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ORIGINAL PAPER

Mapping of QTL for the seed storage proteins cruciferin and napin in a winter oilseed rape doubled haploid population and their inheritance in relation to other seed traits

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Abstract
Key message **Cruciferin (cru) and napin (nap) were negatively correlated and the cru/nap ratio was closely negative correlated with glucosinolate content indicating a link between the two biosynthetic pathways.**
Abstract Canola-type oilseed rape (*Brassica napus* L.) is an economically important oilseed crop in temperate zones. Apart from the oil, the canola protein shows potential as a value-added food and nutraceutical ingredient. The two major storage protein groups occurring in oilseed rape are the 2 S napins and 12 S cruciferins. The aim of the present study was to analyse the genetic variation and the inheritance of napin and cruciferin content of the seed protein in the winter oilseed rape doubled haploid population Express 617 × R53 and to determine correlations to other seed traits. Seed samples were obtained from field experiments performed in 2 years at two locations with two replicates in Germany. A previously developed molecular marker map of the DH population was used to map quantitative trait loci (QTL) of the relevant traits. The results indicated highly significant effects of the year and the genotype on napin and cruciferin content as well as on the ratio of cruciferin to napin. Heritabilities were comparatively high with 0.79 for napin and 0.77 for cruciferin. Napin and cruciferin showed a significant negative correlation (−0.36**) and a close negative correlation of the cru/nap ratio to glucosinolate content was observed (−0.81**). Three QTL for napin and two QTL for cruciferin were detected, together explaining 47 and 35 % of the phenotypic variance. A major QTL for glucosinolate content was detected on linkage group N19 whose confidence interval overlapped with QTL for napin and cruciferin content. Results indicate a relationship between seed protein composition and glucosinolate content.

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Introduction

The meal of Canola-type oilseed rape (*Brassica napus* L.) with a low glucosinolate content in the seed and a low erucic acid content of the seed oil is a valuable feedstuff for animals and a potential protein source for human nutrition (Leckband et al. 2002; Wanasundara 2011) as well as for other non-food uses (Malabat et al. 2001). The oil extracted meal contains about 35–40 % protein (Dimov et al. 2012). Canola research was focused so far on enhancing the seed oil content and improving the oil quality as well as reducing the concentration of undesirable compounds in the meal like, e.g., sinapic acid esters (Hüsken et al. 2005; Zum Felde et al. 2006; Harloff et al. 2012), phytic acid (Lickfett et al. 1999) and fibre content (Bell 1993; Wittkop et al. 2009; Dimov et al. 2012).

The seed storage protein of oilseed rape is composed mainly of cruciferin and napin which account for 60 and 20 %, respectively, of the total protein in mature seeds (Crouch and Sussex 1981; Höglund et al. 1992). The remaining proteins consist mainly of oil body proteins (oleosins, Jolivet et al. 2009) and lipid transfer proteins (Uppström 1995; Malabat et al. 2003). Napin is a 13 kDa low molecular weight basic 2 S albumin. It consists of a large (9 kDa) and a small (4 kDa) subunit, which are connected via disulfide bonds. Napins are encoded by a

multigene-family and the reported copy number varies between 10 (Josefsson et al. 1987) und 16 (Scofield and Crouch 1987) as determined by Southern blot analyses. Cruciferin is a comparatively large (300 kDa), neutral oligomeric 12 S-globulin which belongs to the cupin (small β-barrel) superfamily (Withana-Gamage et al. 2011). It is a hexameric protein which is similar to 11–12 S seed proteins of other species (Rödin et al. 1992 and references therein). The hexameric protein is assembled of two trimers, each comprising three heterogeneous subunits. Each subunit consists of two polypeptides, the heavy α- (acidic, 254–296 amino acids) and the light β- (basic, 189–191 amino acid residues) chains that are linked by one disulfide bond (Rödin et al. 1992; Withana-Gamage et al. 2011). The molecular weight of the α-chains is in the range of 29–33 kDa and that of the light β-chains is 21–23 kDa (Rödin et al. 1992; Kohno-Murase et al. 1995). Three major groups of cruciferin subunits exist (cru1, cru2/3 and cru4), although one of the groups (cru2/3) consists of two very similar subtypes (cru2 and cru3). By Southern blot analysis the gene copy number was estimated to be 3–4 for the cru1 and cru2/3 precursors and 2 for the cru4 precursors (Rödin et al. 1992).

