrossing program, the donor parent was a doubled haploid line (DH5.1) of the old

cultivar 'Mansholts Hamburger Raps' characterised by high erucic acid and high seed glucosinolate content. The recurrent parent was a doubled haploid line (DH11.4) from the canola quality winter rapeseed variety 'Samourai'. The genetic map used in the current study covered 1325 cM of the rapeseed genome using 164 AFLP markers derived from 20 primer pairs **(**Table 3.1) that were distributed across 19 linkage groups (LG) (Ecke personal communication). A total of 26 BC_3 plants with less or equal to 4 donor segments were selected as candidate genotypes for the development of doubled haploid (DH) substitution lines (Marschalek 2003). Fig. 3.1 shows the scheme for the development of the substitution lines. From each of the selected BC_3 plants, 15 seeds were sown in pots filled with compost soil and grown for 3 to 4 weeks in the greenhouse.

3.2.2 Plant materials and marker analysis

DNA was extracted from 0.1 g fresh leave material. BC_4 plants and DH substitution lines were analysed for donor segments that had been present in the parental plant using AFLP markers. A total of 20 AFLP primer pairs were used to analyse the donor segments in the selected plants (Table 3.1). DH substitution lines were generated using microspore culture. DNA extraction, PCR reaction, gel electrophoresis and microspore culture followed the same methods and procedures as described previously (see thesis chapter 2).

3.2.3 Scoring the AFLP gel image

DNA fragment sizes were determined using a 50 to 700 bp sizing standard (LI-COR® Biotechnology, USA). The gel image was electronically stored and opened in Adobe Photoshop for the scoring. In BC_4 families, markers derived from recurrent and donor parent were scored separately. Markers coming from the donor parent were scored from the TIFF-image of the gel based on the presence or absence of the donor parent band in the offsprings. The two parents, DH5.1 (M) and DH11.4 (S), were used as a reference to identify segregating loci with respect to genetic map used for MAS. Markers coming from the recurrent parent can not be scored manually because both heterozygous and homozygous alleles show the band and are visually undistinguishable. Therefore they were scored codominantly using AFLP-Quantar™ Pro 1.0 software (Keygene 2000), which identifies the segregation pattern of the markers based on differences in the band intensity of the fragments in the gel image. The segregation pattern of the progenies for marker loci was expected to follow a 1:1 ratio for heterozygous to homozygous genotypes. Marschalek (2003) depicted the procedure used to run the AFLP-Quantar™ Pro 1.0 software and the efficiency of the software in scoring the codominant markers in early backcross population (BC_2, BC_3) used in the development of the BC_4 population for the current study. Data

organization of the scored markers and selection strategy of plants with target donor segments followed the same procedures as described previously (see thesis chapter 2).

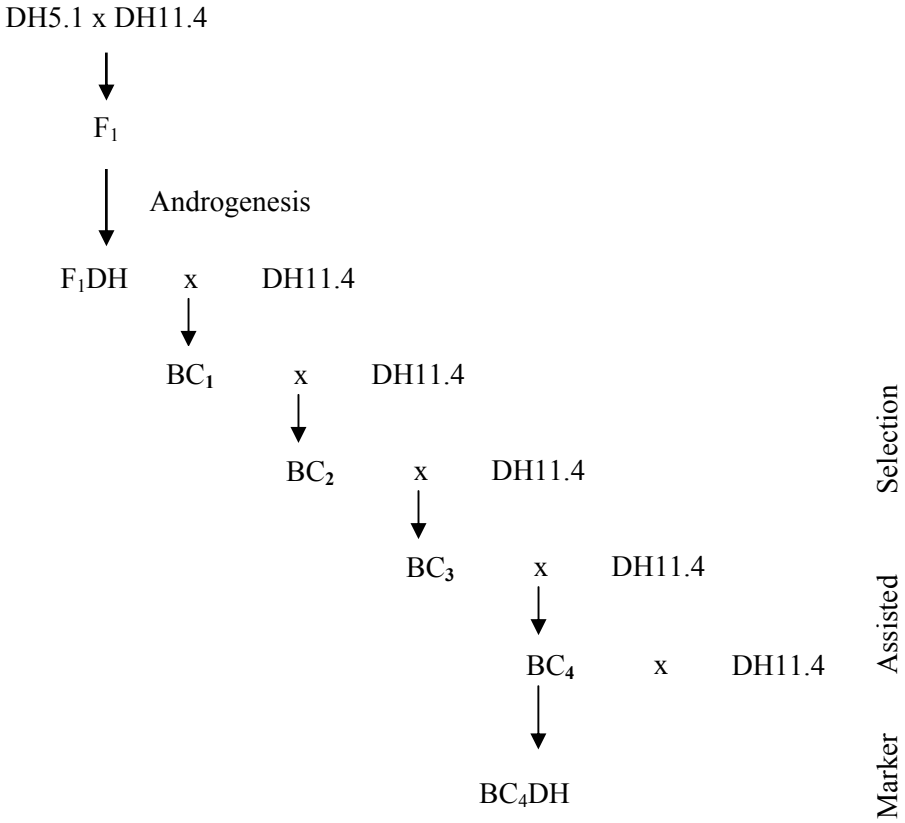


Fig. 3.1 Scheme for the development of substitution lines in the winter rapeseed cross of 'Mansholts Hamburger Raps' x 'Samourai'. All BC generations were subjected to MAS.

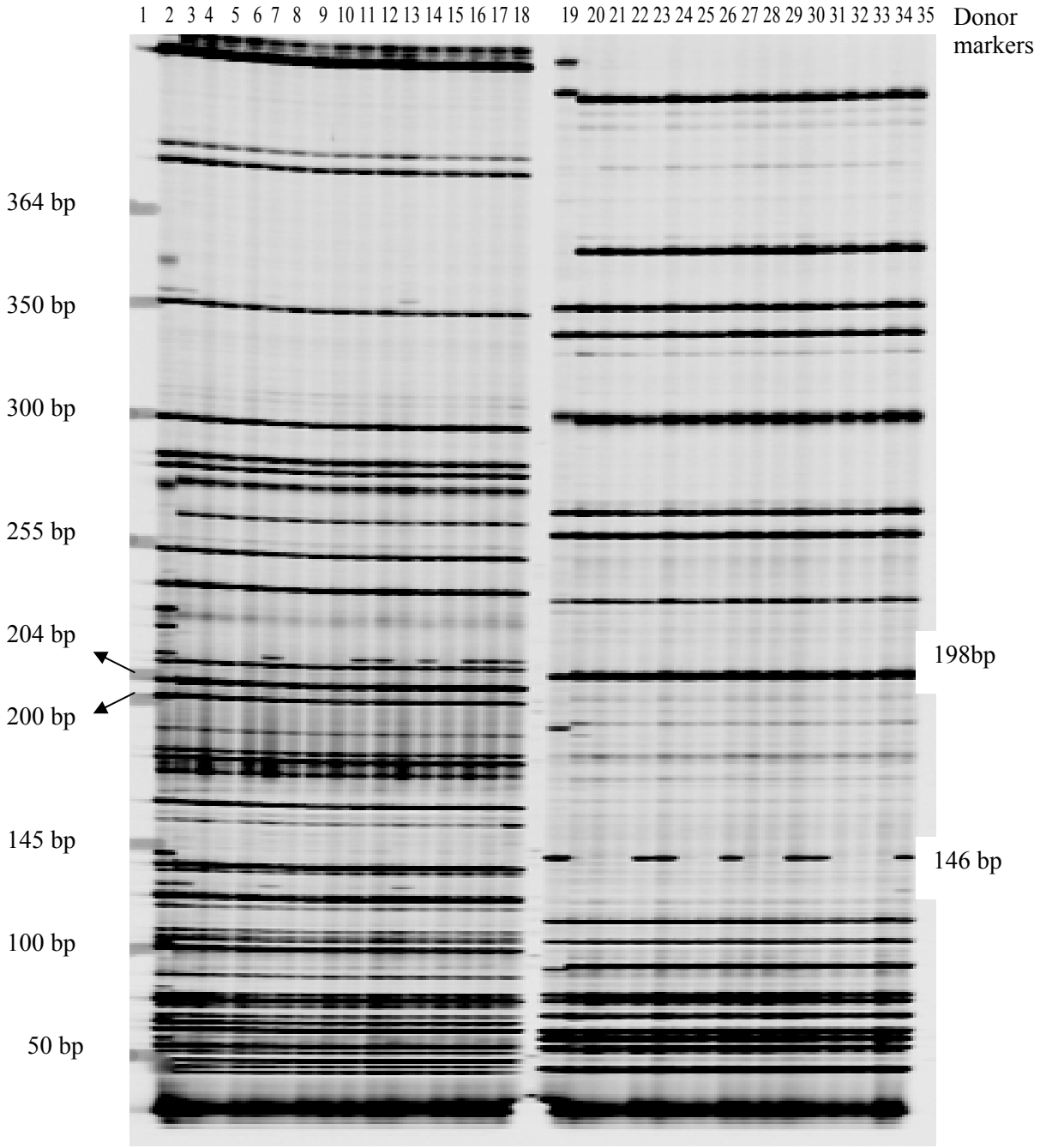
3.3 Result

3.3.1 AFLP markers

The electrophoresis image presented in Fig. 3.2 shows the components of the AFLP patterns in the polyacrylamide gel. The components in the image are (1) molecular weight lane used to facilitate sizing of the markers, (2) lanes with the banding patterns of the parents, DH5.1, and DH11.4, (3) monomorphic bands, and (4) polymorphic dominant marker bands.

3.3.2 Selection in BC_4

From each of 26 selected BC_3 plants 15 backcross progenies were sown. A total of 390 BC_4 genotypes were analysed for heterozygous donor segments of the parental BC_3 plants using 20 primer combinations generating 120 AFLP markers. The mean coverage of the donor segments in the BC_4 population was 50.1 cM ranging from 0 to 104.8 cM.



Lanes
1: Molecular weight, 50 – 700 bp,
2 and 19: Donor parent, DH5.1,
3 and 20: Recurrent parent, DH11.4,
4 -18: Individual plants from the BC_4 family 24.4 primer pairs E33M59
21 -35: Individual plants from the BC_4 family 24.4 primer pairs E40M60

Fig. 3.2 Part of an electrophoresis image adjusted by Adobe Photoshop showing the offsprings of parental BC_4 plant number 24.4 (2 x 15 genotypes) of AFLP markers

The number of donor fragments ranged from 1 to 4 with a mean of 2. In BC_4 generation, a total of 76 genotypes with less or equal to 3 donor segments were selected covering a minimum of 525 cM of the mapped rapeseed genome. The mean of donor segment coverage was 16.2 cM ranging from 0 to 98.2 cM. The largest donor segment was introgressed on linkage group 13 with a length of 95.9 cM. Lines with only one marker of the donor segment were arbitrarily assigned a length of 0 cM since the flanking markers on both sides on the genetic map already showed the recurrent parent genotype so that no minimal length would be determined for the segment. Table 3.2 shows the distribution of the donor segments in the selected BC_4 plants across the linkage group of the rapeseed map.

Table 3.1 Primer pairs used in marker assisted selection

E32M47	E32M59	E33M48	E33M61	E38M59
E32M48	E32M61	E33M49	E33M62	E38M61
E32M49	E32M62	E33M50	E35M60	E38M62
E32M50	E33M47	E33M59	E35M62	E40M60

*see appendix 2.1 for primer sequences

Table 3.2 Length of donor segments (cM) of the selected BC4 plants across the mapped linkage groups (LG)

Mapped linkage groups			Donor segment coverage		
Linkage group	Length (cM)	No. of donor segments†	Min (cM)	Max (cM)	Mean (cM)
1	60.2	2	0.0	0.0	0.0
2	61.5	5	0.0	38.2	18.3
3	48.7	5	6.0	39.1	24.8
5	54.7	3	0.0	3.6	1.2
6	56.0	2	3.3	19.7	11.5
7	5.8	1	0.0	0.0	0.0
8	68.0	2	3.2	18.6	10.9
9	72.5	2	0.0	16.3	8.1
10	83.2	2	11.4	56.3	33.9
11	2.1	1	0.0	0.0	0.0
12	158.0	7	0.0	38.2	6.5
13	101.6	7	20.5	95.9	58.1
14	118.1	5	0.0	59.9	23.4
15	75.2	3	0.0	4.0	1.3
16	105.9	3	0.0	23.7	7.9
17	53.2	2	0.0	22.5	11.3
18	65.3	2	0.0	15.1	7.5
19	58.3	2	0.0	18.6	9.3
20	0.0	1	0.0	0.0	0.0

†number of distinct donor segments on the respective linkage groups

3.3.3 Development of DH substitution lines

The selected BC_4 genotypes were used as microspore donors to generate DH substitution lines using microspore culture. A total of 1736 plantlets were produced from the microspores of 72 selected BC_4 lines. Due to unsuccessful embryo generation, 4 selected BC_4 plants did not produce any plantlets. Moreover, most of the microspore derived plantlets were haploid. Therefore, repeated colchicine treatments of the haploid plants were required to produce doubled haploid plants that can produce seeds (Table 3.3).

Table 3.3 Ploidy levels of the plantlets derived from microspore culture

Ploidy level	No. of plants	Percentage
Diploid	117	6 .8%
Haploid	1046	60.2%
Mixoploid	210	12.1%
Tetraploid	169	9.8%
Dead	194	11.1%
Total	1736	

3.3.4 Marker analysis in the substitution lines

Microspores derived plants were analysed for donor segments with 2 to 10 primer pairs. The number of primer pairs tested among the plants depended on the donor segments previously detected in their respective BC_4 microspore donor plant. From a total of 443 plants analyzed by AFLP markers, only 133 produced seeds. Out of the 133 DH substitution lines two did not contain the donor segment previously detected in their parental BC_4 plant. Table 3.4 shows an overview of the number of donor segments in the 133 DH substitution lines that produced seeds. Donor segment coverage in these DH substitution lines ranged from 0 to 68 cM with a mean of 11 cM. Appendix 3.1 depicts the localization and length of donor segment in the mapped rapeseed genome for each of the DH substitution lines analysed by markers. The estimated length of the donor segment (cM) was based on the minimum size of the segment calculated from the first marker scored for the donor segment to the last marker (outer most markers). Furthermore, a donor segment could be present in more than one DH substitution line. Also in many cases overlapping segments were observed. The donor segments were dispersed across 18 linkage groups giving a minimum coverage of the mapped rapeseed genome of 465.9 cM. Additional 233 DH substitution lines, which had not been analysed by markers, generated seeds. Here only marker information of the parental BC_4 plant was available. Appendix 3.2 shows detailed donor segment information for the selected BC_4 genotypes used as microspore donors to develop DH substitution lines. Appendix 3.3 provides the identification number of DH

substitution lines and their respective parental BC_4 lines. Consequently, the marker information could be integrated with identification number of parental BC_4 lines in appendix 3.2 to infer the donor segment information of DH substitution lines listed in appendix 3.3.

Table 3.4 Number of donor segment fragments in DH substitution lines analysed by MAS

No. of donor segments	Number of plants	Percentage
0	2	1.5%
1	77	42.3%
2	42	31.1%
3	12	8.9%
Total	133	

3.4 Discussion

3.4.1 Degree of polymorphism

The cross used in the development of the substitution lines showed a very low degree of polymorphisms, as a result only few markers coming from the donor parent were produced per primer combination. Hence, the markers were not sufficient to cover a reasonable length of the rapeseed genome. Therefore, additionally AFLP markers coming from the recurrent parent were generated which had to be scored codominantly and finally a total of 1325 cM of the rapeseed genome was covered. Codominant scoring of markers coming from the recurrent parent was carried out using the AFLP Quanta Pro 1 software (Keygene, 2002), which illustrates that AFLP technology allows scoring of the markers codominantly by quantitative measurement of the degree of PCR amplification resulting in a polymorphism of band intensity in the gel image. However, the software was not optimal in the current study to analyse the markers that were codominantly scored in the BC_4 population. Marschalek (2003) found also that very few of the markers (26%) were reliably scored codominantly in early backcrossing generations (BC_2, BC_3) that were used for the development of the BC_4 population in the current study. Therefore, these markers could only be properly scored after the production of the DH substitution lines in which only homozygous donor segments occur.

In another study, Howell et al. (1996) developed substitution lines from the cross of *Brassica napus* cultivars 'Victor' x 'Tapidor' using 158 RFLP marker loci that defined a genetic map of 1204 cM. They have also observed considerably less polymorphisms in their population than a population developed from a cross of conventional cultivar with the resynthesized rapeseed

(Parkin et al. 1995) in which 399 RFLP markers were generated covering 1656 cM of the rapeseed genome. In contrast, the cross of 'Express' x resynthesized rapeseed which was used to develop substitution lines described previously (see thesis chapter 2) possessed a high degree of polymorphism providing more markers and better resolution of the genetic map and information on donor segments through the backcross generations. This resulted in a higher density of the genetic map. A high degree of polymorphisms in the cross could be due to diverse characteristics of the resynthesized genotypes compared to conventional cultivars (see thesis chapter 2). Based on the analysis of the parents, low polymorphism observed by Howell et al. (1996) and the current study could be due to the cross of conventional cultivars used in the development of the substitution lines.

2.4.2 BC_4 and DH substitution lines genome composition

In the BC_4 generation the expected genome composition without MAS would have been 6.25% of the mapped donor genome. In the current study, the average minimum donor genome coverage for BC_4 selected plants was 16.2 cM, which is 1.2% of the mapped donor genome. Donor genome coverage of the selected BC_4 lines was clearly less than expected without MAS. This shows that selection in early generation was in favour of short donor segments to produce lines with only one donor segment. On the other hand the selected lines with the shortest donor segment were represented by only one marker and assigned as 0 cM length although in reality the lines contained more than 0 cM of the donor segment. This was due to the reason that the flanking markers on both sides on the genetic map already showed the recurrent parent genotype and it was not possible to estimate the size of the donor segment. If many lines represented with only one marker for the donor segments, more lines have to be selected in order to cover a reasonable percentage of the donor genome, which is not economical to conduct field trials. However, limiting the number of selected lines can be possible by using previous information on QTL mapping from secondary data sources to select lines with the genome region of interest. On the other hand, for many cultivars mapping information may not be available. Additionally, populations used in the QTL mapping are most likely different and matching markers may not be available between the maps.

Contrary to that, selection of lines with very short donor segments would have an advantage if the lines contain a QTL effect for the target trait, because these lines can be used as addition to the elite cultivars to improve the trait of interest since they contained lower donor segments from the donor parent. Moreover, lines with small segments are more informative for studies such as fine mapping, candidate gene analysis and gene cloning. In the last generation selected plants

have to cover at least a reasonable percentage of the donor genome. A better strategy could be to have a set of selected lines with both overlapping short and long donor segments in order to achieve a good coverage of the donor genome while at the same time achieve a high resolution for QTL mapping.

In the BC_4 generation, the donor segments in the selected plants were covering a minimum of 525 cM of the mapped genome. This is approximately 40% of the mapped donor genome. The rest of the donor genome could not be covered because of the higher number of fragments of donor segments in the candidate BC_3 plants, which contained more than 5 donor segments that were not ideal to be used in the development of the substitution lines. These candidate lines need to be crossed back to the recurrent parent to remove the non target donor segments. Moreover, 60% of the plantlets generated from the microspore culture of the selected BC_4 plants were haploids which required repeated colchicine treatment to produce doubled haploids. However, many haploid plantlets did not develop into doubled haploids therefore some of the plantlets with the target donor segment did not produce seeds. This has reduced the donor genome coverage of the DH substitution lines that could be produced in to 466 cM, which is only 89% of the coverage in the parental BC_4 plants.

In the current study plants with only one donor segment were obtained in the BC_4 generation. However, Ramsay et al. (1996) developed substitution lines with only one segment in BC_2S_1 population from the cross of *Brassica olarecea* L. The reason for the development of substitution lines as early as BC_2 generation could be due to the size of the genetic map of *B. olreacea*, 747 cM, which is nearly half of the genome of the *Brassica napus*. Consequently, species with small size of the genome require fewer generations of backcrossing to remove the donor segments of the donor parent. The other reason could also be the lower numbers of linkage groups in *Brassica olarecea* (9) compared to *Brassica napus* (19) that facilitates greater enrichment of the recurrent parent in earlier generations of backcrossing.

Howell et al. (1996) developed substitution lines in *Brassica napus* from BC_3 plants compared to the current study in which substitution lines were developed in BC_4 generation. This is because Howell et al. (1996) selected candidate lines as early generation as BC_2 by excluding linkage groups 9 and 12 fully; and parts of linkage groups 4, 5 and 15. Furthermore, BC_3 lines were selected without the representation of donor segments in a total of 7 entire linkage groups of the mapped rapeseed genome. By omitting entire linkage groups of the donor genome in early backcross generations, the current study could also have produced substitution lines with only

one donor segment earlier than in the BC_4 generation.

Rae et al. (1999) developed substitution lines in the BC_2 generation from the cross of *B. oleracea* var. *italica* and *Brassica oleracea* var*. alboglabra*. A set of 79 substitution lines were produced in which each line contained 1 to 4 donor segments, together the selected lines represented a maximum of 91% of the donor genome. Moreover, some of the substitution lines contained donor segments as long as 84 cM. In addition, in most of the linkage groups, Rae et al. (1999) made an effort to select larger donor segments to cover a maximum of the genome of the donor parent. However, QTL mapping in substitution lines with large donor segment could mask the QTL effects or underestimate the number and size of QTL. In the current study most of the substitution lines contained smaller segments with a maximum donor segment of 69 cM. Lines with larger donor segments could be crossed back to the recurrent parent to develop the ideal substitution lines with only small segments of the donor parent.

3.4.3 Application of the substitution lines

In rapeseed, Burns et al. (2003) reported several QTL for quality traits using the substitution lines developed from a cross of 'Victor' x 'Tapidor'. In the current study, the two parents used in the development of the substitution line library possessed different quality and other traits. DH5.1 is from an old cultivar with high erucic acid and high glucosinolate content compared to the recurrent parent. Furthermore, phenological, morphological and yield traits also differ between the two parents (Weißleder 1996). Due to the contrasting differences in quantitative traits between the two parents, the DH substitution lines were expected to inherit the donor segment loci with contrasting alleles. Accordingly, the substitution lines developed from the cross of 'Mansholts Hamburger Raps' and 'Samourai' were used for QTL mapping after the lines were tested in multiple field trials. The results of QTL mapping in the DH substitution lines developed in the current study are reported in the next chapters (see thesis chapters 4, 5, 6 and 7).

