GENETIC VARIATION AND INHERITANCE OF PHYTOSTEROL CONTENT IN *BRASSICA NAPUS* L.

DISSERTATION

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1 INTRODUCTION

The growing economic importance of rapeseed (*Brassica napus* L.) and its most recent utilization as a novel source of renewable energy is mainly due to its increased oil content and improved oil quality, high nutritive value protein and enhanced yield and yield stability. Recently, attention has also been focused on minor oil and protein constituents like sterols (Hamama *et al*. 2003), tocopherols (Marwede *et al*. 2004), carotenoids (Shewmaker *et al*. 1999) and sinapate esters (Zum Felde *et al*. 2006). Altering the content and composition of those constituents could give an added value to rapeseed oil, rapeseed meal and their derived products.

As essential cell membrane constituents, sterols are widely distributed in all eukaryotic organisms. Plant cells synthesize a complex array of plant sterols, called phytosterols, with the phytosterol profile varying between species, whereas animal and fungal cells produce only one sterol each, cholesterol and ergosterol, respectively. However, the most common phytosterols are sitosterol, campesterol and stigmasterol, while brassicasterol is typical for *Brassicaceae* family and avenasterol for cereals. Phytosterols are predominantly present in oilseed plants and cereal lipids and their content in vegetables, fruits, nuts and berries is considerably lower (Piironen *et al*. 2003, Piironen *et al.* 2002). Rapeseed oil is, after corn oil, the second richest natural source of phytosterols contributing up to 1% of the crude rapeseed oil (Piironen *et al*. 2000, Gordon and Miller 1997).

For more than half a century the capacity of phytosterols to lower cholesterol absorption and serum LDL-cholesterol level has been well-known (Best *et al*. 1954). Following their consumption, dietary and endogenous cholesterol absorption is being reduced by about 50% (Law 2000). And only two decades ago increased level of plasma cholesterol has been recognized as one of the main risk factors of cardio vascular diseases – the leading cause of mortality in Western countries (Castelli 1984). Since, the cholesterol reduction by health advice and dietary recommendations has been of limited effectiveness, the cholesterol-reducing benefits of phytosterols have been used in ‘functional food’ production, *i.e.* milk and dairy products fortified with natural phytosterols. In most cases, phytosterols are obtained as a by-product during vegetable oil refining but, nevertheless, enhancing the phytosterol content and

modifying their composition in oil seed crops could give an added value to vegetable oils and oil-derived products. Even so, only a limited number of studies report about genetic variation or environmental effects on phytosterol content in oilseed rape (Hamama *et al*. 2003, Abidi *et al*. 1999, Gordon and Miller 1997, Appelqvist *et al*. 1981), whereas possible correlations between phytosterols and other seed quality traits, or genetic inheritance of phytosterol content have not at all been investigated. A probable reasons for this could be that a rather sophisticated extraction and derivatisation method is required for phytosterol identification and that so far there is no gas-chromatographic analysis for their accurate quantification suitable for plant breeding purposes. Quantitative Trait Loci (QTL), on molecular linkage maps of the rapeseed genome, have already been identified for quality traits such as oil, fatty acids, glucosinolates or tocopherols (Qiu *et al*. 2006, Marwede *et al*. 2005, Zhao *et al*. 2005, Ecke *et al*. 1995), however, no QTL for phytosterol content have been mapped so far.

In canola seeds, sinapate esters, like sinapine and sinapoylglucose predominate while sinapate and other sinapate esters, are found only in lower concentrations. As minor rapeseed meal constituents sinapate esters make up 1 to 2% of the seed dry matter (Bell 1993) contributing to the bitter taste, dark colour and low nutritive value of the meal (Shahidi and Naczk 1992), thus compromising the use or rapeseed protein in food and feed industry. A substantial reduction of sinapate esters, which could give an added value to the rapeseed meal, now seems possible considering the available large natural genetic variation in sinapate ester content (Zum Felde *et al*. 2006) along with the success of transgenic approaches to drastically lower the sinapate ester content (Hüsken *et al*. 2005) and the availability of a Near-Infrared Reflectance Spectroscopic (NIRS) calibration for these compounds (Zum Felde *et al*. 2007).

The present research was initiated to develop a high throughput gas-chromatographic method for an accurate assessment of phytosterol content and composition in a large number of seeds of oilseed rape, which would be appropriate for plant breeding purposes. The first aim was to determine genetic variation for total and individual phytosterol content, genetic and genotype *x* environment interaction effects on phytosterol content as well as the heritability of phytosterols in three winter rapeseed doubled haploid populations, grown in different environments. Additionally,

the correlations between phytosterol content and other important seed quality traits were supposed to be analysed. The second main objective was to identify QTL for phytosterol and sinapate ester content in a winter rapeseed doubled haploid population, previously found to show a large variation for sinapate esters (Zum Felde *et al*. 2006) and to assess the possible correlations between phytosterols, sinapate esters and other important seed traits. The third goal was to analyse the genetic variation in phytosterol content in a genetically diverse rapeseed material, including modern cultivars, resynthesized lines and gene bank accessions, grown during different years at different locations and to develop NIRS calibration equations which could provide a good alternative for fast, non-destructive and cost-effective estimation of phytosterol content in seeds.

2 RESULTS AND DISCUSSION (MANUSCRIPT I)

GENETIC VARIATION AND GENOTYPE *X* ENVIRONMENT INTERACTIONS FOR PHYTOSTEROL CONTENT IN *BRASSICA NAPUS* L.

Economic importance of oilseed rape has lately increased largely due to utilisation of its high-grade oil. Apart from improving oil content and oil quality, attention has recently been directed to valuable minor oil-constituents like tocopherols, phytosterols and carotenoids recognised for their antioxidative, cholesterol-lowering and other health-benefiting potentials. Regarding phytosterols, rapeseed oil is, after corn oil, the second richest natural source of phytosterols (Piironen *et al*. 2000), with contents ranging from 0.5 to 1 percent of the oil (Hamama *et al*. 2003). The major objectives of this study were to develop a gas-chromatographic (GC) method for the high throughput analysis of the phytosterol content and composition in seeds of oilseed rape, to determine the genetic variation, genetic and genotype *x* environment effects and the heritability of phytosterol content in three winter rapeseed doubled haploid (DH) populations and to analyse correlations between phytosterols and other economically important seed traits.

The accuracy of the GC method was verified by the three comparative measurements at the Institute for Lipid Research in Münster, the Swedish University of Agricultural Sciences and by participation in an official ring trial of the German association of lipid sciences (DGF). The advantage of the present method is the direct alkaline hydrolysis on the seed meal without quantitative oil extraction, contributing to its simplicity and suitability for the accurate phytosterol analysis in plant breeding programmes. Sitosterol was found to be the most prominent phytosterol, accounting for 50% of the total phytosterol content, followed by campesterol (29%) brassicasterol (13%), avenasterol (4%) and stigmasterol (traces); similar relative contents in rapeseed were observed in studies from Warner and Mounts (1990) and Appelqvist *et al*. (1981). For total and individual phytosterol content large and significant variations were detected in all three DH populations. Total phytosterol content ranged from 2570 to 4150 mg/kg seed and from 4480 to 9380 mg/kg oil, which compares well with the results from previous studies (Hamama *et al*. 2003, Vlahakis and Hazebroek 2000, Gordon and Miller 1997). Predominant and highly significant effects of the

genotypes in comparison to the genotype *x* environment interaction effects, resulted in overall high heritabilities for total phytosterols (0.84 to 0.91), indicating that effective selection for high phytosterol content would be possible with limited effort with respect to the number of test environments. There are currently no indications that increased phytosterol content could be negatively associated with other relevant seed quality traits. The observed negative correlation between oil content and total phytosterol content (r_g=-0.67^{++}) in one population was explained by an indirect effect of erucic acid segregating in this population (see Manuscript II). A positive correlation (r_g=0.59^{++}) between oil and phytosterol content were detected in the second DH population segregating for oleic and linolenic acid content. However, since oil content was not significantly correlated with oleic (r_g=0.14) and linolenic (r_g=-0.14) acid content, this did not provide an explanation for the positive correlation between phytosterols and oil content in this population. In the third DH population, derived from a cross between two 'high erucic acid' parents, there was no correlation between oil and phytosterol content (r_g=0.06). Phytosterol content was not correlated to protein and glucosinolate content indicating that breeding for enhanced phytosterol content and modified composition should be possible without affecting other seed quality traits.

3 RESULTS AND DISCUSSION (MANUSCRIPT II)

MAPPING QTL FOR PHYTOSTEROL AND SINAPATE ESTER CONTENT IN *BRASSICA NAPUS* L.

With regard to the food and feed purposes improved oil and protein quality is considered as an important goal in rapeseed breeding programmes. Seeds of canola quality oilseed rape have been shown to contain high amounts of phytosterols and sinapate esters. Whereas phytosterols are being used to enrich milk- and oil-derived food products, sinapate esters are limiting the utilisation of the rapeseed proteins in the feed industry. Enhancing the phytosterol content of oil and lowering sinapate ester content of the meal could therefore give an added value to the oilseed rape crop. This research was aimed to localise and identify QTL for phytosterol and sinapate ester content in a selected double haploid (DH) winter rapeseed population, previously found to contain a large variation for the two traits.

For total phytosterol content three QTL were detected, explaining 60% of the genetic variance. Two major QTL for total phytosterol content on N8 and N13 were at the same time QTL with largest positive additive effects identified for all individual phytosterols. This was not the case for the third, minor QTL on N18. The correlations among individual phytosterols were all significantly positive, suggesting that most of the variation is influenced by the genetic differences before the separation of the pathways leading to the biosynthesis of individual phytosterols. In this case, likely candidate genes for the QTL on N18 could be the gene for either one of the two key biosynthetic enzymes 3-hydroxy-3-methylglutaryl-CoA reductase and cycloartenol C-24-methyltransferase, which have been reported to considerably influence individual and total phytosterol content (Holmberg *et al*. 2003). In the DH population, the two erucic acid genes segregated, and a close negative correlation was found between erucic acid and phytosterol content (r_s=-0.80**). Following path coefficient analysis, erucic acid revealed a negative direct effect on total phytosterol content (-0.76). Further association between total phytosterol and erucic acid content were confirmed with the QTL analysis. Two of the QTL, with the strongest additive effects, map on linkage groups N8 and N13 within the confidence intervals of the two erucic acid genes (Ecke *et al.* 1995). Apparently, there is either a pleiotropic effect of the

erucic acid genes on the phytosterol content or two major phytosterol genes could be closely linked to the two erucic acid genes. The later hypothesis could be verified by the comparative analysis of phytosterol content in transgenic rapeseed lines expressing the erucic acid *fae*1-gene (James *et al.* 1995) in otherwise low erucic acid background (Han *et al.* 2001). The former hypothesis could be explained by the competition for acetyl-CoA, an early common precursor available only in limited quantities (Fatland *et al.* 2005). It may be easily anticipated that a drastic change in erucic acid content, can significantly modify the phytosterol content, regarding the fact that four molecules of acetyl-CoA are needed to form one molecule of erucic acid (Puyaubert *et al.* 2005) and three molecules of acetyl-CoA are needed to form one molecule of phytosterol (Chappell 1995).

Altogether six QTL for total sinapate ester content were detected, explaining 56% of the genetic variance. A close negative association between erucic acid and total sinapate ester content (r_s=-0.66**) was also confirmed with the QTL analysis, showing that the two QTL, with the strongest additive effect, for total sinapate ester content mapped at a very similar position as the two erucic acid genes. The strong negative correlation between sinapate esters and oil content (r_s=-0.71**) along with the results from path coefficient analysis showing a stronger direct effect of oil on sinapate esters (-0.49) than of erucic acid on sinapate esters (-0.32), may be explained by the competition for plastidic phosphoenolpyruvate (PEP), a common precursor for *de novo* fatty acid and sinapate ester biosynthesis (Fischer *et al.* 1997). Amongst other enzyme activities, pyruvate kinase, which uses the PEP as a substrate, has been found to be enhanced in a *Brassica napus* line with high oil content compared to a near-isogenic line with a low oil content, corroborating the importance of this step in storage lipid biosynthesis (Li *et al.* 2006).

4 RESULTS AND DISCUSSION (MANUSCRIPT III)

GENETIC VARIATION IN PHYTOSTEROL CONTENT IN *BRASSICA NAPUS* L. AND DEVELOPMENT OF NIRS CALIBRATION EQUATIONS

At present, little is known about the genetic variation in phytosterol content of rapeseed genotypes since their accurate identification and quantification require rather complicated extraction and derivatisation sample preparation procedure followed by time-consuming gas-chromatographic (GC) analyses (Dutta and Normen 1998). Even though within the scope of this thesis a more simplified gas-chromatographic method for breeding purposes has been developed, the method is nevertheless destructive, laborious and costly. The objectives of the present work were to study the variation in phytosterol content in a genetically diverse set of 2246 rapeseed lines tested in different years and locations, including resynthesized lines, modern winter cultivars and gene bank accessions, and to develop a Near-Infrared Reflectance Spectroscopic (NIRS) calibration equation which could provide a good alternative for the high throughput and non-destructive estimation of seed phytosterol content.

The phytosterol variation in the complete winter rapeseed germplasm collection was significant, ranging from 2000 to 4800 mg/kg seed. The modern cultivars had the highest phytosterol contents, however, the range of 3600 to 4800 mg/kg seed was rather limited. This may be explained by the fact that the cultivars are rather closely related and that all of them had a low erucic acid content in the seed oil. The resynthesized lines revealed comparably higher phytosterol variation than the cultivars (2100 to 4300 mg/kg seed). The phytosterol content of the resynthesized lines was negatively correlated with erucic acid content (r_s=-0.50**). Explained fraction of variance in cross-validation (R^2_{CV}) ranged from 0.45 for avenasterol to 0.76 for total phytosterols. The prediction of the NIRS calibration, calculated through standard deviation and standard error of cross-validation ratio, was highest for total phytosterols (2.0) implying that the calibration could be quite useful for identifying genotypes with low, medium or high phytosterol content (Fontaine *et al*. 2001). When compared with the resynthesized lines the accessions of the gene bank material showed somewhat limited genetic variation in total phytosterol content (2400 to 4200

mg/kg seed). As found for the resynthesized lines, the erucic acid content in the gene bank collection was negatively correlated with total phytosterol content (r_s=-0.77**).