The amino acid composition of the oilseed rape protein is excellently balanced (Sosulski 1979) with perhaps only a slightly limited amount of lysine (Kohno-Murase et al. 1995). With 3–4 % of sulphur-containing amino acids, the oilseed rape protein is closer to FAO recommendations for humans than any other available vegetable protein (Ohlson and Anjou 1979). Napin has a higher content of the sulphur amino acids cysteine and methionine and of lysine than cruciferin. Hence, reported differences in the amino acid content between oilseed rape genotypes may be indicative for different contents of napin and cruciferin (Malabat et al. 2003). The amino acid composition of the seed storage protein is relevant when the meal is used in animal feeding diets (Kohno-Murase et al. 1995). Genetically modifying the composition of the seed storage proteins therefore should directly affect the amino acid composition. In a transgenic antisense approach, oilseed rape plants were obtained that totally lacked napin as determined by polyacrylamide gel electrophoresis. In those plants reduced napin content was counterbalanced by increased cruciferin content so that the total protein content of the seeds remained unchanged (Kohno-Murase et al. 1994). In a follow up complementary antisense approach the level of cruciferin was reduced which led to an increased napin level and enhanced contents of cysteine, methionine and lysine (Kohno-Murase et al. 1995). As before, the total seed protein content did not change. Malabat et al. (2003) investigated to what extent the conversion from glucosinolate and erucic acid containing cultivars to Canola quality affected the seed storage protein composition. They observed that the former varieties tended to have higher contents of napin and reduced contents of cruciferin. However, the effect of a modified seed storage protein composition on traits like germination, primary and secondary dormancy (Schatzki et al. 2013a), seed longevity in the soil (Nagel et al. 2011; Schatzki et al. 2013a) and fungal disease resistance (Terras et al. 1992; Barciszewski et al. 2000) has yet not been investigated.

There is an increasing commercial interest to investigate and to control the genetic variation of the seed storage protein composition of oilseed rape, because napin and cruciferin have quite different structural, thermal, functional and biological properties which makes them attractive for a number of applications in food and non-food production (Aluko and McIntosh 2001; Malabat et al. 2001; Yoshie-Stark et al. 2006; Wu and Muir 2008; Wanasundara 2011). Furthermore, napin-like proteins are attributed to have antimycotic and antibacterial properties (Terras et al. 1992; Barciszewski et al. 2000 Neumann et al. 1996b, c; Polya 2003). Breeding techniques may be applied to use existing genetic variation in the protein composition and to modify it towards higher cruciferin, respectively higher napin contents. The aim of the present study was to analyse the genetic variation and the inheritance of napin and cruciferin content of the oilseed rape seed protein in a winter oilseed rape doubled haploid population and to determine correlations to other seed traits previously determined in this population (Schatzki et al. 2013b).

Materials and methods

Plant material and field experiments

The seed material was obtained from 229 doubled haploid (DH) lines, which were cultivated in observation plots in field experiments with two replicates in two consecutive seasons, 2008/2009 and 2009/2010, at two locations, Göttingen and Thüle, in northwestern Germany. The DH population was derived from a cross between the inbred line 617 of the German winter oilseed rape cultivar Express (canola quality) and the resynthesised line R53, an interspecific hybrid between *B. oleracea* var. *sabellica* (kale) and *B. rapa* ssp. *pekinensis* (chinese cabbage, see Radoev et al. 2008). R53 has high erucic acid content in the seed oil and high glucosinolate content in the seeds (Schatzki et al. 2013b). The parental genotypes Express 617 and R53 were cultivated together with the DH population in the field. In both field years, seeds were harvested at maturity from the main inflorescence of ten open pollinated plants per genotype and replicate. Seeds from the ten plants were bulked for further analysis. For napin and cruciferin analysis 3 g of seeds from each of the four replicates of a genotype and field year were pooled.

Protein extraction

Then 10 g from each of the mixed seed samples was ground in a coffee mill model Krups F203 for 6 s (3 times 2 s with in between mixing of the meal). From each sample 100 mg of oilseed rape meal was weighed in a 1.5 mL tube and mixed with 1,000 µL deionized water for 10 s and incubated for 3 min in a thermo mixer (model Thermomixer 5436, Eppendorf AG, Barkhausenweg 1, D-22339 Hamburg) with 1,000 rpm at 30 °C. Finally the samples were incubated in an ultrasonic bath (Frequency 48 kHz) for 2 min at 30 °C. Subsequently, the samples were centrifuged for 10 min at 14,000 rpm (model Micro 200, Andreas Hettich GmbH & Co.KG, Föhrenstr.12, D-78532 Tuttlingen) at 4 °C in the cooling chamber and 500 µL was transferred to a new 1.5 mL tube and centrifuged again. 400 µL was transferred to a new 1.5 mL tube and was frozen for further use. 2 weeks before conducting the gel electrophoresis the protein extracts were centrifuged with 14,000 rpm for 5 min at 4 °C and mixed (1:1) with a Laemmli sample buffer (125 mM Tris/HCl, pH 6.8; 20 % (v/v) Glycerol; 4 % (w/v) SDS; 0.1 % (w/v) bromphenol blue; 5 % (v/v) β-mercaptoethanol) and aliquots of 10 µL were frozen for final use. A lyophilisate of purified napin provided by Dr. Klaus Duering (Axara Consulting, Auf dem Rotental 47, D-50226 Frechen) was dissolved in deionized water, mixed (1:1) with Laemmli sample buffer and frozen until final use.

