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QUANTITATIVE GENETIC ANALYSIS OF PHENOLIC ACIDS IN OILSEED RAPE MEAL

Florin-Daniel LIPȘA[1*], Rod SNOWDON[2], Benjamin WITTKOP[2] and Wolfgang FRIEDT[2]

[1] Iasi University of Life Sciences, Faculty of Agriculture, Department of Food Technologies, 3, Mihail Sadoveanu Alley, 700490, Iasi, Romania;
[2] Justus Liebig University, Department of Plant Breeding, Heinrich-Buff-Ring 26-32, 35392-Giessen, Germany;
e-mail: rod.snowdon@agrar.uni-giessen.de, benjamin.wittkop@agrar.uni-giessen.de, wolfgang.friedt@agrar.uni-giessen.de

*Correspondence: flipsa@uaiasi.ro

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ABSTRACT. Rapeseed meal, a by-product of oilseed extraction related to the agri-food and biofuel industries due to its favourable composition of essential amino acids, is currently utilised for animal feed. In this study, 166 doubled haploid (DH) rapeseed lines from the segregating *Brassica napus* doubled haploid population YE2-DH were genetically and chemically analysed for phenolic acids. The relationship between seed colour and phenolic acid fractions in *B. napus* was investigated using these analyses to improve the quality of rapeseed meal. High-performance liquid chromatography assays were used to estimate phenolic acid content, and the outcomes were used to identify quantitative trait loci (QTL). Nine quantitative feature loci for three distinct phenolic acid compounds were mapped to seven linkage groups. A minor QTL for sinapine was located on linkage group C05 in the same interval as the QTL for seed colour. On chromosome A09, two loci for phenolic acids colocalised with the main QTL for seed colour. Closely linked molecular markers for the target traits (seed colour, phenolic acids) identified during this study could be useful tools for breeding new oilseed rape cultivars with lower levels of antinutritive compounds.

Keywords: *Brassica napus*; phenolic acids; meal quality; QTL mapping.

INTRODUCTION

Due to its favourable composition of essential amino acids, oilseed rape/canola (*Brassica napus* L., 2n=38, genome AACC) is a good source of

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vegetable protein (Yang *et al.*, 2015; Cheng *et al.*, 2022). In this context, fibre, dark-coloured tannins and bitter-tasting sinapate esters are of particular importance (TUM, 2019).

Phenolic acids and condensed tannins represent the predominant phenolic compounds in rapeseed, the total content of which is much higher than that in other oilseeds (soybean, cottonseed, peanut). They are responsible for the dark colour, bitter taste, unpleasant odour and astringent properties of rapeseed meal products due to the reduction in protein digestibility caused by their complex formation (Lipsa *et al.*, 2012, Yang *et al.*, 2022).

Phenolic acids are present in rapeseed in free, esterified and insoluble-bound forms and represent benzoic and cinnamic acid derivatives. Soluble phenolic compounds accumulate in rapeseed, mainly in the cotyledons. Sinapic acid accounts for the majority of free phenolic acids, up to 15% of total phenolics in rapeseed meal, and up to 99% of phenolic acids produced from esters and glucosides (Krygier *et al.*, 1982; Naczk *et al.*, 1998; Reungoat *et al.*, 2022).

In terms of quantity, the esterified phenolic acids represent the largest proportion, with the choline ester of sinapic acid, sinapine, accounting for between 70% (Kolodziejczyk *et al.*, 1999) and 80% (Obied *et al.*, 2013; Aludatt *et al.*, 2017) of all sinapic acid esters occurring in rapeseed and can reach contents in rapeseed meal of 1–2% (Rezaeizad *et al.*, 2011).

A reduction in the phenolic compound content is therefore an indispensable prerequisite for the use of rapeseed meal in animal nutrition and in the human protein supply. Extensive studies on the sinapic acid ester content of various *Brassica* species and cultivars have shown considerable variability in this trait (Kozlowska *et al.*, 1990; Milkowski and Strack, 2010). Rapeseed meal has a sinapine content that varies from 5 to 30 mg/g depending on the cultivar, growing environment and location (Shahidi and Naczk, 1995; Tan *et al.*, 2011).