4. Mapping of QTL for oil and fatty acid contents using intervarietal substitution lines in *Brassica napus* L.

4.1 Introduction

Rapeseed is an important oil crop in Europe, China and North America. The value of the crop is determined by its seed oil content and fatty acid composition of the oil. The main fatty acids in rapeseed oil are: oleic (C18:1), linoleic (C18:2), linolenic (C18:3), eicosenic (C20:1) and erucic acid (C22:1). Erucic acid is supposed to be a health risk based on feeding experiment on animals (Roine and Uksila 1959). However, oils high in erucic acid has commercial benefits for use as high temperature lubricants, plasticizers (after minor modification), waxes, water repellents and surface-active agents (Lühs and Friedt 1993). After the release of canola quality rapeseed with low glucosinolate (< 25 μmol per g seed) and low erucic acid (< 2%) contents, rapeseed production increased due to its use in margarines, cooking oil, salad dressings and animal feed. A diet rich in oleic acid may reduce the content of low density lipoprotein cholesterol in blood plasma (Chang and Huang 1998), however, high levels of linolenic acid reduce the oxidative stability of the oil (Thormann et al. 1996) which can reduce the shelf life of the oil. Hence, the important criteria in rapeseed breeding are developing cultivars free from erucic acid, low glucosinolate, high oleic acid and low linolenic acid content for human and animal consumption and high erucic acid for non-food commercial use.

Most important agronomical traits such as oil and fatty acid content are controlled by many genes and vary across environments. Quantitative trait loci (QTL) mapping can help to identify genome region closely linked to the genes that control such traits. Therefore, QTL studies are useful starting points for Marker Assisted Selection (MAS) and positional cloning. Furthermore, plant breeders could use the information of QTL mapping to combine the best genes available in the existing gene pool to improve the trait of interest. Several methods are currently available to map QTL using segregating populations like F_1DH, F_2, BC_1 and RIL, relying on statistical methods (Lander and Botstein 1989; Haley and Knott 1992; Kearsey and Hyne 1994). However, QTL mapping using segregating populations suffer from low power of detection and poor precision of QTL localization (van Ooijen 1992; Kearsey and Farquhar 1998). Moreover, the effects of those few detected QTL are usually overestimated and strongly biased as the population size used for the mapping decreases (Beavis et al. 1994; Hyne et al. 1995; Melchinger et al. 1998; Schön et al. 2004). Additionally, small QTL effects are hardly identified and efforts to detect those requires large populations of up to 1000 lines (Soller et al. 1976; Schön et al. 2004) leading to high cost of genotypic and phenotypic characterisation.

Alternative to a segregating population QTL mapping can be applied in a substitution lines with complementary sets of donor segments in the genetic background of the recurrent parent by which the whole donor genome is represented in a well defined manner (Howell et al. 1996). Substitution lines display high power of QTL detection due to no background effects and no epistasis between donor QTL alleles except in those lines containing more than one donor segments (Eshed and Zamir 1994; 1995). Moreover, localization of QTL can be improved by subdividing a larger donor segment into smaller overlapping segments after further backcrossing to the recurrent parent. After QTL detection only those lines showing significant phenotypic effects can be further characterised and verified which could tremendously decrease the number of lines tested in repeated field trials.

In the current study QTL analysis of oil and fatty acid content was carried out using a DH substitution line library derived from a cross of an old cultivar of oilseed rape 'Mansholts Hamburger Raps' with a modern winter oilseed rape variety 'Samourai'. From selected BC_4 plants substitution lines with homozygous donor segments were produced using microspore culture. QTL identified in this study are compared to those that have been published previously.

4.2 Materials and methods

4.2.1 Plant materials and donor genome coverage

In the development of the substitution lines the donor parent was DH5.1, a doubled haploid line from 'Mansholts Hamburger Raps' ('Mansholt'), an old cultivar with high erucic acid and high glucosinolate contents. A doubled haploid line of the winter oilseed rape variety 'Samourai', DH11.4, was used as a recurrent parent. A total of 366 DH substitution lines had been produced from selected BC_4 genotypes using microspore culture (see thesis chapter 3). Out of the 366 substitution lines produced, 292 lines that had produced sufficient seeds were tested in field trials. Out of the 292 lines tested in the field experiments, 101 DH substitution lines were analysed with markers. 55 of these lines contained 1 donor segment, 31 lines contained 2 donor segments and 15 lines contained 3 donor segments. These 101 lines covered a minimum of 451 cM of the mapped donor genome. Appendix 4.1 depicts details of donor segment coverage (length of donor segment in centi Morgan) of the 101 lines across the mapped linkage groups of the rapeseed genome. The rest of 191 lines were not analysed by markers, however, marker information of BC_4 microspore donor plants were available which could be used to infer to donor segment information of the DH substitution lines (see appendix 3.2 and 3.3). In the 191 lines without marker analysis, 112 lines were derived from BC_4 lines with 1 donor segment, 49

lines from plants with 2 donor segments, and 30 lines from plants with 3 donor segments. The lines represented donor segments from the mapped linkage groups of the rapeseed genome except linkage group 4. However, donor segment coverage across the mapped linkage groups represented only part of the linkage groups with different overlapping segments represented by more than one plants and as indicated in chapter 3 they were not fully cover the donor genome.

4.2.2 Field trials

Field trials were conducted in 2005/06 and a total of 292 substitution lines were sown at Reinshof, the experimental station of the University of Göttingen as randomized block design with two replications. At four additional locations between 230 and 261 lines were tested at the experimental stations of the following companies: Deutsche Saatveredelung (DSV) at Thüle in Northwest Germany, Norddeutschen Pflanzenzucht (NPZ) HG Lembke KG at Hohenlieth in Northern Germany, KWS SAAT AG at Seligenstadt in Southern Germany and SW Seed GmbH at Grund-Schwalheim in central Germany without replication. Plots of the recurrent parent DH11.4 were included in which one plot of DH11.4 was sown for every 9 plots of substitution lines in order to be used as controls. In addition 3 plots of the doubled haploid donor parent DH5.1 were also sown in Reinshof. The plot size varied at the different locations between 2.6 and 8.0 m^2 and the plant density between 27 and 60 plants per m^2. Field management, herbicides and fertilizer applications were done according to the local practices. To generate self-pollinated seeds 3 to 6 plants of each line were covered with plastic bags before flower buds opened. Seeds were harvested from each self-pollinated plant separately. Additionally, ten g bulked seeds were harvested from the terminal racemes and the two uppermost primary branches of five healthy open pollinated plants from each line. Out of 292 lines sown, 288 produced sufficient seed for Near Infrared Reflectance Spectroscopy (NIRS) analysis. Seed oil and fatty acid contents were determined by NIRS using the calibration equation raps2001.eqa developed by Tillmann (2007).

4.2.3 Statistical analysis

Least square means (LSMEANS) of the parameters measured for the test substitution lines and the control were calculated using PROC MIXED of SAS version 9.1 (SAS Institute 2003). The LSMEANS differences between the lines and control were tested using t-tests adjusted according to Dunnet by multiple comparisons (Dunnet 1955). A QTL was detected if the LSMEANS difference of the line was significantly different from the recurrent parent DH11.4 with an alpha level of 0.05. Variance was calculated using PROC GLM of SAS (SAS Institute 2003). The recurrent parent DH11.4 was the common control for all substitution lines. Consequently, the

deviation of the line from the recurrent parent DH11.4 should be due to the introgression of the donor segment in the genetic background of the recurrent parent.

4.3 Results

4.3.1 Oil content

Analysis of variance revealed significant variation for oil content (Table 4.1) and also for oleic acid (4.2) and erucic acid content (Table 4.4) among the lines and locations but not significant variation among the lines for linolenic acid content (Table 4.3). Table 4.5 shows details of QTL detected for four traits. From a total of ten QTL detected for oil content nine showed that the introgression of the donor allele increased oil content with effects ranging from 3.4 to 6.2% while only one QTL was found where the donor allele from DH5.1 decreased oil content (-5.7%). The mean oil content of DH11.4 for all locations was 48.7%. In Reinshof mean oil content for DH5.1 and DH11.4 were 51.5 and 45.4%, respectively. Therefore, more QTL contributed positive effects due to the introgression of the DH5.1 segment in the genetic background of DH11.4.

4.3.2 Fatty acids content

C18: 1 (Oleic acid)

The average oleic acid content for DH11.4 and DH substitution lines for all locations were 59.6 and 59%, respectively. In Reinshof, mean oleic acid content for DH5.1 was lower (41.7%) than the DH11.4 (56.5%). Variance analysis showed significant variation among lines and locations (Table 4.2). A total of four QTL were detected on linkage groups 3, 6, 12 and 19 of which three QTL showed negative effects on oleic acid content ranging from -4.9% to -12.3% as expected since DH5.1 has a lower oleic acid content. However, one QTL on linkage group 19 increased oleic acid content by 13.7% indicating that there are QTL alleles in DH5.1 increasing oleic acid content.

C18: 3 (linolenic acid)

Variance analysis revealed no significant variation among the lines for linolenic acid content (Table 4.3). Nevertheless, a total of 7 QTL were detected for linolenic acid content on linkage groups 2, 3, 5, 7, 12, 14 and 19 (Table 4.5). Mean linolenic acid content for DH11.4 in all locations was 11.54%. In Reinshof, mean linolenic content for DH5.1 and DH 11.4 were 11 and 12.5%, respectively. However, in all cases the DH5.1 allele increased the linolenic acid content with effects ranging from 1.3% to 1.6%.

C22: 1 (erucic acid)

Variance analysis showed highly significant variation for erucic acid content among the lines (Table 4.4). Lines with significantly high erucic acid content have donor segment of specific genome regions either on linkage groups 6 or 12 from the donor parent (Table 4.5). We didn't find lines containing both QTL on linkage groups 6 and 12 which should have showed an erucic acid content equal to the donor parent DH5.1 (53%), however, the sum of erucic acid content in the lines with the QTL on the two linkage groups was nearly equal to that of DH5.1. Correlation between erucic acid and oleic acid content was high (r = -0.85***) while correlation between oil content and erucic acid content was low but highly significant (r = 0.23***).

Table 4.1 Analysis of variance for oil content

Source	DF	SS	MS	F	P
Line	282	4396.24	15.53	3.20	0.001
Location	4	3192.17	798.04	166.60	<0. 0001
Line*location	606	2900.16	4.78	0.44	

Table 4.2 Analysis of variance for oleic acid content

Source	DF	SS	MS	F	P
Line	283	10521.56	37.18	3.64	<0.0001
Location	4	2075.63	518.91	50.80	<0.0001
Line*Location	606	3883.15	6.41	0.63	

Table 4.3 Analysis of variance for linolenic acid content

Source	DF	SS	MS	F	P
Line	283	164.36	0.58	0.78	0.9839
Location	4	306.23	76.56	103.30	<0.0001
Line*Location	606	212.09	0.35	0.47	

Table 4.4 Analysis of variance for erucic acid content

Source	DF	SS	MS	F	P
Line	283	58238.13	205.79	14.35	<0.0001
Location	4	1918.84	479.71	33.45	<0.0001
Line*Location	606	6023.30	9.94	0.69	

Table 4.1 up to Table 4.4, DF: degree of freedom; SS: sum of square;
MS: mean square; F: F variance ratio, P: probability associated with the test statistics

Table 4.5 QTL detected for oil and fatty acid compositions

QTL	DH Line[1]	BC_4 Pl.[2]	Donor segments						QTL eff. (%)	P[9]
			No seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
Oil										
Oil-1	217		1	3	E3259.72M -E3348.001M	20.8	50.0	30.2	3.4	0.033
Oil-2	536[a]	21.4.15	1	5	E3859.289M	39.9	39.9	0.0	4.4	0.039
Oil-3	578[a]	4.4.1	1	6	E3359.564 -E3247.335M	42.0	61.7	19.7	5.7	0.030
Oil-4	434		1	10	E3361.167M	2.6	2.6	0.0	4.3	0.018
Oil-5	1183		2	11	E3259.337S	8.2	8.2	0.0	4.5	0.001
				12	E3247.158M	0.0	0.0	0.0		
Oil-6	156[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	6.2	0.001
Oil-7	1373		1	13	E3359.234M	101.0	101.0	0.0	3.6	0.038
Oil-8	1395[a]	21.4.5	2	14	E3862.229M	4.0	4.0	0.0	-5.7	0.001
				5	E3859.289M	39.9	39.9	0.0		
Oil-9	548[a]	8.4.10	1	15	E3347.160S	75.2	75.2	0.0	4.2	0.063
Oil-10	10[a]	3.4.14	1	18	E3259.194S	62.1	62.1	0.0	5.4	0.071
Oleic acid										
C18:1-1	158[a]	7.4.9	1	3	E3362.143M-E3261.234M	45.1	51.1	6.0	-4.9	0.001
C18:1-2	578[a]	4.4.1	1	6	E3359.564M-E3247.335M	42.0	61.7	19.7	-7.3	0.002
C18:1-3	156[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	-12.3	<0.0001
C18:1-4	1400[a]	9.4.9	1	19	E3359.225M	41.3	41.3	0.0	13.7	<0.0001
Linolenic acid										
C18:3-1	1084[a]	22.4.3	3	2	E3349.155M-E3247.159M	0.0	38.2	38.2		
				10	E3362.211S	19.6	19.6	0.0	1.6	0.016
				16	E3362.204S	105.9	105.9	0.0		
C18:3-2	957		1	3	E3362.143M -E3261.209S	45.1	69.5	24.4	1.2	0.045
C18:3-3	535[a]	21.4.15	1	5	E3859.289M	39.9	39.9	0.0	1.4	0.017
C18:3-4	954		1	7	E3259.149M	28.3	28.3	0.0	1.3	0.054
C18:3-5	1199		1	12	E3247.121S	09.6	09.6	0.0	1.4	0.035
C18:3-6	50[a]	2.4.16	2	14	E3261.218M-E3261.107M	58.2	118.1	59.9	1.4	0.036
			2	16	E3261.370S	0.0	0.0	0.0		
C18:3-7	641[a]	9.4.9	1	19	E3359.225M	41.3	41.3	0.0	1.3	0.021
Erucic acid										
C22:1-1	578[a]	4.4.1	1	6	E3359.564M-E3247.335M	42.0	61.7	19.7	30.2	<0.0001
C22:1-2	156[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	30.4	<0.0001

[1]Identification number (IN) of the DH substitution line with the QTL effect, IN with superscript 'a' means the line was not analysed by markers and the marker information was from the microspore donor plant.

[2]Identification number of the microspore donor BC_4 plant used to generate the DH substitution line

[3]Number of donor segments in the DH substitution line, if the DH line contained more than one donor segments the other segments are listed in the subsequent rows

[4]Linkage group from the genetic map (Uzunova et al. 1995) carrying the donor segment containing the QTL

[5]First and last marker on the donor segment

[6]'Start' indicates where the donor segment starts within the indicated linkage group

[7]'End' depicts where the donor segment ends within the indicated linkage group

[8]Minimal length of the donor segment as defined by the distance between the first and last marker on the genetic map

[9]Significance level of the QTL effect

4.4 Discussion

In the current study a total of 23 QTL were detected for four traits (oil, oleic acid, linolenic acid and erucic acid content) using the DH substitution lines of which ten QTL were detected for oil content. Using a F_1DH population from the same parents to the current study, Ecke et al. (1995) mapped two genes for erucic acid content on linkage groups 6 and 12, and three QTL for seed oil content on linkage groups 6, 10 and 12 using Mapmaker/QTL. The QTL effects for oil content in their study ranged from -1.3 to 1.8 %. Furthermore, using the same F_1DH population, Gül (2002) mapped six QTL for oil content on linkage groups 5, 6, 11, 12, 14 and 15 using the program QTLMapper with a mixed model approach to interval mapping. The QTL effects in his study ranged from -1.1 to 1.14%. However, in the current study, the QTL effects for oil content were higher, ranging from -5.7 to 6. 2%. The reason for the differences in the QTL effects for oil content between the above studies and this study was due to a poor performance of the recurrent parent DH11.4 in the field experiments of the current study, which was used as a control.

The two QTL for oil content detected by Ecke et al. (1995) on linkage groups 6 and 12 were mapped at the same positions as by Gül (2002) on the same linkage groups. Although the QTL effects for oil content differ strongly between the above studies and the present study, the two QTL detected for oil content mapped at the same positions on the same linkage groups. Moreover, a QTL for oil content on linkage group 10 detected by Ecke et al. (1995) was mapped again at the same position as in the current study on the same linkage group. Additionally, a QTL detected for oil content on linkage group 5 by Gül (2002) was also mapped at the same position as in the current study on the same linkage group. Moreover, a QTL for oil content on linkage group 11 detected in the current study was mapped near to the position of QTL for oil content mapped by Gül (2002) on the same linkage group. However, two QTL for oil content detected on linkage groups 14 and 15 in the current study were mapped far apart from the QTL for oil content mapped by Gül (2002) on the same linkage groups.

The two genes mapped by Ecke et al. (1995) affecting erucic acid content were again identified using the substitution lines at the same genome positions on linkage groups 6 and 12 and they were also QTL for oil content. These results indicate that the genes affecting both traits, oil and erucic acid content, are closely linked or are the same genes. Ecke et al. (1995) found an increase of 1 % in oil content per erucic acid allele of 'Mansholt'. In the current study too, the two QTL detected for erucic acid content affected the oil content positively and the effect of each of the QTL for erucic acid on the oil content was relatively higher than any of the other 8 QTL detected for oil content. Accordingly, the QTL for erucic acid content on linkage group 6 increased oil content by 5.7% and the other QTL on linkage group 12 increased oil content by 6.2%.

In a cross between rapeseed varieties 'Major' and 'Stellar' Thormann et al. (1996) identified two QTL affecting erucic acid levels and two QTL affecting linolenic acid content using Mapmaker/QTL. The two QTL affecting erucic acid content were mapped on linkage groups 7 and 15 of their genetic map. Both QTL accounted for 95% of the phenotypic variance and no other regions affecting erucic acid content were detected. The two QTL identified for erucic acid in their study were synonymous with the erucic acid genes detected in the current study.

Howell et al. (1996) used RFLP marker data of 200 BC_1 plants which were used to map two QTL for erucic acid content from the BC_1S_1 population of 'Tapidor' x 'Victor' using interval mapping with Mapmaker/QTL. They reported no other region showing an effect on erucic acid content other than the two QTL detected for erucic acid content (cited after Burns et al. 2003). The two QTL detected in their study might be identical with the QTL detected for erucic acid in the current study and we also didn't find any other genome region affecting the trait.

Burns et al. (2003) identified QTL for oil and fatty acid contents in 22 intervarietal substitution lines derived from a cross of 'Victor' and 'Tapidor' in BC_3 and BC_4 generation. They identified three QTL for erucic acid, seven for oil, four for oleic acid and five for linolenic acid content. In the current study only two QTL were detected on linkage groups 6 and 12 increasing erucic acid content by 30.2 and 30.4%, respectively. Although in the current study we have analysed more substitution lines, 288, instead of only 22 and donor segments were represented on 18 linkage groups of the mapped rapeseed genome instead of only on 10 linkage groups as compared to the study by Burns et al. (2003), we detected only two QTL for erucic acid content. Therefore in the current study there were only two QTL for erucic acid showing additive effects and the sum of the two QTL effects was nearly equal to the erucic acid content of the donor parent (53%) and no other region showed significant effect on erucic acid content. Although 3 QTL were detected for

erucic acid content (Burns et al. 2003), our findings confirm the previous investigations (Harvey and Downey 1964; Stefansson and Hougen 1964; Kondra and Stefanson 1965; Siebel and Pauls 1989; Ecke et al. 1995; and Thormann et al. 1996) that only two genes are responsible for erucic acid content in rapeseed.

In the current study the correlation between erucic acid and oil content was low (r = 0.23) and the correlation coefficient found by Burns et al. (2003) was also low (r = 0.18). This figure is lower than the expected higher correlation based on strong association of the two traits as shown in the QTL mapping. The reason could be that erucic acid content is controlled by only two genes; however, there are QTL for oil content of which many are not linked to erucic acid genes, therefore, a strong correlation was not expected. The strongest correlation that existed with erucic acid content was with oleic acid content (r = - 0.85), which is to be expected since the erucic acid is derived from the elongation of oleic acid by adding two double carbon units. The highest correlation (r = -0.90) was also reported between oleic and erucic acid in the study by Burns et al. (2003) supporting the current study. In the current study, out of four QTL detected for oleic acid content three QTL showed negative effects on oleic acid content and the same QTL affected oil content positively. However, one QTL for oleic acid content with positive effect showed negative effect on oil content. Burns et al. (2003) also detected three QTL for oleic acid content with negative effects and the same donor alleles showed positive effects for oil content and one QTL increased oleic acid with out any effect on oil content supporting the current result.

In the current study nine out of ten QTL detected for oil content contributed positive effects due to the introgression of the donor segment. However, Burns et al. (2003) detected only three QTL out of seven where the donor allele increased oil content. The differences in the direction of the QTL effect for oil content might be due to different populations used in the studies in which there was 2% more oil content in the donor parent ‘Mansholt’ than in the recurrent parent ‘Samourai’ in the current study compared to the population of Burns et al. (2003) in which the donor parent ‘Victor’ contained 0.33% less oil content than 'Tapidor' (calculated from the data of Burns et al. 2003). In the present study for all seven QTL detected for linolenic acid content the donor allele increased linolenic acid content. However, Burns et al. (2003) detected only three QTL out of five increasing linolenic acid content.

Zhao et al. (2005; 2006) mapped 18 QTL with additive effects and additive x additive pairs for oil content in a F_1DH population from a cross between a European cultivar ‘Sollux’ and the

Chinese cultivar 'Gaoyou' using 125 SSR markers in 282 double haploid lines. Comparing the QTL positions in that studies with the QTL positions in the current study was not possible due to absence of alignment between the two maps. Delourme et al. (2006) identified 10 and 14 QTL for oil content from two populations of *Brassica napus* L. with composite interval mapping using QTL cartographer. They have also identified one common QTL for the two populations. The above three studies conferred that more QTL are distributed across the rapeseed genome affecting oil content. Although more QTL have been detected in the segregating population of the above studies, poor precision of QTL localisation was unavoidable (van Ooijen 1992; Kearsey and Farquhar 1998). If QTL mapping information is used for studies like gene cloning, fine mapping or candidate gene analysis; higher precision of QTL localisation is an important factor.