SDS-PAGE

Frozen protein samples, purified napin standard (0.5 µg/µL) and an unstained molecular weight marker (14.4–116 kDa, Lot No.: 00068297, Fermentas GmbH, Opelstraße 9, D-68789 St. Leon-Rot) were gently thawed on ice and subsequently boiled at 95 °C in the thermo mixer with 1,000 rpm and centrifuged at 14,000 rpm for 5 min. From each sample 2 µL was loaded onto precast 4–15 % gradient gels (model Mini-PROTEAN© TGX™, Bio-Rad Laboratories GmbH, Heidemannstr. 164, D-80901 München) with 15 wells running with constant 60 V for 140 min with Laemmli buffer system in a Mini-PROTEAN© chamber. Afterwards the gel was stained with a 0.04 % Coomassie blue (PlusOne Coomassie tablet PhastGel™ Blue R-350, GE Healthcare Deutschland, Oskar-Schlemmer-Str. 11, D-80807 München), 40 % (v/v) ethanol, 10 % (v/v) acetic acid solution for 30 min and washed twice (2 × 1 min) with distilled water. De-staining was performed for 30 min with 20 % (v/v) ethanol and 10 % (v/v) acetic acid and washed once with deionised water for 1 min before the gels were put into de-staining solution with 10 % (v/v) ethanol and 5 % (v/v) acetic acid over night for 15 h.

Evaluation of protein amounts

The protein gels were washed in deionised water for 30 min and subsequently scanned with a dual lens scanner (model Epson Perfection V700 Photo, Seiko Epson Corporation, Tokyo, Japan) with following settings (scan mode: film/positive film, type: 48-bit color, quality: optimal, resolution: 800 dpi, no adjustment and processing was performed). The freely accessible program ImageJ version 1.45 (Rasband 2011) was used with standard settings for gel analysis to integrate peaks of protein bands. Protein amounts in individual bands were calculated from the relation of the peak area of the band to the mean peak area of two purified napin standards on each gel. For identification of the napin protein we used the given napin standard (see lane G and H in Fig. 1) and for identification of cruciferin we used the molecular weights published in the literature

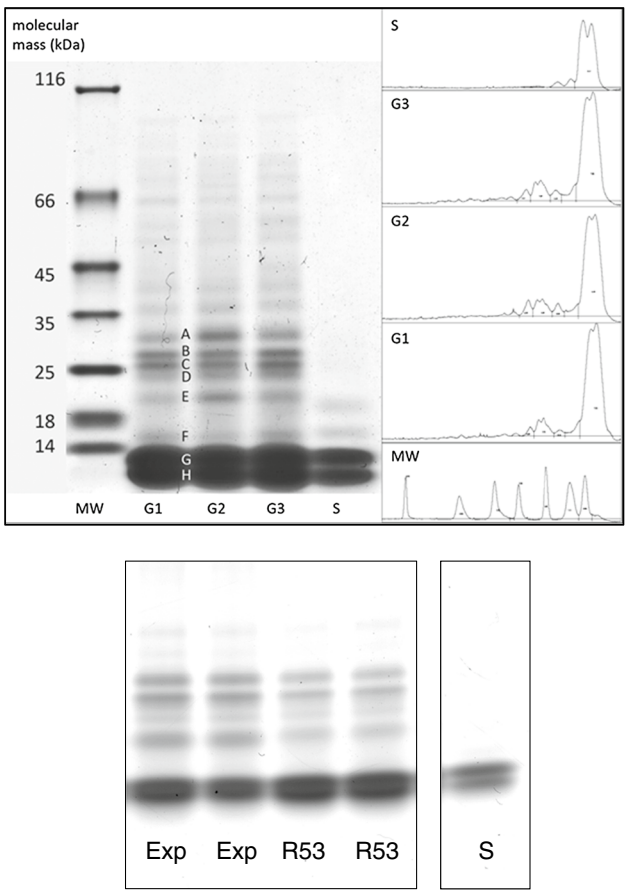

Fig. 1 *Above*, *left* separation of molecular weight marker and oilseed rape seed proteins on a 4–15 % polyacrylamide gel (SDS-PAGE) in presence of β-mercaptoethanol. Proteins were stained with Coomassie Blue and gels were scanned. *Right* corresponding lanes as ImageJ peak output. *MW* molecular weight marker, *G1-3* canola protein extract from seed of three different genotypes, *S* purified napin standard. *Below* Seed protein profiles of the parental lines Express (Exp) and R53 along with the purified napin standard

Table 1 Variance components and heritability for napin-, cruciferin content (µg), cru/nap ratio, glucosinolate- (µmol/g seed at 91 % DM), protein-, oil-, protein in defatted meal- (% at 91 % DM) and sulphur content (% seed DM)

Source of variance	Napin	Cruciferin	Cru/nap ratio	GSL†	Sulphur	Protein†	Oil†	Protein odM†
Year (Y)	0.008**	0.009**	0.002**	0.73	0.0003	0.62	0.93	0.44
Location (L)	–	–	–	0.22	0.0003*	0.30*	0.22*	0.43*
Genotype (G)	0.065**	0.020**	0.026**	269.3**	0.0147**	0.95**	2.82**	1.81**
G × Y	0.034	0.012	0.005	5.15**	0.0003*	0.18**	0.28**	0.22**
G × L	–	–	–	3.05**	0.0001	0.10**	0.16**	0.18**
G × Y × L	–	–	–	5.59**	0.0002+	0.21**	0.29**	0.34**
Residual	–	–	–	32.15	0.0025	0.86	1.29	1.07
h^2	0.79	0.77	0.92	0.98	0.98	0.86	0.93	0.90

+, *, ** Significant at $P = 10$, 5 and 1 %, odM: of defatted meal

† Data taken from Schatzki et al. (2013b)