5 SUMMARY

Improving oil and protein quality for food and feed purposes is considered as an important goal in rapeseed (*Brassica napus* L.) breeding programmes. Seeds of oilseed rape have been shown to contain high amounts of phytosterols and sinapate esters. Whereas phytosterols, known for their cholesterol-lowering properties, are being used to enrich oil- and milk-derived food products, sinapate esters are limiting the utilisation of the rapeseed proteins in the feed industry. Enhancing the phytosterol content of the oil and lowering sinapate ester content of the meal could thus give an added value to the oilseed rape crop.

This research was initiated to develop a gas-chromatographic (GC) method for the accurate analysis of phytosterol content and composition in seeds of oilseed rape. The study was further aimed to determine the genetic variation, the genotype *x* environment interactions and the heritability of phytosterol content in three winter rapeseed doubled haploid (DH) populations grown in different environments and to analyse the correlations between phytosterols and other seed quality traits. The subsequent goal was to localise QTL for phytosterol and sinapate ester content in a DH population, previously found to contain a large variation for these two traits. The final objective was to study the variation in phytosterol content in a genetically diverse set of rapeseed lines and to develop a Near-Infrared Reflectance Spectroscopic (NIRS) calibration equation for high throughput and non-destructive phytosterol evaluation.

The advantage of the developed GC method is the direct alkaline hydrolysis on the seed meal without quantitative oil extraction, contributing to its simplicity and suitability for the accurate phytosterol analysis in plant breeding programmes. In the three DH populations of winter oilseed rape sitosterol and campesterol were detected as the two major phytosterols followed by brassicasterol, avenasterol and stigmasterol. Large genetic differences were found with total phytosterol contents ranging from 2570 to 4150 mg/kg seed. Predominant genetic effects and high heritabilities ranging from 0.71 to 0.97 were detected for total and individual phytosterol content in all three populations, indicating that an effective selection and breeding for enhanced phytosterol genotypes would be possible. Up to now there are

no indications that increased phytosterol content could be negatively associated with other economically important traits like oil, protein and glucosinolates.

The DH population showing the largest variation in total phytosterol content, segregated for the two erucic acid genes. A close negative correlation was found between erucic acid and phytosterol content (r_s=-0.80**). Two of the three QTL detected for total phytosterol content, mapped on the same linkage groups and within the confidence intervals of the two major erucic acid genes, suggesting that the erucic acid genes exert a negative pleiotropic effect on phytosterol content or, that two major phytosterol genes are closely linked to the two erucic acid genes. The first hypothesis could be explained by competition for cytoplasmic acetyl-CoA, an early precursor for phytosterols biosynthesis, required also for the elongation of oleic acid to erucic acid. For total sinapate ester content six QTL were detected and two of the QTL with the strongest additive main effects, mapped on the same linkage groups and in the same region as the two major erucic acid genes. Again, a close negative correlation was found between erucic acid and total sinapate ester content (r_s=-0.66**), which may be explained by the competition for plastidic phosphoenolpyruvate, a common precursor for *de novo* fatty acid and sinapate ester biosynthesis.

A genetically diverse set of 2246 winter rapeseed samples, from different years and locations, including breeding lines, resynthesized rapeseed lines and modern cultivars was used to developed NIRS calibrations. The phytosterol variation in the complete winter rapeseed germplasm collection ranged from 2000 to 4800 mg/kg seed. Modern cultivars had the highest phytosterol contents, with a range of 3600 to 4800 mg/kg seed. Resynthesized rapeseed lines revealed a comparatively higher phytosterol variation than the cultivars (2100-4300 mg/kg seed). The phytosterol content of the resynthesized lines was negatively correlated with erucic acid content (r_s=-0.50**). The NIRS calibrations showed an explained fraction of variance in cross-validation (R^2_{CV}) ranging from 0.45 for avenasterol to 0.76 for total phytosterols. The ratio standard deviation/SECV was highest for total phytosterols (2.0), indicating that the calibration could be quite useful for distinguishing genotypes with low, medium or high phytosterol contents. Further increases in seed phytosterol content can be expected from crosses among adapted high phytosterol genotypes. The NIRS calibrations should be useful for germplasm screening for genotypes with extreme

phytosterol contents or unusual phytosterol composition and in breeding programmes aimed at increasing the total phytosterol content in rapeseed.

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7 MANUSCRIPT I

GENETIC VARIATION AND GENOTYPE *X* ENVIRONMENT INTERACTIONS FOR PHYTOSTEROL CONTENT IN *BRASSICA NAPUS* L.

ABSTRACT

Phytosterols are natural plant oil constituents known since more than 50 years for their LDL-cholesterol-lowering properties. Functional food products enriched with phytosterols have already been developed and marketed. Enhancing the phytosterol content of oil crops and modifying their composition could give an added value to vegetable oils and derived products. The major objectives of this study were to develop a gas-chromatographic method for the high throughput analysis of the phytosterol content and composition in seeds of oilseed rape, to determine the genetic variation, the genotype *x* environment interactions and the heritability of phytosterol content in three doubled haploid populations of winter oilseed rape and to analyse correlations between phytosterols and other seed quality traits. With the gas-chromatographic analyses sitosterol and campesterol were detected as the two major phytosterols followed by brassicasterol, avenasterol and stigmasterol. Large and highly significant variations were found in total and individual phytosterol content as well as for oil, protein and glucosinolate content in all three populations. The analysis of variance showed predominant genetic effects for total and the individual phytosterol content in comparison to the genotype *x* environment interaction effects, which resulted in overall high heritabilities ranging from 0.71 to 0.97 in all three populations. Phytosterol content was not correlated to protein and glucosinolate content. However, positive, negative and no correlations for oil content with phytosterols, found in three double haploid populations, require further investigations. The large genetic variations along with the observed high heritabilities indicate that breeding for enhanced phytosterol content and modified composition should be possible without affecting other seed quality traits.

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is one of the largest oilseed crops worldwide. Scientific interest in rapeseed and its economic importance has lately increased largely due to the use of the high-grade oil for food purposes and as a source for the production of biodiesel. Crude rapeseed oil contains a number of essential compounds like tocopherols, phytosterols and carotenoids (Mag 1990). Regarding phytosterols, rapeseed oil has been recognised, after corn oil, as the second richest natural source of phytosterols followed by sunflower, cottonseed, soybean and olive oil, cereal grains and nuts (Piironen *et al*. 2000, Gordon and Miller 1997, Warner and Mounts 1990). The phytosterol content in rapeseed oil typically ranges between 0.5 and 1% (Hamama *et al*. 2003, Raymer 2002) and has been found to be twice as high as the phytosterol content in soybean and sunflower oil (Vlahakis and Hazebroek 2000, Cole *et al*. 1998).

Phytosterols are polyisoprenoids and essential components of all eukaryotic membranes. Whereas animal and fungi contain only one major sterol – cholesterol and ergosterol, respectively, plants have a variety of more than 40 different phytosterols (Law 2000). Their structural common feature is that they are all derivatives of a tetra-cyclic nucleus with a flexible side chain. The configuration of the sterol nucleus and the alkylation of the side chain distinguish different phytosterols. The most abundant phytosterols are: sitosterol, campesterol and stigmasterol. Other phytosterols, like avenasterol are synthesised earlier in the biosynthetic pathway and usually occur only in relatively smaller amounts, while brassicasterol is typical for the *Brassicaceae* family. As primary metabolites (Chappell 1995), phytosterols play a vital role in membrane-associated metabolic processes, they are phytohormone precursors and thus involved in important growth and developmental processes (Hartmann 1998). Phytosterols occur either in the free form, or they are esterified with fatty acids and phenolic acids, or conjugated with glucose (phytosteryl glycosides), which may also be acylated (acylated phytosteryl glycosides). Quantitatively, different phytosterol forms may vary with tissue and plant species. Phytosterols esterified with fatty acids have been incorporated into milk and dairy products as functional ingredients with cholesterol-lowering properties, thereby reducing the risk of cardiovascular diseases (Weststrate and Meijer 1998).

Phytostanols are saturated phytosterols known for their capacity of lowering LDL-cholesterol level even more effectively than phytosterols (Salo *et al*. 2003). Phytostanols are quite less abundant in plants than phytosterols and mainly found in cereal lipids (Dutta and Appelqvist 1996) but they can technically be produced by hydrogenation of phytosterols. Sitostanols esterified with polyunsaturated fatty acids most efficiently reduce the intestinal cholesterol absorption and the serum cholesterol concentration, without being absorbed (Nissinen *et al*. 2002). It has also been postulated that avenasterol inhibits oxidative degradation of unsaturated fatty acids at high temperatures (Gordon and Magos 1983).

Similarly to tocopherols, phytosterols are obtained as by-products during oil refining from vegetable oils like soybean, rapeseed and sunflower (Piironen *et al*. 2000). Increasing the phytosterol content in seeds of oilseed rape could give an added value to its oil. However, at present nothing is known about the inheritance of phytosterols in rapeseed the genetic variation in phytosterol content. The probable reasons for this could be that a rather sophisticated extraction and derivatisation method is required for phytosterol identification and that so far there is no gas-chromatographic analysis for their accurate quantification suitable for plant breeding purposes. The major objectives of this study were to develop a gas-chromatographic method for high throughput analysis of phytosterol content and composition in seeds of oilseed rape, to determine the genetic variation, the genotype *x* environment interactions and the heritability of phytosterol content in three different doubled haploid populations of winter oilseed rape, grown in different environments. Furthermore, the correlations between phytosterol content and other economically important seed quality traits were studied.

MATERIALS AND METHODS

Plant material and field experiments

Three doubled haploid (DH) populations were grown in different environments over a period of several years. Population I consisted of 148 doubled haploid DH lines, derived from a cross between two DH lines obtained from two winter rapeseed cultivars, the French cultivar ‘Samourai’ (low in erucic acid and glucosinolates) and

the old Dutch cultivar ‘Mansholt’s Hamburger Raps’ (high in erucic acid and glucosinolates). All DH lines were tested in a field trial without additional N-fertilizer in a randomised block design with two replicates during two years at two locations. In 1999 the two locations were two fields at Reinshof (4 km southwest of Göttingen, Germany) with different soil types. In 2000 one location was Reinshof and the other Weende (5 km northwest of Göttingen). Seeds from three open-pollinated plants were harvested and bulked for the analysis (Gül 2002). Population II consisted of 49 DH lines obtained from a cross between the high oleic acid mutant line ‘19508’ and the low linolenic mutant line ‘2293E’. The population was grown in 2000 in a randomised block design with two replicates at three different locations: Reinshof, Weende and Hohenlieth (northwest of Kiel, Germany). One self-pollinated plant per plot was used for the analysis. Population III was composed of 284 DH lines derived from the cross between the old German cultivar ‘Sollux’ and the old Chinese landrace ‘Gaoyou’. Both cultivars have a high erucic acid and high glucosinolate content. The DH population was grown, together with the two parental lines, in 2000 at four locations, two in Germany (Reinshof and Weende) and two in China: Xian (western China) and Hangzhou (eastern China) in a randomised complete block design with two replicates. The population showed a large segregation for oil content (Zhao *et al.* 2005). From this population, 20 lines each with lowest and with highest oil content and equal erucic acid content were selected and seeds from five self-pollinated plants per plot were bulked and used for analysis.

Analysis of phytosterol content and other quality traits

A capillary column gas-liquid chromatographic (GC) method was developed and used for an accurate assessment of phytosterol content and composition in a large number of seed samples. The method was based on the modified sample preparation methodology for quantitative analysis of tocopherols (Ulberth *et al*. 1992). Phytosterol extraction and preparation for the GC was performed directly on the seeds in three major steps: alkaline hydrolysis, extraction and derivatisation to trimethyl-silyl ethers. Seed material (200 mg) was measured on an analytical balance (0.1 mg accuracy, M2P Sartorius, Göttingen, Germany) and placed in polypropylene tubes with screw caps (11.5 cm length; 0.9 cm diameter, Sarstedt, Nümbrecht, Germany)

with one stainless steel rod (1.1 cm length; 0.4 cm diameter) per tube. 200 µl of internal standard solution was added, prepared by dissolving cholesterol (99% purity, Sigma-Aldrich, Germany) in hexane-ethanol (3:2) solution at a concentration of 0.1% (w/v). Other phytosterol standards: sitosterol (40% purity) and stigmasterol (95% purity) were purchased from Sigma-Aldrich, Germany. Brassicasterol was obtained from Dr. Paresh Dutta (Department of Food Science, Swedish University of Agricultural Sciences in Uppsala) and avenasterol was identified by comparison of the retention time from chromatograms provided by Dr. Paresh Dutta and Dr. Ludger Brühl (Institute for Lipid Research, Münster, Germany). Since stigmasterol was present only in minute average amounts (0.01 g/kg seed or 0.4% from the total phytosterol amount), it is not shown separately, but was considered when calculating the total phytosterol content. Alkaline hydrolysis was performed with 2 ml of potassium hydroxide (Merck, Darmstadt, Germany) dissolved in ethanol (2%; w/v). The samples were homogenised for 60 seconds in a Mini-Bead-Beater-8 (BioSpec Products, Inc., OK, USA), with speed chosen to be as high as possible without destroying the tubes, and left for 15 minutes at 80°C in a water bath. Phytosterols were extracted by briefly vortexing with 1 ml hexane and 1.5 ml water. The upper hexane layer with phytosterols were transferred to a new tube and left on a hot plate at 37.5°C over night to evaporate. 100 µl of hexane was added to the dried pellet, transferred to vials together with 50 µl of silylating agent (10% w/w N-methyl-N-trimethyl-silyl-heptafluor(o)butyramid in trimethylchlorosilane) and left in the oven for 15 minutes at 105°C±3°C. Capillary gas-liquid chromatograph (PerkinElmer 8420, San Jose, CA, USA), equipped with an autosampler, flame ionization detector and split injector, was used with medium polarity, fused silica capillary column (SE-54, 50 m long, 0.1 µm film thickness, 0.25 mm internal diameter coated with 5%-phenyl-1%-vinyl-methylpolysiloxane, IVA Analysentechnik, Meerbusch, Germany). The following optimised conditions were used: initial oven temperature of 240°C was increased at 5°C/min to final oven temperature of 265°C and held for 20 minutes. Total analytical time was 25 minutes. Injection and detection temperature was set at 320°C. Hydrogen (carrier gas) pressure was set at 150 kP.

