Genetic variation and genotype×environment interactions for phytosterol content in rapeseed (*Brassica napus* L.)

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Abstract

Phytosterols are natural plant oil constituents known for their LDL-cholesterol-lowering properties since more than 50 years. An impressive number of human studies have shown their effectiveness in reducing coronary mortality rate. This has led to the development of functional food products with an enhanced phytosterol content. So far there have been no attempts to enhance the phytosterol content of oil crops and thus give an added value to vegetable oils and derived products. The main objectives of the present study to assess the genetic variation and genotype×environment interactions of phytosterol content among three winter rapeseed doubled haploid (DH) populations grown in different environments and to study the correlations among phytosterols and other seed quality traits. Sitosterol and campesterol were identified as the two major phytosterols followed by *brassica*sterol, avenasterol and stigmasterol. Large and highly significant variations were found for individual and total phytosterol content in all three populations. The variance components for phytosterol content showed for all three populations a predominant effect of the genotype in comparison to the genotype×environment interaction effects, resulting in overall high heritabilities. Total phytosterol content was negatively correlated to oil content only in the first DH population. No, or only weak correlations to protein and glucosinolate content were found. The identified genetic variation along with the observed high heritabilities indicate that an effective breeding for increased phytosterol content would be possible, without affecting other seed quality traits.

Key words: phytosterols, genetic variation, genotype×environment interaction, heritability, gas chromatography

Introduction

Oilseed rape (*Brassica napus* L.) is the most important oilseed crop in Europe and the second largest worldwide. Its scientific interest and economic importance has increased due to the improved quality of seed oil used for consumption as well as because of its use as a source of renewable energy. Rapeseed oil has been recognised as one of the phytosterol's richest natural source (Gordon and Miller 1997). Phytosterols are polyisoprenoids with the common structure derived from a tetracyclic nucleus with a side chain. Structural variations arise from different configurations of the sterol nucleus and the alkylation of the chain. The most abundant phytosterols are: sitosterol, campesterol and stigmasterol. Other phytosterols, like avenasterol, are synthesised earlier in the biosynthetic pathway and they usually occur in relatively smaller amounts, while *brassica*sterol is typical for the *Brassicaceae* family. Phytosterols are playing a vital role in membrane-associated metabolic processes, they are phytohormone precursors and thus involved in important growth and developmental processes (Hartmann 1998). Their LDL-cholesterol-lowering properties and effectiveness in reducing coronary mortality rate has led to the development of functional food products with an enhanced phytosterol content (Piironen *et al.* 2000). In the present study a high throughput gas chromatographic method for the analysis of phytosterols in seeds of oilseed rape was developed. The main objectives were to assess the genetic variation and genotype×environment interactions of phytosterol content among three winter rapeseed doubled haploid (DH) populations grown in different environments and to study the correlations among phytosterols and other seed quality traits.

Materials and Method

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). The DH lines were tested in a field trial in a randomised block design with two replications during two years at two locations. In 1999 the two locations were two fields at Reinshof (4 km south west of Göttingen, Germany) with different soil types. In 2000 one location was Reinshof and the other Weende (5 km north west 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 replications and at three different locations: Reinshof, Weende and Hohenlieth (north west of Kiel, Germany). One plant per plot was used for the analysis.

Population III included 284 DH lines derived from the cross between the old German cultivar 'Sollux' and the old

Chinese landrace 'Gaoyou'. Both cultivars have high erucic acid and high glucosinolate content. The population was grown in 2000 at four locations, two in Germany (Reinshof and Weende) and two in China: Xian (west China) and Hangzhou (east China) in a randomised complete block design with two replications. 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 were selected. Seeds from five plants per plot were bulked and used for analysis.