Table 2 Min, max and mean values for napin-, cruciferin content (µg), cruciferin/napin ratio, glucosinolate- (µmol/g seed at 91 %), protein-, oil-, protein in defatted meal- (% at 91 % DM) and sulphur content (% seed DM) of the DH population and the parental lines

	Napin	Cruciferin	Cru/nap ratio	GSL†	Sulphur	Protein†	Oil†	Protein odM†
Min	0.80	0.23	0.13	16.8	0.30	18.1	39.6	34.2
Max	2.22	1.26	1.05	81.5	0.83	24.3	48.3	41.6
Mean	1.50	0.53	0.38	42.6	0.52	21.1	44.1	37.8
LSD5%	0.36	0.22	0.13	6.6	0.06	1.1	1.4	1.3
Express 617	1.38	0.53	0.38	16.7	0.41	18.0	45.7	33.1
R53	1.60	0.35	0.22	41.8	0.58	20.6	42.6	35.9

LSD5%: least significant difference at $P = 5$ %, odM: of defatted meal

† Data taken from Schatzki et al. (2013b)

Table 3 Spearman-rank correlation coefficients for napin-, cruciferin contents and other seed traits

	Napin	Cruciferin	Cru/nap	GSL†	Sulphur	Protein†	Oil†	Prot odM†	TSD†
Cruciferin	−0.36**	–	–	–	–	–	–	–	–
Cru/nap	−0.72**	0.89**	–	–	–	–	–	–	–
GSL†	0.76**	−0.62**	−0.81**	–	–	–	–	–	–
Sulphur	0.79**	−0.61**	−0.83**	0.97**	–	–	–	–	–
Protein†	0.36**	−0.11	−0.26**	0.36**	0.45**	–	–	–	–
Oil†	−0.23**	0.23**	0.30**	−0.39**	−0.48**	−0.66**	–	–	–
Prot odM†	0.28**	0.04	−0.10	0.13*	0.19**	0.77**	−0.06	–	–
TSD†	−0.19**	0.06	0.13	−0.14*	−0.15*	−0.13	0.10	−0.10	–
TKW†	0.18**	0.03	−0.06	0.16*	0.15*	0.03	−0.13*	−0.08	−0.08

GSL glucosinolate content, *TSD* total seed dormancy, *TKW* thousand kernel weight

*, ** Significant at $P = 5$ and 1 %, respectively

† Original data taken from Schatzki et al. (2013b)

(Schwenke et al. 2000) indicated by the lanes A, B, C, D in Fig. 1.

Sulphur content and other traits

Sulphur content (% seed dry matter) was determined by NIRS using the calibration raps2009.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Teichstr. 35, D-34130 Kassel, http://h1976726.stratoserver.net/cms (28 Oct 2013). Data for other seed quality traits were taken from a previous paper (see Tables 1, 2, 3; Schatzki et al. 2013b).

Statistics

Analysis of variance and calculation of heritabilities (h^2) were performed by using PLABSTAT software (Utz 2011) considering the years, locations, replicates and genotypes as random. Mean values of the genotypes over the years were used to calculate Spearman's rank correlation

coefficients between traits. $^{+}$, *, ** denotes significant at $P = 10$, 5 and 1 %, respectively.

Molecular marker map and QTL mapping

The framework map developed by Radoev et al. (2008) was improved and extended and finally consisted of 229 markers including 80 SSR and 149 AFLP markers. The mean distance of all markers covering 21 linkage groups was 9.5 cM, the standard deviation was 5.7 cM and the maximum distance was 26.6 cM (Kosambi). Total map size was 2,283 cM. Linkage groups were named according to the N-nomenclature (Parkin et al. 1995). Linkage groups N1 to N10 of this nomenclature correspond to linkage groups A1 to A10 and N11 to N19 to C1 to C9 in the new A/C nomenclature proposed by the Steering Committee of the Multinational Brassica Genome Project (http://www.brassica.info/resource/maps/lg-assignments.php, 28 Oct 2013).

Quantitative trait loci (QTL) mapping was performed using the freely available software QTL Network 2.1 (Yang et al. 2009) with a significance level of $P = 5$ % for declaring a QTL. Permutation analyses were performed (1,000 permutations) to determine the critical F value threshold for each trait. Additive × additive epistatic interactions between loci were also evaluated by using QTL Network 2.1

Results

The separation of seed proteins extracted from the oilseed rape meals on denaturing polyacrylamide gels (SDS-PAGE) with a gradient of 4–15 % worked quite satisfactorily (Fig. 1). In routine analysis, only those gels were evaluated which showed a clear separation of the protein bands. The lane with the napin standard (S in Fig. 1) showed clear separation of the two napin bands, which represent after SDS treatment and under reducing conditions the large and small subunit (cf. Introduction). There were also bands with a larger molecular weight (14–18 kDa), indicating that the napin standard was not 100 % pure. The band of the smaller protein was also detectable in the extract samples of the DH population. The cruciferin bands A to E in Fig. 1 were identified according to Schwenke et al. (2000). Under reducing conditions 5 cruciferin bands were identified, consisting of α-chains (27–31 kDa) and β-chains (18–21 kDa). Since it was not clear if oleosins (20 kDa, Jolivet et al. 2009) were present in the extract, protein band E was excluded from the calculation of the total cruciferin amount.