Seed oil, protein, glucosinolates, expressed on seed dry matter basis, were determined using the Near-Infrared Reflectance Spectroscopy (NIRS) with the calibration equation raps2001.eqa developed by Tillmann (2007).

Statistical analysis

Analysis of variance was performed with the PLABSTAT software (Utz 2007) using the following model:

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

where: Y_{ijk} was observation of genotype *i* in environment *j* in replicate *k;* μ was the general mean; g_i, e_j and r_{jk} were the effects of genotype *i*, environment *j* and replicate *k* in the environment *j*, respectively; ge_{ij} was the genotype *x* environment interaction of genotype *i* with environment *j* and ε_{ijk} was the residual error of genotype *i* in environment *j* in replicate *k*. The genotypes, environments and replicates were considered as random variables.

Broad-sense heritability (h^2) of mean values over environments was generated using PABSTAT (Utz 2007), following Hill *et al*. (1998) from the components of variance:

$$h^2 = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_{ge}}{E} + \frac{\sigma^2_\varepsilon}{ER}}$$

where: σ^2_g, σ^2_{ge} and σ^2_ε are variance components for *g*, *ge*, and ε, and *E* and *R* are number of environments and replicates, respectively. Genetic correlation coefficients were calculated using PLABCOV (Utz 1994).

RESULTS AND DISCUSSION

Chemical analysis of phytosterols

The developed gas chromatography was used for the identification of sitosterol, campesterol, brassicasterol, avenasterol and stigmasterol and the analysis of their content in seeds of oilseed rape (Figure 1). Phytosterol identification and quantification was based on the internal standard method (peak area and retention time) and reference samples. Depending on the different plant material and their phytosterol composition various potential internal standards have been proposed: 5α-cholestane, cholesterol, cholestanol, sitostanol, betulin, etc. (Aitzetmüller *et al*. 1998). In the present study, like in a number of other studies (Hamama *et al.* 2003, Piironen *et al*. 2002), cholesterol was used as internal standard, despite the small

amounts present in rapeseed (Appelqvist *et al*. 1981). The incentive for doing so was, that cholesterol is structurally very similar to phytosterols and hence shows the same extraction characteristics, it was completely dissolved in the hexane-ethanol mixture and there were no other peaks with the same retention time in the chromatogram. An example of a chromatogram with the different phytosterols is shown in Figure 1.

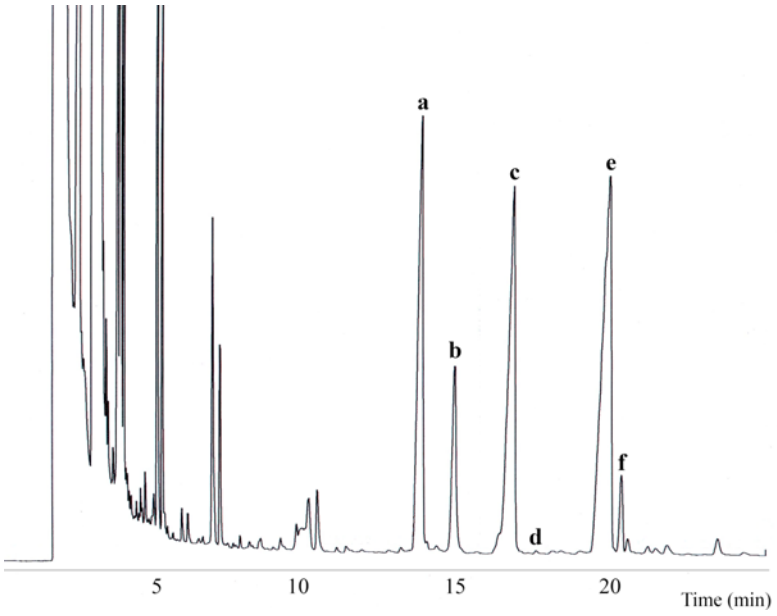

Figure 1. Gas chromatogram of major phytosterols in a seed sample of the cv. Linetta with cholesterol as internal standard. Peaks: **a** – cholesterol; **b** – brassicasterol; **c** – campesterol; **d** – stigmasterol; **e** – sitosterol; **f** – avenasterol.

The accuracy of the method was verified using a seed sample of the cultivar Linetta analysed by the Department of Food Science, Swedish University of Agricultural Sciences in Uppsala and by the Institute for Lipid Research in Münster, Germany. The accuracy of the method was also confirmed by analysing oil samples of an official ring trial organized by the German association of lipid sciences (data not shown).

One of the advantages of the present method compared to others (Dutta and Normen 1998, Fiebig *et al*. 1998) was the direct alkaline hydrolysis performed on the seed meal avoiding a separate step of quantitative oil extraction. The simplified method is therefore suitable for the analysis of a large number of samples as it is the case in breeding programs. The alkaline hydrolysis as performed in the present study allowed the quantification of free phytosterols and phytosterol fatty acid esters. In

rapeseed oil, the content of phytosterol fatty acid esters have been shown to be two times higher than the content of free phytosterols (Verleyen *et al*. 2002, Johansson and Appelqvist 1978). In contrast to this, Gordon and Miller (1997) reported almost twofold higher amounts of free phytosterols than esterified phytosterols in canola oil.

On the other hand, in this study, the content of total phytosterols was underestimated by failing to detect the content of phytosterol glycosides and acylated phytosterol glycosides. This analysis would require an additional acid-hydrolysis step (Normen *et al.* 1999, Yang *et al.* 2003), which would lead, due to the strong acidic conditions, to phytosterol destruction (Piironen *et al*. 2000).

Genetic variation

In all three DH populations five individual phytosterols were identified: sitosterol, campesterol, brassicasterol, avenasterol and stigmasterol. The analysis of variance revealed highly significant genetic variation in total and individual phytosterol content in all three DH populations (Table 1). Average total phytosterol content between all three populations ranged from 3.1 to 3.7 g/kg seed. The largest range of the total phytosterol content within populations was ascertained for population I with 2.6 to 4.1 g/kg seed. For all three populations the range of phytosterol content in the oil was 4.5 to 9.4 g/kg. This range compares well with phytosterol variation detected in nine canola commercial lines ranging from 4.6 to 8.1 g/kg oil with an average phytosterol content of 5.8 g/kg oil (Vlahakis and Hazebroek 2000). Gordon and Miller (1997) have analysed two commercial rapeseed cultivars and found a somewhat smaller average phytosterol content of 6.9 g/kg oil. The largest ranges of individual phytosterols were ascertained in population I for the two most prominent phytosterols sitosterol (1.3–2.1 g/kg seed) and campesterol (0.6–1.5 g/kg seed), which is in accordance with the largest variation in total phytosterol content within this population. A tenfold variation in avenasterol content was observed in population III. Appelqvist *et al*. (1981) found a large difference of avenasterol content in two summer canola cultivars.

Table 1. Variation in phytosterol content (g/kg seed) in three DH populations of *Brassica napus* L.

	Sitosterol			Campesterol			Brassicasterol			Avenasterol			Total		
	Population														
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Mean	1.65	1.98	1.71	0.90	1.04	0.97	0.42	0.53	0.35	0.13	0.10	0.14	3.11	3.66	3.18
Min	1.25	1.66	1.38	0.63	0.73	0.70	0.33	0.45	0.21	0.06	0.05	0.04	2.57	2.95	2.83
Max	2.14	2.21	1.95	1.53	1.32	1.30	0.55	0.61	0.48	0.30	0.18	0.45	4.10	4.15	3.72
F-value[§]	10[**]	6[**]	7[**]	14[**]	8[**]	14[**]	14[**]	7[**]	30[**]	7[**]	3[**]	5[**]	11[**]	9[**]	6[**]
LSD 5%	0.15	0.15	0.15	0.11	0.14	0.13	0.03	0.04	0.05	0.05	0.04	0.09	0.26	0.25	0.23

[§]F-value from the analysis of variance for genetic variation among DH lines;
[**] significant at p=0.01.

In all three populations sitosterol was the most prominent phytosterol, accounting for more than 50% of the total phytosterol content (Table 2), followed by campesterol (28-31%), brassicasterol (11-15%) and avenasterol (3-4%). Similar relative mean contents of individual phytosterols in rapeseed were also observed in other studies (Hamama *et al*. 2003, Gordon and Miller 1997, Warner and Mounts 1990, Appelqvist *et al*. 1981). Considerable variation in relative phytosterol composition was found within the populations indicating the possibility to develop material with a modified phytosterol composition in breeding programs. From a nutritional point of view the contents of sitosterol should be high and the contents of campesterol should be low. Although they have the same cholesterol lowering effects, sitosterol is taken up in the small intestine to a somewhat lower extent compared to campesterol (Miettinen 2001).

Table 2. Relative content of phytosterols (total phytosterols=100%) in three DH populations of *Brassica napus* L.

	Sitosterol			Campesterol			Brassicasterol			Avenasterol		
	Population											
	I	II	III	I	II	III	I	II	III	I	II	III
Mean	53	54	54	29	28	31	13	15	11	4	3	4
Min	47	50	45	24	24	22	10	12	6	2	2	1
Max	60	59	61	37	33	40	17	18	16	10	5	15
F-value[§]	14[**]	8[**]	10[**]	13[**]	6[**]	20[**]	16[**]	6[**]	30[**]	7[**]	4[**]	5[**]
LSD 5%	2.0	2.1	3.1	1.9	2.5	2.9	1.0	1.2	1.6	1.5	1.1	2.7

[§]F-value from the analysis of variance;
[**]significant at p=0.01.

In all three populations the analysis of variance revealed predominant and highly significant effects of the genotypes, in comparison to the genotype *x* environment interaction effects, on total and individual phytosterol content (Table 3). Only in

population III the variance components showed a larger effect of the environment on avenasterol and on total phytosterol content, which probably results from the considerably different test environments in China and Germany. The average amount of avenasterol content measured at the locations in Germany (0.21 g/kg seed) was more than three times higher than the content measured at the locations in China (0.06 g/kg seed), whereas the total phytosterol content in Germany (3.40 g/kg seed) was only slightly higher compared to China (2.99 g/kg seed). In a previous study (Zhao *et al*. 2005), where the same population was analysed for oil content, a higher average oil content for all DH lines was observed for the locations in Germany (51.5%) compared to those in China (44.5%). However, to date very little is known about the influence of specific environmental factors on phytosterol content. In one study a 2.5-fold variation in phytosterol content was detected in 12 commercial soybean lines, grown in three different temperature regimes (Vlahakis and Hazebroek 2000). It was shown that total phytosterol content increased at elevated temperatures, while its composition significantly changed with proportionally more campesterol at the expense of sitosterol. In another study eleven canola genotypes, grown during one year at two locations in the mid-Atlantic region of the United States showed no effect of the environment on total phytosterol content (Hamama *et al*. 2003).

Table 3. Components of variance for phytosterol content (g/kg seed) in three DH populations of *Brassica napus* L.

Variance component	Sitosterol			Campesterol			Brassicasterol			Avenasterol			Total		
	Population														
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
σ^2_g [#]	25.6**	12.2**	16.3**	19.1**	18.3**	29.2**	1.6**	1.6**	8.1**	2.1**	0.6**	4.5**	90.9**	63.2**	35.6**
σ^2_e [§]	4.0**	1.1**	0.8*	2.8**	8.1**	14.6**	0.4**	0.2**	1.1**	1.4**	1.3**	8.2**	7.3**	26.4**	49.7**
σ^2_{ge} [¶]	2.7**	2.4*	4.7**	1.9**	4.7**	3.8**	0.1**	0.2*	0.3^{+}	1.0**	0.5**	3.3**	9.5**	7.7*	7.7*
σ^2_ε [¥]	17.1	17.1	13.5	8.2	9.2	10.0	0.7	1.6	1.74	0.8	0.6	2.5	52.1	50.7	40.3

**, *, $^{+}$ significant at p=0.01, p=0.05 and p=0.1, respectively; F-value from the analysis of variance;
[#] σ^2_g genetic variance;
[§] σ^2_e environmental variance;
[¶] σ^2_{ge} variance of genotype *x* environment interaction;
[¥] σ^2_ε residual error.

High heritabilities, found in all three DH populations, of individual and total phytosterols confirmed the strong genetic component for phytosterol content (Table 4). The heritabilities were as high for oil (0.88-0.91) and glucosinolate content (0.89-0.95) and somewhat lower for protein content (0.70-0.83) in all three

populations. Heritabilities in population II were as high as in other populations, although only one self-pollinated plant per plot was used for the analysis. Marwede *et al*. (2004) analysed tocopherol content in first two populations and found, as a result of large genotype *x* environment interactions and a large experimental error, much lower heritabilities (0.41 in population I and 0.34 in population II).

Table 4. Heritability of phytosterol content and other quality traits in three DH populations of *Brassica napus* L.

Population	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total	Oil	Proteins	Glucosinolates
I	0.90	0.93	0.93	0.86	0.91	0.90[#]	0.76[#]	0.95[#]
II	0.82	0.88	0.86	0.71	0.89	0.88[§]	0.70[§]	0.91[§]
III	0.85	0.93	0.97	0.80	0.84	0.91	0.83	0.89

[#]data provided by Gül (2002);
[§]data provided by Marwede *et al*. (2004).