QTL position that could be mapped using a segregating populations is most likely in the confidence intervals of 10's of cM (van Ooijen 1992; Darvasi 1993; Kearsey and Farquhar 1998), which could be a large genome region to obtain higher precision of QTL localisation. In the substitution lines the interval to which a QTL can be mapped depends on the size of the donor segment in the lines. In the current study, the precision of QTL localization could be narrowed down to a few centi Morgan using substitution lines with small overlapping donor segment. Substitution lines with high precision of QTL localisation could be used in the analysis of candidate genes and cloning of the target genes.

5. Mapping of QTL for protein and glucosinolate using intervarietal substitution lines in *Brassica napus* L.

5.1. Introduction

Rapeseed is primarily grown as oil crop in the temperate areas. Rapeseed meal that remains after the oil has been extracted contains a high amount of protein that can be used in animal feeding. Although rapeseed meal production is only one-fifth compared to the leading soybean meal, it is the world second leading source of protein (Oil World 2007). Furthermore, nutrition studies showed that addition of rapeseed protein to sausages improved taste, give a good texture and characteristic aroma (Yoshie-Stark 2006). Nevertheless, rapeseed meal contains unfavourable components like glucosinolates, which limit the use in animal feeding. Glucosinolates are sulphur rich plant secondary products that are toxic to animals and fungi (Chew 1988). However, modern canola cultivars contain a low seed glucosinolate content (≤ 25 µmol per seed) and a low erucic acid content (< 2%). On the other hand, research on glucosinolate genetics and biosynthesis became a favourite system for ecological studies to analyse plant-insect interactions because a number of herbivorous insect species are restricted to glucosinolate containing plants (Zhang et al. 2006). In the last decades, rapeseed breeding criteria for animal feeding targeted developing of varieties with low seed glucosinolate content. Using naturally occurring variation seed glucosinolate content has been drastically reduced to less than 10 µmol g^{-1} seed (Ransey et al. 1999). Quantitative traits like protein and glucosinolate contents vary continuously, affected by environment and genotype by environment interactions.

QTL mapping become one of the favourite method to study the effects, numbers and positions of QTL affecting a trait in order that the information generated from these studies could be incorporated into the classical crop improvement programmes. In many studies segregating populations like F_2, BC, RIL, and F1DH were used for QTL mapping. Using a segregating population of *Brassica napus* L. 4 QTL (Uzunova et al. 1995) and 5 QTL (Toroser et al. 1995) were mapped for seed glucosinolate content. Gül (2002) mapped 5 QTL for protein content using a F_1DH population of *Brassica napus* L. However, QTL mapping in a segregating population has many drawbacks such as low power of detection and poor precision of QTL localization (van Ooijen 1992; Kearsey and Farquhar 1998; Melchinger 1998; see also thesis chapter 2 and 3). In contrast to a segregating population, QTL mapping can be applied in a set of substitution lines with well defined segments of the donor parent in the genetic background of the recurrent parent. These lines show a high power of QTL detection and accurate localization of QTL due to low background effects (Eshed and Zamir 1994; 1995).

In the current study QTL mapping for protein and glucosinolate content was carried out using a substitution line library derived from a cross of an old cultivar 'Mansholts Hamburger Raps' with a modern homozygous winter oilseed rape variety 'Samourai'. In addition, a relationship between QTL for protein content to QTL for oil content was investigated. QTL identified in the present study are compared to those published.

5.2. Materials and methods

5.2.1. Plant materials and genome coverage

In the development of the substitution lines the donor parent was a doubled haploid line DH5.1 from an old cultivar 'Mansholts Hamburger Raps' (Mansholt) with high erucic acid and high glucosinolate content. The recurrent parent was a doubled haploid DH11.4 line from canola quality winter oilseed rape variety 'Samourai'. DH substitution lines were developed from selected BC_4 plants using microspore culture. A total of 366 DH substitution lines had been produced (see thesis chapter 3) of which 292 lines that had produced sufficient seeds were tested in field trials. Out of 292 lines tested in the field experiments, 101 lines were analysed with markers. 55 of these lines contained 1 donor segment, 31 lines contained 2 donor segments and 15 lines contained 3 donor segments. The 101 lines covered a minimum of 451 cM of the mapped rapeseed genome. The rest of 191 lines were not analysed by markers, however, marker information of BC_4 microspore donor plants were available which could be used to infer to donor segment information of the DH substitution lines (see appendix 3.2 and 3.3). In the 191 lines without marker analysis, 112 were derived from BC_4 lines with 1 donor segment, 49 lines from plants with 2 donor segments, and 30 lines from plants with 3 donor segments. The lines represented parts of donor segments from the mapped linkage groups of rapeseed genome except linkage group 4.

5.2.2. Field trials

In year 2005/06, 292 substitution lines were sown at Reinshof as randomized block with two replications. At four additional locations between 230 and 261 lines were tested without replication at the experimental stations of the companies: Deutsche Saatveredelung (DSV) at Thüle in Northwest Germany, Norddeutschen Pflanzenzucht (NPZ) HG Lembke KG at Hohenlieth in Northern Germany, KWS SAAT AG at Seligenstadt in Southern Germany, and SW Seed GmbH at Grund-Schwalheim in central Germany. Plots of the recurrent parent 'DH11.4' were used as controls by sowing one plot for each 9 plots of substitution lines. In addition 3 plots of the double haploid donor parent 'DH5.1' were also sown in location

Reinshof. The plot size varied at the different locations between 2.6 and 8.0 m^2 and the plant density between 27 and 60 plants per m^2. Field management, herbicides and fertilizer applications were done according to the local practices. To secure self-pollinated seeds 3 to 6 plants of each line were covered with plastic bags before flower buds opened. Seeds were harvested from each self-pollinated plants separately and additionally ten g-bulked seeds were harvested from the terminal racemes and the two uppermost primary branches of five healthy plants of open pollinated lines. Of the 292 lines sown, 288 produced enough seeds for Near Infrared Reflectance Spectroscopy (NIRS). Analysis of seed samples obtained from the field experiments were performed at the University of Göttingen in the department of crop science, section of plant breeding using NIRS calibration equation raps2001.eqa developed by Tillmann (2007).

5.2.3. Statistical analysis

Least square means (LSMEANS) of the parameters measured for the test substitution lines and the control were calculated using PROC MIXED procedure of SAS (SAS Institute 2003). Multiple comparisons of LSMEANS differences of the lines from the control were tested using t-tests adjusted according to Dunnet (Dunnet 1955). A QTL was detected if the LSMEANS difference of the line was significantly different from the recurrent parent DH11.4 with an alpha level of 0.05. The recurrent parent 'DH11.4' was the common control for all DH substitution lines. Consequently, the deviation of the line from the DH11.4 should be due to the introgression of the donor segment in the genetic background of the recurrent parent. ANOVA was performed using PROC GLM procedure of SAS. The Pearson correlation coefficients were calculated with PROC CORR procedure of SAS to determine correlation among the estimated phenotypic values.

5.3 Results

A total of 3 significant correlations were identified between 3 traits (Table 5.1). A strong significant correlation between oil and protein content ($r = 0.9$) was observed. The correlation between glucosinolate and oil content was weak, although significant. The analysis of variance revealed a significant difference between the lines for the analysed traits. Therefore, the DH substitution lines differ due to the introgression of the donor segment for the analysed traits. ANOVA revealed significant variation for protein and glucosinolate content among the lines and locations (Table 5.2 and Table 5.3).

5.3.1 Protein content

In Reinshof, mean protein content of the donor and recurrent parent were 23.5 and 24.3%, respectively. For all locations, mean protein content of the lines and the recurrent parent were 22.5 and 24.9, respectively. A total of 5 QTL were detected for protein content of which 4 QTL showed a negative effect on oil content (Table 5.4). The donor parent contained lower amount of protein content compared to the recurrent parent, therefore, due to the introgression of the donor segments into the lines, negative QTL effects were expected.

5.3.2 Glucosinolate content

The mean seed glucosinolate content of the donor and the recurrent parent were 78 and 25.4 μmol g^{-1} seed, respectively, for location Reinshof. For all locations, mean glucosinolate content of the recurrent parent and the lines were 20.6 and 22.4 μmol g^{-1} seed, respectively. A total of 9 QTL were detected for total seed glucosinolate content showing positive QTL effects due to the replacement of a DH11.4 allele by a DH5.1 allele as expected (Table 5.5).

Table 5.1 Correlation coefficient among the traits

	Oil	Protein	GSL
Oil	1.00	-0.90***	-0.12**
Protein		1.00	0.18*
GSL			1.00

*and *** statistically significance level at =0.05 and =0.001, respectively

Table 5.2 Analysis of variance for protein content

Source	DF	SS	MS	F	P
Line	283	2149.66	7.60	1.40	0.0015
Location	4	1493.25	373.31	68.64	<.0001
Line*Location	606	1641.94	2.71	0.50	

Table 5.3 Analysis of variance for glucosinolate content

Source	DF	SS	MS	F	P
Line	283	58274.70	205.92	3.20	<.0001
Location	4	1560.74	390.18	6.07	<.0001
Line*Location	606	14472.30	23.88	0.37	

Table 5.2 and 5.3 DF: degree of freedom; SS: sum of square; MS: mean square; F: F variance ratio, P: probability associated with the test statistics

Table 5.4 Relationships between QTL for protein and oil content

QTL	DH Line[1]	BC_4 Pl[2]	Donor segments						Protein		Oil	
			No. Seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)	QTL effect[9] (%)	P[10]	QTL effect[11] (%)	P[12]
pro-1/oil-1	252		1	3	E3259.72M -E3348.001M	20.8	51.0	30.2	-3.9	0.018	3.4	0.033
oil-2	536[a]	21.4.15	1	5	E3859.289M	39.9	39.9	0.0	-	-	4.4	0.039
oil-3	578[a]	4.4.1	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	-	-	5.7	0.030
oil-4	434		1	10	E3361.167M	02.6	02.6	0.0	-	-	4.3	0.018
pro-2/oil-5	1183		2	11	E3259.330M	8.2	8.2	0.0	-3.6	0.010	4.5	0.001
				12	E3247.158M	0.0	0.0	0.0				
pro-3	144		1	12	E3361.001M	101.6	101.6	0.0	-3.6	0.054		
oil-6	156[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	-	-	6.2	0.001
oil-7	1373		1	13	E3359.234M	101.0	101.0	0.0	-	-	3.6	0.038
pro-4/oil-8	1386[a]	21.4.5	2	14	E3862.229M	4.0	4.0	0.0	3.1	0.048	-5.7	0.001
				5	E3862.229M	39.9	39.9	0.0				
pro-5/oil-9	548[a]	8.4.10	1	15	E3347.160S	75.2	75.2	0.0	-3.4	0.025	4.2	0.063
oil-10	10[a]	3.4.14	1	18	E3259.194S	62.1	62.1	0.0	-	-	5.4	0.071

[1]Identification number (IN) of the DH substitution line with the QTL effect, IN with superscript 'a' means the line was not analysed by markers and the marker information is from the microspore donor plant. [2]Identification number of the microspore donor BC_4 plant used to generate the DH substitution line

[3]Number of donor segments in the DH substitution line, if the DH line contained more than one donor segment the other segment is listed in the subsequent row.

[4]Linkage group from the genetic map (Uzunova et al. 1995) carrying the donor segment [5]First and last marker on the donor segment

[6]'Start' indicates where the donor segment starts within the indicated linkage group

[7]'End' depicts where the donor segment ends within the indicated linkage group

[8]Minimal length of the donor segment as defined by the distance between the first and last marker on the genetic map

[9]QTL effect for protein content [10]Significance level of the QTL effect for protein content

[11]QTL effect for oil content [12]Significance level of the QTL effect for oil content

Table 5.5 QTL detected for seed glucosinolate content

QTL	DH Line[1]	BC_4 Pl[2]	Donor segments						QTL effect µmol/g	P
			No. seg.[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
gsl-1	124		1	2	E3361.138M	61.5	61.5	0.0	25.2	<0.0001
gsl-2	158[a]	7.4.9	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0	11.6	0.0110
gsl-3	589[a]	4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	26.5	<0.0001
gsl-4	265		2	9	E3859.105M	72.5	72.5	0.0	20.3	<0.0001
			2	14	E3261.218M	58.2	58.2	0.0		
gsl-5	769		1	12	E3247.202S	132.7	132.7	0.0	15.7	0.0050
gsl-6	264		1	14	E3261.218M -E3261.107M	58.2	118.1	59.9	28.3	<0.0001
gsl-7	1036		1	16	E3261.370S -E3560.312S	0.0	14.1	14.1	22.2	<0.0001
gsl-8	258		1	18	E3861.459M -E3859.243S	84.2	99.3	15.1	25.8	<0.0001
gsl-9	1400[a]	9.4.9	1	19	E3359.225M	41.3	41.3	0.0	19.5	<0.0001

†The definitions of superscripts are the same as in the Table 5.4

5.4. Discussion

5.4.1 QTL for protein content and their relationship to QTL for oil content

A total of ten QTL were detected for oil content as previously described (see thesis chapter 4). Out of five QTL identified for protein content three QTL showed positive effects on oil content while decreasing protein content. One QTL showed a positive effect for protein content, however, the same QTL showed a negative effect on oil content, which revealed that these four QTL for protein content are either closely linked or identical to QTL for oil content. The complementary effects between the QTL for oil and protein content are in good agreement with the strong negative correlation (r = 0.9) observed between the two traits. The reason for the strong negative correlation between protein and oil content is likely be due to common substrate and energy used in the two biochemical pathways leading to a competition between protein and oil synthesis.

Gül (2002) identified six QTL for oil content in a F_1DH population from the same parents as in the current study using QTLMapper version with a mixed model approach to interval mapping. Out of six QTL identified for oil content four were closely linked to QTL for protein content, of which two QTL were increasing oil content while the same QTL were coupled with a decrease in protein content. In the current study a total of six QTL for oil content and one QTL for protein content were detected on separate linkage groups independently without a complementary effect on the other trait. Gül (2002) has also identified two QTL for oil content and one QTL for protein content on separate linkage groups, unlinked to QTL for the other traits. In another study, Zhao et al. (2005, 2006) mapped eighteen QTL with additive effects and additive x environment

effects for oil content in a F_1DH population from a cross between a European cultivar 'Sollux' and the Chinese cultivar 'Gaoyou'. Zhao et al. (2006) identified six and nine epistatic locus pairs, which had pleiotropic effects on protein and oil contents, respectively. However, they have also identified five QTL and eight epistatic locus pairs for oil content independently without effects on protein content. Therefore, there is a clear indication of unlinked QTL for oil and protein content although the two traits are strongly correlated.

In the current study, a QTL position on linkage group 11 for protein content was mapped near to a QTL position for protein content detected by Gül (2002) on the same linkage group. However, two QTL detected for protein content on linkage groups 14 and 15 in the current study were not located at the same positions as QTL by Gül (2002) on the same linkage groups. In the current study, QTL detected for oil content on linkage groups 6 and 12 were not linked to protein content. These QTL were correlated with a high erucic acid content and are the two additive genes for erucic acid content (Ecke et al. 1995; see also thesis chapter 3). However, another QTL on linkage group 12 apart from the erucic acid gene, pro-3, was identified showing a negative effect on protein content without a significant effect on oil content.

Siebolt et al. (2000) identified QTL in association with high protein content and low oil content in a BC_3 population of soybean. In another study, Shoemaker et al. (1996) identified QTL for protein and oil content in corresponding genome regions using RIL of soybean cultivars revealing the strong association of the traits. In the current study, several QTL for oil content and a QTL for protein content were identified independently without effect on other trait, which could be used in rapeseed breeding to increase oil content without decreasing protein content.

5.4.2 QTL for glucosinolate content

The levels of seed glucosinolate content in the substitution lines of the current study are regulated by at least 9 QTL. The replacement of 'Samourai' alleles by alleles of the high glucosinolate parent 'Mansholt' increased glucosinolate content between 12 to 28 µmol g^{-1} seed. Using F_1DH population from the same parents as the current study, Uzunova et al. (1995) mapped four QTL on linkage groups 2, 9, 16, and 18 for seed glucosinolate content. In another study, Toroser et al. (1995) mapped five QTL for seed glucosinolate content in a F_1DH population from a cross of 'Major' x 'Stellar', out of which two QTL were mapped on linkage groups 1 and 20 of their genetic map. They have partially integrated their genetic map with the RFLP map used by Uzunova et al. (1995). Accordingly, Toroser et al. (1995) postulated that the

two QTL on linkage groups 16 and 18 identified by Uzunova et al. (1995) were equivalent to the QTL on linkage groups 20 and 1 identified by Toroser et al. (1995), respectively.

Furthermore, Howell et al. (2003) mapped seven QTL for seed glucosinolate content in two populations of *Brassica napus* L. crosses of 'Victor' x 'Tapidor' and 'Bienvenu' x 'Tapidor' using MAPMAKER/QTL. They extended the comparative analysis by using map alignments between the maps of Parkin et al. (1995), Ferreira et al. (1994) and Uzunova et al. (1995). Out of the seven QTL identified by Howell et al. (2003), 3 major QTL assigned as GLN1 on N 9, GLN2 on N 12, and GLN3 on N 19 corresponded to the 3 major QTL identified by Uzunova et al. (1995) on linkage groups 16, 9, and 18, respectively. Besides, QTL for seed glucosinolate content assigned as gsl-4, gsl-7, and gsl-8 in the current study were mapped at the same position as QTL for seed glucosinolate content identified by Uzunova et al. (1995) on linkage groups 9, 16, and 18, respectively. However, the positions of QTL mapped in both studies on linkage groups 2 (gsl-2) were far from one another indicating two different QTL.

In a total of four studies that analysed five crosses of high glucosinolate vs. low glucosinolate rapeseed genotypes 4, 5, 4, 4 and 3 QTL were detected influencing seed glucosinolate content. The above studies demonstrated differences in the number of QTL detected which could be due to differences in population size, genome coverage of the genetic map used for the mapping, method of QTL detection and differences in likelihood threshold levels assigned to detect the QTL. However, commonly the above segregating populations showed lower power of QTL detection than the current study in which 9 QTL were detected for the seed glucosinolate content using the substitution lines.

Howell et al. (2003) hypothesized that differences in the number of subsets of QTL detected for glucosinolate content between the experiments and populations could be due to the presence of latent low glucosinolate alleles in high glucosinolate parents. In the current study alleles contributing low seed glucosinolate content from the donor parent were not identified, however, the hypothesis is consistent with the result from another study where we analysed BC_3F_3 populations of a cross of 'Express' x 'R239', a resynthesized rapeseed, in which some of the BC_3F_3 lines contained a considerably low amount of seed glucosinolats than the double zero quality parent 'Express', though, the resynthesized rapeseed contained a higher seed glucosinolate content (data not shown). Out of 5 QTL identified by Toroser et al. (1995), at one QTL, assigned as GSL-3 on linkage group 18 of their genetic map, the alleles of the parent 'Major' reduced glucosinolate content, although 'Major' is a high glucosinolate cultivar

indicating that cultivars high in glucosinolate content can also possess alleles low in glucosinolate content.

The QTL for glucosinolate content mapped on linkage groups 9, 16 and 18 by Uzunova et al. (1995) were assigned as major QTL in the above studies and they were repeatedly identified by other studies in different populations of rapeseed (Toroser et al. 1995, Howell et al. 2003). In the current study it was difficult to differentiate between major and minor QTL by looking at the QTL effects. For instance, the QTL effect on linkage group 2 was high (26 μmol^{-1} g seed) while the effects of the major QTL on linkage groups 9 (20.3 μmol^{-1} g seed), 16 (22.2 μmol^{-1} g seed) and 18 (25.8 μmol^{-1} g seed) were lower. The same problem was observed for another QTL on linkage group 6 (gsl-3), which was also identified as a minor QTL by Gül (2002). There were only two DH substitution lines available with donor segments carrying the QTL on linkage groups 2 and 6, respectively. Therefore mean adjustment for only a single line gave biased estimation of the effects and therefore it was not possible to clearly differentiate between some of the minor and major QTL. However, three QTL assigned as gsl-2, gsl-5 and gsl-9 on the linkage groups 3, 12 and 19, respectively, were identified as the minor QTL, because they revealed lower effects on seed glucosinolate content compared to the effects of the three major QTL.

5.4.3 Glucosinolate content and yield traits

The low glucosinolate allele was introgressed into modern *Brassica napus* L. cultivars from 'Bronowski', an agronomically inferior cultivar (Toroser et al. 1995). Sharpe and Lydiate (2003) identified 29% loci with 'Bronowski' alleles in the genome of 'Tapidor' a canola quality rapeseed variety. In another study, Quijada et al. (2006) found that in winter rapeseed seed yield was linked in coupling to a QTL allele for high glucosinolate content. They hypothesized that the donor segments from 'Bronowski' probably contributed to the reduced agronomical performance of 'Tapidor' compared to 'Bienvenu' a high glucosinolate and good yielding winter rapeseed. The pedigree analysis showed that alleles of ‘Bienvenu’ were introgressed into F_5 lines of low erucic acid ’Turret’ x ‘Liho’ and ’Bronowski’ to produce cultivar with low glucosinolate content, ‘Tapidor’, but with average yield and oil content. The problem of low yield due to introgression of the genome of ‘Bronowski’ into modern cultivars could be associated to a generally lower yield of spring rapeseed over winter rapeseed. Another consequence of the introgression of parts of the 'Bronowski' genome to the winter rapeseed may be that a QTL for low glucosinolate content is linked to QTL for winter survival, which caused a reduction in winter hardiness of modern cultivars (Röbbelen and Thies 1980). In the current study the donor parent DH5.1, an old

cultivar with high glucosinolate and high erucic acid content showed higher oil content, grow taller, healthier and showed better winter survival than the recurrent parent, a canola quality rapeseed variety (data not shown, see thesis chapter 6). Most probably, many agronomically important traits are linked in repulsion phase to the quality alleles such as low glucosinolate that could lead to their removal during breeding of canola quality cultivars. Marker technologies are vital tools to split these linkages and combine favourable alleles at multiple loci of low glucosinolate content to high seed yield and other agronomically important traits. Moreover, the identification of many QTL for seed glucosinolate content in the current study will open a new selection strategy to compensate the yield penalty of canola cultivars due to the introgression of low glucosinolate allele.

5.4.4 Future perspectives

The development of double haploid substitution lines through backcrossing and microspore culture produced uniform lines homozygous for the donor segments, which can be propagated by self pollination. The lines can be tested in multiple environment and multiple years for further studies and characterization of the phenotypic traits that could not be identified in the current study. These lines can be used in verifying the QTL detected in the present study using only those lines with significant QTL effects. Therefore, the analysis can generate high precision of QTL localization, which could be again narrowed down using lines with overlapping donor segments. The development of the substitution lines in the current study helped to identify and localize more QTL with small effects than could have been identified using a segregating population. QTL to QTL interaction can be identified for those lines containing two or more donor segments, which could be verified using the lines with a single donor segment of the same genome region and QTL effect. This method can provide a more precise estimation of the interaction between the QTL.