The analyses of variance indicated highly significant effects of the year and the genotype on napin and cruciferin content as well as on the ratio of cruciferin to napin (cru/nap ratio, see Table 1). The variance components of the genotype by year interaction for these traits include the residual error and were about half the size of the genotypic effects. The genotype by year interaction was comparatively much lower for the cruciferin to napin ratio and the heritability was with 0.92 surprisingly high. Highly significant effects of the genotype were also found for all other traits. Genotype by year interaction was significant for glucosinolate, sulphur, protein, oil and protein content in the defatted meal. Heritabilities were high for glucosinolate-, sulphur- and oil content of the seeds.

A large variation among the 229 genotypes was detected for napin- and cruciferin content ranging from 0.8 to 2.2 and from 0.2 to 1.3 µg, respectively (see Table 2). The cruciferin to napin ratio ranged from 0.13 to 1.05. Both parental genotypes had a much higher napin than cruciferin content as apparent from the low cruciferin to napin ratio. The cru/nap ratio mean of the DH population was as high as the value of the higher parent Express 617. Large variations were also observed for all other traits. The frequency distribution for napin showed a rather normal distribution (Fig. 2). For cruciferin and the cruciferin to napin ratio, the frequency distribution was skewed towards lower values (Fig. 2). In the DH population, significant correlations were discovered between napin content and all other traits (Table 3). Napin content was negatively correlated with cruciferin (Fig. 3). It was positively correlated with protein and negatively correlated with oil content (Table 3; Fig. 3). The opposite was observed for cruciferin content. A close positive correlation between glucosinolate and napin content and a close negative correlation between glucosinolate and cruciferin content were found (Fig. 3). Sulphur content was positively correlated with glucosinolate, napin and protein content but negatively correlated with cruciferin content. Furthermore, napin content was positively correlated with thousand kernel weight and negatively correlated with total seed dormancy.

The QTL mapping revealed three QTL for napin content and two QTL for cruciferin content (Table 4). The three QTL for napin content explained together 47 % of the phenotypic variance. The two QTL for cruciferin content explained 35 % of the phenotypic variance. Together with the epistatic effects the genetic main effects explained 39 % of the phenotypic variance. In total 61 % of the phenotypic variance of the cruciferin to napin ratio was explained by the variance of the additive and epistatic effects. The QTL for napin and/or cruciferin content were located on linkage groups N2, N16 and N19, whereby the QTL on N-19 had the largest effect on napin and cruciferin content.

For the mapped QTL, the estimated additive effect (a) is shown as the substitution of one R53 allele by an Express 617 allele. For example, at the QTL Na-1 of linkage group N2 the substitution of the R53 allele by an Express 617

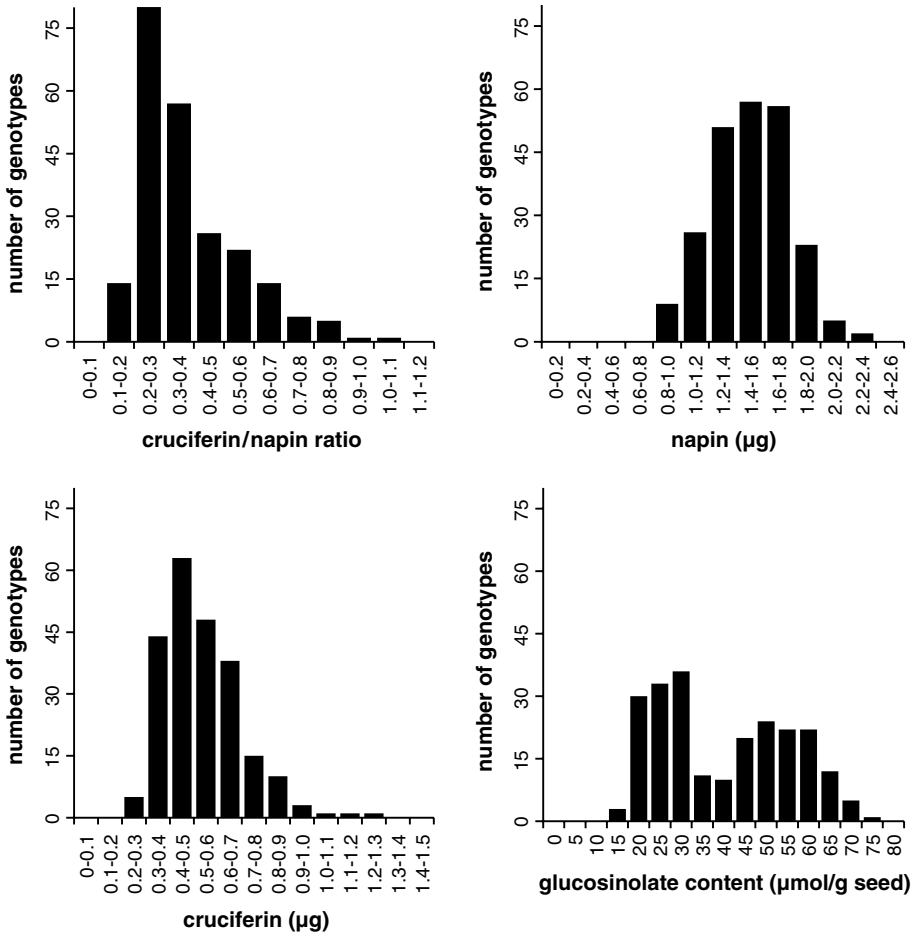

Fig. 2 Frequency distributions of napin, cruciferin and glucosinolate contents and of the cruciferin to napin ratio in the DH population of 229 genotypes (mean values over 2 years)