Correlations among traits

Genetic and phenotypic correlations among different phytosterols and other quality traits were mostly of similar size and sign (Table 5). In population I and II positive correlations were detected between all individual phytosterols, while in population III most of the individual phytosterols were not correlated to each other, with the exception of a negative correlation between campesterol and brassicasterol (r_g=-0.78^{++}). However, in all three DH populations both major phytosterols sitosterol and campesterol were positively correlated with total phytosterol content. Oil content was negatively correlated with total phytosterol content (r_g=-0.67^{++}) only in population I. This has been explained by the segregation of erucic acid, with no evidence that could be found for a direct effect of oil content on phytosterol content in this population (Amar *et al.* 2007). A positive correlation (r_g=0.59^{++}) between oil and phytosterol content was detected in population II, segregating for oleic and linolenic acid content (data not shown). In this population, total phytosterol content was to a low extent negatively correlated with oleic acid content (r_g=-0.34^{++}) and positively correlated with linolenic acid content (r_g=0.21^{+}). Since oil content was not significantly correlated with oleic (r_g=0.14) and linolenic (r_g=-0.14) acid content, this does not provide an explanation for the positive correlation between phytosterols and oil content in this population. Comparing different canola varieties with their transgenic counterparts having a modified fatty acid composition, Abidi *et al*. (1999) found that total phytosterol content was twofold decreased in high oleic acid low

linolenic acid lines, whereas high stearic acid lines had higher levels of phytosterols than the control. No, or only weak correlations to protein and glucosinolate content were found, while protein and oil content were in all three populations highly negatively correlated. Positive and small correlation was detected between protein and glucosinolate content.

Table 5. Coefficients of genetic r_g (upper part) and phenotypic r_p (lower part) correlations for phytosterol content and other quality traits in three DH populations of *Brassica napus* L.

	Sitosterol			Campesterol			Brassicasterol			Avenasterol			Total phytosterols			Oil			Protein			Glucosinolates		
	Population																							
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Sitosterol				0.58^{++}	0.56^{++}	0.06	0.16^{+}	0.42^{++}	-0.02	0.29^{++}	0.34^{+}	-0.31^{+}	0.87^{++}	0.84^{++}	0.63^{++}	-0.64^{++}	0.42^{++}	-0.28^{+}	0.10	-0.02	0.01	0.25^{++}	0.02	-0.33^{++}
Campesterol	0.60**	0.55**	0.09				0.38^{++}	0.50^{++}	-0.78^{++}	0.38^{++}	0.37^{++}	0.09	0.88^{++}	0.90^{++}	0.59^{++}	-0.50^{++}	0.54^{++}	0.21^{+}	-0.03	0.05	-0.16	0.06	-0.17^{+}	-0.25^{+}
Brassicasterol	0.20*	0.45**	0.01	0.40**	0.43**	-0.76**				0.07	-0.13	0.13	0.40^{++}	0.61^{++}	-0.17	-0.32^{++}	0.50^{++}	-0.07	0.11^{+}	-0.12	0.08	-0.16^{+}	-0.10	0.19^{+}
Avenasterol	0.28**	0.26	-0.28	0.36**	0.32*	0.07	0.09	-0.10	0.14				0.50^{++}	0.42^{++}	0.29^{+}	-0.39^{++}	0.35^{+}	0.21^{+}	0.17^{+}	-0.25^{+}	0.12	0.21^{++}	-0.05	0.06
Total phytosterols	0.88**	0.85**	0.66**	0.88**	0.89**	0.60**	0.43**	0.58**	-0.14	0.48**	0.37**	0.30				-0.67^{++}	0.59^{++}	0.06	0.08	-0.03	-0.08	0.17^{+}	-0.11	-0.33^{++}
Oil	-0.55**	0.29*	-0.23	-0.47**	0.49**	0.20	-0.28**	0.39**	-0.08	-0.35**	0.21	0.26	-0.60**	0.48**	0.07				-0.60^{++}	-0.49^{++}	-0.91^{++}	-0.34^{++}	-0.11	-0.21^{+}
Protein	0.01	0.00	-0.04	-0.06	-0.01	-0.19	0.02	-0.09	0.08	0.13	-0.12	-0.02	0.00	-0.03	-0.15	-0.60**	-0.60**	-0.89**				0.25^{++}	0.34^{+}	0.34^{++}
Glucosinolates	0.20*	0.01	-0.32*	0.03	-0.16	-0.29	-0.19*	-0.11	0.17	0.19*	-0.06	-0.02	0.12	-0.11	-0.39*	-0.32**	-0.09	-0.22	0.24**	0.28	0.39			

$^{+}$ coefficient is larger than the standard error;
$^{++}$ coefficient is two times larger than the standard error;
* significant at p=0.05;
** significant at p=0.01.

CONCLUSIONS

The large genetic differences and the high heritabilities of the total phytosterol content indicate that an effective selection for high phytosterol genotypes in a cultivar development program would be possible with a comparatively low effort with respect to the number of required test environments. There is no evidence that an increase of phytosterol content would be associated with a change in other relevant seed quality traits like oil, protein and glucosinolate content, although for oil content that should be confirmed with additional crosses.

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8 MANUSCRIPT II

MAPPING QTL FOR PHYTOSTEROL AND SINAPATE ESTER CONTENT IN *BRASSICA NAPUS* L.

ABSTRACT

Improving oil and protein quality for food and feed purposes is an important goal in rapeseed (*Brassica napus* L.) breeding programmes. Seeds of oilseed rape have been shown to contain high amounts of phytosterols and sinapate esters. While phytosterols, known for more than 50 years for their cholesterol-lowering properties, are being used to enrich food products, sinapate esters contribute to the bitter taste, low digestibility and dark colour of the rapeseed meal and are thus limiting its utilisation in the feed industry. Increasing the phytosterol content of the oil and lowering sinapate ester content of the meal could increase the value of the oilseed rape crop. The objective of this study was to identify QTL for phytosterol and sinapate ester content in a winter rapeseed doubled haploid population, previously found to have a large variation for the two traits. In the population, which also segregated for the two erucic acid genes, a close negative correlation was found between erucic acid and phytosterol content (r_s=-0.80**). For total phytosterol content three QTL were detected, explaining 60% of the genetic variance. The two QTL, with the strongest additive effects, mapped on linkage groups N8 and N13 within the confidence intervals of the two major erucic acid genes. For total sinapate ester content six QTL were detected, explaining 56% of the genetic variance. Again, a close negative correlation was found between erucic acid and total sinapate ester content (r_s=-0.66**) and the QTL with the strongest additive main effects mapped on linkage groups N8 and N13 within the confidence intervals of the two major erucic acid genes. The results suggest that the two erucic acid genes exert a negative pleiotropic effect on phytosterol and total sinapate ester content. Possible reasons for this are discussed based on known biosynthetic pathways.

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is the major oilseed crop in temperate regions and ranks second among oilseed crops produced worldwide (http://faostat.fao.org). Scientific interest in oilseed rape research has recently increased due to its diversified utilization in food and feed production and its growing economic importance as a novel source of renewable energy, mainly as biodiesel (Mittelbach and Gangl 2001). Besides improving its yield, yield stability and increasing the oil content, attention has recently been paid to minor seed constituents like tocopherols (Marwede *et al*. 2004) and sinapate esters (Zum Felde *et al*. 2006, Hüsken *et al*. 2005a). Phytosterols are another group of important, minor constituents, contributing from 0.5 to 1% of the crude rapeseed oil, establishing the rapeseed oil as one of the richest natural sources of phytosterols (Piironen *et al*. 2000). Phytosterols have been known for more than half a century for their LDL-cholesterol-lowering properties (Best *et al*. 1954). Two decades ago, increased level of plasma cholesterol has been recognized as one of the main risk factors of cardio vascular diseases – the leading cause of mortality in Western countries (Castelli 1984). One of the main mechanisms for cholesterol reduction is prevention of cholesterol absorption by its replacement with phytosterols in the intestinal-micellar phase (Trautwein *et al*. 2003, Nissinen *et al*. 2002). These observations have led to the development of a new type of 'functional food', including milk and dairy products, enriched with phytosterols as bioactive components. Following consumption, phytosterols reduce the absorption of dietary and endogenous cholesterol by about 50% (Law 2000).

In contrast to mammals and fungi, which contain only cholesterol and ergosterol, respectively, plants have a complex mixture of different phytosterols. In rapeseed, major phytosterols are sitosterol, campesterol, brassicasterol and avenasterol, while stigmasterol and cholesterol occur only in relatively small amounts (Appelqvist *et al*. 1981). Apart from being essential membrane constituents, regulating their fluidity and permeability, phytosterols also regulate membrane-bound enzyme activities and signal transduction events (Hartmann 1998). As plant hormone-precursors they play a crucial role in plant growth and developmental processes like cell division and polarity and morphogenesis (Schaller 2004, Lindsey *et al.* 2003). Phytosterols are products of the isoprenoid biosynthetic pathway occurring

exclusively in the cytoplasm and consisting of more than 25 enzyme-catalysed reactions (Benveniste 2002).

Only few studies report about genetic variation in phytosterol content in seeds of oilseed rape. The analysis of phytosterol composition in 10 different oil types showed that rapeseed oil had the second highest phytosterol content next to corn oil (Gordon and Miller 1997). Analysing phytosterol content of 12 different spring canola varieties, Abidi *et al*. (1999) reported concentrations ranging from 7659 to 14023 mg/kg oil. In another study, three winter rapeseed doubled haploid populations were tested in replicated field experiments (Amar *et al.* 2007a). Among the doubled haploid lines a twofold variation was found in the total phytosterol content, ranging from 4475-9380 mg/kg oil.

Besides being cultivated for its high oil content, the oil-extracted rapeseed meal is a valuable animal feed containing up to 40% of high nutritive value protein with near optimal amino acid composition (Yoshie-Stark *et al*. 2005). Though the utilization of rapeseed protein in food production has been prospected, its realization still depends on further quality improvements (Leckband *et al*. 2002). In this regard, the high contents of phenolic acid esters in the meal are considered as critical. In canola seeds, phenolic compounds are predominantly sinapate esters, whereas the most prominent one is sinapoylcholine (sinapine) followed by sinapoylglucose. Sinapate and other sinapate esters are found only in lower concentrations. Sinapate and the derived esters make up 1 to 2% of the seed dry matter (Bell 1993) and contribute to the bitter taste, astringency and low nutritive value of rapeseed meal by forming complexes with amino acids and thus inhibiting digestive enzymes of protein hydrolysis (Shahidi and Naczk 1992, Kozlowska *et al*. 1990). Classical breeding for reduced sinapate ester content seems promising, considering the available large genetic variation along with reported medium to high heritabilities (Zum Felde *et al*. 2006) and the availability of a NIRS calibration (Zum Felde *et al*. 2007). Moreover, transgenic approaches to drastically lower sinapate ester content in rapeseed have also been successful (Hüsken *et al*. 2005a, Hüsken *et al.* 2005b).

Quantitative Trait Loci (QTL) for different seed quality traits such as oil content, fatty acid composition, glucosinolates and tocopherols have been identified earlier on molecular linkage maps of the rapeseed genome (Qiu *et al*. 2006, Marwede

et al. 2005, Zhao *et al*. 2005, Ecke *et al*. 1995, and references therein). However, QTL for phytosterol and sinapate ester content have not been mapped so far. The objective of this study was to identify QTL for phytosterol and sinapate ester content in a winter rapeseed doubled haploid population segregating for erucic acid and previously found to show a large variation for the two traits (Amar *et al*. 2007a, Zum Felde *et al*. 2006).

MATERIALS AND METHODS

Plant material and field experiment

The mapping population consisted of 148 doubled haploid (DH) lines derived from a cross between two DH lines obtained from the old Dutch cultivar 'Mansholt's Hamburger Raps' (high contents of erucic acid and glucosinolates) and the modern French cultivar 'Samourai' (low contents of erucic acid and glucosinolate). The DH lines were grown in a field trial during two consecutive years at two locations in a randomised block design with two replicates. Each plot consisted of a double row with around 80 plants/plot. In 1999, the two locations were two fields at Reinshof (4 km southwest of Göttingen, Germany) with different soil types. In 2000, one location was Reinshof and the other was Weende (5 km northwest of Göttingen). The two parental lines were grown only in 1999 on one of the fields at Reinshof with four replicates. Seeds harvested from three open pollinated plants were bulked for the analysis (Gül 2002).

Analysis of phytosterol content and other quality traits

A gas-liquid chromatographic (GC) method was developed and used for assessment of phytosterol content and composition as described in Amar *et al*. (2007a).

Seed oil content, expressed on seed dry matter basis, and erucic acid content, were determined by using Near-Infrared Reflectance Spectroscopy (NIRS) with the calibration equation raps2001.eqa developed by Tillmann (2007). From the selected samples, erucic acid content was determined by GC and results were used to adjust a systematic bias of the NIRS data. The contents of sinapine, sinapoylcholine, other sinapate (sinapate and minor sinapate esters grouped together) and total sinapate

esters were determined using the NIRS calibration equations developed by Zum Felde *et al.* (2007). Samples with the highest and the lowest total sinapate ester content were also analysed by HPLC (Dr. Baumert, Halle, Germany) and results showed conformity with the NIRS results (n=20; R^2=0.81).