6. Mapping of QTL for flowering time, plant height and winter survival using intervarietal substitution lines in *Brassica napus* L

6.1 Introduction

Introduction of crops that can better withstand adverse climatic conditions has become important breeding criteria to diversify crop production. Flowering time, drought tolerance and winter survival are important traits of regional and seasonal adaptation. For example change in light intensity and temperature variation through seasons and geographic locations, are the main factors affecting flowering time (Li et al. 2006). Generally, flowering time is accelerated by longer days and higher temperature (Balasubramanian et al. 2006). Flower initiation in winter rapeseed cultivars requires vernalization with cold temperature of usually 4 ^{o}C in early stage of growth. Flowering time can also influence plant maturity (Buzza 1995). Plant height elucidates the status of the plant and yield potential. Differences in plant height may contribute to differences in lodging resistance and in such cases plant breeders may select shorter genotypes to cope with lodging problems (Becker et al. 1999). Winter survival is also an important characteristic for crops growing in cold climate zones covered by snow in winter season and it may be affected by genetic variation for other cold-regulated traits, such as freezing tolerance and vernalization responsive flowering time (Kole et al. 2002). Traits related to adaptation of crops are an important focus for breeding programs of crops diversification.

Traits that are related to a wide range of adaptations such as begin of flowering, end of flowering, duration of flowering, winter survival and plant height vary continuously because many genes control them. QTL studies are useful starting points for Marker Assisted Selection (MAS) because they can localize quantitative trait loci and can estimate the genetic effects of the loci. Using segregating populations, three QTL were mapped for flowering time in *Brassica napus* L. (Ferreira et al. 1995; Osborn et al. 1997), of which two QTL corresponded with QTL identified for flowering time in *Brassica rapa* L. (Teutonico and Osborn 1995). Butruille et al. (1999) identified seven QTL for flowering time and four QTL for plant height in a backcross population of *Brassica napus* L. Moreover, Teutonico et al. (1995) identified four QTL for relative freezing tolerance in *Brassica rapa* L. However, QTL mapping in segregating populations have many drawbacks such as low power of detection and poor precision of QTL localization (van Ooijen 1992; Kearsey and Farquhar 1998; Melchinger 1998; see thesis chapter 1 and 2). As an alternative to segregating populations, QTL can be mapped in a set of substitution lines with small segments of a donor parent in the genetic background of a recurrent

parent. A total of 14 QTL were mapped for flowering time in 76 substitution lines derived from a cross between *Brassica olearacea* var *italica* and var. *alboglabra* (Rae et al. 1999).

In the current study QTL mapping of five quantitative traits namely begin of flowering, end of flowering, duration of flowering, plant height and winter survival were carried out using a substitution line library derived from a cross of an old cultivar of oilseed rape 'Mansholts Hamburger Raps' with a modern winter oilseed rape variety 'Samourai'. Relationships between these five agronomical traits and two quality traits (oil and protein content) were also examined. QTL identified in this study are compared to those that have been published previously.

6.2 Materials and methods

6.2.1 Plant materials and donor genome coverage

In the development of substitution lines the donor parent was DH5.1, a doubled haploid line from 'Mansholts Hamburger Raps', an old cultivar with high erucic acid and high glucosinolate contents. A doubled haploid line of the winter oilseed rape variety 'Samourai', DH11.4, was used as the recurrent parent. A total of 366 DH substitution lines had been produced from selected BC_4 genotypes using microspore culture (see thesis chapter 3). Out of the 366 substitution lines, 292 lines that had produced sufficient seeds for the experiments were tested in the field trials. Out of the 292 lines tested in the field experiments 101 DH substitution lines had been analysed with markers. 55 of these lines contained 1 donor segment, 31 lines contained 2 donor segments and 15 lines contained 3 donor segments. The donor segments in the 101 lines covered a minimum of 451 cM of the mapped rapeseed genome. The rest of 191 lines had not been analysed by markers, however, marker information of the BC_4 microspore donor plants were available which can be used to infer which donor segments may be present in DH substitution lines. Of the 191 lines, 112 lines were derived from BC_4 plants with 1 donor segment, 49 lines from plants with 2 donor segments, and 30 lines from plants with 3 donor segments. The BC_4 parental plants used for the microspore culture contained donor segments on all linkage groups of the genetic map of the rapeseed genome except on linkage group 4.

6.2.2 Field trials

Field trials were conducted in 2005/06 using 292 lines. All lines were sown at Reinshof, the experimental station of the University of Göttingen in randomized block design with two replications. At four additional locations between 230 to 261 lines were sown at the experimental stations of companies at Thüle (DSV) in Northwest Germany, Hohenlieth (NPZ) in Northern Germany, Seligenstadt (KWS) in Southern Germany and Grund-Schwalheim (SW Seed GmbH)

in Southwest Germany. Plots of the recurrent parent DH11.4 were used as controls by sowing one plot of DH11.4 for every 9 plots of substitution lines. In addition 3 plots of the double haploid donor parent 'DH5.1' were also sown in Reinshof. The plot size varied at the different locations between 2.6 and 8.0 m^2 and the plant density between 27 and 60 plants per m^2. Field management, herbicides and fertilizer applications were done according to the local practices.

Table 6.1 depicts detailed methods used for measuring the traits in up to five locations. To score winter survival, plant density of each genotype was scored before winter and after winter and the difference between the two scorings was calculated as winter survival.

Table 6.1 Quantitative traits investigated in up to five environments

Abb.	Trait	Method of measurement	Environment tested†
BOF	Begin of flowering	Number of days after sowing when 50 % of plants have the first 3 open flower	S, T, R, G, H
EOF	End of flowering	Number of days after sowing until opening of the last corolla in the main raceme	R, T, G
DOF	Duration of flowering	Calculated by subtracting BOF from EOF	R, T, G
Plh	Plant height	Average plant height measured at plant maturity from the soil to the tip of the main raceme in centimetre (cm)	S, T, R, G, H
WS	Winter survival	Status of plants before and after winter were scored by scaling from 1 to 9, 1 is susceptible to winter 9 is strong winter survival	S, T, R, G, H

†Abbreviation of the environment in which the traits were tested: Reinshof (R), Hohenlieth (H), Thüle (T), Seligenstadt (S) and Grund-Schwalheim (G).

6.2.3 Statistical analysis

LSMEANS of the parameter measured for the test substitution line and the control was calculated using PROC MIXED procedure of SAS (SAS Institute 2003). Multiple comparisons of estimated differences of the lines from the control were tested using t-tests adjusted according to Dunnet (Dunnet 1995). A QTL was detected if the LSMEANS difference of the line was significantly different from the recurrent parent DH11.4 with an alpha level of 0.05. The recurrent parent 'DH11.4' was the common control for the substitution lines. Consequently, the deviation of the line from the DH11.4 should be due to the substitution of the donor segment in the genetic background of the recurrent parent. ANOVA was calculated using PROC GLM procedure. Pearson correlation coefficients among the traits were calculated using PROC CORR procedure.

6.3 Results

6.3.1 Trait correlations and variance analysis

Pearson correlations among the five traits analysed in this study and previously analysed three quality traits (see thesis chapter 4 and 5) revealed a total of 26 significant correlations. The correlation between winter survival and oil content was positive but negative to protein content (Table 6.2). Mapping of QTL for oil and protein content were described previously (see thesis chapter 4 and 5). Winter survival was positively correlated to plant height, however, negatively correlated to the traits related to flowering time (BOF, EOF and DOF). In addition, oil content was negatively correlated to flowering time. Plant height was positively correlated to oil content, however, negatively correlated to protein content and duration of flowering. Variance analysis revealed significant variation for traits related to flowering time, plant height and winter survival among the lines and locations (Table 6.3).

6.3.2 Begin of flowering

Mean begin of flowering of the lines and the recurrent parents were 239.6 and 240 days after sowing, respectively, for all location. In Reinshof, mean begin of flowering for recurrent and donor parents were 240.3 and 241 days, respectively. Table 6.4 summarizes the mean of lines, donor and recurrent parents for location Reinshof and all locations. A total of 13 QTL were detected for begin of flowering distributed across 11 linkage groups of the mapped rapeseed genome. All of the detected QTL effects showed a decrease in the days to begin of flowering ranging from 2.5 to 4 days (Table 6.5) as compared to days to begin of flowering of the recurrent parent.

Table 6.2 Correlation coefficients among quantitative traits

	BOF	EOF	DOF	Plh	Oil	Protein	Glucosinolate
WK	-0.49***	-0.33***	-0.12*	0.42***	0.31***	-0.36***	0.23*
BOF		0.51***	-0.02	-0.55***	-0.40***	0.41***	0.08
EOF			0.82***	-0.57***	-0.49***	0.48***	-0.13**
DOF				-0.33***	-0.31***	0.29***	0.19**
Plh					0.46***	-0.44***	0.21**
Oil						-0.90***	-0.12**
Protein							0.18*

†Significance thresholds for r values, * *P*= 0.05, ** *P*= 0.01, ***P*= 0.001

Table 6.3 Mean squares from analysis of variance

	Lines (287)†	Location (4)	Lines*Location (782)
WK	0.78***	24.25***	0.30
BOF	14.21***	7424.12***	4.91
DOF	9.37***	5295.83***	5.42
BOF	6.25***	592.77***	2.86
Plh	137.87***	41189.78***	46.87

†Numbers in parenthesis are degrees of freedom.
Significance levels, * *P*=0.05, ** *P*=0.01, ***P*=0.001

Table 6.4 Mean of parents and lines for phenological traits

Traits	Unit	DH11.4	Lines	DH11.4[g]	DH5.1[g]	Lines[g]
WK	Score (1-9)	7.8	8.4	8.3	9.0	8.5
BOF	days	240.0	239.6	240.3	241.0	238.9
EOF	days	275.0	269.7	275	268.0	271.7
DOF	days	35.0	30.0	34.8	27.4	32.9
Plh	cm	128.0	133.5	122.7	130.0	132.8

[g] Location Reinshof

Table 6.5 QTL detected for begin of flowering

QTL	DH Line[1]	BC_4 pl.[2]	Donor segments						QTL effect (days)	P[9]
			No. Seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L[8] (cM)		
BOF-1	996[a]	16.4.11	1	1	E3362.130S	48.5	48.5	0.0	-3.17	0.020
BOF-2	1108		1	2	E3349.155M -E3247.159M	0.0	38.2	38.2	-3.17	0.020
BOF-3	158[a]	7.4.9	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0	-2.97	0.001
BOF-4	960		1	3	E3261.209S	69.5	69.5	0.0	-3.17	0.020
BOF-5	350[a]	4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	-2.83	0.010
BOF-6	1187		2	8	E3359.64M	34.4	34.4	0.0	-3.97	0.001
				10	E3362.250M	53.7	53.7	0.0		
BOF-7	24[a]	2.4.6	1	9	E3859.105M	72.5	72.5	0.0	-3.12	0.030
BOF-8	223[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	-2.83	0.010
BOF-9	877		1	12	E3247.158M -E3348.58M	0.0	7.3	7.3	-2.69	0.030
BOF-10	241[a]	7.4.7	1	17	E3261.166M -E3861.237M	30.7	53.2	22.5	-3.92	0.001
BOF-11	59		1	18	E3861.459M -E3250.339M	84.2	99.3	15.1	-2.87	0.001
BOF-12	194		1	19	E3862.371M -E3349.461S	0.0	18.6	18.6	-3.40	0.040
BOF-13	1145[a]	21.4.3	1	20	E3349.199M	0.0	0.0	0.0	-3.17	0.020

[1]Identification number (IN) of DH substitution lines with QTL effect: within the column, IN with superscript 'a' means the lines were not analysed by markers but marker information from the microspore donor plant was available.

[2]Identification number of the microspore donor BC_4 plant used to generate the DH substitution line

[3]Number of donor segments in the DH substitution line. If it contained more than one donor segment the other segments are listed in subsequent rows

[4]Linkage group from the genetic map (Uzunova et al. 1995) carrying the donor segment containing the QTL

[5]First and last marker on the donor segment

[6]'Start' indicates where the donor segment starts within the indicated linkage group

[7]'End' depicts where the donor segment ends within the indicated linkage group

[8]Minimal length (in centi Morgan) of the donor segment as defined by the distance between the first and last marker on the genetic map

[9]Significance level of the QTL effect

6.3.3 End of flowering

Mean end of flowering of the lines and the recurrent parent were 269.7 and 275 days after sowing, respectively, for all locations. In Reinshof, mean end of flowering of recurrent and donor parents were 275 and 268.3 days, respectively. A total of 18 QTL were detected for end of flowering distributed across 14 linkage groups of the rapeseed genome (Table 6.6). All of the detected QTL effects showed a decrease in days for end of flowering ranging from 2.9 to 6.6 days as compared to days for end of flowering of the recurrent parent.

Table 6.6 QTL detected for end of flowering

QTL	DH Line[1]	BC_4 Pl.[2]	Donor segments						QTL effect (days)	P[9]
			No. Seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
EOF-1	1612[a]	16.4.11	1	1	E3362.130S	48.5	48.5	0.0	-4.5	0.030
EOF-2	1172		1	2	E3349.155M - E3247.159M	0.0	38.2	38.2	-3.6	0.001
EOF-3	316		1	2	E4060.199M -E3361.138M	57.9	61.5	3.6	-5.2	0.001
EOF-4	960		1	3	E3261.209S	69.5	69.5	0.0	-3.2	0.020
EOF-5	158[a]	7.4.9	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0	-3.0	0.001
EOF-6	169		1	5	E4060.559M	70.8	70.8	0.0	-5.9	0.001
EOF-7	114[a]	4.4.1	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	-6.2	0.001
EOF-8	1175		1	8	E3359.001M -E3359.002M	10.2	37.6	27.4	-5.2	0.001
EOF-9	1187		2	8	E3359.64M	34.4	34.4	0.0	-6.3	0.001
				10	E3362.250M	53.7	53.7	0.0		
EOF-10	24[a]	2.4.6	1	9	E3859.105M	72.5	72.5	0.0	-3.1	0.030
EOF-11	1186		1	10	E3362.250M	53.7	53.7	0.0	-6.6	0.001
EOF-12	869[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	-5.5	0.001
EOF-13	877		1	12	E3247.158M -E3348.58M	0.0	7.3	7.3	-4.4	0.010
EOF-14	476		1	14	E3261.107M	118.1	118.1	0.0	-5.3	0.020
EOF-15	196		1	17	E3261.166M -E3861.237M	30.7	53.2	22.5	-4.4	0.010
EOF-16	59		1	18	E3861.459M -E3250.339M	84.2	99.3	15.1	-2.9	0.001
EOF-17	194		1	19	E3862.371M -E3349.461S	0.0	18.6	18.6	-3.4	0.040
EOF-18	1145[a]	21.4.3	1	20	E3349.199M	0.0	0.0	0.0	-3.2	0.020

[†]See table 6.5 for definition of superscripts

6.3.4 Duration of flowering

Mean duration of flowering of the lines and the recurrent parent were 30 and 35 days for all locations, respectively. In Reinshof, the mean of duration of flowering of recurrent and donor

parents were 34.8 and 27.4 days, respectively. A total of 6 QTL were detected for duration of flowering and mapped on linkage groups 2, 3, 6, 18, and 19 of the mapped rapeseed genome (Table 6.7). All of the detected QTL effects showed a decrease in days for duration of flowering ranging from 4 to 6.3 days as compared to the recurrent parent.

Table 6.7 QTL detected for duration of flowering

QTL	DH Line[1]	BC_4 pl.[2]	Donor segments						QTL effect (days)	P[9]
			No. seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
DOF-1	316		1	2	E4060.199M -E3361.138M	57.9	61.5	3.6	-4.15	0.061
DOF-2	158[a]	7.4.9	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0	-4.15	0.061
DOF-3	589[a]	4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	-6.33	0.003
DOF-4	260		1	18	E3861.459M -E3859.243S	84.2	99.3	15.1	-5.63	0.025
DOF-5	694[a]	9.4.9	1	19	E3359.225M	41.3	41.3	0.0	-4.15	0.061

†See table 6.5 for definition of superscripts

Table 6.8 QTL detected for plant height

QTL	DH Line[1]	BC_4 pl[2]	Donor segments						QTL effect (cm)	P[9]
			No. seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
Plh-1	1105[a]	16.4.11	1	1	E3362.130S	48.5	48.5	0.0	12.97	0.040
Plh-2	1108		1	2	E3349.155M -E3247.159M	0.0	38.2	38.2	17.97	0.001
Plh-3	316		1	2	E4060.199M -E3361.138M	57.9	61.5	3.6	13.55	0.001
Plh-4	217		1	3	E3259.72M -E3348.001M	20.8	51.0	30.2	13.41	0.001
Plh-5	960		1	3	E3261.209S	69.5	69.5	0.0	13.37	0.050
Plh-6	169		1	5	E4060.559M	70.8	70.8	0.0	12.21	0.050
Plh-7	100[a]	4.4.7	1	6	E3359.564M -E3247.335M	42	61.7	19.7	10.69	0.001
Plh-8	1187		2	8	E3359.64M	34.4	34.4	0.0	17.57	0.001
				10	E3362.250M	53.7	53.7	0.0		
Plh-9	24[a]	2.4.6	1	9	E3859.105M	72.5	72.5	0.0	17.3	0.001
Plh-10	1186		1	10	E3362.250M	53.7	53.7	0.0	17.17	0.001
Plh-11	144		1	12	E3361.001M	101.6	101.6	0.0	13.9	0.030
Plh-12	769		1	12	E3247.202S	132.7	132.7	0.0	12.08	0.030
Plh-13	875		1	12	E3247.158M -E3247.121S	0.0	9.6	9.6	11.53	0.020
Plh-14	1373		1	13	E3359.234M	101.0	101.0	0.0	13.31	0.010
Plh-15	280[a]	10.4.2	1	14	E3261.218M -E3359.109M	58.2	80.5	22.3	12.84	0.010
Plh-16	548[a]	8.4.10	1	15	E3347.160S	75.2	75.2	0.0	15.31	0.001
Plh-17	1433[a]	20.4.8	1	16	E3261.370S -E3862.461M	0.0	23.7	23.7	12.77	0.050
Plh-18	196		1	17	E3261.166M -E3861.237M	30.7	53.2	22.5	11.7	0.030
Plh-19	260		1	18	E3861.459M -E3859.243S	84.2	99.3	15.1	16.3	0.001
Plh-20	367		1	19	E3862.371M	0.0	0.0	0.0	13.97	0.030

†See table 6.5 for definition of superscripts

6.3.5 Plant height

For plant height significant variations among the lines were observed based on analysis of variance. Mean plant height of the lines and recurrent parent were 133.5 and 128 cm, respectively, for all five locations. In Reinshof, mean plant height of recurrent and donor parents were 122.7 and 130 cm, respectively. A total of 20 QTL were detected across 16 linkage groups of the mapped rapeseed genome. All QTL effects showed increase in plant height ranging from 10.7 to 17.9 cm (Table 6.8) as compared to the recurrent parent.

6.3.6 Winter survival

The recurrent parent was susceptible to winter while the donor parent was resistance and has better winter survival. Mean of winter survival for the lines and recurrent parent were 8.42 and 1.2, respectively, for all five locations. In Reinshof, mean of winter survival of recurrent and donor parents were 1.1 and 9, respectively. A total of 13 QTL were detected for winter survival distributed across 9 linkage groups of the mapped rapeseed genome (Table 6.9).

Table 6.9 QTL detected for winter survival

QTL	DH Line[1]	BC_4 Pl[2]	Donor segments						QTL effect (1 – 9)	P[9]
			No. seg[3]	LG[4]	Flanking markers[5]	Start[6] (cM)	End[7] (cM)	L.[8] (cM)		
WS-1	18[a]	3.4.7	1	2	E3349.155M	0.0	0.0	0.0	8.1	0.006
WS-2	316		1	2	E4060.199M -E3361.138M	57.9	61.5	3.6	8.1	0.046
WS-3	217		1	3	E3259.72M -E3348.001M	20.8	51.0	30.2	7.9	0.002
WS-4	169		1	5	E4060.559M	70.8	70.8	0.0	8.0	0.030
WS-5	100[a]	4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7	8.1	0.001
WS-6	221		2	12	E3247.181S -E3250.192S	59.1	61.2	2.1	7.8	<0.001
				2	E3861.189M	26.7	26.7	0.0		
WS-7	223[a]	3.4.5	1	12	E3862.129M	139.8	139.8	0.0	8.1	0.046
WS-8	280[a]	10.4.2	1	14	E3261.218M -E3359.109M	58.2	80.5	22.3	8.1	0.046
WS-9	196		1	17	E3261.166M -E3861.237M	30.7	53.2	22.5	8.1	0.046
WS-10	59		1	18	E3861.459M -E3250.339M	84.2	99.3	15.1	8.2	0.018
WS-11	210[a]	3.4.14		18	E3259.194S	62.1	62.1	0.0	8.1	0.046
WS-12	451[a]	1.4.13	1	19	E3862.371M -E3349.461S	0.0	18.6	18.6	8.1	0.046
WS-13	641[a]	9.4.9	1	19	E3359.225M	41.3	41.3	0.0	8.1	0.046

†See table 6.5 for definition of superscripts

6.4 Discussion

A total of 69 QTL were identified for 5 analysed traits in which 13 QTL were identified for begin of flowering, 5 for duration of flowering, 18 for end of flowering, 20 for plant height and

13 QTL for winter survival. Several QTL were identified in this study for all traits because of using substitution lines for the QTL mapping. Substitution lines contain few donor segments in the genetic background of the recurrent parent thus they do not hinder the detection of several QTL as compared to segregating populations. Ferreira et al. (1995) used a segregating population from a cross of the winter rapeseed 'Major' and the spring cultivar 'Stellar' to map QTL for days to flowering using MAPMAKER/QTL. They identified 1 major QTL on linkage group 9 and, additionally two minor QTL on linkage groups 12 and 16 on their genetic map. In another study, Weißleder (1996) and Gül (2002) mapped QTL for agronomically important traits in a segregating population from the same parents as the current study. Weißleder (1996) detected 5 QTL for begin of flowering, 3 QTL for duration of flowering, 1 QTL for end of flowering and 3 QTL for plant height. Gül (2002) identified 5 QTL for begin of flowering, 5 QTL for duration of flowering, 4 QTL for end of flowering and 4 QTL for plant height. Therefore, compared to this study, QTL mapping in segregating populations underestimated the number of QTL affecting the traits.