Although the available cultivars sometimes show significant differences in phenolic content and sinapine concentration, the development of newer cultivars with reduced sinapine content by classical breeding has been less successful and very time consuming. A promising way to reduce sinapine content is the genetic suppression of the two key enzymes, UDP-glucose:sinapic acid glucosyltransferase (SGT) and sinapoylglucose:choline sinapoyltransfe rase (sinapine synthase; SCT), in the sinapine biosynthesis pathway using antisense regulation (Milkowski *et al.*, 2004; Corso *et al.*, 2020). Such targeted genetic engineering approaches provide hope that the levels of phenolic compounds in canola can be sustainably reduced. An improvement in the nutritional value and possibly an increased use of rapeseed meal products in animal and human nutrition would be the result. Other ways to reduce phenolic acids from rapeseed meal are the use of biological methods, such as enzyme addition and microbial fermentation, to remove antinutritional factors. Enzyme-assisted methods and microbial fermentation have shown outstanding effects on the removal of antinutritional factors and are also considered potential strategies for rapeseed commercial feed production (Yang *et al.*, 2022).

To identify closely linked molecular markers associated with quantitative trait loci involved in the acquisition and content variability of phenolic acids in rapeseed meal, the purpose of this study was to establish screening and analysis techniques for these compounds in *B. napus* seed from a doubled haploid population with 166 lines.

MATERIALS AND METHODS

The materials and methods are described in detail in a previous article of the authors (Lipsa *et al.*, 2012). For a better understanding of the steps that are followed in the present study we present the structure of the chapter and the most important data for the genetic analysis of phenolic acids.

Plant material

A *B. napus* population of 166 doubled haploid lines, descended from a cross between the true-breeding line '1012/98' (yellow seeds) and the German winter oilseed rape cultivar 'Express' (black seeds), was used to create a genetic map. Both parental lines presented low erucic acid and glucosinolate content in the seed (00 quality).

To conduct a quantitative trait loci (QTL) study of seed colour and related features, the population was cultivated over three seasons in various sites. The plants deposition and seed storage are similar with the those presented by Lipsa *et al.* (2012).

Seed colour and phenolic acid content evaluation

Utilising the NIR System 6500 and WinISI II software, seed colour was quantified based on levels of digital reflectance utilising near-infrared reflectance spectroscopy (NIRS) as describe by Lipsa *et al.* (2012).

Sample preparation and extraction of phenolic acid fractions from seed meal

The sample preparation for phenolic acid is similar with sample preparation for condensed tannins (Lipsa *et al.*, 2012) Then the dried rapeseed meal samples (500 mg, 3 repetitions per line) were extracted twice for better results. sing solid-phase extraction (SPE) techniques modified from Naczk and Shahidi (2004) and Sun *et al.* (2006), the phenolic acids were eluted with water after crude rapeseed meal extract was added to activated, preconditioned C18 mini-columns

Chromatographic conditions and HPLC quantification of proanthocyanidins

For the quantification of the phenolic acids via HPLC, procyanidin B2 (Extrasynthese, France) was used as the internal standard. Using a Merck-Hitachi low-pressure gradient system, HPLC analysis for the quantification of phenolic acid fractions from all 166 DH lines was carried out

Genetic linkage map and QTL analysis

Genetic linkage map was created using a crucial logarithm of odds (LOD) score of 3.0 and a maximum likelihood distance of 35 cM. TL analysis was carried out using the composite interval mapping technique.