Statistical analysis

Analysis of variance was performed with the PLABSTAT software (Utz 2007) using the following model:

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

where: Y_{ijk} was observation of genotype i in environment j in replicate k; μ was the general mean; g_i, e_j and r_{jk} were the effects of genotype i, environment j and replicate k in the environment j, respectively; ge_{ij} was the genotype x environment interaction of genotype i with environment j and ε_{ijk} was the residual error of genotype i in environment j in replicate k. The genotypes, environments and replicates were considered as random variables. Broad-sense heritability (h^2) of mean values over environments was calculated from the components of variance using PLABSTAT (Utz 2007), as described in Hill *et al*. (1998):

$$h^2 = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_{ge}}{E} + \frac{\sigma^2_\varepsilon}{ER}}$$

where: σ^2_g, σ^2_{ge} and σ^2_ε are variance components for g, ge, and ε, and E and R are number of environments and replicates, respectively. Spearman's rank correlation coefficients were calculated using PLABSTAT (Utz 2007). Direct and indirect path coefficients of the path coefficient analysis were calculated as described in Lynch and Walsh (1998):

$$r_{yi} = P_{yi} + \sum_{\substack{i'=1 \\ i' \neq i}}^{k} r_{ii'} P_{yi'} \quad \text{for } i \neq 1$$

where: r_{yi} is the correlation coefficient between the i-th causal variable (X_i) and effect variable (y), $r_{ii'}$ is the correlation coefficient between the i-th and i'-th causal variables, P_{yi} is the path coefficient (direct effect) of the i-th causal variable (X_i), $r_{ii'}P_{yi'}$ is the indirect effect of the i-th causal variable via the i'-th causal variable. To determine the direct effect, square matrices of the Spearman's rank correlation

coefficients between independent traits in all possible pairs were inverted and multiplied by the correlation coefficients between the independent and dependent traits. The total phytosterol and total sinapate ester content were considered as effect variables, whereas oil and erucic acid as causal variables.

Molecular marker map

The QTL were mapped on a framework map (Uzunova *et al*. 1995) comprising 185 evenly distributed markers selected from a previously established primary RFLP and AFLP map. The primary map comprised 482 markers distributed on 20 linkage groups and covered 1745 cM (Kosambi 1944) of the rapeseed genome. Molecular marker genotypic data and means for phytosterol phenotypic data over all environments of the segregating DH population were used for composite interval mapping performed with the PLABQTL (Utz 2007). Putative QTL were detected by using a LOD score threshold of 2.82 corresponding to a 5% probability of falsely declaring a QTL anywhere in the genome.

RESULTS

Trait variation and heritability in the DH population

The DH population showed a large and significant variation in the total and individual phytosterol content. Together, four different phytosterols were quantified: sitosterol, campesterol, brassicasterol and avenasterol (Table 1). Sitosterol was the most prominent phytosterol, accounting for 53% of the total phytosterol content, followed by campesterol (29%), brassicasterol (14%), avenasterol (4%) and stigmasterol present only in amounts less than 1% (data not shown). Total phytosterol content ranged from 2570 to 4104 mg/kg seed. The largest ranges of individual phytosterols were ascertained for sitosterol (1251-2138 mg/kg seed) and campesterol (631-1533 mg/kg seed). Large and significant variations were also found in the oil, erucic acid and total sinapate ester content (Table 1), as well as for the individual groups of sinapate esters (data not shown). Overall high heritabilities were determined for all phytosterol traits as well as for oil, erucic acid and total sinapate ester content. The parental line Samourai had a higher phytosterol and total sinapate ester content

compared to Mansholt's, whereas Mansholt's had a higher erucic acid and a higher oil content.

Table 1. Genetic variation in phytosterol, total sinapate ester content (mg/kg seed) and other seed quality traits in the DH mapping population and the DH parental lines.

	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total phytosterols	Total sinapate esters	Oil (%)	Erucic acid (%)
Mean	1648	903	415	129	3107	7990	55	25
Min	1251	631	333	61	2570	6240	50	0
Max	2138	1533	548	303	4104	9910	60	42
F-value[§]	10**	14**	14**	7**	11**	8**	12**	47**
LSD 0.05	147	108	31	51	262	730	2	6
h^2	0.90	0.93	0.93	0.86	0.91	0.87	0.92	0.98
Mansholt's	1617	1029	383	188	3226	5443	58	49
Samourai	1896	1552	469	207	4132	6942	55	0

[§] F-value from analysis of variance for genetic variation in the DH lines;
** significant at p=0.01.

Correlations between traits and path coefficient analysis

Very strong and positive correlations were observed between total phytosterol and all individual phytosterols (Table 2). All individual phytosterols were positively correlated with each other. A significant positive correlation was also found for phytosterols and total sinapate esters (r_s=0.59**). Total phytosterol content was negatively correlated with oil (r_s=-0.58**) and erucic acid content (r_s=-0.80**). Between total sinapate esters and oil (r_s=-0.71**) and total sinapate esters and erucic acid (r_s=-0.66**) strong negative correlations were also observed.

Table 2. Spearman's rank correlation (r_s) between different seed quality traits in the DH mapping population.

	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total phytosterols	Oil	Erucic acid
Campesterol	0.53**						
Brassicasterol	0.14	0.37**					
Avenasterol	0.22**	0.31**	0.09				
Total phytosterols	0.84**	0.86**	0.38**	0.40**			
Oil	-0.55**	-0.44**	-0.23**	-0.36**	-0.58**		
Erucic acid	-0.69**	-0.68**	-0.28**	-0.45**	-0.80**	0.69**	
Total sinapate esters	0.61**	0.42**	0.20*	0.31**	0.59**	-0.71**	-0.66**

*, ** significant at p=0.05 and p=0.01, respectively.

Since oil content was positively correlated with erucic acid content (r_s=0.69**), the question arose whether total phytosterol content was directly affected by oil content or indirectly via erucic acid content. Therefore, the correlations were further investigated by path coefficient analysis to partition the correlations into direct and indirect effects (Table 3). Although there was a significant negative correlation between oil and phytosterol content, the results of the path coefficient analysis showed no direct effect of oil content on this trait (-0.05). Nevertheless, the observed strong direct effect of erucic acid on total phytosterol content (-0.76) is in congruence with the highly significant negative correlation between the two traits. At the same time, path analysis revealed a negative indirect effect of oil on total phytosterol content only via erucic acid content (-0.53). A stronger direct effect of oil on total sinapate ester content (-0.49) than of erucic acid on total sinapate ester content (-0.32) is in accordance with the stronger negative correlation between oil and total sinapate esters than between erucic acid and total sinapate esters. Likewise, erucic acid content showed a stronger indirect effect on total sinapate esters via oil (-0.34) than oil on total sinapate esters via erucic acid (-0.22).

Table 3. Direct and indirect effect of oil and erucic acid on total phytosterol and total sinapate ester content.

Trait	Direct effect on		Indirect effect via			
			Erucic acid	Oil	Erucic acid	Oil
	Total phytosterols	Total sinapate esters	On total phytosterols		On total sinapate esters	
Oil	-0.05	-0.49	-0.53	-	-0.22	-
Erucic acid	-0.76	-0.32	-	-0.04	-	-0.34
Residual effect	0.60	0.66	0.60		0.66	

Regression analysis

The DH population segregated for erucic acid content. As expected for a trait inherited by two genes, three classes with no (eeee), medium (EEee, eeEE) and high erucic acid content (EEEE) could be distinguished (Figure 1a). Regression curve displayed a 23% reduction of phytosterol content from 3699 to 2728 mg/kg seed when erucic acid content increased from 0 to 42% in the DH lines. Similar effect of erucic acid content could be observed on total sinapate ester content (Figure 1b), which showed a 20% decrease from 9087 to 7259 mg/kg seed when erucic acid content increase from 0 to 42%.

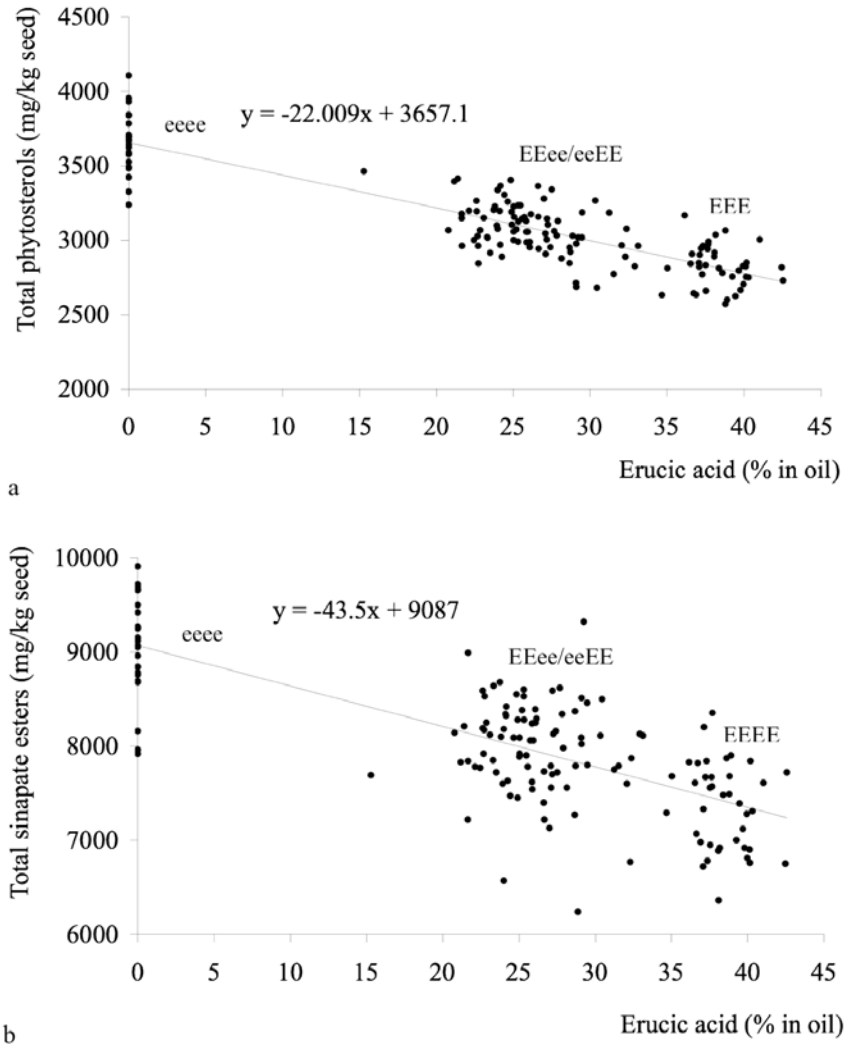

Figure 1. Regression analysis between erucic acid and phytosterol content (a) and total sinapate ester content (b).

QTL for phytosterol content

Two large and one small QTL were identified for total phytosterol content, with positive additive effects explaining 54% of the phenotypic and 60% of the genetic variance (Table 4). The two QTL with the largest effects were located on linkage groups N8 and N13 (Figure 2). The third smaller QTL was located on linkage group N18. Between four and eight QTL were detected for individual phytosterols, which together explained between 48% and 68% of the total genetic variance. QTL for sitosterol and campesterol content were detected on N8 in the same marker interval and at a similar position as the QTL for total phytosterol content. For brassicasterol and avenasterol, QTL were found on N8 in neighbouring marker intervals at distances

of 2 cM and 28 cM, respectively. Equally, a QTL for sitosterol was detected on N13 in the same marker interval at a similar position as the QTL for total phytosterol content. In marker intervals adjacent to this, QTL were found for campesterol, brassicasterol and avenasterol in distances ranging from 8 to 10 cM. All QTL for individual phytosterols on linkage groups N8 and N13 had positive signs indicating that the presence of the Samourai alleles at these QTL positions increased phytosterol content. Additional QTL for individual phytosterols with negative and positive effects were found on a number of other linkage groups (Table 4). On the linkage group N3 there was a QTL affecting campesterol content and at the same position there was a QTL for avenasterol with an opposite effect. Likewise, another QTL for campesterol content localised on N4, mapped in 2 cM distance to a QTL for brassicasterol with an opposite effect.

Table 4. Mapped QTL and their most likely positions for total and individual phytosterol content.

Trait	N[a]	D[b]	Marker interval	LOD score	a[c]	Vp%[d]	Vg%[d]	Total Vp%[e]	Total Vg%[e]
Total phytosterols	8	62	MG21-GATA.H3	5.9	205	47	52	54	60
	13	136	OPAG10.630-RP318a.E1	22.9	172	34	37		
	18	0	OPAG4.620-MG87	4.7	61	8	8		
Sitosterol	6	8	RP1454.E1-WG7E10.H1	16.7	-70	30	33	61	68
	8	58	MG21-GATA.H3	32.2	91	39	43		
	10	26	RP1470.H1-WG7B3.H1	4.0	22	4	4		
	13	134	OPAG10.630-RP318a.E1	30.2	76	29	33		
Campesterol	3	76	RP1142.H1-RP1117a.E4	11.8	31	8	8	44	48
	4	42	RP1230.H1-RP1117a.E1	7.6	44	14	15		
	8	60	MG21-GATA.H3	21.1	74	31	34		
	13	24	WG9A2.E1-RP1117a.E2	10.6	28	6	7		
	13	146	RP318a.E1-RP1365.H3	9.2	54	19	21		
Brassicasterol	3	28	OPAI16.1420-RP1422.E1	10.6	8	7	8	48	52
	4	44	RP117a.E1-WG4A4.H1	4.9	-22	33	36		
	8	64	GATA.H3-OPS7.970	8.5	12	13	14		
	10	8	MG25-MG26	10.2	-10	10	11		
	11	22	RP984.H2-MG40	5.8	8	7	8		
	13	128	RP1218.H1-OPAG10.630	6.1	11	11	12		
	15	48	OPT9.862-RP981.H1	3.7	8	7	7		
	19	56	RP1100.E1-RP825.H1	10.7	9	7	7		
Avenasterol	1	40	GATA.H1-RP1126.H1	9.5	22	27	32	50	58
	3	76	RP1142.H1-RP1117a.E4	2.7	-20	25	29		
	8	36	OPA15.896-MG21	4.1	15	6	19		
	13	144	RP318a.E1-RP1365.H3	3.0	12	10	12		
	14	12	OPA18.600-cRT21.E1	3.0	-16	13	15		
	17	10	RP1202.H1-RP318b.E1	5.7	-7	4	4		

[a] linkage group;
[b] distance from the first marker of the linkage group in cM;
[c] additive effect of the mapped QTL for phytosterol content (mg/kg seed) estimated for the substitution of a 'Mansholt's allele by a 'Samourai' allele;
[d] proportion of phenotypic (Vp) or genetic (Vg) variance explained by the additive effect of the QTL;
[e] proportion of total phenotypic (Vp) or total genetic (Vg) variance explained by the QTL, adjusted (Utz *et al.* 2000, Utz 2007).