Weißleder (1996) mapped QTL for begin of flowering on linkage groups 2, 4, 8 and 9 at the same positions as Gül (2002) on the same linkage groups. Besides, in the current study too, these four QTL were mapped at the same positions as in the studies by Weißleder (1996) and Gül (2002) on the same linkage groups. Additionally, QTL for begin of flowering identified by Weißleder (1996) on linkage group 14 but not by Gül (2002) was again detected in the current study and mapped at the same position as by Weißleder (1996) on the same linkage group. However, QTL for begin of flowering identified by Weißleder (1996) and Gül (2002) on linkage group 4 was not detected in the current study because there were no substitution lines with donor segments from linkage group 4. QTL position for end of flowering on linkage group 9 detected by weißleder (1996) and Gül (2002) was mapped at the same position as the QTL position in the current study on the same linkage group. Moreover, QTL for end of flowering on linkage group 3 detected by Gül (2002) was also again detected in the current study at the same position on the same linkage group. However, another QTL for end of flowering detected by Gül (2002) on linkage groups 7 and 16 were not detected in the current study on the same linkage groups. The QTL position for duration of flowering on linkage group 2 detected by weißleder (1996) and Gül (2002) was also mapped at the same position as in the current study on the same linkage group. However, the rest of four QTL for duration of flowering in the current study were not mapped at the same positions or were not identified on the same linkage groups as in the study by Weißleder (1996) and Gül (2002). For instance QTL for duration of flowering on linkage group 3, which was detected by Gül (2002) was not mapped at the same position as in the current

study. QTL for duration of flowering on linkage group 8 detected by Gül (2002), and another QTL on linkage groups 9 and 15 detected by Weißleder (1996) were not also identified in the current study on the same linkage groups.

In the current study, mean plant height of recurrent and donor parents at the location Reinshof were 122.7 and 130 cm, respectively. Weißleder (1996) reported mean plant height of 117.9 and 120.7 cm for recurrent and donor parents, respectively, in double row data analysis of year 1992/93 and 1993/94. Mean plant height for recurrent and donor parents were 133.3 and 140 cm, respectively, in the study by Gül (2002). The QTL effects for plant height in the study by Weißleder (1996) ranged from -10 to 9.2 cm and from -6.5 to 6.6 cm in the study by Gül (2002). In the current study the QTL effects for plant height ranged from 10.7 to 17.9 cm. As indicated above, there was not much difference in the mean plant height between the two parents used in this current study to explain the higher effects of the QTL found in the current study. A possible explanation of the higher QTL effect in the substitution line might be the combination of the QTL in 'Mansholt' lead to negative epistatic effects on plant height, however, when some of these QTL were again combined in the genetic background of 'Samourai' they showed a higher additive effects increasing plant height more than the parents. All the 3 QTL identified for plant height by Weißleder (1996) were mapped at the same positions as 3 out of 4 QTL mapped by Gül (2002). Although the QTL effects for plant height differ between the current study and the above studies, the three QTL identified by Weißleder (1996) and Gül (2002) were mapped at the same positions as in the current study.

Quijada et al. (2006) identified QTL for seed yield, plant height and flowering time from crosses of two old rapeseed cultivars and a modern canola quality variety using QTL cartographer. They detected 4 QTL for flowering time and 17 QTL for plant height. In the current study from the total of 20 QTL detected for plant height, 15 QTL were mapped in the same genome regions as QTL for flowering time (begin of flowering, duration of flowering and end of flowering). QTL for flowering time mapped in the same genomic regions as QTL for plant height are probably due to pleiotropic effects of the flowering genes on plant height or vice versa. However, the rest of 5 QTL for plant height were mapped independently from the QTL for flowering time. For the five QTL plant height increased due to the introgression of the donor segments in the substitution lines. In the study by Quijada et al. (2006) five QTL were detected for plant height that were not linked to the QTL for flowering time, however, QTL effects showed a decrease in plant height contrary to the current study.

Duration of flowering was positively correlated to end of flowering but not to begin of flowering. This result was not in line with the study by Gül (2002) in which negative correlation between duration of flowering and begin of flowering was observed. Weißleder (1996) found also negative correlation between duration of flowering and begin of flowering from the analysis of drill plots, however, from double row analysis of field trials 1992/93, Weißleder (1996) did not find correlation between duration of flowering and begin of flowering but with end of flowering supporting the current study, and the current study did also the analysis of phenological traits from double rows.

Correlation analysis has also revealed early flowering and short duration of flowering are positively correlated with winter survival and increase in plant height. This is to be expected, because plants with better winter survival can grow faster after winter and are better able to compete in nutrient uptake. Therefore they grow taller and flower earlier. In *Brassica napus* L. fall frosts adversely affect quality while a spring frost can kill or set back the vegetative stage of the plant resulting in yield reduction (Johnson-Flanagan et al. 1991). Winter kills mostly the parts of the plant that are above ground but also may damage the root system. If the roots do not regenerate, plants can die and only few plants are able to survive the winter and re-grow in spring. Moreover, those few re-grow plants may require more time to regenerate and develop fully which can result into late begin of flowering and late end of flowering. Plants with damaged roots due to winter damage may unable to compete with the winter hardy neighbouring plants in nutrition uptake. Therefore insufficient nutrient uptake by winter susceptible plants can result in a decrease in plant height. Plant height, early flowering, early end of flowering, short duration of flowering and winter survival were positively correlated to oil content but negatively to protein content.

The donor parent DH5.1 is better adapted to winter survival than the recurrent parent DH11.4. Therefore, most of the substitution lines showed better winter survival which might be due to the introgression of donor segments into the substitution lines. The donor parent, DH5.1 is an old cultivar with high erucic acid and high glucosinolate contents. DH5.1 might have developed better survival mechanisms to cold winters due to a longer adaptation as an old cultivar than the recurrent parent which is relatively new cultivar released in the late 80's. In the recent study, Sharpe and Lydiate (2003) identified 'Bronowski' alleles at 29% loci of RFLP loci in the genome of the canola quality rapeseed variety 'Tapidor'. The recurrent parent used in the current study is also a canola quality rapeseed variety with low erucic acid and low glucosinolate content, which is related to 'Bronowski', a spring cultivar that is sensitive to frost and the genome of the initial

low glucosinolate genotype in the breeding of canola quality (low glucosinolate and low erucic acid content) rapeseed varieties.

In the current study correlation analysis showed that content of seed glucosinolate were positively correlated to winter survival. The introgression of parts of the 'Bronowski' genome into modern cultivars may be the cause for a reduction in winter hardiness (Röbbelen and Thies 1980). Frost resistance appeared as the main factor influencing winter survival of winter cereals and also winter oilseed rapeseed (Rapacz and Markowski 1999). Kole et al. (2002) found strong association of QTL for winter survival and freezing tolerance and mapped them very close to each other in *Brassica rapa*. In another study, Rapacz and Markowski (1999) found significant correlations between frost resistance and field survival in older high glucosinolate cultivars of *Brassica napus* L. They did not find such correlation for canola quality rapeseed cultivars. The result implied canola quality rapeseed varieties might not have a strong resistance to winter as old cultivars which are characterised by high glucosinolate content. However, most of the modern cultivars became more tolerant to frost and can better survive the winter due to intensive selection during the development of canola quality varieties (Röbbelen and Thies 1980). Although, both parents used in the current study are winter types which are well adapted to winter, the level of winter survival might vary between them in which the recurrent parent "Samourai" was affected more than the donor parent.

Teutonico et al. (1995) mapped QTL for freezing tolerance in a F_1DH population of the cross 'Major' x 'Stellar' using MAPMAKER/QTL. They detected 4 QTL for acclimated freezing tolerance. Freezing tolerance is the capacity of plants to survive subfreezing temperatures and is the main component of winter survival (Teutonico et al. 1995). Alleles from the winter parent 'Major' contributed a positive additive effect for freezing tolerance. In the current study, a total of 13 QTL were detected for winter survival due to the introgression of donor segments of the donor parent DH5.1 to the substitution lines. Out of 13 QTL for winter survival 7 were the same QTL or were closely linked to the QTL for flowering time. Kole et al. (2002) also found QTL for winter survival near to homologous loci for flowering time in *Brassica rapa* and *Brassica napus.* In *Brassica napus* they found two QTL for winter survival very close to QTL for flowering time (VFN1), which had a major effect on flowering time and one QTL for winter survival close to a minor QTL for flowering time. This close relationship between loci for winter survival and flowering time might be due to the effect of winter survival on plant morphology as discussed above.

Winter survival is greatly affected by environmental factors such as diseases, pests, inadequate, excessive or unbalanced soil fertility and poor drainage conditions. Moreover, the absence of snow cover during the coldest period of the winter decreases plant survival rates (Sovero 1993). Winter survival in oilseed rape depends on plant development in autumn, on biochemical processes of cold hardening and agro climatic factors. Rapeseed overwintering also depends on crop density, because the higher the crop density the smaller the number of surviving plants (Velicka et al. 2005). It is more difficult to study winter survival under field conditions without the bias of the above parameters, which will make the estimation and analysis more complex. The QTL identified in the current study may have been assorted with the above parameters, which can also affect winter survival. However, verifying these parameters under the field conditions might be difficult.

7. Erucic acid gene affects phytosterol and sinapate esters in intervarietal substitution lines of *Brassica napus* L.

7.1 Introduction

Vegetable oils are rich sources of minor bioactive components like phytosterols (sterols) (Abidi et al. 1999; Piironen et al. 2000; Harker et al. 2003). Sterols are constituents of plant oils that play important roles in eukaryotes, serving as components of cell membranes and as precursors to steroid hormones. Plant sterols have a structure that is similar to cholesterol. Addition of phytosterols in human diets were proved to reduce blood cholesterol in people with mildly elevated cholesterol levels, therefore, they can lower the risk of cardiovascular disease (Miettinen et al. 1995; Westrate et al. 1998). Phytosterols have also been used by cosmetic industry as emulsifiers (Clark 1996). More than 40 structurally and functionally related sterols have been identified from natural sources like vegetable oils (Khripach et al. 2000). The most common representatives of phytosterols are sitosterol, stigmasterol and campesterol (4-desmethylsterols) (Harker et al. 2003). However, other sterols like brassicasterol and avenasterol occur in many plant materials. Brassicasterol is abundant in oil of *Brassica* crops. Amar et al. (2007a) mapped QTL for individual and total phytosterols in a population of *Brassica napus* L. Oil extracted from rapeseed contains the highest sterol content compared to oils from olive, palm, cottonseed, rice, soybean and sunflower (Piironen et al. 2000). Gül and Seker (2006) also observed higher concentration of sterols in rapeseed oil as compared to olive oil. Higher concentration of brassicasterol, campesterol, ß-sitosterol and total phytosterols were also reported in rapeseed oil than in the oils of sunflower and soybean by Cole et al. (1998), and Vlahakis and Hazebroek (2000), This suggests the use of canola oil as a promising source of phytosterols.

Rapeseed oil naturally has a high erucic acid content which may pose health problems (Roine and Uksila 1959). Using traditional selection methods, canola varieties with low seed glucosinolate and low erucic acid contents were released. High erucic acid oils are used as lubricants and also after minor modification as plasticizers, waxes, water repellents and surface-active agents (Lühs and Friedt 1993). Content of erucic acid in rapeseed is controlled by two additive genes (Harvey and Downey 1964; Stefansson and Hougen 1964; Kondra and Stefanson 1965; Siebel and Pauls 1989). Using a F_1DH population of *Brassica napus* L. Ecke et al. (1995) mapped two genes affecting erucic acid content. Using another population of *Brassica napus* L., Thormann et al. (1996) had also mapped the two genes.

Rapeseed meal that remains after extraction of the oil is rich in protein and can be used in animal feeding. However, the use of rapeseed meal is limited due to undesirable compounds in the seed like glucosinolates, tannins of the black seed coat, and phenolic acid esters (Sosulski 1979; Ismail et al. 1981). The most abundant phenolic compounds in canola seeds are sinapoylcholine (sinapine) and sinapoylglucose. Additionally, minor contents of sinapate and other sinapate esters (SE) were also reported (Kozlowska et al. 1990; Shahidi and Naczk 1992).

Seed dry matter of sinapate and other derived phenolic esters range between 1 to 2 % in canola seeds (Bell 1993), which is about 30 times higher than in soybean (Kozlowska et al. 1990; Shahidi and Naczk 1992). These compounds are known to inhibit digestive proteolytic enzymes of protein hydrolysis and may form complexes with proteins, which can lower the digestibility of rapeseed meal (Kozlowska et al. 1990, Shahidi and Naczk 1992; Naczk et al.1998). Sinapate derived esters contribute to the bitter taste and dark colour of the rapeseed meal (Sosulski 1979; Ismail et al. 1981). Furthermore, their presence in the diet of certain strains of hens that lay brown-shelled eggs leads to an unacceptable fishy odour in the eggs (Pearson et al. 1980; Butler et al. 1982). Therefore breeding to lower components of sinapate esters is required to improve rapeseed meal. Large genetic variation in sinapate esters was observed among rapeseed varieties (Velasco and Möllers 1998; zum Felde et al. 2007). However, no specific breeding programmes have started yet to lower sinapate esters content in rapeseed (zum Felde et al. 2006).

Analysis of effects of fatty acid contents on components of phytosterols and sinapate esters can provide vital information for breeders in order to achieve simultaneous objectives that cope with present quality standards. The objective of this study was to analyse the effects of the erucic acid genes on the contents of phytosterols, sinapate esters and other quality traits using substitution lines developed from a cross of an old cultivar ‘Mansholts Hamburger Raps’ with ‘Samourai’.

7.2 Materials and methods

7.2.1 Plant materials and genome coverage

Substitution lines were developed using a doubled haploid line DH5.1 derived from ‘Mansholts Hamburger Raps’, an old cultivar with high erucic acid and high glucosinolate contents as donor parent. The recurrent parent was a doubled haploid line, DH11.4, from the winter oilseed rape variety ‘Samourai’ which is of double low seed quality. A total of 366 DH substitution lines were produced from selected BC_4 genotypes of which 292 lines, which had produced sufficient seeds were tested in field trials. Out of 292 lines, 101 lines had been analysed by markers. The donor segments in these lines covered a minimum of 451 cM of the mapped rapeseed genome.

The rest of the lines had not been analysed by markers, however, marker information of the parental BC_4 plants were available to infer donor segments in these lines.

7.2.2 Field trials

Field trials were conducted in 2005/06 using 292 DH substitution lines. All lines were sown at Reinshof (4 km South of Göttingen) as randomized block design with two replications. At four additional locations between 230 and 261 lines were tested without replication. These locations were the experimental stations of the following companies: Deutsche Saatveredelung at Thüle (Northwest Germany), Norddeutschen Pflanzenzucht HG Lembke KG at Hohenlieth (Northern Germany), KWS SAAT at Seligenstadt (Southern Germany) and SW Seed GmbH at Grund-Schwalheim (central Germany). Plots of the recurrent parent 'DH11.4' were sown as a control in each 10^{th} plot. In addition 3 plots of the double haploid donor parent 'DH5.1' were also sown at location Reinshof. The plot size varied at the different locations between 2.6 and 8.0 m^2 and the plant density between 27 and 60 plants per m^2. Field management, herbicides and fertilizer applications were done according to local practices. To secure self-pollinated seeds 3 to 6 plants of each line were covered with plastic bags before flowers opened. Seeds were harvested from each self-pollinated plant separately and additionally ten g bulked seeds were harvested from the terminal racemes and the two uppermost primary branches of five healthy open pollinated plants from each line. Of the 292 lines sown, 288 produced sufficient seeds for Near Infrared Reflectance Spectroscopy analysis (NIRS). Seed oil, protein, glucosinolate, fatty acid, sinapate esters and individual phytosterol content were determined by NIRS (Tillmann 2007; zum Felde et al. 2007). Additionally, out of 288 lines tested in the field trials, fatty acid contents of 56 lines were verified by gas chromatography (Thies 1971).

7.2.3 Statistical analysis

Least square means (LSMEANS) of the lines and the recurrent parent across locations were calculated using PROC MIXED procedure of SAS (SAS Institute 2003) to identify the genes for erucic acid. Multiple comparisons of LSMEANS differences of the lines to the recurrent parent were tested with t-tests adjusted according to Dunnet (Dunnet 1955). The two genes for erucic acid content (Ecke et al. 1995) could be identified in the substitution lines and assigned to linkage group 6 or 12 on the genetic map of Uzunova et al. (1995) based on the donor segment with high erucic acid content in the lines. To identify effects of the two erucic acid genes on other traits, LSMEANS differences of phenotypic values of the lines with one erucic acid gene to lines free from erucic acid were tested with t-test adjusted according to Dunnet. A strong association of a trait with erucic acid content was declared, if LSMEANS differences of the two

groups of lines, lines with high erucic acid and lines free from erucic acid, were significant for the trait at an alpha level of 0.05. Spearman correlation coefficients among traits were determined by PROC CORR procedure of SAS.

7.3 Results

7.3.1 Erucic acid

The mean erucic acid content was 7.6 % (Table 7.1) ranging between 0.84% and 42.4% for all lines in five location. The two erucic acid genes were detected on linkage groups 6 and 12 in the substitution lines as previously described (Ecke et al. 1995, see thesis chapter 4). Out of 288 lines analysed, 6 lines were identified with erucic acid gene on linkage group 6 and 11 lines with gene on linkage group 12. The mean contents of erucic acid were 30.2 and 30.4%, in the lines with the genes on linkage groups 6 and 12, respectively. Figure 7.1 shows the genome positions of the two genes for erucic acid and oil content on linkage groups 6 and 12. Comparison of lines with erucic acid genes and lines free from erucic acid content revealed that the two genes increased oil content, however did not affect protein and glucosinolate contents (Table 7.2). However, the two genes showed significant difference between themselves for protein content. The correlation analysis revealed negative relationship between erucic acid and total phytosterols (Table 7.3).

7.3.2 Phytosterols

The mean content of total phytosterols was 3935 mg kg^{-1} seeds ranging from 3064 to 3984 mg kg^{-1} seeds. Erucic acid genes on linkage groups 6 and 12 decreased the total phytosterol content by 469 and 622 mg kg^{-1} seeds, respectively, compared to erucic acid free lines (Table 7.2). The distribution of total phytosterol content was skewed towards higher contents (Figure 7.2). Comparison of the two genes showed that lines with the erucic acid gene on linkage group 12 on average have 153 mg kg^{-1} seed more total phytosterols content than the gene on linkage group 6. The content of campesterol was also affected by the erucic acid genes in which the effects of the genes on linkage groups 6 and 12 reduced campesterol content by 217.6 and 165.5 mg kg^{-1} seeds, respectively. Sitosterol and avenasterol contents were also negatively affected by high erucic acid content (Table 7.2). A negative correlation (r = - 0.85***) was obtained between erucic acid and total phytosterols content confirming the negative effects of the two QTL for erucic acid on the components of phytosterol (Table 7.3). However, positive correlations were observed between campesterol, avenasterol, sitsoterol and total phytosterols contents (Table 7.4).

7.3.3 Sinapate esters

The mean of total sinapate ester content was 7.1 mg sinapate g^{-1} seed ranging from 4.9 to 8.6 mg sinapate g^{-1} seed. The distribution of total sinapate ester content in the lines was again skewed towards higher contents, P= 0.001 (Figure 7.3). The erucic acid genes again affected total sinapate ester content negatively. The erucic acid genes on linkage groups 6 and 12 decreased total sinapate ester content by 1.26 and 0.99 mg sinapate g^{-1} seed, respectively. Sinapoylglucose and other sinapate esters contents were also decreased significantly due to effects of the two erucic acid genes but not sinapine content (Table 7.2). The components of sinapate esters like sinapine, sinapoylglucose, other sinapate esters, and total sinapate esters were positively correlated (Table 7.5).

Table 7.1 Traits mean value in all locations and Reinshof only

Traits	DH lines	DH11.4	DH lines†	DH11.4†	DH5.1†
Oil	45.92	48.74	45.75	45.41	51.54
Protein	22.79	24.9	22.99	24.43	23.50
Glucosinolate	22.43	20.55	20.56	25.43	78.23
Erucic acid	7.62	0.48	9.51	0.45	53.42
Sinapoylglucose	2.30	2.20	2.46	2.38	1.58
Sinapine	6.09	6.10	6.21	6.11	6.61
Total Sinapate esters	7.11	6.63	6.96	6.73	5.97
Other Sinapate esters	1.38	1.34	1.36	1.26	0.52
Campesterol	1157.24	1114.92	1148.14	1088.25	728.48
Sitosterol	1971.31	1954.43	1858.05	1826.65	1451.05
Avensterol	69.69	52.55	77.59	70.99	76.33
Total phytosterols	3935.15	3626.98	3630.39	3481.77	2547.61

† for location Reinshof

Table 7.2 Relationships between genes for erucic acid and other quality traits

Traits/Genotype classes			Diff	P	Traits/Genotype classes			Diff	P	Traits/Genotype classes			Diff	P
Eru6	Eru12	Zero			Eru6	Eru12	Zero			Eru6	Eru12	Zero		
Erucic acid (%)					Total phytosterol (mg/kg)					Total SE (mg/g)				
31.07		0.39	30.68	<0.0001	3115.81		3737.98	-469.23	<0.0001	5.49		6.75	-1.26	<0.0001
	30.46	0.39	30.07	<0.0001		3268.75	3737.98	-622.17	<0.0001		5.76	6.75	-0.99	<0.0001
31.07	30.46		0.61	0.5030	3115.81	3268.75		-152.94	0.0300	5.49	5.76		-0.26	0.1700
Oil (%)					Campesterol (mg/kg)					Sinapine (mg/g)				
47.27		45.29	1.97	0.0400	945.61		1163.24	-217.63	<0.0001	5.79		6.11	-0.32	0.0800
	50.01	45.29	4.72	<0.0001		997.78	1163.24	-165.46	<0.0001		5.92	6.11	-0.19	0.1800
47.27	50.01		-2.75	0.020	945.61	997.78		-52.17	0.1000	5.79	5.92		-0.13	0.5100
Protein (%)					Sitosterol (mg/kg)					Sinapoylglucose (mg/g)				
24.43		23.10	1.33	0.1183	1719.32		1981.50	-262.19	<0.0001	1.64		2.32	-0.68	<0.0001
	22.17	23.10	-0.93	0.1491		1730.42	1981.50	-251.08	<0.0001		1.85	2.32	-0.48	0.0001
24.43	22.17		2.26	0.0097	1719.32	1730.42		-11.11	0.6900	1.64	1.85		-0.21	0.2900
Glucosinolate (%)					Avenasterol (mg/kg)					Other sinapine ester (mg/g)				
21.49		20.4	1.04	0.9375	52.69		69.08	-16.39	0.0100	1.05		1.38	-0.34	<0.0001
	17.68	20.4	-2.77	0.4808		44.26	69.08	-24.82	0.0010		1.12	1.38	-0.27	<0.0001
21.49	17.68		3.81	0.3530	52.69	44.26		8.43	0.0300	1.05	1.12		-0.07	0.4400

Eru6 and Eru12 are the phenotypic values in the lines with one gene for erucic acid content on linkage groups 6 and 12, respectively (see Figure 7.1). Zero is phenotypic values of the trait in the lines free from erucic acid content. Diff is the difference between the phenotypic values of lines with one gene for erucic acid content (Eru6 or Eru12) minus the phenotypic values of lines free of erucic acid content (Zero), or between the Eru12 and Eru6.