RESULTS

Phenolic acid content and seed colour variation

In the phenolic acid fraction (*Figure 1*), two major peaks were detected at retention times of 12.72 (F1PA2) and 14.37 min (F1PA1). These peaks have been identified by the co-migration of standard substances and probably correspond to sinapoylglucose and sinapine, the major phenolic compounds in rapeseed (Mabon *et al.*, 1999; Vuorela *et al.*, 2003; Milkowski *et al.*,

2004; Zum Felde, 2007). Furthermore, two signals were detected at retention times of 19.47 (F1PA3) and 20.19 min (F1PA4). Phenotype information for various phenolic acid fractions and seed colour for all 166 DH lines, along with a summary of their unique features, are presented in *Table 1*. The genotypes of the double haploid population's seed colours ranged from 2.9 (bright yellow) to 8.1 (black) in terms of optical light reflectance, while the phenolic acid content ranged from 601.7 to 1466.1 mg/100 g of oil-free rapeseed meal.

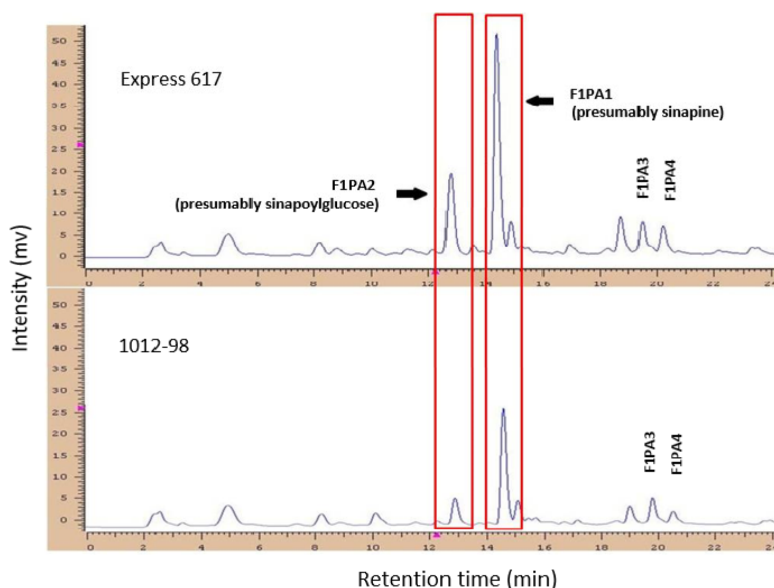

Figure 1 – High-performance liquid chromatography chromatograms (280 nm) of phenolic acid fractions from the black- and yellow-seeded parents ('Express 617' and '1012-98,' respectively). The red areas show the major peaks for the phenolic acids, presumably sinapoylglucose and sinapine

Table 1 – Seed colour and total and individual phenolic acid contents for the YE2 double haploid population and mean values from the yellow- ('1012-98') and black-seeded parental lines ('Express 617')

Seed trait or compound		Min.	Max.	Mean	SD	Parental lines	
						1012-98	Express 617
Seed colour[1)]		2.9	8.1	6.0	1.1	2.8	8.3
Total phenolic acids[2)]		601.7	1466.1	1012.5	164.8	352.5	968.5
Phenolic acids[2)]	F1PA1 (pr. Sinapine)	376.2	936.1	629.2	106.5	239.0	636.5
	F1PA2 (pr. Sinapoylglucose)	39.0	359.6	178.8	58.9	70.9	202.5
	F1PA3	50.0	247.3	123.0	44.0	60.8	80.4
	F1PA4	9.0	179.7	81.4	36.2	43.9	69.0

[1)] Seed colour as absorbance value (1 = white, 10 = black)
[2)] Phenolic acid content in mg/100 g dried rapeseed meal
pr. – presumably

The most predominant phenolic compound was represented by sinapine (F1PA1), with a quantity ranging from 376.2 to 936.1 mg/100g dried meal, followed by sinapoylglucose (F1PA2) with 39.0 to 359.6 mg/100g defatted meal. The minimum content among the phenolic acids was found in the case of an unidentified peak F1PA4 with 9.0 to 179.7 mg/100 g of oil-free meal.