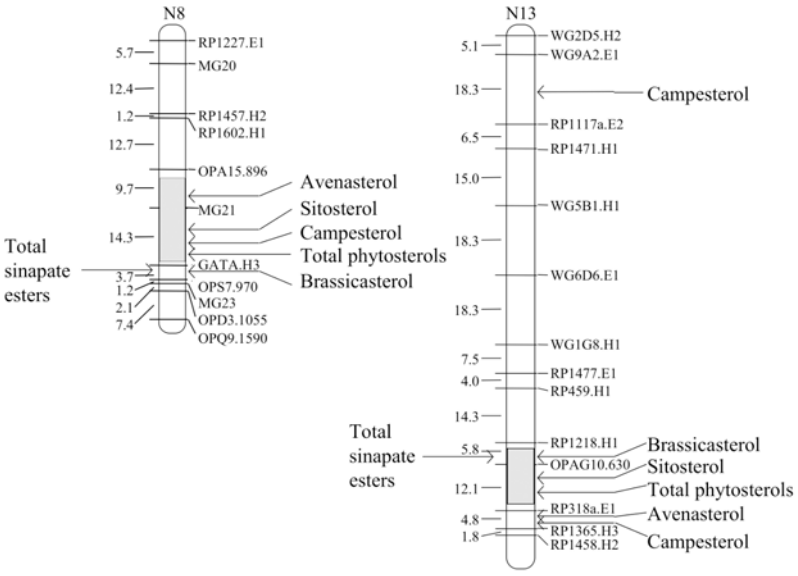

Figure 2. The framework maps of linkage groups N8 and N13 with the mapped phytosterol QTL. The distances between markers are given in cM (Kosambi 1944). The grey areas are representing the confidence intervals of 3 LOD score units around the most likely position of the erucic acid genes.

QTL for sinapate ester content

Two large and four smaller QTL were identified for total sinapate ester content, with additive effects explaining 47% of the phenotypic and 56% of the genetic variance (Table 5). The two QTL with the largest additive effects were located on linkage groups N8 and N13 (Figure 2) in marker intervals adjacent to those of the QTL for total phytosterol content. For N8 and N13 the distance between the marker positions were 2 cM and 8 cM, respectively. The two QTL had a positive additive main effect, indicating that the ‘Samourai’ allele at these positions increased total sinapate ester content. Between five and twelve QTL were detected for the individual sinapate esters on 15 different linkage groups, which together explained between 60% and 71% of the total genetic variance. No QTL were detected on linkage groups N1, N3, N10, and N17. The QTL for total sinapate ester content on N8 mapped at 4 cM distance to the QTL for sinapoylglucose and to the QTL for other sinapate esters (the confidence intervals of these QTL were overlapping). The QTL for total sinapate ester content on N13 mapped at 4 cM and 8 cM distance to the QTL for sinapoylglucose and for other sinapate esters, respectively. Interestingly, QTL for sinapine were mapped neither on N8 nor on N13. On linkage group N7 there was a QTL affecting sinapoylglucose content and at 10 cM distance there was a QTL for sinapine with an opposite effect.

Table 5. Mapped QTL and their most likely positions for sinapate ester content.

Trait	N[a]	D[b]	Marker interval	LOD score	a[c]	Vp %[d]	Vg %[d]	Total Vp%[e]	Total Vg%[e]
Total sinapate esters	5	64	OPC19.1090-WG4C5.H1	6.9	-111	5	6	47	56
	6	76	WG1F6.H1-OPB5.910	3.8	-182	10	11		
	7	48	TG2B4.E1-MG11	2.7	-163	10	11		
	8	64	GATA.H3-OPS7.970	15.6	433	43	50		
	13	128	RP1218.H1-OPAG10.630	17.5	328	28	33		
	15	46	OPT9.862-RP981.H1	3.2	142	7	8		
Sinapine	4	0	WG6F10.H1-RP1230.H1	10.9	106	10	11	62	71
	5	64	OPC19.1090-WG4C5.H1	15.7	-210	28	32		
	6	24	WG6A11.H1-RP1068.E1	2.6	-88	6	7		
	7	0	RP1146.H3-RP830.E1	12.9	177	22	25		
	7	50	MG11-RP1457.H1	3.0	-142	14	16		
	9	4	WG3F7.H1-RP1175.H1	4.7	-72	4	5		
	9	72	RP1253.E1-TG2F9.H1	6.7	109	9	10		
	11	22	RP984.H2-MG40	7.6	-206	27	31		
	12	64	RP1565.E1-OPA18.820	3.1	127	12	14		
	12	124	WG7A8.H1-WG4E12.H1	18.0	-191	23	26		
	16	74	MG9-RP1087.H1	3.5	93	6	7		
	19	36	RP1100.E1-RP825.H1	13.7	-129	13	15		
Sinapoylglucose	2	68	RP1146.H1-MG16	3.6	-68	4	5	57	68
	6	38	RP1068.E1-WG1F6.H1	5.1	-108	10	12		
	7	10	RP1122.H1-OPD3.1190	8.3	-120	14	17		
	8	60	MG21-GATA.H3	27.9	263	39	47		
	13	132	OPAG10.630-RP318a.E1	20.2	251	36	43		
	14	6	OPA18.600-cRT21.E1	3.0	-79	5	6		
Other sinapate esters	6	60	WG1F6.H1-OPB5.910	2.6	-73	8	10	52	60
	8	60	MG21-GATA.H3	29.3	131	32	39		
	13	136	OPAG10.630-RP318a.E1	13.6	104	21	26		
	14	2	OPA18.600-cRT21.E1	4.3	-41	4	5		
	18	0	OPAG4.620-MG87	6.7	70	14	16		

[a] linkage group;
[b] distance from the first marker of the linkage group in cM;
[c] additive effect of the mapped QTL for sinapate ester content (mg/kg seed) estimated for the substitution of a 'Mansholt's allele by a 'Samourai' allele;
[d] proportion of phenotypic (Vp) or genetic (Vg) variance explained by the additive effect of the QTL;
[e] proportion of total phenotypic (Vp) or total genetic (Vg) variance explained by the QTL, adjusted (Utz *et al*. 2000 and Utz 2007).

DISCUSSION

Natural rapeseed populations contain around 50% erucic acid in the seed oil. The detection of low erucic acid mutants led to a complete conversion to low erucic acid

cultivars in the 1970s in Canada and in Europe (Shahidi 1990). Nowadays, the so called Canola or double zero (’00’) quality type cultivars with low erucic acid content in the seed oil and low glucosinolate content in the seed meal are exclusively being cultivated in most rapeseed growing areas of the world. This conversion to low erucic acid seed oil quality has not been reported to affect any other seed constituents than the fatty acid composition and the oil content (Ecke *et al*. 1995). This is rather surprising, since erucic acid is formed via consecutive elongation of oleoyl-CoA with malonly-CoA (Figure 3). In a first round oleoyl-CoA (18:1-CoA) is elongated to eicosenoyl-CoA (20:1-CoA) and in a second round, eicosenoyl-CoA is elongated to erucoyl-CoA (22:1-CoA). Unlike to the synthesis of fatty acids with chain lengths shorter than 18 carbons in the plastids, the elongation of oleoyl-CoA occurs in the cytoplasmic cell compartment. There, malonyl-CoA is generated through carboxylation of acetyl-CoA. Then again, cytoplasmic acetyl-CoA is also required for the biosynthesis of a plethora of other compounds, e.g. isoprenoids, phenolics, flavonoids, stilbenoids, alkaloids, anthocyanins, etc. (Fatland *et al*. 2005). Among those, phytosterols are present in the highest concentration in the seed (0.3%; Table 1).

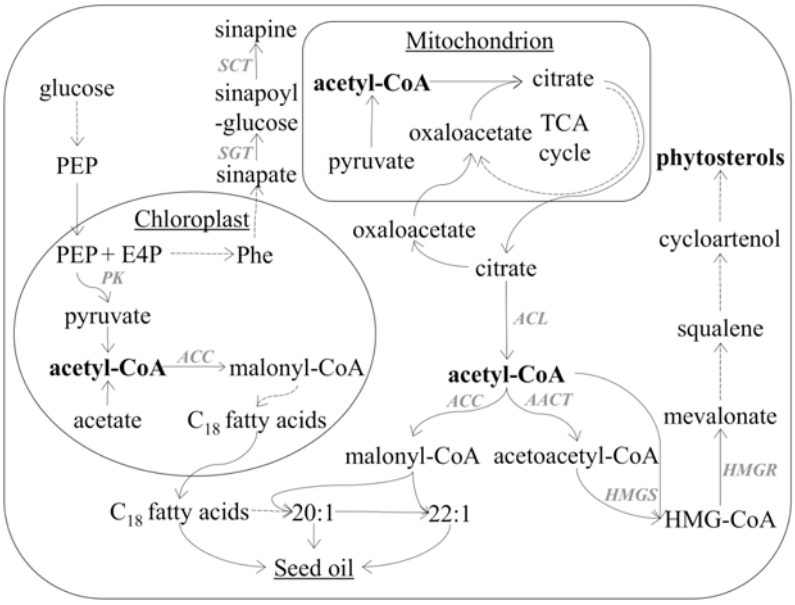

Figure 3. Scheme of putative phytosterol, fatty acid and sinapate ester biosynthetic pathway in plants (adapted from Milkowski 2004, Fatland *et al.* 2005, Ruuska *et al.* 2002, Fischer *et al.* 1997). Solid and dashed arrows represent single and multiple enzymatic reactions, respectively. TCA – tricarboxylic acid cycle; HMG-CoA – 3-hydroxy-3methylglutaryl-CoA; PEP – phosphoenolpyruvate; E4P – erythrose-4-phosphate; Phe – phenylalanine; ACL – ATP-citrate lyase; ACC – acetyl-CoA carboxylase; AACT – acetoacetyl-CoA thiolase; HMGS – HMG-CoA synthase; HMGR – HMG-CoA

reductase; PK – pyruvate kinase; SGT – sinapate glucosytransferase; SCT – choline sinapoyltransferase.

Considering the fact, that up to 25% of the seed may consist of erucic acid and that four molecules of acetyl-CoA are required for the synthesis of one molecule of erucic acid, it can be anticipated that a major change in erucic acid content could have an effect on contents of other seed constituents synthesized from cytoplasmic acetyl-CoA. Indeed, the results of the present study show a close negative association between total phytosterol content and erucic acid content (Table 2). The results from the path coefficient analysis confirmed the direct negative effect of erucic acid on total phytosterol content (Table 3). Further evidence for the association between erucic acid content and total phytosterol content can be drawn from the fact that the two largest QTL for total phytosterol content on linkage groups N8 and N13 mapped within the confidence intervals of the two erucic acid genes (Figure 2). Their additive effects were positive, indicating that the alleles increasing the total phytosterol content were coming from the low erucic acid parent ‘Samourai’. The two erucic acid genes were mapped earlier in the present population on linkage groups LG6 and LG12 Ecke *et al*. (1995), which are corresponding to linkage groups N8 (*B. rapa*) and N13 (*B. oleracea*) mapped by Parkin *et al*. (1995). Obviously, there is a pleiotropic effect of the erucic acid genes on phytosterol content, which may be explained by the competition for cytoplasmic acetyl-CoA – an early essential precursor for both compounds (Figure 3) and presumably available only in limited quantities (Fatland *et al*. 2005). Otherwise, it could be also possible that major genes for phytosterol content are closely linked to the erucic acid genes. This could be checked easily by analysing transgenic rapeseed expressing the erucic acid *fae*1-gene (James *et al*. 1995) in an otherwise low erucic acid background (Han *et al*. 2001).

The two major QTL for total phytosterol content on N8 and N13 were at the same time QTL for all individual phytosterols. All the confidence intervals were overlapping, except for the QTL for avenasterol on N8. This was not the case for the third minor QTL for total phytosterol content on N18. It may be that the effect of this QTL was too small to cause a significant effect. QTL found for sitosterol, campesterol and avenasterol, at N18 in the same marker interval and at the same position, were below the critical LOD score value (data not shown). At this time, it can only be speculated about the gene behind the QTL on N18 and its function. Results from

transgenic plants show, that over expression or inhibition of the key biosynthetic enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; Figure 4) and cycloartenol C-24-methyltransferase (SMT1), influence the amount of total phytosterol content in *Nicotiana tabacum* and *Arabidopsis thaliana* seed and leaf tissue (Schaller 2004, Harker *et al*. 2003a, Holmberg *et al.* 2003). Hence, the genes could be likely candidates for the QTL on N18. Transgenic approaches have been successful in modifying phytosterol composition and accumulation in seeds. Ectopic expression of the HMGR gene in transgenic tobacco led to 3.2-fold and 10-fold increased phytosterol levels in seed and leaf tissue, respectively (Harker *et al*. 2003b). Overexpression of the SMT1 gene in tobacco seed tissue increased the amount of total phytosterols up to 44% and modulated the phytosterol composition; sitosterol proportion was increased by up to 50% and levels of isofucosterol (sitosterol precursor) and campesterol increased by up to 80% (Holmberg *et al*. 2002).