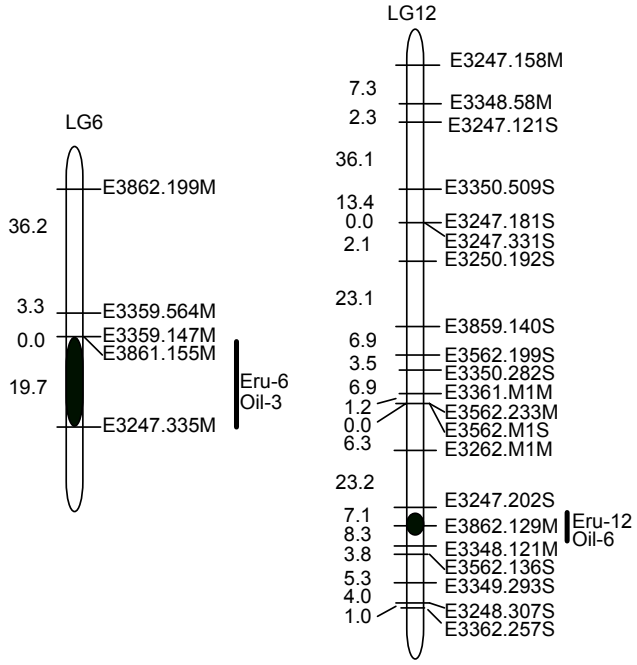


Figure 7.1 The framework maps of linkage groups 6 and 12 (Uzunova et al. 1995) with mapped QTL for erucic acid and oil content in the substitution lines

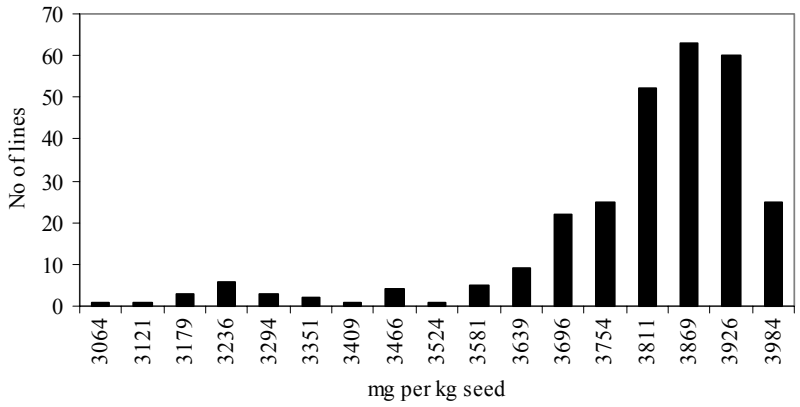


Fig. 7.2 Distribution of total phytosterols content

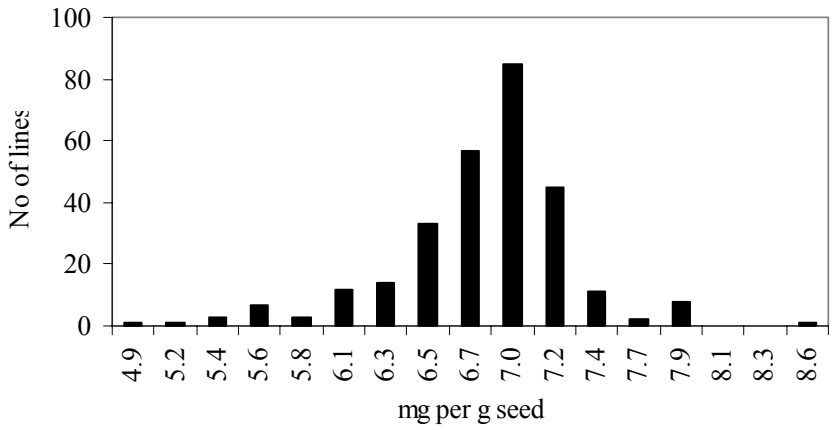


Fig. 7.3 Distribution of total sinapate ester content

Table 7.3 Spearman correlation among traits (LSMEANS values) over five locations

	Protein	GSL	C16:0	C18:1	C18:2	C18:3	C22:1	Total-SE	Total Phytosterol
Oil	-0.71 ***	-0.30 *	-0.66 ***	-0.32 *	-0.32 *	-0.58 ***	0.30 *	-0.27 *	-0.11
Protein		0.44 ***	0.13	-0.23	-0.23	0.08	0.24	-0.26	-0.52 ***
Glucosinolate (GSL)			0.03	-0.01	-0.01	-0.12	0.18	-0.11	-0.26
C16:0				0.53 ***	0.53 ***	0.86 ***	-0.56 ***	0.64 ***	0.55 ***
C18:1					0.72 ***	0.47 ***	-0.66 ***	0.63 ***	0.70 ***
C18:2						0.47 ***	-0.66 ***	0.63 ***	0.70 ***
C18:3							-0.66 ***	0.60 ***	0.63 ***
C22:1								-0.74 ***	-0.85 ***
Total Sinapate esters (SE)									0.78 ***

Table 7.4 Spearman correlation among traits (LSMEANS values) over five locations

	Protein	Glucosinolate	C22:1	Camp	Sito	Ave	Total Phytosterols
Oil	-0.71 ***	-0.30 *	0.30 *	-0.10	-0.45 ***	0.28 *	-0.11
Protein		0.44 ***	0.24	-0.50 ***	-0.08	-0.81 ***	-0.52 ***
Glucosinolate			0.18	-0.30 *	-0.20	-0.34 **	-0.26
C22_1				-0.85 ***	-0.87 ***	-0.49 ***	-0.85 ***
Campesterol (Camp)					0.77 ***	0.73 ***	0.96 ***
Sitosterol (Sito)						0.33 *	0.78 ***
Avenasterol (Ave)							0.76 ***

Table 7.3 and Table 7.4 statistically significant at * P=0.05, ** P=0.01, *** P=0.001

7.4 Discussion

The two genes detected for erucic acid content on linkage groups 6 and 12 in the current study were the same genes mapped by Ecke et al. (1995) and Thormann et al. (1996). Genes for erucic

acid content decreased the components of sinapate esters and total phytosterol contents. The correlation analysis revealed highly significant association between erucic acid and total phytosterols content (r = -0.85***) and also with total sinapate ester content (r = -0.83***, Table 7.3). Individual phytosterols like campesterol, avenasterol and sitosterol contents were also negatively affected by erucic acid alleles. Amar et al. (2007a) found also a strong negative correlation between erucic acid and individual phytosterols content. This implies that the genes for erucic acid content are likely closely linked to QTL for individual phytosterol and sinapate esters content, or are the same genes leading to pleiotropic effects. However, distinguishing closely linked genes from pleiotropic effect can be difficult.

Table 7.5 Spearman correlation among traits (LSMEANS values) over five locations

	Protein	Glucosin-olate	C22:1	Sinapoyl-glucose	Sinapine	Other SE	Total Sinapate ester
Oil	-0.71 ***	-0.30 *	0.30 *	0.09	-0.25	-0.31 *	-0.27 *
Protein		0.44 ***	0.24	-0.52 ***	-0.08	-0.15	-0.26
Glucosinolate			0.18	-0.23	0.19	-0.33 *	-0.11
C22_1				-0.63 ***	-0.35 **	-0.63 ***	-0.74 ***
SinGlc					0.12	0.59 ***	0.67 ***
Sinapine						0.26	0.63 ***
Other Sinapate esters (SE)							0.71 ***

Statistically significant at * P=0.05, ** P=0.01, *** P=0.001

The negative correlation between erucic acid content and phytosterol content might be due to the fact that cytoplasmic acetyl-CoA is a common precursor for the biosynthesis of phytosterol and long chain fatty acids (Fatland et al. 2002). The two erucic acid genes did not affect seed glucosinolate and protein contents, however, significantly increased oil content (Ecke et al. 1995).

The erucic acid gene on linkage group 12 (eru12) affected total phytosterol content more than the gene on linkage group 6 (eru6). The relative increase of phytosterol content compared to the effect by eru6 gene might not be the direct effect of the eru12 gene since both genes contributed the same amount of erucic acid content and were expected to react equally to other traits; however, this difference could most probably be due to another gene residing near eru12 increasing phytosterol content. Amar et al. (2007a) mapped multiple QTL on linkage groups 6

and 12 for individual phytosterol contents, therefore, most probably these QTL react differently on the traits based on which linkage group they were located. Moreover, protein content was affected negatively by the eru12 gene and decreased by 2.26% compared to the effect of the eru6 gene. This might also happen due to additional QTL located near eru12 and eventually, one of the QTL for protein content was identified on linkage group 12 (pro-3, see thesis chapter 5) in which the combination of the genes (eru12 and pro3) probably decrease protein content. Furthermore, the eru12 gene increased oil content by 2.7% higher than the eru6 gene, which could most probably be the involvement of another QTL for oil content near the erucic acid gene on linkage group 12. The negative contribution of the gene near the eru12 on protein content and simultaneously positive effect on oil content came in consistent with the negative relationship between oil and protein content (see thesis chapter 5) because the two traits share the same substrate and energy in the two biochemical pathway of their synthesis.

Since the individual phytosterol and erucic acid content are negatively correlated, genotypes with high erucic acid content might not be used as breeding materials for high phytosterols content. However, this could also imply that as the components of phytosterol increase, the content of erucic acid decrease, which is a wished scenario for development of high phytosterol cultivars with lower erucic acid content. The positive correlation obtained between fatty acid compositions like C18:1, C18:2 and components of phytosterol but negative correlation with C22:1 can provide a breeding strategy that can simultaneously increase C18:1, C18:2 and individual phytosterols content.

Using 19 modern canola cultivars Gül and Seker (2006) found low variation for phytosterol contents. They identified the highest total phytosterols content in the variety ‘Adder’ with a mean of 3937 mg kg^{-1} seeds. The substitution lines used in the present study contained a mean of 3935 mg kg^{-1} seeds nearly equal to the highest cultivar identified by Gül and Seker (2006). Abidi et al. (1999) analysed 12 canola varieties of which one genetically modified line has elevated phytosterol content while the rest of the genotypes were not varying in phytosterols content implying a low genetic pool of breeding materials in conventional modern canola cultivars. Vlahakis and Hazebroek (2000) found strong interactions of genotypes and locations for phytosterol contents in soybean germplasms, which can complicate achievements using conventional breeding approach. Therefore the limited variability in phytosterols levels in many varieties could unlikely lead to increase in content of phytosterols using conventional breeding approach. Therefore, genetically modified canola varieties might be used to increase components of phytosterol (Abidi et al. 1999).

In the current study the correlations between campesterol, sitosterol, avenasterol and total phytosterol content were positive. The same positive correlations between individual phytosterols and total phytosterols content were also observed in other studies (Gül and Seker 2006; Amar et al. 2007a; Amar et al. 2007b). This can be explained by the biochemical pathway leading the individual sterols in which 24-methylene sterol is a common precursor that controls a branching point of the biosynthesis pathways to campesterol, avenasterol and sitosterol (Hartman 1998; Schrick at al. 2004) and avenasterol is a precursor for sitosterol (Schrick at al. 2004).

In the current study, erucic acid gene affected sinapate esters negatively. Erucic acid gene on linkage groups 6 and 12 decreased content of total sinapate ester by 1.26 and 0.99 mg sinapate per g seed, respectively. This is most likely due to close linkage of the genes for erucic acid and sinapate esters or probably due to pleiotropic effects of the same gene. The mean content of total sinapate esters was 7.1 mg sinapate g^{-1} seed in the substitution lines. zum Felde et al. (2007) found mean of 7.43 mg sinapate g^{-1} seed in three different segregating populations of winter rapeseed of which one of the population was derived from the same parents used in the development of the substitution lines of the current study. In contrast to individual phytosterol content, large genetic variation was found for sinapate esters in the three populations of winter rapeseed (zum Felde et al. 2007). Higher variation can lead to a promising genetic pool to lower sinapate esters needed to improve the quality of rapeseed meal. Additionally higher heritability was found in two of the three populations analysed by zum Felde et al. (2007) implying possibility of success to lower sinapate esters using conventional breeding programme. The negative correlation between erucic acid and sinapate esters found in the current study can lead to a competition between the two traits if breeders wished to have cultivar with low erucic acid and low sinapate esters. However, if the genes affecting both traits are closely linked separating of the two genes can be achieved using recombination.

The correlations between total sinapate ester, sinapine, sinapoylglucose, and other sinapate esters were positive. This implies reduction of sinapate esters can be achieved simultaneously for all four components encouraging breeding programme to lower sinapate esters. In the three populations of rapeseed, zum Felde et al. (2007) had also observed positive correlation between the components of sinapate esters. Using transgenic T2 plants with low sinapate esters content, Hüsken et al. (2005a; 2005b) found also positive correlation between individual sinapate esters. The correlation among individual sinapate eater can be explained by the biochemical pathway in which sinapoylglucose is the direct precursor of both sinapine and other sinapate eater.

Total sinapate esters and phytosterols contents were positively correlated which is not good for a breeding programme that might set up simultaneous aim of increasing content of phytosterols and decreasing sinapate esters content. The negative correlation between erucic acid and sinapate esters content implies that cultivars with higher erucic acid content might have the gene for lowering contents of sinapate esters. zum Felde et al. (2006; 2007) found the old cultivar used in the current study, 'Mansholts' Hamburger Raps' as a potential material for breeding program to lower sinapate ester. Our finding again confirms their findings because the lowest sinapate esters were identified in the substitution lines with defined segments on linkage groups 6 and 12 of the donor parent 'Mansholts' Hamburger Raps' in which these donor segments are also genes for erucic acid content, a distinct trait of this donor parent as compared to canola cultivars.

8. Conclusions

In this thesis, the development of intervarietal substitution lines and QTL mapping were presented and discussed. The first library of intervarietal substitution lines was developed from the cross of 'Express', used as the recurrent parent, and 'R239', a resynthesized rapeseed, used as the donor parent. The second substitution lines library was developed from the cross of 'Mansholts Hamburger Raps' and 'Samourai'. The main findings and conclusions are:

- In the development of the substitution lines, marker assisted selection and backcrossing approaches helped to select lines with small segments of the donor parent in the genetic background of the recurrent parent. Substitution lines with only single donor segments were developed faster than in classical backcrossing approach because of the use of marker assisted selection.

- The detection of QTL was performed using differences in the least square means of the phenotypic value of the substitution lines from the recurrent parent and tested for statistical significance. A significant difference between a substitution line and the recurrent parent implies that the donor segment carries the QTL responsible for the effect on the trait because the background of the substitution line is from the recurrent parent except for the introgressed genome region. The development of the substitution lines in the current study helped to identify and localize more QTL with small effects than could have been identified using a segregating population.

- QTL were detected for oil content with small effects revealing that many additive genes are responsible for the effect. Negative correlation was observed between oil and protein content due to the competition for substrate and energy in two biochemical pathways of their synthesis. Corresponding QTL regions were also detected for oil and protein content, which are likely identical QTL or QTL residing near to each other. Independent QTL were also identified for each of the traits which could be useful in breeding cultivars with high oil content without decreasing protein content. Several QTL were identified for seed glucosinolate content, which could help us to understand more about the genetic architecture of glucosinolate content in rapeseed.

- The donor parent showed better winter survival than the recurrent parent and this trait was also reflected on most of the substitution lines due to the introgressed donor segments. Several QTL were also identified which were responsible for the trait.

Therefore, agronomically important alleles are still found in the old cultivars that could be introgressed into the modern cultivars.

- The two erucic acid genes which were mapped to control erucic acid content in *Brassica napus* L. were again identified using the substitution lines. The effect of each of the two genes on other quality traits like individual phytosterol and sinapate esters was examined. The two erucic acid genes decreased individual phytosterol and sinapate esters. The individual phytosterol and erucic acid content were negatively correlated; therefore, genotypes with low erucic acid content are suitable breeding materials to increase phytosterols content. The negative correlation between erucic acid and sinapate esters content implies that cultivars with higher erucic acid content might have genes for lowering contents of sinapate esters.

In the substitution lines the interval to which a QTL can be mapped depends on the size of the donor segment. In the current study using the substitution lines the precision of QTL localisation could be narrowed down to a few centi Morgan using lines with small overlapping donor segments.

Substitution lines library reveals high precision of QTL localisation and is a valuable approach to produce materials that can be used for gene cloning and candidate gene studies. Further backcrossing of selected lines will allow fine mapping to study complex traits. The development of substitution lines is a universally applicable method in many plant species using marker assisted selection and backcrossing approaches over a successive period of time to generate useful information about complex traits.

The development of double haploid substitution lines through backcrossing and microspore culture produced uniform lines with homozygous donor segments, which can be propagated by self-pollination. The lines can be tested in multiple environments and multiple years for further studies and characterization of the phenotypic traits that could not be identified in the current study. They can also be used in verifying the QTL detected in the present study using only those lines with the significant QTL effects.

Using substitution lines, QTL to QTL interactions can be identified by comparing those lines containing two or more donor segments to the lines with only a single donor segment of the

same genome region. This method can provide a more precise estimation of the interactions between the QTL.

Summary

Rapeseed *(Brassica napus* L.) belongs to the Brassicaceae family and is widely grown as oil crop in temperate areas of North America, Europe and China. Rapeseed meal that remains after the extraction of oil is rich in protein that can be used in animal feeding. Better understandings of the genetics of agronomically important traits in rapeseed are important prerequisites in breeding programs. In addition to classical quantitative genetics, QTL mapping can be a valuable tool to understand the number, size and effects of quantitative trait loci affecting a trait (QTL). In the current study, intervarietal substitution lines were developed and were used for QTL mapping. Substitution lines comprise a set of lines with complementary donor segments in the genetic background of a recurrent parent covering the genome of the donor parent. A set of intervarietal substitution lines represents a genetically well characterised material that is suitable in the analysis of genome wide effects of allelic variation. These lines have a common genetic background except for the introgressed region of the donor segment. The first objective of the current study was the development of two intervarietal substitution lines libraries from crosses of rapeseed varieties. The second objective was to map QTL for agronomically important traits using one of the intervarietal substitution lines libraries.

The first library was developed using 'Express' as the recurrent parent and 'R239', a resynthesized rapeseed, as the donor parent. A map with 220 AFLP markers covering 1327 cM of the rapeseed genome was used in the development of the substitution lines. After 4 generations of backcrossing to the recurrent parent and marker assisted selection, a total of 27 BC_4 plants with donor segments covering 60% of the mapped rapeseed genome were selected. From the selected BC_4 plants, double haploid plants were developed using microspore culture in order to produce doubled haploid substitution lines with homozygous donor segments. At the moment, seed materials are being multiplied in greenhouse to produce sufficient seeds for next year field trials.

The second substitution line library was developed using 'Mansholts Hamburger Raps' as the donor parent and 'Samourai' as the recurrent parent. 164 AFLP markers that cover 1325 cM of the rapeseed genome were used for marker assisted selection in the development of the substitution lines. A total of 26 genotypes were selected in BC_4 and 15 seeds from each plant were sown to produce 390 genotypes. The 390 genotypes were analysed by AFLP markers. A total of 76 genotypes were selected with donor segments covering 525 cM of the mapped rapeseed genome. The 76 selected BC_4 plants were used to produce doubled haploid lines with homozygous donor segments using microspore culture. Out of 1736 microspore-derived plantlets

produced, 366 double haploid lines with seeds set were obtained. Out of the 366 genotypes, 292 genotypes, which had produced sufficient seeds were tested in field trials at up to five locations in Germany and used to map QTL for agronomically important traits. Out of the 292 lines tested in the field experiments, 101 DH substitution lines were analysed with markers. Out of the 101 lines, 55 lines contained 1 donor segment, 31 lines contained 2 donor segments and 15 lines contained 3 donor segments. The donor segments of these 101 lines covered a minimum of 451 cM of the mapped rapeseed genome. The rest of 191 lines were not analysed by markers, however, marker information of BC_4 microspore donor plants were available.

Analysis of variance revealed significant variation for oil, protein, oleic acid, erucic acid, and glucosinolate content among the lines. Moreover, a significant variation was also observed for begin of flowering, end of flowering, duration of flowering, plant height and winter survival. QTL effects due to the introgression of the donor segments were determined by multiple comparisons of least square means differences of the phenotypic values of lines to the recurrent parent and were tested by t-test for significance levels. On average, the donor parent 'Mansholt' contained 2 % more seed oil than the recurrent parent 'Samourai'. A total of 10 QTL were mapped for oil content with QTL effects ranging from -5.7 to 6.2% of which 9 QTL increased oil content in the substitution lines. The donor parent contained lower oleic acid content compared to the recurrent parent. For oleic acid content 4 QTL with QTL effects ranging from -12.3 to 13.7% were identified of which 3 QTL decreased oleic acid content.

For protein content a total of 5 QTL were identified with QTL effects ranging from -3.9 to 3.1% and except one all QTL effects showed a decrease in protein content. Four out of five QTL for protein content also affected oil content in the opposite directions of the effects on protein content implying a close linkage of the QTL or pleiotropic gene effects. The reason for the complementary relation could be due to the same substrates being used in the synthesis of oil and protein. However, one QTL for protein content independent of any effects on oil content was also identified. The donor parent is characterized by high glucosinolate content. A total of 9 QTL were identified and all of the QTL effects showed an increase in glucosinolate content ranging from 12 to 28 µmol/g seed due to the replacement of 'Samourai' alleles by alleles of 'Mansholt'.