No significant correlation was found between seed colour and phenolic acid fractions F1PA2 (presumably sinapoylglucose), F1PA3 and F1PA4. A weak correlation ($r=0.37^{*}$) was found between seed colour and phenolic acid F1PA1 (presumably sinapine). There are no data in the literature on unexpected correlations between the different phenolic acids and the mentioned traits. The largest proportion of phenolic acids in rapeseed was represented by sinapic acid or its esters (70–85%). These were predominantly found in the cotyledons and only as traces in the seed coat (0.06–0.24%). Among the phenolic acids, F1PA1 was correlated with F1PA2, and F1PA2 was correlated with both F1PA3 and F1PA4.

QTL analysis

Each QTLs' contribution to phenotypic variance is shown in *Table 2*, along with its chromosomal location, LOD score and additive effect (partial R^2).

Localisation of QTL for seed colour

For the seed colour trait, three QTLs were localised on linkage groups A09, C01 and C05 (*Figure 2*, *Table 2*), explaining a total of 66.8% of the phenotypic variance (R^2). The positive alleles of all three QTLs originated from the yellow-seeded parent '1012-98.' There was a highly significant QTL on linkage group N9, with a maximum LOD value of 6.4 and an additive effect of 0.45%. This QTL explained 40.9% of the observed partial phenotypic variance (part. R^2) and played an important role in phenotype expression.

The QTL in linkage group N11 (LOD=7.6) had a smaller effect on phenotype expression (R2=19.0%; part. R2=19.8%) with an additive effect of 0.29%, while the QTL on linkage group N15 (LOD=3.9) explained only 12.2% of the observed partial phenotypic variance, with an additive effect of 0.23%.

Localisation of QTL for phenolic acids

For three different phenolic acid compounds (F1PA1, F1PA2, F1PA3), nine quantitative feature loci were mapped to seven linkage groups (*Figure 2*).

For trait F1PA1 (presumably sinapine), two major QTL ($R^2 \geq 10\%$) were located on linkage groups C01 and C05. These two QTLs had a clearly comparable effect on phenotype expression, with 13.6 and 12.8%. The two QTLs explained 21.1% of the phenotypic variance, and the LOD curves reached two maxima of 5.3 and 4.9, respectively.

For trait F1PA2 (presumably sinapoylglucose), three QTLs were localised to linkage groups A02, A09 and C01 at a LOD threshold of 3.0. The total phenotypic variance corresponded to 39.7%. The individual QTL explained 11.2 (A09), 16.8 (C01) and 40.0% (A02) of the observed phenotypic variance (R^2), and 5.6 (A09), 14.9 (C01) and 32.2% (A02) of the partial phenotypic variance (part. R^2). LOD values differed

between 4.2 for linkage group A09 and 18.4 for linkage group A02.

The individual QTL for trait F1PA3 described between 8.8 and 16.0% of the observed phenotypic variance in the mapping population. Overall, 34.2% of the phenotypic variance was explained by these four QTLs. The locus with the highest partial phenotypic variance (part. R^2=6.4%) and a LOD value of 6.3 was located on linkage group C03. Linkage group A09 contained the second QTL (LOD=4.9; part. R2=12.1%); the third was on linkage group C09 (LOD=4.4; part. R2=8.6%), and the fourth was on linkage group C06 (LOD=3.3; part. R2=8.6%).

Chromosomal QTL distribution in the YE2-DH population

In linkage group A02 (*Figure 2*), the main QTL for F1PA2 content (presumably sinapoylglucose) explained 40% of the phenotypic variance for these traits and had a statistically significant LOD value. The QTL on A02 had a very large effect on antinutritive phenolic acids but did not have any effect on seed colour because phenolic acids were predominantly localised in the cotyledons. For trait F1PA2, a total of three QTLs were localised on linkage groups A02, A09 and C01 at a LOD threshold of 3.0.

In linkage group A09, a 10 cM region with a strong effect on two different seed traits was detected. Here, the QTL for seed colour and phenolic acids (F1PA2, F1PA3) were colocalised. Among all QTL, the one with the strongest effect on seed colour explained 40.9% of the observed partial phenotypic variance (part. R^2) and played an important role in phenotype expression.