allele led to an increase in napin content of 0.06 µg. The results show that in two out of three cases, the Express 617 allele led to a reduction in napin content. This is consistent with the result that Express 617 has lower napin content than R53 (cf. Table 2; Fig. 1). The QTL for napin and cruciferin content on linkage groups N2 and on N19 had clearly overlapping confidence intervals. Their signs of the additive effects had different directions, indicating that at these loci the cruciferin and napin content were affected simultaneously in different directions. On linkage group N16 there was a QTL which obviously only affected the napin content. Carefully checking the data did not reveal a QTL for cruciferin content below the significance level in this region of linkage group N16. A number of QTL were also identified for glucosinolate, oil and protein content of the seeds. The frequency distribution (Fig. 2) and the scatter plot of the glucosinolate content plotted against napin and cruciferin content (Fig. 3) indicated a bimodal 1:1 segregation of the glucosinolate content which can be explained by segregation of the major QTL for glucosinolate content on linkage group N19. Interestingly, QTL for cruciferin, napin, oil, protein and glucosinolate content were located in the same region around 30 cM of linkage group 19 (see also Fig. 4). QTL GSL-2 on linkage group 19 had the largest effect on the glucosinolate content. The negative sign of the additive effect confirms that the allele from the low glucosinolate parent Express 617 led to a reduction in seed glucosinolate content. However, simultaneously, there is a reduction in napin and protein content (cf. QTL Na-3 and Pro-2) and an increase in cruciferin and oil content (cf. QTL Cru-2 and Oil-4).

Discussion

The seed proteins of oilseed rape consist mainly of cruciferin, napin, oleosin and lipid transfer proteins (Raab et al. 1992; Uppström 1995; Malabat et al. 2003). Oleosins are associated with the oil bodies and are removed by defatting of the meal (Huang 1996; Malabat et al. 2003). After defatting of the seed meal of 64 *Brassica napus* genotypes and fractionation of the proteins according to their size on a gel filtration column, Malabat et al. (2003) identified three main peaks corresponding to cruciferin, napin and lipid transfer protein. The cruciferin and the napin content of the seed protein ranged from 32 to 53 and from 25 to

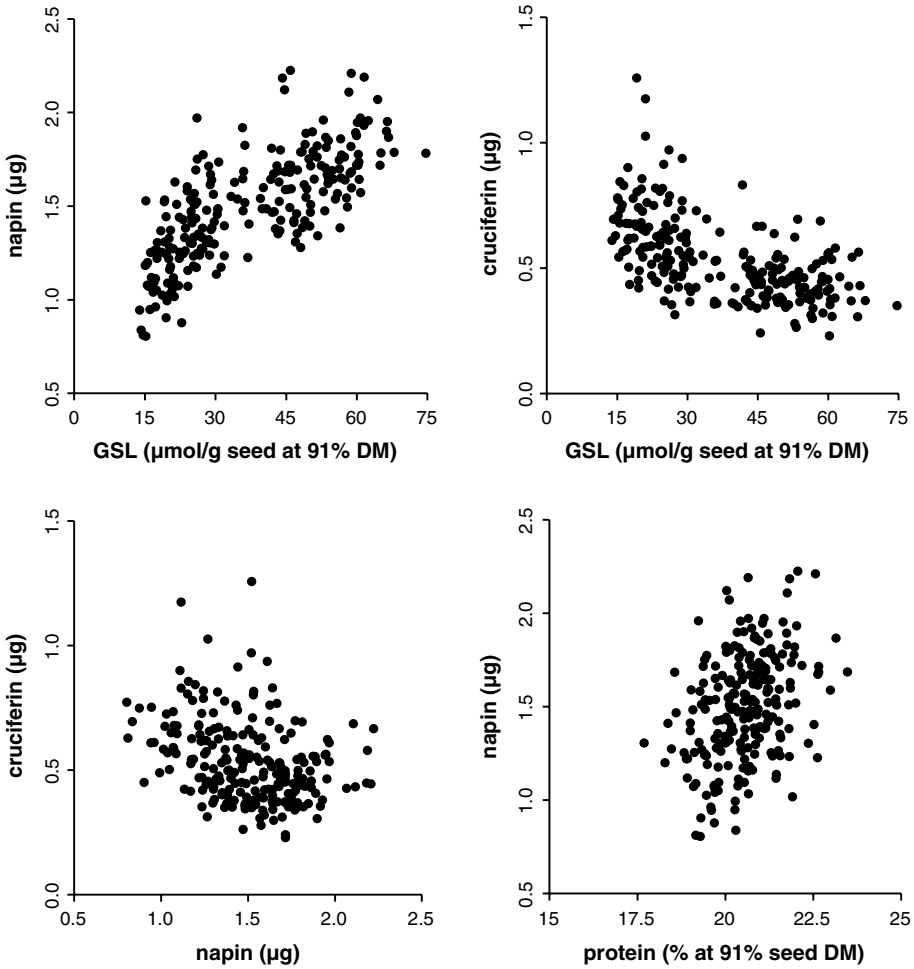

Fig. 3 Scatterplots of seed quality traits (*GSL* glucosinolate)