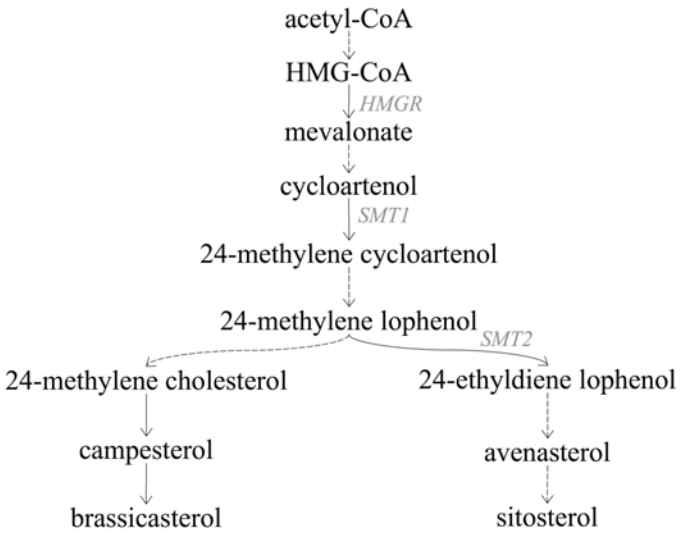

Figure 4. Scheme of phytosterol biosynthetic pathway in plants (adapted from Holmberg *et al.* 2003, Benveniste 2002). HMG-CoA – 3-hydroxy-3methylglutaryl-CoA; HMGR – HMG-CoA reductase; SMT1 – cycloartenol C-24-methyltransferase; SMT2 – SAM-24-methylene lophenol C-24-methyltransferase.

The correlations among individual phytosterols were all positive (Table 2), suggesting that most of the variation is caused by genetic differences before the separation of the two pathways leading to brassicasterol and sitosterol (Figure 4). Nevertheless, one QTL for campesterol on N3 with a positive additive effect mapped at the same position as a QTL for avenasterol with a negative additive effect (Table 4). A candidate gene for this QTL is the SAM-24-methylene lophenol C-24-

methyltransferase gene (SMT2) regulating phytosterol biosynthesis either towards campesterol and brassicasterol or towards avenasterol and sitosterol (Figure 4). The overexpression of SMT2 transgenic *Arabidopsis* plants led to an increased accumulation of sitosterol (from 65% to 75% of total phytosterols) at the expense of campesterol (from 11% to 4%) and brassicasterol (from 2% to 0.5%), whilst its co-suppression resulted in higher campesterol levels at the expense of sitosterol (Schaeffer *et al*. 2001). Likewise, there was a QTL for campesterol on N4 that mapped at a distance of only 2 cM to a QTL for brassicasterol. Still, little is known so far about the gene(s) coding the C22-desaturase type enzymes responsible for the conversion of campesterol to brassicasterol (Schaller 2004).

The close negative association between total sinapate ester and erucic acid content (Table 2) were confirmed by the results from the QTL analysis, which showed that the two major QTL for total sinapate ester content mapped at a very similar position as the erucic acid genes (Table 5). The confidence intervals of the erucic acid genes (Ecke *et al*. 1995) and of the two QTL for total sinapate ester content were overlapping (data not shown). This also explains the negative association between total sinapate ester content and oil content (indirect effect of oil via erucic acid content). However, path coefficient analysis also revealed a direct effect of oil content on total sinapate ester content (Table 3). From known biochemical pathways there is no obvious connection between the biosynthesis of sinapate esters and erucic acid. Starting from phenylalanine, sinapate esters are synthesized in the cytoplasmic cell compartment via the phenylalanine/hydroxycinnamate pathway (Figure 3; Milkowski *et al*. 2004). It can only be speculated, that plastidic phosphoenolpyruvate (PEP), as a common precursor for *de novo* fatty acid and phenylalanine biosynthesis (Fischer *et al*. 1997) in the DH population, is limiting and hence may explain the negative association between sinapate esters and oil content. Amongst other enzyme activities, pyruvate kinase (PK; Figure 3) has been found to be enhanced in a *Brassica napus* line with high oil content compared to a near-isogenic line with a low oil content, corroborating the importance of this step in storage lipid biosynthesis (Li *et al.* 2006). Zum Felde *et al*. (2006) analysed the same DH population as in the present study, but reported only a slightly negative, non-significant correlation between oil content and total sinapate ester content. This may be due to the fact, that the seed material used in the present study was derived from a low N-fertilizer variant, which led to overall

higher oil contents. Even so, for two additional DH populations analyzed by Zum Felde *et al*. (2006), no significant association between oil and sinapate ester content were reported; both populations were low in erucic acid content. Clearly, further studies are needed to elucidate the association between total sinapate ester content and oil content as well as erucic acid content. The effects of the two major QTL on N8 and on N13 were limited to sinapoylglucose and to the other sinapate esters; for sinapine content no significant QTL were found in these regions.

Sinapoylglucose is the direct precursor for sinapine. This step is catalysed by the choline sinapoyltransferase (SCT, Figure 3; Milkowski *et al*. 2004). The QTL for sinapine on N7, at position 0 cM with a positive effect, mapped 10 cM away from QTL for sinapoylglucose with a negative effect, indicating that they may be identical. A candidate for this QTL could be the *SCT* gene (Milkowski *et al*. 2004).

CONCLUSIONS

The present study provides convincing evidence that the two erucic acid genes exert a pleiotropic effect on phytosterol and total sinapate ester content in seeds of oilseed rape. Canola quality type rapeseed has a higher total sinapate ester and phytosterol content than high erucic acid rapeseed material. These results have recently been confirmed by using a set of intervarietal substitution lines derived from the same parents as in this study (Kebede 2007). The present findings have consequences when screening *Brassica* genetic resources for germplasm with reduced contents of sinapate esters or increased phytosterol contents (Zum Felde *et al*. 2007, Amar *et al*. 2007b). In this case some adjustment should be performed depending on the erucic acid content of the seed oil. It is likely that the two erucic acid genes exert a pleiotropic effect also on other secondary compounds synthesized from cytoplasmic acetyl-CoA. Beside the two major QTL, additional QTL for total and individual phytosterol and sinapate ester content were identified, which may be useful in future candidate gene mapping projects. The utilization of an additional DH population not segregating for erucic acid content for mapping QTL for phytosterol and sinapate ester content should increase the power of detection for QTL with smaller effects. Alternatively, conditional QTL mapping, taking the variation in the erucic acid content into account

could be performed with the present DH population (Zhao *et al.* 2006).

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9 MANUSCRIPT III

GENETIC VARIATION IN PHYTOSTEROL CONTENT IN *BRASSICA NAPUS* L. AND DEVELOPMENT OF NIRS CALIBRATION EQUATIONS

ABSTRACT

Rapeseed oil is one of the richest natural sources of phytosterols, known to reduce the serum-LDL-cholesterol level, one of the major cardiovascular disease risk factors. Increasing the phytosterol content in rapeseed could give an added value to the oil and derived products. Our aim was to analyse the genetic variation in phytosterol content and composition in genetically diverse winter rapeseed material, including modern cultivars (n=27), gene bank accessions (n=558) and resynthesized lines (n=101). A second objective was to develop Near-Infrared Reflectance Spectroscopic (NIRS) calibration equations for the high throughput and non-destructive estimation of phytosterol content. NIRS spectra from a diverse set of seed samples of winter oilseed rape (old and new cultivars, breeding lines, resynthesized rapeseed) from different years and locations were recorded and samples were then analysed by gas-chromatography. The phytosterol content of modern cultivars tested in the field at six locations in Germany varied between 3565 and 4800 mg/kg seed. Among the resynthesized lines, tested in the field during two to three years, a slightly larger variation ranging form 2079 to 4329 mg/kg seed was found. The NIRS calibration equations showed a high fraction of explained variance in cross validation (R^2_{cv}) of 0.76 for total phytosterol content. The standard error of cross validation (SECV) was 218 mg/kg. The NIRS calibration was used to predict the phytosterol content in seed samples from the gene bank accessions grown together during one year in the field. The variation in this material ranged from 2370 to 4230 mg/kg seed. Results need to be confirmed by gas-chromatographic analyses. The results indicate that modern rapeseed cultivars contain already high phytosterol contents and that further increases can be expected by making crosses among this adapted material. NIRS calibrations can be developed for phytosterol content in seeds of rapeseed and they will be helpful in germplasm screening and breeding programmes.

INTRODUCTION

LDL-cholesterol-lowering effect of plant sterols, also called phytosterols, has been known for more than 50 years (Best *et al*. 1954). Since high serum LDL-cholesterol level has been identified as the main risk factor for cardiovascular diseases in Western countries, efforts were undertaken by the food industry to develop functional food products enriched with natural phytosterols, which would then have a health benefiting effect. Today, milk and milk-derived products fortified with natural phytosterols are already available in many countries. In most cases, vegetable oils are being used as a source for the phytosterols extraction. Plants contain more than 40 different phytosterols (Law 2000). High phytosterol contents are found in the seeds of oilseed plants and among the crops, rapeseed has been identified as the one with the highest contents (Piironen *et al*. 2000, Normen *et al*. 1999). In rapeseed the most prominent phytosterols are sitosterol and campesterol followed by brassicasterol and avenasterol; stigmasterol and cholesterol occur only in small quantities (Appelqvist *et al*. 1981). Increasing phytosterol content and modifying its composition (Harker *et al*. 2003, Holmberg *et al*. 2002) in the seeds of oil crops could give an added value to the oil and derived products. However, at present, little is known about the genetic variation in phytosterol content and composition in a larger collection of rapeseed genotypes. Amar *et al*. (2007a) have reported a variation in total phytosterol content ranging from 2570 to 4150 mg/kg seed for three different doubled haploid populations of winter rapeseed tested in different environments. For all three populations the range of phytosterol content in the oil was 4480-9380 mg/kg oil. This compares well with the results from previous studies where the phytosterol content in rapeseed oil was reported to range between 0.5 and 1% (Hamama *et al*. 2003, Raymer 2002, Vlahakis and Hazebroek 2000).

The limited number of reports on the genetic variation in phytosterol content of rapeseed may be caused by the fact that a rather sophisticated extraction and derivatisation method followed by gas-chromatographic analyses is required for accurate phytosterol identification and quantification (Dutta and Normen 1998). Although recently a more simplified gas-chromatographic method for breeding purposes has been developed (Amar *et al*. 2007a), this is nevertheless, destructive, laborious, time-consuming and costly. Although not so accurate, the application of

Near-Infrared Reflectance Spectroscopy (NIRS) could provide a good alternative for fast, non-destructive and cost-effective estimation of seed phytosterol content. Seed quality traits like oil, protein, glucosinolates, sinapate esters and fatty acid content have previously been reported to be determined by NIRS (Zum Felde *et al.* 2007, Velasco and Becker 1998a, Velasco and Becker 1998b, Tkachuk 1981). However, to our knowledge there is so far no report about the development of NIRS calibrations for phytosterol content in crop plants.

This research was initiated to study the variation in phytosterol content and composition in a genetically diverse set of modern winter rapeseed cultivars, resynthesized rapeseed lines as well as in the IPK Gaterslebener gene bank (www.ipk-gatersleben.de) collection of winter rapeseed material. An additional aim was to develop NIRS calibration equations for high throughput analysis of phytosterol content in seeds, which could be used in rapeseed breeding programmes for pre-selection of genotypes with high or low phytosterol content or unusual phytosterol composition.

MATERIALS AND METHODS

Plant material and field experiments

The analyzed seed material was derived from (1) 27 modern winter rapeseed cultivars tested in 2004/05 at six different environments situated in different German federal states (Bundes- und EU-Sortenversuch Winterraps). The field experiment was conducted as a randomised complete block design with four replicates for each cultivar at a different location. The seed samples were taken after combined harvesting of the yield plots. The samples from the four replicates of each cultivar were equally mixed and thus produced subsample was used for the analysis, (2) resynthesized rapeseed lines tested in two (n=71) to three years (n=30; 2001 to 2003 and 2005) in the field at Reinshof near Göttingen (Germany), (3) 148 doubled haploid (DH) lines derived from a cross between two DH lines obtained from two winter rapeseed cultivars, the French cultivar ‘Samourai’ (low in erucic acid and glucosinolates) and the old Dutch cultivar ‘Mansholt’s Hamburger Raps’ (high in erucic acid and glucosinolates). The DH lines were tested in a field trial in a

randomised block design with two replicates during two years (1999 and 2000) at two locations near Göttingen (Gül 2002), (3) 49 DH lines obtained from a cross between the high oleic acid mutant line '19508' and the low linolenic mutant line '2293E' (Marwede *et al*. 2004). The lines were grown in 2000/01 in a randomised block design with two replicates at three different locations in central and northern Germany, (4) 40 DH lines derived from the cross between the old German cultivar 'Sollux' and the Chinese landrace 'Gaoyou', both high in erucic acid and glucosinolate content. The DH lines were grown in 2000/01 at four locations, two in central Germany and two in China (Eastern and Western China), in a randomised complete block design with two replicates (Zhao *et al.* 2005), (5) 558 winter rapeseed accessions of the gene bank Gatersleben (www.ipk-gatersleben.de) were tested with one replication in 1998/99 in the field at Reinshof near Göttingen. Seeds samples from material (2) to (5) were obtained after bagging plants at flowering to secure self-pollination.

Analytical method

A previously developed gas-chromatographic (GC) method (Amar *et al.* 2007a) was used for the analysis of individual and total phytosterol content and composition in the seeds of above described samples.

NIRS calibration development

The above-described collection of 2246 rapeseed samples (3-5 g of seeds per sample) was scanned with NIRS monochromator model 6500 (NIRSystems, Inc., Silver Spring, MD, USA), using ring cups (internal diameter 4.7 cm) with a sample cup autochanger. The spectra were collected between 400 and 2498 nm, registering the absorbance values (R) as log (1/R) at 2 nm intervals for each sample. NIRS spectral data were matched with reference data obtained from GC analysis. The calibration equation was developed with spectral absorbance information using WinISI II Project Manager 1.50 (Infrasoft International, Hosham, West Sussex, UK). Modified partial least squares regression analysis (MPLS) and cross-validation techniques were used. The maximum number of factors suggested by the software was 16 and there were 4 cross validation groups. The derivative was 1, the gap was 4 and first and second

smooth were 4 and 1, respectively. The results of the calibration were checked observing the *t*-outliers with $t > 2.0$, GH- and X-outliers > 10. The number of outlier elimination passes was 2. Samples with $t > 2.0$ were deleted from the sample file. A lower than usual *t*-outlier value of 2 was chosen because the GC analyses were not repeated. Calibration performance was assessed by SEC (standard error of calibration), R_C^2 (explained variance of calibration), SECV (standard error of cross-validation) and R_{CV}^2 (explained variance of cross-validation). The calibration was used to calculate standard deviation (SD) and mean for the calibration set, whereas the estimated range of the calibration (Est. Min and Est. Max) was calculated on the basis of mean±3SD from the measurement range.