In the same place as the QTL for seed colour on linkage group A09, there was a major QTL for ADF (acid detergent fibre), NDF (neutral detergent fibre) and ADL (acid detergent lignin) content (Bekele, 2008).

Table 2 – A summary of the quantitative trait loci in the double haploid population's genetic map for seed colour and phenolic acid components

Trait		Marker interval	Chromosome position (cM)	LOD Score	R^2 (%)	Partial R^2 (%)
Seed colour		Ni4D09 / SSR1_144	A09 / 26	6.38***	16.2	40.9
		E32M62_197 / E35M62_151	C01 / 48	7.61***	19.0	19.8
		Ol10D02_A / E35M60_153	C05 / 4	3.89*	10.2	12.2
Phenolic acids	F1PA1 (presumably sinapine)	tt1-1 / mr000228	C01 / 36	5,25**	13,6	12,5
		Ol10D02_A / E35M60_153	C05 / 6	4.94**	12.8	11.6
	F1PA2 (presumably sinapoylglucose)	E35M60_305 / sn012964	A02 / 36	18,39***	40,0	32,2
		Ni4D09 / SSR1_144	A09 / 24	4,24**	11,2	5,6
		tt1-1 / mr000228	C01 / 36	6,65***	16,8	14,9
	F1PA3	Ni4D09 / SSR1_144	A09 / 24	4,91**	12,7	12,1
		Na10E02 / E40M60_334	C03 / 16	6,25***	15,9	16,4
		E33M59_271 / Ol12E03	C06 / 56	3,32	8,8	8,6
		E35M62_169 / E33M48_186	C09 / 38	4,38**	11,4	8,6

* significant at p=0.05 by permutation analysis; ** significant at p=0.01; *** significant at p =0.001
R^2 (%): phenotypic variation; Partial R^2 (%): partial phenotypic variation