45 %, respectively. For most of the varieties, cruciferin proved to be the major protein and in some genotypes the cruciferin to napin ratio reached 2 (Malabat et al. 2003). However, Malabat et al. (2003) also identified genotypes with napin as predominant storage protein (cruciferin to napin ratio of around 0.7). The genotypes with a higher napin than cruciferin content mostly proved to be high in glucosinolate content. Previously, Raab et al. (1992) reported for seven winter oilseed rape cultivars a cruciferin to napin ratio ranging from 0.7 to 4.9, as determined after size exclusion chromatography. In the present study, the two genotypes R53 and Express 617 had a higher napin than cruciferin content with a cruciferin to napin ratio of 0.2 and 0.4 (Table 2), as determined after polyacrylamide gel electrophoresis. This comparatively low ratio may be specific for the genotypes but may also be explained by a suboptimal extraction of the cruciferins in the present study, which as globulins have a better solubility in a salty extraction buffer (Uppström 1995). Furthermore, since it was not clear if protein band E (cf. Fig. 1) was definitely a cruciferin band, it was disregarded in the calculation of the total cruciferin content.

However, as found in previous studies (Kohno-Murase et al. 1994, 1995) cruciferin and napin content were negatively correlated to each other in the doubled haploid population. Furthermore, a close positive correlation between napin content and glucosinolate content has been found in the present study (Table 3) which is corroborating the results of Malabat et al. (2003) obtained for 64 *Brassica napus* genotypes. Vice versa a similar close negative correlation between cruciferin and glucosinolate content was observed (Table 3). This result indicates a simultaneous up-regulation of glucosinolate and napin biosynthesis. Both, glucosinolates as well as napins are rich in sulphur. Hence, the results also suggest that sulphur supply in relation to nitrogen availability has not been limiting during seed growth and maturation. In their review paper Tabe et al. (2002) pointed out that at a given level of nitrogen supply the availability of sulphur may influence the composition of the seed protein fraction. When N supply is sufficient, variations in S supply can result in the adjustment of the relative abundance of specific S-rich or S-poor proteins. Results from grain legumes and cereals show that at ample nitrogen supply and under limited sulphur availability the

Table 4 Mapped QTL and their most likely positions for napin- (nap) and cruciferin contents (cru) and other seed traits

QTL	LG	Position (cM)	CI (cM)	a	h^2 (a)	*F* value	*F* threshold value	V(A)/V(P)	V(I)/V(P)	V(G)/V(P)
Nap-1	N2	43.5	31.0–47.4	0.06	0.06	15.0	13.6	0.47	–	0.47
Nap-2	N16	49.1	37.4–60.2	−0.07	0.05	25.8				
Nap-3	N19	31.0	27.0–38.5	−0.18	0.37	131.6				
Cru-1	N2	53.4	35.5–79.9	−0.04	0.05	14.8	13.2	0.35	0.04	0.39
Cru-2	N19	34.5	30.0–38.5	0.08	0.30	98.6				
Cru/Nap-1	N2	45.5	36.5–47.4	−0.05	0.09	31.3	12.7	0.55	0.06	0.61
Cru/Nap-2	N16	40.4	28.0–49.1	0.03	0.03	18.4				
Cru/Nap-3	N19	35.5	31.0–38.5	0.10	0.44	184.4				
GSL-1	N6	80.2	70.1–92.2	2.37	0.002	16.0	12.4	0.74	–	0.74
GSL-2	N19	33.5	32.0–35.5	−13.43	0.721	587.6				
Pro-1	N7	35.7	21.7–51.5	−0.42	0.10	17.9	13.8	0.15	–	0.15
Pro-2	N19	30.0	20.0–38.5	−0.24	0.05	14.4				
Oil-1	N12	0.0	0.0–6.0	0.45	0.07	19.0	13.3	0.47	–	0.47
Oil-2	N13	24.7	21.7–27.3	−0.98	0.28	86.4				
Oil-3	N13	131.5	123.3–138.4	0.38	0.03	20.5				
Oil-4	N19	31.0	23.0–41.5	0.43	0.08	21.7				

LG linkage group, *CI* confidence interval, *a* additive effect, $h^2(a)$ heritability of additive effect, *V(A)/V(P)* variance of additive effects/phenotypic variance, *V(I)/V(P)* variance of epistatic effects/phenotypic variance—position of epistatic effects not shown, *V(G)/V(P)* variance of genetic main effects/phenotypic variance. For the mapped QTL, the estimated additive effect (a) is shown as the substitution of an R53 allele by an Express 617 allele