Statistical analysis

Analysis of variance was performed by the PLABSTAT software (Utz 2007) using the following model:

$$Y_{ij} = \mu + g_i + l_j + gl_{ij} + \varepsilon_{ij}$$

where: Y_{ij} is observation of genotype *i* at location *j*; μ is the general mean; g_i, l_j are the effects of genotype *i* and location *j*, respectively; gl_{ij} is the genotype *x* environment interaction of genotype *i* with location *j* and ε_{ij} is the residual error of genotype *i* at the location *j*. The locations are considered as random and the genotypes as fixed factors.

Means and confidence intervals, at a comparison alpha level of 0.05, were calculated for each resynthesized rapeseed line with SAS Proc Mixed, version 8.0 (SAS Institute 2000).

RESULTS AND DISCUSSION

Variation in phytosterol content among modern cultivars

The modern winter rapeseed cultivars showed a significant variation in individual and total phytosterol content (Table 1). The cultivar with the lowest total phytosterol content was Savannah (3565 mg/kg seed) and the one with the highest Sansibar (4800 mg/kg seed). Similar variation between cultivars was found for the individual phytosterols, where a twofold variation was observed only for avenasterol. Based on

the oil content of the seed samples, as determined by NIRS, the phytosterol content in the oil ranged from 0.7% to 1.1%. Some of the cultivars were hybrids, which nevertheless didn't differ in phytosterol content from the line cultivars. The variation in phytosterol content among the cultivars is somewhat lower than the variation within three different doubled haploid populations (2570 and 4150 mg/kg seed) as described by Amar *et al*. (2007a). The relatively limited range may be explained by the fact that the genetic basis of the cultivars is probably quite narrow and that some lines and hybrids may even be closely related. Furthermore, all cultivars were low in erucic acid, which has been found earlier to be negatively correlated with phytosterol content (Amar *et al*. 2007b). The about 25% variation in total phytosterol content indicates that by crossing cultivars with a high phytosterol content, recombinant lines with an further increased phytosterol content may be identified among the progenies. Rapeseed lines high in phytosterol content (e.g. Sansibar, Aurum, etc.) could be utilised for the production of cold pressed oil rich in phytosterols, without, otherwise required, addition of natural phytosterols.

Table 1. Variation in phytosterol content (mg/kg seed) in a set of 27 modern rapeseed cultivars; the results are presented as the mean over six locations and four replicates.

Genotype	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total phytosterols
Savannah[L]	1816	1161	445	134	3565
Oase[L]	1803	1193	485	92	3580
Monarch[L]	2074	1178	267	72	3600
Brise[L]	1872	1399	296	70	3641
Ramano[L]	1944	1381	312	77	3721
Californium[L]	1882	1377	377	89	3735
Pacific[L]	1809	1439	399	86	3741
Missouri[L]	1939	1254	420	128	3749
Caracas[L]	1878	1288	523	71	3770
Ibex[L]	1857	1352	441	148	3804
Catalina[L]	1899	1313	520	89	3830
NK Bravour[L]	2052	1199	488	90	3839
Elektra[H]	1936	1365	458	94	3861
Talent[H]	1939	1406	481	85	3919
Expert[L]	2003	1552	310	79	3950
ES Astrid[L]	2125	1537	303	106	4078
Express[L]	1991	1542	489	93	4125
Maxima[H]	2100	1456	494	80	4140
Tenno[H]	2109	1426	496	111	4152
Taurus[H]	2070	1597	419	80	4175
Trabant[H]	2120	1611	375	89	4204
Matrix[H]	2095	1677	413	82	4277
Amigo[H]	2144	1653	376	109	4292
SW Gospel[L]	2225	1718	274	95	4319
NK Fair[L]	2271	1641	313	84	4320
Aurum[L]	2355	1741	322	111	4538
Sansibar[L]	2463	1708	498	121	4800
Mean	2029	1451	407	95	3990
Range	1803–2463	1161–1741	267–523	70–148	3565–4800
LSD 5%[f]	107	84	20	17	170
F-value[¥]	19[**]	36[**]	134[**]	11[**]	27[**]

[H L] – hybrid and line cultivar, respectively;
[f]LSD 5% – least significant difference at the level of probability p=0.05;
[¥]F-value – from the analysis of variance; [**]significant at p=0.01.

Variation in phytosterol content among resynthesized rapeseed lines

Total phytosterol content of the 101 resynthesized rapeseed lines varied between 2079 and 4329 mg/kg seed (Table 2). The confidence intervals indicated significant differences between the fourteen lines with the highest and the fourteen lines with the lowest phytosterol content. The resynthesized lines showed an about 50% variation

for total phytosterol and sitosterol content and an about 80% variation for campesterol, brassicasterol and avenasterol content. This compares favourably to the variation found among the modern cultivars (Table 1), which ranged between 30 to 50%. The resynthesized rapeseed lines with the highest phytosterol content turned out to have a low erucic acid erucic acid content, except for the lines R18, H30 and H144 (Table 2) which had an about 25% erucic acid content in the seed oil (data not shown). All resynthesized lines with the low phytosterol content belonged to the group with medium and high erucic acid content. The Spearman's rank correlation between both traits was r_s=-0.50**. These findings are consistent with earlier results obtained from a doubled haploid population segregating for erucic acid content (Amar *et al*. 2007b).

Table 2. Variation in phytosterol content (mg/kg seed) in a set of 101 resynthesized rapeseed lines; the results with 14 lowest and highest phytosterol content are presented as the mean over four environments, unbalanced.

Genotype	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total phytosterols
H240[2]	1292	527	198	48	2079
H357 [2]	1448	376	350	19	2196
H61[2]	1477	623	229	4	2354
L35[2]	1474	547	297	25	2360
H210[2]	1392	577	412	34	2435
S4[2]	1318	601	418	96	2449
G89[3]	1442	644	336	32	2458
S8a[2]	1555	536	279	67	2460
S2[2]	1400	623	347	82	2473
H31[2]	1530	531	367	19	2474
G53[2]	1525	560	369	21	2476
R31[2]	1390	664	412	27	2495
L90[2]	1465	893	106	32	2516
S17[2]	1279	775	414	48	2520
R18[2]	1927	1219	478	62	3716
S39[2]	2325	1070	291	51	3737
R19[2]	1947	1379	374	55	3778
H72[2]	1939	1466	263	113	3784
S31[2]	2109	1212	467	45	3841
L239[2]	2069	1204	515	86	3895
G43[3]	2220	1344	285	63	3917
H143[2]	2228	1241	447	60	3979
S30[3]	2191	1492	233	72	3994
R8[3]	2278	1272	419	123	4099
H30[3]	2305	1191	510	129	4142
H144[2]	2257	1333	475	58	4143
H48[3]	2173	1572	429	139	4316
H250[2]	1973	1833	435	72	4329
Mean	1743	920	348	58	3079
Range	1279–2325	376–1833	106–583	19–140	2079–4329
CI[f] for 2/3 years	262/214	228/186	79/64	45/37	513/419
F-value[¥]	4.23	6.04	7.66	1.99	4.47

[2], [3] – the resynthesized line was tested in 2 and 3 years, respectively;
[f]CI – confidence interval at the level of probability 0.05;
[¥]F-value – from SAS analysis.

Development of NIRS calibration

A total of 2446 seed samples were analyzed with the GC for phytosterol content, their NIRS spectra recorded and used for the development of the NIRS calibration

equation. Following removal of outliers between 2002 and 2041 sample spectra were used for calibration development. Table 3 shows the variation in total and individual phytosterol content of the calibration set. Explained fraction of variance in calibration (R_C^2), representing a correlation between GC and NIRS data, ranged from 0.78 for total phytosterols to 0.46 for avenasterol. Slightly lower explained fraction of variance in cross-validation (R_{CV}^2) than R_C^2, ranging from 0.76 for total phytosterols to 0.45 for avenasterol, indicates that the calibration set is quite homogenous and that the calibration will not become much more robust when new samples are included. The standard error of calibration (SEC) and the standard error of cross-validation (SECV) were quite low for total phytosterols and sitosterol (SECV/mean=7%, 8%, respectively) but higher for campesterol, brassicasterol and avenasterol (SECV/mean = 13%, 12%, 34%, respectively). SECV was in the range of 218 mg/kg seed (total phytosterols) to 34 mg/kg seed (avenasterol). The NIRS prediction calculated through the SD/SECV ratio was highest for total phytosterols (2.0). Sitosterol and campesterol had the same SD/SECV ratio (1.7), followed by brassicasterol (1.6), while avenasterol had the smallest SD/SECV ratio (1.4). According to Fontaine *et al.* (2001) the SD/SECV ratio of 2 and above indicates that the calibration is already quite useful for identifying genotypes with low, medium or high content, whereas if the ratio is below 2 the use of the calibration is limited. Nevertheless, a better SD/SECV ratio could be obtained by increasing the SD or reducing the SECV. With more than 2000 spectra, the present calibration sets are rather large and certainly contain an above average number of samples with medium phytosterol contents. Reducing the number of those less informative samples to achieve a more even distribution of the samples to the phytosterol classes would increase the SD. Furthermore, the analyses of samples have been performed during a period of about two years and none of the samples have been analyzed in duplicate in order to obtain more accurate results, neither by NIRS nor by GC; doing this the SECV should be definitely reduced. Then again, duplicate measurement of samples is not a typical situation in the breeding programmes. The removal of more outliers would also improve the calibration, but undoubtedly would result in a lower performance of independent validation with seed samples derived from environments not included in the present calibrations. Such an independent validation still needs to be done with the calibrations developed in this study.

Table 3. Variation in total and individual phytosterol content as measured by the GC and NIRS-calibration and cross-validation in the calibration sample collection.

Phytosterols	GC		Calibration					Cross-validation	
	Mean	SD	Est. Min (mg/kg)	Est. Max (mg/kg)	No.	R_C^2	SEC (mg/kg)	R_{CV}^2	SECV (mg/kg)
Total phytosterols	3260	445	1926	4594	2011	0.78	209	0.76	218
Sitosterol	1753	233	1054	2452	2041	0.65	138	0.64	140
Campesterol	951	208	325	1576	2012	0.66	121	0.65	124
Brassicasterol	430	82	183	676	2022	0.61	51	0.60	52
Avenasterol	99	46	0	236	2002	0.46	34	0.45	34

SD – standard deviation from the analysis of variance; No. – number sample spectra considered in calibration; Est. Min and Est. Max – estimated minimum and maximum of calibration; SEC – standard error of calibration; SECV – standard error of cross-validation; R_C^2 and R_{CV}^2 – explained fractions of variance in calibration and cross-validation, respectively.

In the calibration set, total phytosterol content was significantly positively correlated with sitosterol, campesterol, brassicasterol and avenasterol content (Table 4). The close correlation between total phytosterol content and sitosterol as well as campesterol could indicate that the NIRS calibration for the individual phytosterols may not be very specific but could be based on the correlations among those traits. This may perhaps be tested by analyzing a sample set with identical total phytosterol content and with varying sitosterol and campesterol contents.

Table 4. Spearman's rank correlation (r_s) between the individual and total phytosterol content in the calibration set of 2011 samples.

	Sitosterol	Campesterol	Brassicasterol	Avenasterol
Campesterol	0.54**			
Brassicasterol	0.40**	0.17**		
Avenasterol	-0.08**	0.20**	0.01	
Total phytosterols	0.87**	0.82**	0.48**	0.21**

** significant at p=0.01.

Variation in phytosterol content among gene bank accessions as determined by NIRS

The phytosterol content of altogether 558 winter rapeseed accessions of the gene bank material were analysed with the NIRS calibration equations (Table 5). Total phytosterol content showed a relatively large variation (2370-4230 mg/kg seed), which, nevertheless, was not larger than the one observed for the resynthesized material (see above) and the three doubled haploid populations (Amar *et al.* 2007a).

Table 5. Variation in phytosterol content (mg/kg seed) in a calibration set of 558 accessions from the genebank material.

Genotype	Sitosterol	Campesterol	Brassicasterol	Avenasterol	Total phytosterols
Mean	1921	1107	401	85	3361
Range	1372–2276	649–1495	97–569	0–147	2370–4230
SD[f]	201	198	57	27	456

[f]SD-standard deviation.

The largest variation among gene bank accessions was ascertained for two minor phytosterols avenasterol (0-147 mg/kg seed) and brassicasterol (97-569 mg/kg seed). This large variation for the smaller phytosterol components should be confirmed by the GC analysis. Samples with confirmed extreme contents of individual phytosterols are valuable for extending the NIRS calibrations and for further genetic studies. As found for the resynthesized lines, the erucic acid content was negatively correlated with total phytosterol content (r_s=-0.77**) in the gene bank collection.

CONCLUSIONS

The natural variation in total phytosterol content among adapted and non-adapted winter rapeseed germplasm seems to be limited to contents ranging from 2000 to 4800 mg/kg seed. Modern winter rapeseed cultivars contain already high phytosterol contents. Further increases can be expected by making crosses among the adapted material. A wider variation may be found within other gene pools like spring rapeseed, Chinese and Indian rapeseed or even within the ancestral species of rapeseed, *B. oleracea* and *B. rapa*. The NIRS calibrations for phytosterol content should be useful for germplasm screening and in breeding programmes aimed at increasing the phytosterol content in rapeseed. The availability of the NIRS calibrations and the previously observed high heritabilities of phytosterol content (Amar *et al*. 2007a) indicate, that an effective breeding for high phytosterol genotypes should be possible without too much effort.

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