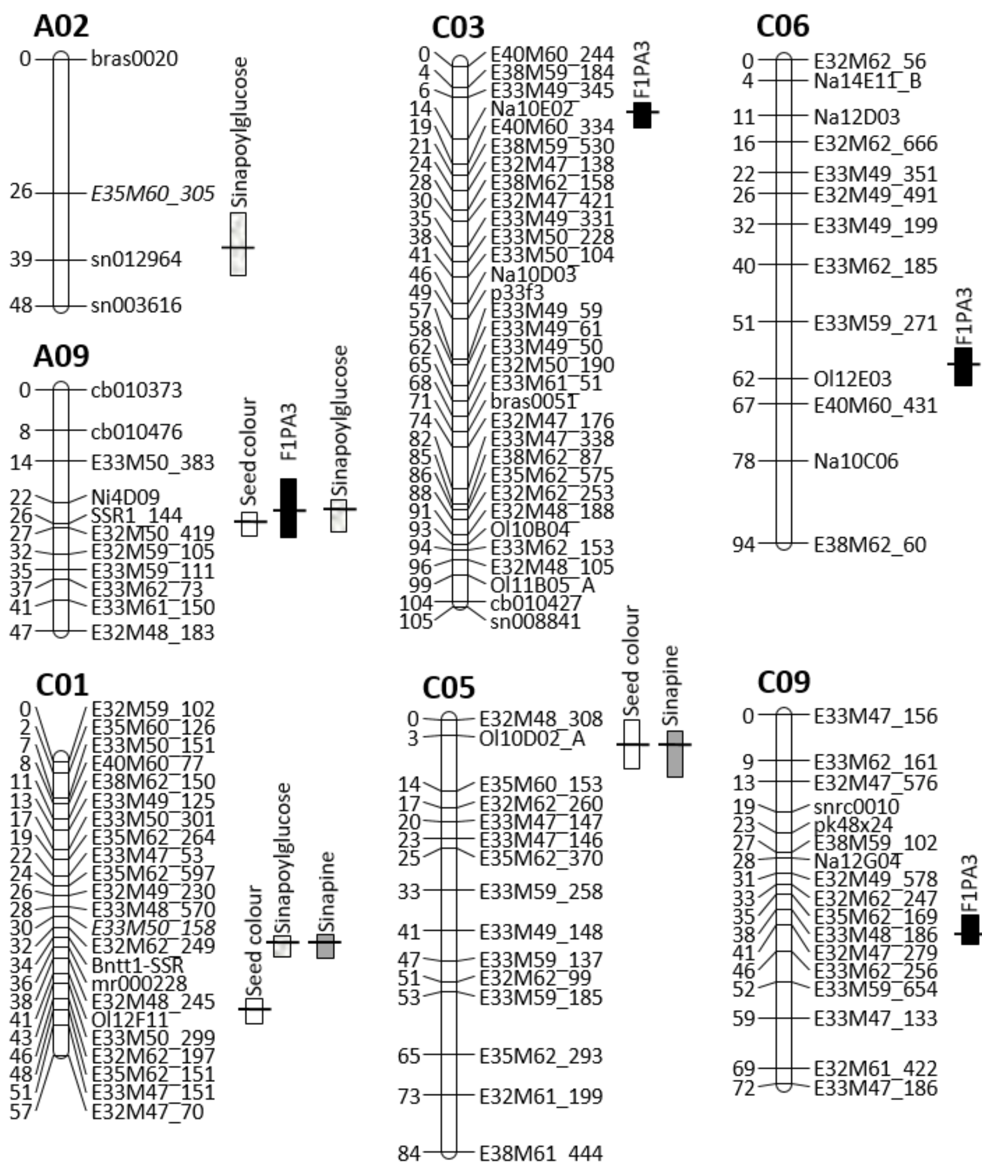

Figure 2 – Localisation of QTL for seed colour and phenolic acids on *Brassica napus* chromosomes of the mapping population

The SSR marker tt1_1 was located in the middle of linkage group NC01 of the population Express 617 × 1012-98 and was potentially tightly coupled to the tt1 gene in *B. napus* (Stein *et al.*, 2013). The QTL for F1PA1 (putative sinapin) and F1PA2 (putative sinapoylglucose) content colocalised and were located on linkage group C01 in close proximity to marker tt1_1. The

major QTL for the phenolic acid F1PA3 was located in linkage group C03 and explained 15.9% of the phenotypic variance. *Figure* 2 shows linkage group C05 of the *B. napus* DH population. In this linkage group, QTLs were distributed practically along the entire chromosome, influencing the quality traits of the canola seeds. The mapped QTL for seed colour and phenolic acid F1PA1 (presumably sinapine) colocalised in the upper part of the chromosome. In the middle region of chromosome C05, the main QTL for oil content was localised (data not shown in the paper). In linkage groups C06 and C09, two QTLs for F1PA3 content were detected, explaining 8.8 and 11.4% of the phenotypic variance.

DISCUSSION

The use of rapeseed meal or rapeseed extraction meal as a high-quality protein source for animal and human nutrition is an alternative to soybean meal. However, the use of rapeseed extraction meal as a complete feed is still limited due to antinutritive or undesirable ingredients (glucosinolates, sinapins, tannins and fibre components), which impair digestibility and protein availability and are also responsible for a bitter taste, especially when used in the feeding of monogastric livestock (chicken, pig) (Cheng *et al.*, 2022).

Breeding and selection of yellow-seeded canola genotypes represents the opportunity to reduce antinutritional phenolic substances, while increasing oil and/or protein content, and to achieve better digestibility and protein availability, as well as higher acceptance of canola meal as animal feed.

In the course of the present work, 166 lines of a DH population from the cross Express 617 (black-seeded) × 1012-98 (yellow-seeded) were analysed for total and individual phenolic acid content using an HPLC assay and, as expected, a wide variation in quantification was observed.