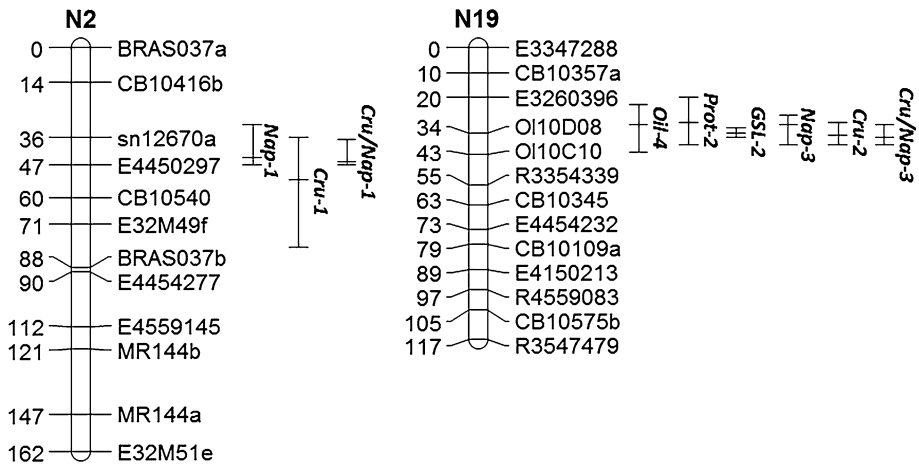

Fig. 4 Linkage groups N2 and N19 showing relevant QTL for napin- (Nap), cruciferin content (Cru), cruciferin to napin ratio (Cru/Nap), glucosinolate- (GSL), protein- (Prot) and oil content (oil)

synthesis of sulphur amino acid proteins is very much decreased (Tabe et al. 2002 and references therein). Zhao et al. (1993) showed that sulphur fertilisation had no significant effect on the seed protein content in 00-quality oilseed rape. However, sulphur deficiency, in particular at high nitrogen fertiliser levels, reduces the seed protein content and its quality in terms of sulphur amino acid (Hawkesford and De Kok 2006; Zhao et al. 1993). Especially, the content of sulphur rich napins is reduced (Zhao et al. 1993 and references therein).

The aromatic and indole glucosinolate molecules contain two and the methionine derived aliphatic glucosinolate molecules contain three sulphur atoms. Sulphur makes up 15–20 % of the molecular weight of the glucosinolates and the glucosinolate sulphur makes up 10–30 % of the total seed sulphur content (Falk et al. 2007). There is a close relationship between seed sulphur and seed glucosinolate content. Hence, glucosinolate content of the seeds can be determined by analysis of its sulphur content (Schnug and Kallweit 1987). Finding this close correlation, it has been anticipated that there is little variation in the amount of sulphur bound in the seed storage proteins. However, the results of the present study show that there is a quite close positive correlation between napin and glucosinolate

content. This close correlation is also demonstrated by the overlapping confidence intervals of the major QTL for glucosinolate (GSL-2) and napin content (Nap-3) on linkage group N19 (Fig. 4), explaining most of the phenotypic variation of these two traits (72 and 37 %, respectively). When examining the same segregating bi-parental doubled haploid population, albeit tested in different years and locations, Radoev et al. (2008) similarly reported the major QTL for glucosinolate content to be located at a similar position on linkage group N19 as in this study. Furthermore, they detected a minor QTL for oil content in this region of linkage group N19. However, the minor QTL for protein content (Pro-2, cf. Table 4) was not detected in their study.

For napin and cruciferin content 3 respectively 2 QTL were detected, which together explained 47 and 35 % of the phenotypic variance. Earlier Southern blot analyses (cf. Introduction) suggested for the napins a copy number ranging from 10 to 16 and for the different cruciferin precursors a copy number ranging from 2 to 4. Those genes may be clustered closely together on one linkage group and they may not all be functional. Genome sequence analysis of *Brassica napus* and its ancestor species will reveal exact copy number and organization of the napin and cruciferin gene families. However, searching the *Brassica rapa* and *Brassica oleracea* genome database (BRAD; http://brassicadb.org/brad/, 28 Oct 2013) for homologs of published cruciferin and napin sequences did not give any high-scoring segment pairs for linkage groups N2, N16 and N19, suggesting that the napin and cruciferin genes are not responsible for the observed changes in cruciferin and napin contents in the doubled haploid population.

The biological significance of the presence of two different classes of seed storage proteins in the seeds of oilseed rape remains to date unclear. Varying contents of napin and cruciferin of seeds may influence their germination (Müntz 1998; Neumann et al. 1996a), dormancy and seed longevity in the soil (Schatzki et al. 2013a). Furthermore, the expression of napins in seeds of *Brassica napus* L. is regulated by the expression of the transcriptional activator ABI3 and by endogenous or externally applied ABA (Ezcurra et al. 2000). This suggests a possible role of napins in seed maturation and hence possibly also in dormancy and germination related processes (Brocard-Gifford et al. 2003). However, only a loose negative correlation to total seed dormancy was observed (cf. Table 3). In summary, the results show an up-regulation of napin biosynthesis in the presence of glucosinolates and confirm previous results (Malabat et al. 2003) that the conversion to Canola type cultivars with low glucosinolate content in the seeds has led to a reduction of napin content and a concomitant increase of cruciferin content in the seeds.

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Conflict of interest None of the authors have any conflicts of interest associated with this study.

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