Analytical Method: A gas-liquid chromatographic method was developed and used for assessment of phytosterol content and composition. Phytosterol extraction was performed directly on the seeds in three major steps: alkaline hydrolysis, extraction and silylation. Seed material was placed in tubes with a stainless steel rod, 200 µl of internal standard (cholesterol) solution and 2 ml of KOH. The samples were homogenised in a bead beater and left for 15 minutes at 80°C in a water bath. Phytosterols were extracted with 1 ml hexane and 1.5 ml of water and left on a hot plate at 37.5°C to evaporate. 100 µl of hexane was added, transferred to vials together with 50 µl of silylating agent (5 ml of N-Methyl-N-trimethyl-silyl-heptafluor(o)butyramid in 500 µl of Trimethylchlorosilane) and left in the oven for 15 minutes at 105°C. A gas-liquid chromatograph model Perkin Elmer 8420 equipped with an autosampler, flame ionization detector and split injector was used along with a medium polarity, fused silica capillary column SE-54, 50 m long. The following optimised conditions were used: 240°C initial oven temperature, 5°C/min ramp rate, 265°C final oven temperature, 20 min second isothermal time, 320°C injection and detection temperature, 150 kP hydrogen pressure.

Near-infrared reflectance spectroscopy (NIRS) was applied to determine the oil, protein, glucosinolate, fatty acid and sinapate ester content of the seed samples.

Statistical Analysis: Analysis of variance was performed by the PLABSTAT software (Utz 2006) 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 replication *k;* µ was the general mean; g_i, e_j and r_{jk} were the effects of genotype *i*, environment *j* and replication *k* in the environment *j*, respectively; ge_{ij} was the genotype×environment interaction of genotype *i* with environment *j* and ε_{ijk} was the residual error of genotype *i* in environment *j* in replication *k*. The genotypes, environments and replicates were considered as random variables.

Broad-sense heritability (h^2) for mean values over environments was calculated using PABSTAT (Utz 2006), 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

In all three DH populations five individual phytosterols were identified: sitosterol, campesterol, *brassica*sterol, avenasterol and stigmasterol. The analysis of variance revealed highly significant genetic variation of total and individual phytosterol content in all three doubled haploid populations (Table 1). Means of the populations for total phytosterol content ranged from 3.1 to 3.7 g/kg seed. The largest range of total phytosterol content within the populations was ascertained for the first population (2.6-4.1 g/kg seed). Sitosterol, campesterol and *brassica*sterol content largely varied in population I, while a tenfold variation of avenasterol content was observed in population III. Sitosterol was the most prominent phytosterol, contributing on average 54% to the total phytosterol content, followed by campesterol (29%), *brassica*sterol (13%) and avenasterol (4%). Since stigmasterol was a minor component in the phytosterol mixture, with a negligible content of around 0.01 g/kg (0.4%), it is not shown separately but was considered when calculating the total phytosterol content.

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

	*Brassica*sterol			Campesterol			Sitosterol			Avenasterol			Total		
	Population														
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Mean	0.42	0.53	0.35	0.90	1.04	0.97	1.65	1.98	1.71	0.13	0.10	0.14	3.11	3.66	3.18
Min	0.33	0.45	0.21	0.63	0.73	0.70	1.25	1.66	1.38	0.06	0.05	0.04	2.57	2.95	2.83
Max	0.55	0.61	0.48	1.53	1.32	1.30	2.14	2.21	1.95	0.30	0.18	0.45	4.10	4.15	3.72
LSD5%	0.03	0.04	0.05	0.11	0.14	0.13	0.15	0.15	0.15	0.05	0.04	0.09	0.26	0.25	0.23

In all three populations the analysis of variance revealed predominant and highly significant effects of genotypes in comparison to the genotype×environment interaction effects on total and individual phytosterol content (Table 2). Only in population III the environmental effect on the total phytosterol content was higher than the genetic effect, probably as a result from the very different test environments. High heritabilities were found in all three populations confirming the strong genetic component for phytosterol content (Table 2). The heritabilities were as high for oil (0.88-0.91) and glucosinolate content (0.89-0.95) and comparatively lower for protein content (0.70-0.83) in all three populations.