Traits such as days to begin of flowering, end of flowering and duration of flowering were reduced due to the replacement of the recurrent parent alleles by alleles of the donor parent. A total of 13 QTL were identified for begin of flowering reducing days to begin of flowering by

2.5 to 4 days compared to the recurrent parent. For end of flowering 18 QTL were identified decreasing the days to end of flowering from 2.9 to 6.6 days. For duration of flowering, a total of 5 QTL were detected decreasing duration of flowering by 4 to 6 days. A total of 20 QTL were detected for plant height and for all QTL the donor allele contributed to an increase in plant height ranging from 10.7 to 18 cm. The donor parent survived winter better than the recurrent parent. 13 QTL were detected for winter survival due to the introgression of donor segments. Correlation analysis revealed early flowering and short duration of flowering are positively correlated with winter survival and increased plant height. Plant height, early flowering, early end of flowering, short duration of flowering, winter survival and oil content were positively correlated to each other but negatively correlated to the protein content.

Erucic acid genes were also examined in relation to content of phytosterols and sinapate esters. Erucic acid genes on linkage groups 6 and 12 decreased total sinapate ester content by 1.26 and 0.99 mg sinapate g^{-1} seed, respectively, compared to erucic acid free lines. Moreover, erucic acid genes on linkage groups 6 and 12 decreased total phytosterol content by 469 and 622 mg kg^{-1} seeds, respectively, compared to erucic acid free lines. The correlation analysis also revealed highly significant association between erucic acid and total phytosterols contents (r = -0.85***).

Zusammenfassung

Raps (*Brassica napus* L.) gehört zur Senf-Familie und wird überwiegend als Ölfrucht in gemäßigten Gebieten Nordamerikas, Europas und Chinas angebaut. Rapsschrot, welches nach der Gewinnung von Öl verbleibt, ist reich an Protein, das in der Tierfütterung verwendet werden kann. Ein besseres Wissen über die Genetik von wichtigen agronomischen Merkmalen ist eine bedeutende Vorbedingung in Züchtungsprogrammen. Zusätzlich zur klassischen quantitativen Genetik, kann die QTL-Kartierung ein wertvolles Verfahren sein, um die Anzahl, und die Effekte von QTL (Quantitative trait loci), die ein quantitativ vererbtes Merkmal kontrollieren, besser zu verstehen. In der vorliegende Arbeit wurden „intervarietal substitution lines" entwickelt und für QTL-Kartierungen verwendet. Ein Serie von Substitutionslinien enthält Linien mit komplementären Donorsegmente in dem gemeinsamen genetischen Hintergrund des rekurrentes Elters die zusammen das gesamte Donorgenom abdecken. Substitutionslinien dienen als genetisch charakterisiertes Material, um genomweit die Effekte der allelen Variation zu analysieren. Diese Linien haben einen gemeinsamen genetischen Hintergrund von dem nur die eingeschränkten Donorsegmente abweichen. Das erste Ziel der vorliegende Arbeit war die Entwicklung von zwei Serien von Substitutionslinien aus den Kreuzungen von unterschiedliche Rapssorten. Das zweite Ziel war einen Set an Substitutionslinien für die QTL-Kartierung in agronomische wichtigen Merkmalen zu verwenden.

Die erste Serie wurde aus der Kreuzung 'Express' x 'R239' mit 'Express' als rekurrentem Elter und 'R239', einem Resyntheseraps, als Donorelter entwickelt. Eine genetische Karte mit 220 AFLP Marker, die 1327 cM des Rapsgenoms abdeckt, wurde in der Entwicklung der Substitutionslinien verwendet. Nach 4-facher Rückkreuzung zum rekurrenten Elter und markergestützte Selektion, wurden insgesamt 27 BC_4 Genotypen, deren Donorsegmente 60% des kartierten Rapsgenoms abdecken, ausgewählt. Aus Mikrosporen der ausgewählten BC_4 Genotypen wurden doppelhaploide (DH) Linien über Mikrosporenkultur entwickelt, um Substitutionslinien mit homozygoten Donorsegmenten zu produzieren. Das Pflanzenmaterial wird im Moment im Gewächshaus vermehrt damit nächstes Jahr Feldversuchen durchgeführt werden können.

Die zweite Serie von Substitutionslinien wurde aus der Kreuzung 'Mansholts Hamburger Raps' x 'Samourai' mit 'Mansholt' als Donorelter und 'Samourai' als rekurrentem Elter entwickelt. Es wurden 164 AFLP Marker, die 1325 cM des Rapsgenoms abdecken, für die markergestützte Selektion in den Substitutionslinien verwendet. Insgesamt wurden 26 BC_3 Genotypen ausgewählt und 15 Samen von jedem Genotypen ausgesät, um 390 BC_4-Nachkommen zu

erhalten. Die 390 Genotypen wurden mit den AFLP Marker analysiert und 76 Linien ausgewählt deren Donorsegmente 525 cM vom Rapsgenom abdecken. Diese 76 BC_4 Genotypen wurden als Spenderpflanzen für die Mikrosporenkultur verwendet, um DH-Linien mit homozygoten Donorsegmente zu produzieren. Aus 1736 aus Mikrosporen hergestellte Pflänzchen konnten 366 DH-Linien mit Samenansatz gewonnen worden. Aus den 366 Genotypen wurden 292 Genotypen, die genügend Samen erzeugt hatten, in Feldversuchen an bis zu fünf Orten in Deutschland geprüft um QTL für wichtige agronomische Merkmale zu kartieren. Von den 292 geprüften Linien waren 101 DH-Linien schon mit Marker untersucht. Von diesen enthielten 55 Linien 1 Donorsegment, 31 Linien 2 Donorsegmente, und 15 Linien 3 Donorsegmente. Die Segmente in den 101 Linien deckten insgesamt 451 cM des kartierten Rapsgenoms ab. Die restlichen 191 Linien waren nicht mit Marker untersucht worden, aber Markerinformation der BC_4 Spenderpflanzen war verfügbar.

In der Varianzanalyse zeigten sich signifikante Unterschiede für Öl-, Protein-, Ölsäure-, und Glucosinolatgehalt unter den Linien. Außerdem wurden signifikante Unterschiede für Blühbeginn, Blühende, Blühdauer, Pflanzenhöhe und Auswinterung beobachtet. QTL-Effekte auf Grund der Introgression der Donorsegmente wurden durch Vergleiche von „Least Square Means“ von Unterschieden der phänotypischen Werte der Linien vom rekurrenten Elter bestimmt und mit einem T-test geprüft.

Durchschnittlich hatte der Donorelter 'Mansholt' einen um 2 % höheren Ölgehalt als der rekurrente Elter 'Samourai'. Insgesamt 10 QTL wurden für Ölgehalt mit QTL-Effekten von -5,7 bis 6,2% identifiziert, von denen bei 9 QTL das Donorsegment den Ölgehalt erhöhte. Der Donorelter hatte einen niedrigeren Ölsäuregehalt im Vergleich zum rekurrente Elter. Insgesamt 4 QTL mit QTL-Effekten von -12,3 bis 13,7% konnten für dieses Merkmal identifiziert werden, von denen bei 3 QTL das Donorallel den Ölsäuregehalt verminderte.

Für Proteingehalt konnten insgesamt 5 QTL mit QTL-Effekten von -3,9 bis 3,1% identifizierte werden. Alle QTL-Effekte mit einer Ausnahme zeigten eine Abnahme im Proteingehalt. Vier aus fünf QTL für das Markmal Proteingehalt betrafen auch den Ölgehalt wobei die Effekte auf den Ölgehalt gegenläufig zu den Effekten auf den Proteingehalt waren. Dies deutet auf eine enge Kopplung von QTL für Öl- und Proteingehalt oder aber einen pleiotropen Effekte entsprechender Gene hin. Der Grund für die negative Beziehung zwischen Protein und Ölgehalt könnte in der Verwendung derselben Substrate in der Synthese von Öl und Protein liegen. Ein QTL für Proteingehalt konnte identifiziert werden der keine Wirkung auf den Ölgehalt hat. Der

Donorelter besitzt einen hohen Glucosinolatgehalt. Insgesamt wurden 9 QTL für dieses Merkmal identifiziert. Bei allen diesen QTL erhöht das Donorallel den Glucosinolatgehalt mit Effekten zwischen 12 und 28 µmol/g Samen.

Insgesamt 13 QTL wurden für Blühbeginn identifiziert bei denen das Donorallel den Blühbeginn zwischen 2,5 und 4 Tagen reduziert. Für das Blühende wurden 18 QTL identifiziert wobei das Donorallel die Zeit bis zum Blühende jeweils um 2,9 bis 6,6 Tage reduziert. Für Blühdauer wurden insgesamt 5 QTL identifiziert. Auch hier reduziert das Donorallel jeweils die Blühdauer um 4 bis 6 Tage. Insgesamt 20 QTL wurden für die Pflanzenhöhe identifiziert. Die Donorallele bewirkten jeweils eine Zunahme der Pflanzenhöhe um 10,7 bis 18 cm. Der Donorelter überlebte den Winter besser als der rekurrente Elter. Insgesamt 13 QTL wurden für Winterhärte in den Substitutionlinien identifizierte. Eine Korrelationsanalyse zeigte, dass früher Blühbeginn, kurze Blühdauer, Winterhärte und Pflanzenhöhe positiv zueinander und zum Ölgehalt korreliert sind, aber negativ mit den Proteingehalt.

Die beiden Erucasäuregene von Raps wurden in Bezug auf Phytosterol- und Sinapinestergehalte untersucht. Die beiden Gene auf den Kopplungsgruppen 6 und 12 verminderten den Gesamtgehalt an Sinapinestern um 1,26 bzw. 0.99 mg Sinapin/g Samen im Vergleich zu erucasäurefreien Linien. Außerdem, verminderten die Erucasäuregene den Gesamtgehalt an Phytosterolen um 469 bzw. 622 mg/kg Samen im Vergleich zu erucasäurefreien Linien. Die Korrelationsanalyse zeigte auch eine enge Beziehung zwischen Erucasäure und Gesamtphytosterolgehalt mit einem Korrelationskoeffezienten von r = -0.85***.

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Thesis chapter 2: Development of intervarietal substitution lines in the cross of 'Express' x 'R239' a resynthesized rapeseed using marker assisted selection

Thesis chapter 3 Development of substitution lines in the *Brassica napus* L. cross of 'Mansholts Hamburger Raps' x 'Samourai' using marker assisted selection

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Appendices

Appendix 2.1 Primer sequences

Eco primers	Sequences
E32	5'CTG CGT ACC AAT TCA AC 3'
E33	5'CTG CGT ACC AAT TCA AG 3'
E35	5'CTG CGT ACC AAT TCA CA 3'
E38	5'CTG CGT ACC AAT TCA CT 3'
E40	5'CTG CGT ACC AAT TCA GC 3'
Mse primers	
M47	5'GAT GAG TCC TGA GTA ACA A 3'
M48	5'GAT GAG TCC TGA GTA ACA C 3'
M49	5'GAT GAG TCC TGA GTA ACA G 3'
M50	5'GAT GAG TCC TGA GTA ACA T 3'
M51	5'GAT GAG TCC TGA GTA ACC A 3'
M59	5'GAT GAG TCC TGA GTA ACT A 3'
M60	5'GAT GAG TCC TGA GTA ACT C 3'
M61	5'GAT GAG TCC TGA GTA ACT G 3'
M62	5'GAT GAG TCC TGA GTA ACT T 3'

Appendix 3.1 Donor segments in the DH substitution lines analysed by markers

DH Line[1]	BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
2	2.4.16	2	3	E3261.234M	51.0	51.0	0.0
			14	E3359.109M -E3261.107M	80.5	118.1	37.6
9	2.4.16	2	14	E3261.218M -E3359.109M	58.2	80.5	22.3
			16	E3261.370S	0.0	0.0	0.0
43	19.4.6	3	3	E3259.72M -E3362.143M	20.8	45.1	24.3
			5	E3259.176S -E4060.559M	76.3	79.9	3.6
			12	E3361.001M	101.6	101.6	0.0
44	19.4.6	3	5	E3259.176S-E4060.559M	76.3	79.9	3.6
			12	E3361.M001M -E3862.129M	101.6	139.8	38.2
			16	E3362.293S	4.7	4.7	0.0
47	2.4.16	2	9	E3859.105M	72.5	72.5	0.0
			14	E3859.200M -E3261.107M	93.5	118.1	24.6
49	19.4.1	3	10	E3862.149M	14.0	14.0	0.0
			12	E3361.001M	101.6	101.6	0.0
			17	E3862.77M	0.0	0.0	0.0
55	2.4.16	2	14	E3261.218M -E3261.107M	58.2	118.1	59.9
			16	E3261.370S	0.0	0.0	0.0
59	5.4.7	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
			18	E3861.459M -E3250.339M	84.2	99.3	15.1
75	19.4.6	2	5	E3259.176S-E4060.559M	76.3	79.9	3.6
			12	E3361.001M -E3862.129M	101.6	139.8	38.2
76	19.4.6	2	5	E3259.176S-E4060.559M	76.3	79.9	3.6
			12	E3247.202S -E3862.129M	132.7	121.2	7.1
78	18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
79	18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
85	5.4.7	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
89	19.4.4	1	3	E3362.143M -E3348.001M	45.1	51	5.9
98	7.4.10	2	8	E3261.001S	10.2	10.2	0.0
			17	E3261.166M	30.7	30.7	0.0
111	18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
117	18.4.10	2	17	E32/M50-F-449-S	10.7	10.2	0.0
			18	E3861.459M -E3250.339M	84.2	99.3	15.1
119	5.4.7	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
123	5.4.7	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
124	18.4.11	1	2	E3361.138M	61.5	61.5	0.0
128	18.4.11	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
140	19.4.6	3	3	E3362.143M	45.1	45.1	0.0
			5	E3259.176S	76.3	76.3	0.0
			12	E3247.202S	132.7	132.7	0.0
141	19.4.6	2	3	E3362.143M	45.1	45.1	0.0
			5	E3259.176S -E4060.559M	67.2	70.8	3.6
142	19.4.6	3	3	E3362.143M	45.1	45.1	0.0
			5	E3259.176S -E4060.559M	67.2	70.8	3.6
			12	E3247.202S	132.7	132.7	0.0

Appendix 3.1 (continued)

DH Line[1]	BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
144	19.4.1	1	12	E3361.001M	101.6	101.6	0.0
155	4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7
169	6.4.2	1	5	E4060.559M	70.8	70.8	0.0
172	1.4.3	2	12	E3349.293S	153.0	153.0	0.0
			19	E3862.371M -E3349.461S	0.0	18.6	18.6
186	19.4.6	2	3	E3259.72M -E3362.143M	20.8	45.1	24.3
			12	E3361.001M -E3862.129M	101.6	139.8	38.2
189	19.4.6	3	3	E3259.72M -E3362.143M	20.8	45.1	24.3
			12	E3361.001M -E3862.129M	101.6	139.8	38.2
			16	E3362.293S	4.7	4.7	0.0
194	1.4.13	1	19	E3862.371M -E3349.461S	0.0	18.6	18.6
196	7.4.10	1	17	E3261.166M -E3861.237M	30.7	53.2	22.5
198	7.4.10	2	14	E3261.218M	58.2	58.2	0.0
			17	E3862.327S -E3861.237M	43.7	53.2	9.5
202	18.4.10	2	12	E3247.158M -E3250.192S	0.0	61.2	61.2
			18	E3861.459M	84.2	84.2	0.0
205	7.4.10	1	17	E3862.327S	43.7	43.7	0.0
207	7.4.10	2	1	E3261.47M	54.5	54.5	0.0
			17	E3862.327S -E3861.237M	43.7	53.2	9.5
208	7.4.10	2	15	E3861.214M	50.4	50.4	0.0
			17	E3862.327S -E3861.237M	43.7	53.2	9.5
212	18.4.10	2	12	E3247.121S -E3250.192S	9.6	61.2	51.6
			12	E3247.158M	0.0	0.0	0.0
213	18.4.10	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
216	19.4.4	1	3	E3259.72M -E3348.001M	20.8	51	30.2
217	19.4.4	1	3	E3259.72M -E3348.001M	20.8	51	30.2
221	18.4.10	2	2	E3861.189M	26.7	26.7	0.0
			12	E3247.181S -E3250.192S	59.1	61.2	2.1
241	7.4.7	1	17	E3261.166M -E3861.237M	30.7	53.2	22.5
242	24.4.10	1	10	E3361.167M	2.6	2.6	0.0
252	19.4.4	1	3	E3259.72M -E3348.001M	20.8	51	30.2
254	19.4.4	2	3	E3259.72M -E3348.001M	20.8	51	30.2
			12	E3361.001M	101.6	101.6	0.0
255	18.4.15	1	12	E3250.192S	61.2	61.2	0.0
256	18.4.15	2	12	E3250.192S	62.2	62.2	0.0
			18	E3861.459M -E3859.243S	84.2	99.3	15.1
257	18.4.15	2	12	E3250.192S	62.2	62.2	0.0
			18	E3861.459M -E3859.243S	84.2	99.3	15.1
258	18.4.15	1	18	E3861.459M -E3859.243S	84.2	99.3	15.1
259	18.4.15	1	18	E3250.339M	99.3	99.3	0.0
260	18.4.15	1	18	E3861.459M -E3859.243S	84.2	99.3	15.1
261	2.4.16	1	14	E3261.218M -E3859.200M	58.2	93.5	35.3

Appendix 3.1 (continued)

DH Line[1]	BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
262	2.4.16	3	3	E3261.234M	51	51	0.0
			14	E3261.218M -E3859.200M	58.2	93.5	35.3
			16	E3261.370S	0.0	0.0	0.0
264	2.4.16	1	14	E3261.218M -E3261.107M	58.2	118.1	59.9
265	2.4.16	2	9	E3859.105M	72.5	72.5	0.0
			14	E3261.218M	58.2	58.2	0.0
269	2.4.1	1	9	E3859.105M	72.5	72.5	0.0
285	19.4.4	1	12	E3361.M001M	101.6	101.6	0.0
293	18.4.10	3	12	E3247.121S	9.6	9.6	0.0
			12	E3247.158M	0.0	0.0	0.0
			18	E3250.339M	99.3	99.3	0.0
294	18.4.10	3	12	E3247.121S -E3250.192S	9.6	61.2	51.6
			12	E3247.158M	0.0	0.0	0.0
			18	E3861.459M -E3250.339M	84.2	99.3	15.1
296	2.4.16	2	14	E3261.107M	118.1	118.1	0.0
			16	E3261.370S	0.0	0.0	0.0
316	18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
341	13.4.4	1	8	E3362.001M -E3262.002M	45.9	64.5	18.6
342	13.4.4	1	8	E3362.001M -E3262.002M	45.9	64.5	18.6
364	1.4.3	1	19	E3862.371M -E3349.461S	0.0	18.6	18.6
367	1.4.3	1	19	E3862.371M	0.0	0.0	0.0
368	1.4.3	1	12	E3349.293S	153.0	153.0	0.0
373	7.4.7	2	1	E3862.196M	48.5	48.5	0.0
			17	E3261.166M -E3861.237M	30.7	53.2	22.5
389	18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
397	19.4.6	3	3	E3259.72M -E3362.143M	20.8	45.1	24.3
			5	E4060.559M	70.8	70.8	0.0
			12	E3361.001M	101.6	101.6	0.0
434	24.4.3	1	10	E3862.82M	2.6	2.6	0.0
476	14.4.15	1	14	E3261.107M	118.1	118.1	0.0
483	11.4.5	1	11	E3259.337S -E3259.330M	8.2	8.5	0.3
484	11.4.5	2	11	E3259.337S -E3259.330M	8.2	8.5	0.3
			12	E3247.158M	0.0	0.0	0.0
496	18.4.10	2	12	E3247.121S	9.6	9.6	0.0
			13	E3247.158M	0.0	0.0	0.0
499	18.4.10	2	12	E3247.181S -E3250.192S	59.1	61.2	2.1
			18	E3861.459M -E3250.339M	84.2	99.3	15.1
506	4.4.1	0					
508	4.4.1	0					
526	22.4.4	1	11	E3262.75S -E3259.330M	8.0	8.5	0.5
527	22.4.4	3	2	E3349.155M	0.0	0.0	0.0
			14	E3259.70M	18.4	18.4	0.0
			18	E3262.186S	46.7	46.7	0.0

Appendix 3.1 (continued)

DH Line[1]	BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
540	14.4.15	1	14	E3261.107M	118.1	118.1	0.0
544	14.4.15	2	12	E3247.158M -E3247.121S	0.0	9.6	9.6
			14	E3261.107M	118.1	118.1	0.0
564	11.4.5	2	11	E3259.337S -E3259.330M	8.2	8.5	0.3
			12	E3247.158M	0.0	0.0	0.0
625	22.4.4	2	11	E3262.75S -E3259.330M	8.0	8.5	0.5
			15	E3349.107M	41.7	41,7	0.0
646	20.4.8	1	16	E3261.370S -E3862.461M	0.0	23.7	23.7
660	22.4.4	1	14	E359.70M -E4060.447M	18.4	39.0	20.6
671	9.4.4	2	6	E3359.147M	45.3	45.3	0.0
			13	E3247.186S -E3247.314M	10.8	31.3	20.5
768	19.4.1	1	17	E3862.77M	0.0	0.0	0.0
769	19.4.1	1	12	E3247.202S	132.7	132.7	0.0
864	18.4.15	2	12	E3250.192S	61.2	61.2	0.0
			18	E3250.339M -E3859.243S	99.3	99.3	0.0
875	14.4.15	1	12	E3247.158M -E3247.121S	0.0	9.6	9.6
877	14.4.15	1	12	E3247.158M -E3348.58M	0.0	7.3	7.3
887	15.4.12	1	9	E3247.394M	0.0	0.0	0.0
918	25.4.6	2	2	E4060.199M -E4060.199M	57.9	59.1	1.2
			13	E3348.323M	28.6	28.6	0.0
951	11.4.11	2	7	E3259.149M	28.3	28.3	0.0
			12	E3247.158M -E3247.121S	0.0	9.6	9.6
953	11.4.11	1	12	E3247.158M -E3247.121S	0.0	9.6	9.6
954	11.4.11	1	7	E3259.149M	28.3	28.3	0.0
957	12.4.2	1	3	E3362.143M -E3261.209S	45.1	69.5	24.4
960	12.4.2	1	3	E3261.209S	69.5	69.5	0.0
962	12.4.2	1	3	E3362.143M -E3261.209S	45.1	69.5	24.4
963	12.4.2	1	3	E3347.106M E3261.209S	59.9	69.5	9.6
1026	12.4.2	1	3	E3347.106M E3261.209S	59.9	69.5	9.6
1034	20.4.8	1	16	E3862.461M	23.7	23.7	0.0
1036	20.4.8	1	16	E3261.370S -E3560.312S	0.0	14.1	14.1
1057	23.4.16	1	2	E3359.368M	39.9	39.9	0.0
1107	23.4.8	2	2	E3861.189M	26.7	26.7	0.0
			10	E3362.250M	53.7	53.7	0.0
1108	23.4.8	1	2	E3349.155M -E3247.159M	0.0	38.2	38.2
1109	23.4.8	1	2	E3349.155M -E3247.159M	0.0	38.2	38.2
1111	23.4.8	2	2	E3349.155M -E3861.189M	0.0	26.7	26.7
			10	E3362.250M	53.7	53.7	0.0
1112	23.4.8	1	2	E3349.155M -E3861.189M	0.0	26.7	26.7
1113	23.4.8	2	2	E3861.189M -E3247.159M	26.7	38.2	11.5
			10	E3362.250M	53.7	53.7	0.0
1115	23.4.8	2	2	E3359.368M -E3247.159M	34.2	38.2	4.0
			10	E3362.250M	53.7	53.7	0.0