Phenolic acids represent the predominant phenolic compounds in canola seed, with sinapine accounting for approximately 85% of all occurring sinapic acid esters in canola (Qiao and Classen, 2003). HPLC examination of a crude methanolic extract revealed two major peaks. With reference to Qiao and Classen (2003), these peaks are thought to correspond to sinapine (peak F1PA1) and sinapoylglucose (peak F1PA2), the two most abundant phenolic acids in canola meal. QTL analysis showed that the major QTL for F1PA1 content was located at the same position on chromosome C01, with a locus for F1PA2 content. In other words, there is probably a gene mutation at this location in the genome that results in a simultaneous reduction of both phenolic acids in the yellow-seeded parent. At the same locus, there are major QTL for total flavonoid and proanthocyanidin content and adjacent QTL for seed colour and oligomeric (F2PA3) and polymeric proanthocyanidins (F3PA4) (Lipsa *et al.*, 2012). Because a copy of the potential candidate gene tt1 is also suspected here because of a neighbouring SSR marker (Bntt1), a mutation in a tt1 gene copy would be a possible explanation for these QTL. tt1 is a WIP zinc finger transcription factor with direct effects on endothelial cell formation. Mutations in this gene in

Arabidopsis are expressed by a thin, transparent seed coat.

A minor QTL for F1PA1 content (presumably sinapin) is located on linkage group C05 in the same interval (2–8 cM) with the QTL for seed colour. On chromosome A09, two loci for F1PA2 and F1PA3 colocalised with the main QTL for seed colour (part. R2 = 40.9%), together with additional QTL for ADF, NDF and ADL content, and with a QTL with a small effect on flavonoid content (Bekele, 2008; Lipsa *et al.*, 2012; Gacek *et al.*, 2021). With regard to breeding for improvement of rapeseed meal quality, this result is significant because it demonstrates an association between seed coat thickness, phenolic acid content (F1PA1, F1PA2, F1PA3) and other antinutritive substances. Marker-assisted selection with reference to this locus could achieve substantial reductions in phenolic acid and fibre components in canola meal, independent of seed colour.

The QTL on linkage groups C01 and C05 for phenolic acids (F1PA1, F1PA2), seed colour and other antinutritional traits coincide with the positions of two copies of the gene BnSGT1-(sinapoylglucose transferase) in the YE2-DH population (Lipsa *et al.*, 2012). SGT1 is a major gene involved in sinapate ester biosynthesis and is expressed during seed formation. The obtained results suggest a possible interaction between Bntt1 and BnSGT1 in the regulation of phenylpropanoid biosynthesis in the seed coat.

Today, to identify more closely linked molecular markers than the reported SSR markers, advanced resources are available, including a single nucleotide polymorphism (SNP) map for the YE2 population (Stein *et al.*, 2013) that could be used to assign QTL by comparison to the new reference genome from Express 617 (Lee *et al.*, 2020). This information could be used to more closely identify sequence-annotated markers flanking the QTL and possibly narrow down the candidate genes.

CONCLUSIONS

Two distinct peaks of phenolic acids were detected during the HPLC characterisation of crude phenolic extracts. These are thought to represent the phenolic acids sinapine (F1PA1) and sinapoylglucose (F1PA2), the two most common in rapeseed meal. F1PA1 and F1PA2 QTLs, as well as a seed colour QTL, were clustered together on chromosome C01. The primary QTL for seed colour (part. R^2=40.9%) and additional QTL for total flavonoid compounds, ADF, NDF and ADL content were all located on the same region of chromosome A09. This region also contained two loci for F1PA2 (probably sinapoylglucose) and F1PA3. Because a correlation was observed between seed coat thickness and amounts of phenolic acids and other antinutritive chemicals, this research has important implications for breeding to improve the quality of rapeseed meal. More precise mapping of the major QTL using dense, sequence-annotated SNP markers, for example, from a denser SNP map of the YE2 population published by Stein *et al.* (2013), may help identify sequence-annotated markers flanking the QTL. Using closely linked SNPs for MAS could significantly

reduce antinutritional phenolic acids and fibre components in rapeseed meal, independent of seed colour.

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