Table 2. Components of variance for phytosterol content (g/kg seed) and heritability in three DH populations of *Brassica napus* L.

Variance component	*Brassica*sterol			Campesterol			Sitosterol			Avenasterol			Total		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
σ^2_g	1.6**	1.6**	8.1**	19.1**	18.3**	29.2**	25.6**	12.2**	16.3**	2.1**	0.6**	4.5**	90.9**	63.2**	35.6**
σ^2_e	0.4**	0.2**	1.1**	2.8**	8.1**	14.6**	4.0**	1.1**	0.8*	1.4**	1.3**	8.2**	7.3**	26.4**	49.7**
σ^2_{ge}	0.1**	0.2*	0.3$^+$	1.9**	4.7**	3.8**	2.7**	2.4*	4.7**	1.0**	0.5**	3.3**	9.5**	7.7*	7.7*
σ^2_ε	0.7	1.6	1.74	8.2	9.2	10.0	17.1	17.1	13.5	0.8	0.6	2.5	52.1	50.7	40.3
h^2	0.93	0.86	0.97	0.93	0.88	0.93	0.90	0.82	0.85	0.86	0.71	0.80	0.91	0.89	0.84

**significant at p = 0.01; F-test from the analysis of variance
* significant at p = 0.05
$^+$ significant at p = 0.1

Genetic correlation coefficients among different quality traits are presented in Table 3. In the first two populations positive correlations were found between campesterol and *brassica*sterol, sitosterol and avenasterol, respectively. A negative genetic correlation between campesterol and *brassica*sterol existed only in the third population.

Table 3. Coefficients of genetic correlation for phytosterol content and other quality traits in three DH populations of *Brassica napus* L.

	*Brassica*sterol			Campesterol			Sitosterol			Avenasterol			Total		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Campesterol	0.38^{++}	0.50^{++}	-0.78^{++}												
Sitosterol	0.16$^+$	0.42^{++}	-0.02	0.58^{++}	0.56^{++}	0.06									
Avenasterol	0.07	-0.13	0.13	0.38^{++}	0.37^{++}	0.09	0.29^{++}	0.34$^+$	-0.31$^+$						
Total	0.40^{++}	0.61^{++}	-0.17	0.88^{++}	0.90^{++}	0.59^{++}	0.87^{++}	0.84^{++}	0.63^{++}	0.50^{++}	0.42^{++}	0.29$^+$			
Oil	-0.32^{++}	0.50^{++}	-0.07	-0.50^{++}	0.54^{++}	0.21$^+$	-0.64^{++}	0.42^{++}	-0.28$^+$	-0.39^{++}	0.35$^+$	0.21$^+$	-0.67^{++}	0.59^{++}	0.06
Protein	0.11$^+$	-0.12	0.08	-0.03	0.05	-0.16	0.10	-0.02	0.01	0.17$^+$	-0.25$^+$	0.12	0.08	-0.03	-0.08
Glucosinolates	-0.16$^+$	-0.10	0.19$^+$	0.06	-0.17$^+$	-0.25$^+$	0.25^{++}	0.02	-0.33^{++}	0.21^{++}	-0.05	0.06	0.17$^+$	-0.11	-0.33^{++}

$^+$ coefficient is larger than the standard error
$^{++}$ coefficient is two times larger than the standard error

Total phytosterol content was in all populations closely positive correlated with the most prominent phytosterols. Only in the first population total phytosterol content was negatively correlated to oil content. Quite contrary to this, the second population showed a close positive correlation between phytosterol and oil content, while no correlation at all could be found in population III. No, or only weak correlations to protein and glucosinolate content were found. The identified genetic variation of phytosterol content along with the observed high heritabilities allows an effective breeding for enhanced phytosterol content without affecting other seed quality traits.

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