Appendix 3.1 (continued)

DH Line[1]	BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
1126	11.4.11	1	12	E3247.158M	0.0	0.0	0.0
1155	26.4.2	3	6	E3359.147M	45.3	45.3	0.0
			13	E3859.64M -E3362.112M	71.4	103.7	32.3
			13	E3249.154M	8.4	8.4	0.0
1157	26.4.2	1	13	E3249.154M -E4060.524M	8.4	76.7	68.3
1158	26.4.2	1	13	E3859.64M -E3362.112M	71.4	103.7	32.3
1172	23.4.8	1	2	E3349.155M -E3247.159M	0.0	38.2	38.2
1175	23.4.3	1	8	E3359.001M -E3359.002M	10.2	37.6	27.4
1182	11.4.5	2	11	E3259.337S -E3259.330M	8.2	8.5	0.3
			12	E3247.158M	0.0	0.0	0.0
1183	11.4.5	2	11	E3259.337S	8.2	8.2	0.0
			12	E3247.158M	0.0	0.0	0.0
1186	23.4.3	1	10	E3362.250M	53.7	53.7	0.0
1187	23.4.3	2	8	E3359.64M	34.4	34.4	0.0
			10	E3362.250M	53.7	53.7	0.0
1199	14.4.15	1	12	E3247.121S	9.6	9.6	0.0
1201	14.4.15	1	12	E3247.158M -E3247.121S	0.0	9.6	9.6
1202	14.4.15	1	12	E3247.158M -E3247.121S	0.0	9.6	9.6
1303	25.4.8	1	13	E3859.64M -E4060.524M	71.4	76.7	5.3
1332	26.4.2	1	13	E3359.234M -E3362.112M	101.0	103.7	2.7
1360	9.4.4	1	13	E3247.193S	105.8	105.8	0.0
1373	25.4.8	1	13	E3359.234M	101.0	101.0	0.0
1380	25.4.8	2	2	E4060.199M	57.9	57.9	0.0
			13	E3348.323M -E3248.68M	28.6	55.1	26.5
1538	26.4.2	1	13	E3362.118M -E3362.112M	57.7	103.7	46.0
1548	26.4.2	2	13	E3249.154M -E3362.118M	8.4	57.7	49.3
			13	E3859.64M -E3359.234M	71.4	101.0	29.6

[1]Identification number of the DH substitution line

[2]Identification number of the parental BC_4 plant

[3]Number of donor segments in the DH substitution line

[4]'LG' is the identification number of the linkage group (Uzunova et al. 1995)

[5]First and last marker on the donor segment

[6]'Start' indicates where the donor segment starts within the indicated linkage group

[7]'End' depicts where the donor segment ends within the indicated linkage group

[8]Centimorgan length of the donor segment between the first and last markers within the linkage group indicated and calculated as centi Morgan length of the 'End' minus the 'Start' of the donor segment within the linkage group.

Appendix 3.2 Donor segments in the selected BC_4 plants used as microspore donors to generate DH substitution lines

BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
1.4.3	1	19	E3862.371M -E3349.461S	0.0	18.6	18.6
		19	E3862.371M -E3349.461S	0.0	18.6	18.6
2.4.1	2	14	E3261.218M -E3261.107M	58.2	118.1	59.9
		16	E3261.370S	0.0	0.0	0.0
2.4.6	1	9	E3859.105M	72.5	72.5	0.0
2.4.16	2	14	E3261.218M -E3261.107M	58.2	118.1	59.9
		16	E3261.370S	0.0	0.0	0.0
3.4.5	1	12	E3862.129M	139.8	139.8	0.0
3.4.7	1	2	E3349.155M	0.0	0.0	0.0
3.4.12	2	18	E3259.194S	62.1	62.1	0.0
		19	E3862.371M	0.0	0.0	0.0
3.4.14	2	18	E3259.194S	62.1	62.1	0.0
		19	E3862.371M	0.0	0.0	0.0
4.4.1	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7
4.4.7	1	6	E3359.564M -E3247.335M	42.0	61.7	19.7
5.4.7	1	18	E3861.459M -E3250.339M	84.2	99.3	15.1
6.4.2	1	5	E4060.559M	79.9	79.9	0.0
7.4.7	1	17	E3261.166M -E3861.237M	30.7	53.2	22.5
7.4.8	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0
7.4.9	1	3	E3362.143M -E3261.234M	45.1	51.1	6.0
8.4.10	1	15	E3347.160S	75.2	75.2	0.0
9.4.2	2	13	E3247.186S -E3247.314M	10.8	31.3	20.5
		19	E3359.225M	41.3	41.3	0.0
9.4.9	1	19	E3359.225M	41.3	41.3	0.0
10.4.2	1	14	E3261.218M -E3359.109M	58.2	80.5	22.3
10.4.10	3	9	E3859.105M	69.8	69.8	0.0
		14	E3261.218M -E3359.109M	58.2	80.5	22.3
		18	E3250.339M	99.3	99.3	0.0
11.4.5	2	11	E3259.330M	8.5	8.5	0.0
		12	E3247.158M -E3348.58M	0.0	7.3	7.3
11.4.11	2	7	E3259.149M	28.3	28.3	0.0
		12	E3247.158M -E3348.58M	0.0	7.3	7.3
11.4.15	2	7	E3259.149M	28.3	28.3	0.0
		11	E3259.330M	8.5	8.5	0.0
12.4.2	1	3	E3362.143M -E3261.209S	45.1	69.5	24.4
13.4.3	1	8	E3362.M1M -E3262.M2M	48.2	66.8	18.6
13.4.4	1	8	E3362.M1M -E3262.M2M	48.2	66.8	18.6
13.4.10	1	15	E3259.167M -E3349.107M	37.7	41.7	4.0
14.4.8	2	2	E3861.189M -E3247.159M	26.7	38.2	11.5
		14	E3261.107M	118.1	118.1	0.0
14.4.9	2	2	E3861.189M -E3247.159M	26.7	38.2	11.5
		18	E3861.459M	84.2	84.2	0.0

Appendix 3.2 (continued)

BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
14.4.15	2	12	E3247.158M -E3348.58M	0.0	7.3	0.0
		14	E3261.107M	118.1	118.1	0.0
15.4.9	1	9	E3247.394M -E3249.409S	0.0	16.3	16.3
15.4.12	1	9	E3247.394M -E3249.409S	0.0	16.3	16.3
16.4.1	2	1	E3362.130S	48.5	48.5	0.0
		3	E3259.72M -E3347.106M	20.8	59.9	39.1
16.4.11	1	1	E3362.130S	48.5	48.5	0.0
18.4.3	2	12	E3262.M1M	109.1	109.1	0.0
		18	E3861.459M -E3250.339M	84.2	99.3	15.1
18.4.10	3	12	E3247.158M -E3348.58M	0.0	7.3	7.3
		18	E3861.459M -E3250.339M	84.2	99.3	15.1
		12	E3262.M1M	109.1	109.1	0.0
18.4.11	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
18.4.12	1	2	E4060.199M -E3361.138M	57.9	61.5	3.6
18.4.15	2	12	E3250.192S	61.2	61.2	0.0
		18	E3861.459M -E3250.339M	84.2	99.3	15.1
19.4.1	2	12	E3361.M1M -E3862.129M	101.6	139.8	38.2
		17	E3862.77M	0.0	0.0	0.0
19.4.4	2	3	E3259.72M - E3348.M1M	20.8	51.1	30.3
		12	E3361.M1M	101.6	101.6	
19.4.5	2	5	E4060.559M	79.9	79.9	0.0
		17	E3862.77M	0.0	0.0	0.0
19.4.6	3	3	E3259.72M -E3362.143M	20.8	45.1	24.3
		5	E4060.559M	79.9	79.9	0.0
		12	E3361.M1M -E3862.129M	101.6	139.8	38.2
20.4.8	1	16	E3261.370S -E3862.461M	0.0	23.7	23.7
20.4.10	2	16	E3261.370S -E3862.461M	0.0	23.7	23.7
		6	E3359.564M – E3359.147M	42.0	45.3	3.3
21.4.3	1	20	E3349.199M	0.0	0.0	0.0
21.4.5	2	5	E3859.289M	39.9	39.9	0.0
		14	E3862.229M	4.0	4.0	0.0
21.4.9	1	13	E3249.154M -E3247.314M	8.4	31.3	23.5
21.4.15	1	5	E3859.289M	39.9	39.9	0.0
22.4.1	3	2	E3349.155M -E3247.159M	0.0	38.2	38.2
		6	E3247.335M	61.7	61.7	0.0
		14	E3862.229M -E4060.447M	1.6	36.6	35.0
22.4.4	3	2	E3349.155M	0.0	0.0	0.0
	3	11	E3259.330M	8.5	8.5	0.0
	3	14	E3862.229M -E4060.447M	4.0	39.0	35.0
22.4.7	3	6	E3359.564M -E3247.335M	42.0	61.7	19.7
		10	E3362.211S	19.6	19.6	0.0
		16	E3362.204S	105.9	105.9	0.0

Appendix 3.2 (continued)

BC_4 Pl.[2]	No. Seg.[3]	LG[4]	First and last marker[5]	Start[6] (cM)	End[7] (cM)	Length[8] (cM)
22.4.9	2	6	E3359.564M -E3247.335M	42.0	61.7	19.7
	2	14	E3259.70M -E4060.447M	18.4	39.0	20.6
23.4.3	2	8	E3359.64M -E3359.M2M	36.7	39.9	3.2
	2	10	E3362.250M	53.7	53.7	
23.4.8	2	2	E3349.155M -E3247.159M	0.0	38.2	38.2
	2	10	E3362.250M	53.7	53.7	0.0
23.4.16	2	8	E3359.64M -E3359.M2M	36.7	39.9	3.2
	2	10	E3362.250M	53.7	53.7	
24.4.3	1	10	E3862.82M -E3861.148M	2.6	58.9	56.3
24.4.5	2	10	E3862.82M -E3862.149M	2.6	14.0	11.4
	2	15	E3347.160S	75.2	75.2	0.0
24.4.7	2	10	E3862.82M -E3247-M1M	2.6	43.1	40.5
	2	15	E3347.160S	75.2	75.2	0.0
24.4.8	1	19	E3359.225M	41.3	41.3	0.0
24.4.10	1	10	E3862.82M -E3861.148M	2.6	58.9	56.3
25.4.6	2	2	E4060.199M	57.9	59.1	1.2
	2	13	E3348.323M -E3359.234M	28.6	101.0	72.4
25.4.8	1	13	E3348.323M -E3359.234M	28.6	101.0	72.4
26.4.1	2	13	E3249.154M -E3362.118M	8.4	57.7	49.3
	2	15	E3261.142M	2.6	2.6	0.0
26.4.2	2	6	E3359.564M -E3359.147M	42.0	45.3	3.3
	2	13	E3249.154M -E3362.112M	8.4	103.7	95.3
26.4.7	2	6	E3359.564M -E3359.147M	42.0	45.3	3.3
	2	13	E3348.323M -E3362.112M	28.6	103.7	75.1
26.4.12	2	13	E3249.154M -E4060.524M	8.4	76.7	68.3
	2	15	E3261.142M	2.6	2.6	0.0

* See appendix 3.1 for the descriptions of superscripts

Appendix 3.3 Parental BC_4 plants of the DH substitution lines not analysed with markers

DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]
8	2.4.16	459	3.4.14	978	8.4.10	1326	26.4.2
10	3.4.14	464	14.4.10	983	8.4.10	1340	15.4.12
12	3.4.12	470	14.4.8	990	16.4.11	1351	25.4.6
16	3.4.7	473	9.4.9	991	16.4.11	1354	16.4.4
17	3.4.7	498	18.4.10	994	16.4.11	1356	16.4.4
18	3.4.7	500	3.4.14	996	16.4.11	1361	22.4.3
24	2.4.6	504	24.4.7	997	16.4.11	1366	12.4.2
26	3.4.7	506	4.4.1	1000	16.4.4	1367	12.4.2
27	3.4.7	518	19.4.5	1001	16.4.4	1377	25.4.6
50	2.4.16	534	21.4.15	1005	16.4.4	1378	25.4.8
57	5.4.7	535	21.4.15	1006	16.4.4	1385	21.4.5
58	5.4.7	536	21.4.15	1007	16.4.4	1386	21.4.5
61	5.4.7	538	21.4.15	1008	9.4.9	1391	21.4.11
72	3.4.12	539	14.4.15	1024	21.4.9	1395	21.4.5
88	13.4.10	548	8.4.10	1030	22.4.1	1397	21.4.5
90	19.4.4	549	14.4.10	1037	20.4.8	1399	9.4.9
91	19.4.4	553	11.4.15	1051	14.4.8	1400	9.4.9
96	7.4.10	560	11.4.15	1053	14.4.8	1402	23.4.15
100	4.4.7	576	18.4.3	1054	14.4.8	1413	23.4.3
108	10.4.10	578	4.4.1	1065	22.4.3	1428	23.4.8
114	4.4.1	589	4.4.7	1069	22.4.3	1433	20.4.8
122	5.4.7	608	13.4.4	1073	8.4.10	1439	16.4.11
132	18.4.11	613	9.4.9	1078	14.4.10	1507	23.4.3
156	3.4.5	627	11.4.15	1084	22.4.3	1508	23.4.3
158	7.4.9	641	9.4.9	1093	16.4.1	1509	23.4.3
162	2.4.16	656	8.4.10	1094	16.4.1	1514	23.4.3
182	24.4.8	659	22.4.4	1099	16.4.1	1515	23.4.3
187	19.4.6	674	8.4.10	1103	16.4.1	1520	23.4.3
191	19.4.6	677	18.4.3	1105	16.4.1	1522	9.4.9
192	13.4.10	683	11.4.5	1116	8.4.10	1528	16.4.4
197	7.4.10	689	14.4.10	1118	8.4.10	1530	16.4.4
203	10.4.10	694	9.4.9	1119	8.4.10	1533	21.4.5
210	3.4.14	704	19.4.5	1122	22.4.3	1537	26.4.2
214	18.4.10	708	19.4.5	1131	11.4.15	1545	26.4.2
223	3.4.5	711	13.4.4	1133	11.4.15	1550	21.4.9
227	10.4.10	717	19.4.5	1137	26.4.1	1563	26.4.1
244	24.4.10	718	19.4.5	1140	26.4.1	1565	26.4.1
263	2.4.16	720	1.4.13	1141	26.4.1	1568	23.4.8
280	10.4.2	728	7.4.10	1142	26.4.1	1572	26.4.7
287	18.4.3	772	3.4.14	1145	21.4.3	1580	15.4.9
291	7.4.9	785	7.4.10	1148	21.4.3	1589	26.4.1
317	1.4.13	786	7.4.10	1150	26.4.7	1594	23.4.15
318	1.4.13	789	4.4.7	1165	16.4.11	1610	15.4.12

Appendix 3.3 (continued)

DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]	DH Line[1]	BC_4 Pl.[2]
339	3.4.12	795	18.4.10	1167	16.4.11	1612	16.4.11
350	4.4.7	809	7.4.10	1196	11.4.15	1617	23.4.8
356	2.4.6	814	3.4.5	1204	22.4.9	1619	16.4.4
362	1.4.3	831	3.4.5	1209	25.4.8	1633	12.4.2
372	10.4.2	835	3.4.5	1213	25.4.8	1648	23.4.16
379	7.4.8	842	4.4.1	1220	21.4.3	1663	23.4.8
386	19.4.4	863	3.4.5	1223	21.4.3	1691	26.4.1
387	10.4.10	869	3.4.5	1225	21.4.3	1704	22.4.4
390	1.4.13	873	22.4.7	1228	21.4.3	1722	20.4.15
409	4.4.7	900	22.4.9	1250	26.4.1		
410	10.4.2	926	14.4.8	1292	21.4.9		
430	18.4.3	932	20.4.8	1298	25.4.8		
437	13.4.4	941	11.4.15	1306	16.4.1		
447	24.4.7	944	11.4.15	1314	6.4.1		
449	13.4.3	971	8.4.10	1315	16.4.1		
451	1.4.13	975	8.4.10	1316	16.4.1		

[1]Identification number of the DH substitution line

[2]Identification number of the microspore donor BC_4 plant used to generate the DH lines

Appendix 4.1. Donor genome representation in 101 DH substitution lines analysed by markers across the mapped linkage groups

LG[1]	First and last marker[2]	Start[3] (cM)	End[4] (cM)	Length[5] (cM)
1	E3862.196M	48.5	48.5	0.0
1	E3261.47M	54.5	54.5	0.0
2	E3349.155M	0.0	0.0	0.0
2	E3349.155M -E3861.189M	0.0	26.7	26.7
2	E3349.155M -E3247.159M	0.0	38.2	38.2
2	E3861.189M	26.7	26.7	0.0
2	E3861.189M -E3247.159M	26.7	38.2	11.5
2	E3359.368M -E3247.159M	34.2	38.2	4.0
2	E4060.199M	57.9	57.9	0.0
2	E4060.199M -E4060.199M	57.9	59.1	1.2
2	E4060.199M -E3361.138M	57.9	61.5	3.6
2	E3361.138M	61.5	61.5	0.0
3	E3259.72M -E3362.143M	20.8	45.1	24.3
3	E3259.72M -E3348.001M	20.8	51.0	30.2
3	E3362.143M	45.1	45.1	0.0
3	E3362.143M -E3348.001M	45.1	51.0	5.9
3	E3362.143M -E3261.209S	45.1	69.5	24.4
3	E3347.106M E3261.209S	59.9	69.5	9.6
3	E3261.209S	69.5	69.5	0.0
5	E3259.176S -E4060.559M	67.2	70.8	3.6
5	E4060.559M	70.8	70.8	0.0
7	E3259.149M	28.3	28.3	0.0
8	E3359.64M	34.4	34.4	0.0
8	E3362.001M -E3262.002M	45.9	64.5	18.6
9	E3247.394M	0.0	0.0	0.0
9	E3859.105M	72.5	72.5	0.0
10	E3862.82M	2.6	2.6	0.0
10	E3862.149M	14.0	14.0	0.0
10	E3362.250M	53.7	53.7	0.0
11	E3259.337S -E3259.330M	8.2	8.5	0.3
12	E3247.158M	0.0	0.0	0.0
12	E3247.158M -E3348.58M	0.0	7.3	7.3
12	E3247.158M -E3247.121S	0.0	9.6	9.6
12	E3247.121S	9.6	9.6	0.0
12	E3247.121S -E3250.192S	9.6	61.2	51.6
12	E3247.181S -E3250.192S	59.1	61.2	2.1
12	E3250.192S	62.2	62.2	0.0
12	E3361.001M	101.6	101.6	0.0
12	E3361.001M -E3862.129M	101.6	139.8	38.2
12	E3247.202S	132.7	132.7	0.0
12	E3247.202S -E3862.129M	132.7	139.8	7.1

Appendix 4.1. continued

LG[1]	First and last marker[2]	Start[3] (cM)	End[4] (cM)	Length[5] (cM)
13	E3247.158M	0.0	0.0	0.0
13	E3249.154M	8.4	8.4	0.0
13	E3249.154M -E4060.524M	8.4	76.7	68.3
13	E3247.186S -E3247.314M	10.8	31.3	20.5
13	E3348.323M	28.6	28.6	0.0
13	E3348.323M -E3248.68M	28.6	55.1	26.5
13	E3362.118M -E3362.112M	57.7	103.7	46.0
13	E3859.64M -E4060.524M	71.4	76.7	5.3
13	E3859.64M -E3359.234M	71.4	101.0	29.6
13	E3859.64M -E3362.112M	71.4	103.7	32.3
13	E3359.234M	101.0	101.0	0.0
13	E3359.234M -E3362.112M	101.0	103.7	2.7
14	E3259.70M	18.4	18.4	0.0
14	E3261.218M	58.2	58.2	0.0
14	E3261.218M -E3359.109M	58.2	80.5	22.3
14	E3261.218M -E3859.200M	58.2	93.5	35.3
14	E3261.218M -E3261.107M	58.2	118.1	59.9
14	E3859.200M -E3261.107M	93.5	118.1	24.6
14	E3261.107M	118.1	118.1	0.0
16	E3261.370S	0.0	0.0	0.0
16	E3261.370S -E3560.312S	0.0	14.1	14.1
16	E3261.370S -E3862.461M	0.0	23.7	23.7
16	E3362.293S	4.7	4.7	0.0
16	E3862.461M	23.7	23.7	0.0
17	E3862.77M	0.0	0.0	0.0
17	E32/M50-F-449-S	10.7	10.2	0.0
17	E3261.166M -E3861.237M	30.7	53.2	22.5
17	E3862.327S -E3861.237M	43.7	53.2	9.5
18	E3262.186S	46.7	46.7	0.0
18	E3861.459M -E3250.339M	84.2	99.3	15.1
18	E3250.339M -E3859.243S	99.3	99.3	0.0
19	E3862.371M	0.0	0.0	0.0
19	E3862.371M -E3349.461S	0.0	18.6	18.6

[1]Linkage group according to Uzunova et al. (1995)

[2]First and last markers on the indicated donor segment

[3]Start indicates where the donor segment starts within the indicated linkage group

[4]End depicts where the donor segment ends within the indicated linkage group

[5]Minimum length of the donor segment as defined by the distance between the first and the last marker on the segment.

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04/1998 -09/1998	German language course in Goethe Institute, Goettingen, and awarded DSH certificate
09/1989 -08/1993	Studied Agricultural Economics at Haromaya University, Ethiopia and awarded Bsc. in Agriculture
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