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Identification of seed-related QTL in *Brassica rapa*

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Abstract

To reveal the genetic variation, and loci involved, for a range of seed-related traits, a new F2 mapping population was developed by crossing *Brassica rapa* ssp. *parachinensis* L58 (CaiXin) with *B. rapa* ssp. *trilocularis* R-o-18 (spring oil seed), both rapid flowering and self-compatible. A linkage map was constructed using 97 AFLPs and 21 SSRs, covering a map distance of 757 cM with an average resolution of 6.4 cM, and 13 quantitative trait loci (QTL) were detected for nine traits. A strong seed colour QTL (LOD 26) co-localized with QTL for seed size (LOD 7), seed weight (LOD 4.6), seed oil content (LOD 6.6), number of siliques (LOD 3) and number of seeds per silique (LOD 3). There was only a significant positive correlation between seed colour and seed oil content in the yellow coloured classes. Seed coat colour and seed size were controlled by the maternal plant genotype. Plants with more siliques tended to have more, but smaller, seeds and higher seed oil content. Seed colour and seed oil content appeared to be controlled by two closely linked loci in repulsion phase. Thus, it may not always be advantageous to select for yellow-seededness when breeding for high seed oil content in Brassicas.

Additional key words: F2 mapping population; genetic linkage map; quantitative trait loci; yellow seeds.

Introduction

The haploid *Brassica rapa* genome consists of ten chromosomes (n = 10) and is one of the parents of the amphidiploids species *Brassica napus*. Domestication led to a wide variety of forms, such as the leafy vegetables, root vegetables, and oil types.

The seed of *B. rapa* is mainly used for oil production, although the meal remaining after oil extraction is also of economic interest as fodder. Seed yield is the resultant of a few determining components, *i.e.,* the number of siliques per unit area, branch number, number of seeds per silique, and seed size (Snowdon *et al*., 2007). The oil yield can be improved by increasing the seed oil content or by increasing the seed yield (Chen & Heneen, 1992). In *Brassica* seed coat colour varies from yellow to black with intermediates. In general, the yellow-seeded varieties have higher oil content (Badani *et al*., 2006). As most of the commercial varieties are black, breeding for the yellow seeded-varieties through available natural variation or synthesized variation is developed (Jönsson, 1975; Rahman, 2001). Use of molecular markers linked to seed-related traits will facilitate the breeding of favourite varieties in *Brassica*.

In *B. rapa* it has been shown that seed coat colour is controlled by one major locus (Lou *et al*., 2007), a single gene with the black allele dominating over the yellow allele (Chen & Heneen, 1992) or by multiple genes (Schwetka, 1982). Most reports support the idea

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Abbreviations used: AFLP (amplified fragment length polymorphism); LOD (log of odds); QTL (quantitative trait loci); SSR (simple sequence repeat).

that seed colour is mainly controlled by the maternal genotype (Ahmed & Zuberi, 1971; Stringam, 1980; Chen & Heneen, 1992; Rahman & McVetty, 2011). Recently the seed coat colour gene has been detected in *B. rapa* using map-based cloning (Li *et al*., 2012).

The aim of this work was to do quantitative trait loci (QTL) analysis of seed-related traits using a new F2 population of *B. rapa*. The Cai Xin parent is L58, a vegetable type with black seed coat colour. The other parent, R-o-18, is an oil type line with yellow seed coat colour, which has been used as susceptible parent in F2 populations for genetic analysis of disease resistance to *Turnip mosaic virus* and *Xanthomonas campestris* (Rusholme *et al*., 2007; Soengas *et al*., 2007). This line has become a reference line for *B. rapa* research, as it is also used for microsatellite development (Lowe *et al*., 2004), and TILLING purposes (McCallum *et al*., 2000). To evaluate the population segregation, some morphological traits have also been analysed.

Materials and methods

Population and plant growth

An F2 population was made by crossing *B. rapa* L58 (♀) × R-o-18(♂) and selfing the F1 plants. L58 is the Cai Xin parent, a vegetable type originating from China (*B. rapa* ssp. *parachinensis*) (Wu *et al*., 2007) and R-o-18, is a doubled haploid Yellow Sarson oil type (*B. rapa* ssp. *trilocularis*) originating from India (M. Koornneef). The number of 190 F2 seeds were used as starting material. Individual F2 plants were grown in 19 cm diameter black plastic pots filled with Lentse potgrond soil (Hortimea Group, Lent, The Netherlands) in a temperature-controlled greenhouse (21°C) with artificial day length extension to 16 hours. No cold treatment or vernalization was used for the germination and flowering. The inflorescences were covered with plastic bags to prevent cross-pollination.

Phenotyping

The population and its parental lines were phenotyped for 14 traits: flowering time, plant height and leaf number until first open flower, branch number, total leaf number, total plant height, number of siliques per plant, silique length and number of seeds per silique were measured or recorded in F2 plants. The *B. rapa* vegetable types have a typical morphology, a short stem with many leaves positioned very close to each other. Plant height and leaf number until first open flower measurements can reveal these characteristics. F3 seeds were used for analysing seed weight, seed size, seed colour, seed oil content, and total seed protein content.

To measure seed size, 10 F3 seeds of each line were randomly taken, digitally photographed and analyzed by ImageJ 1.390 (http://rsb.info.nih.gov). Seed colour of F3 seeds was scored by eye and ranked into 13 different classes ranging from yellow (1) to black (13). Silique length was determined on harvested siliques of F2 plants as the average length of three ripe siliques at random. The mean number of seeds per silique on F2 plants was determined by averaging three ripe siliques. Seed oil was extracted by a crude method of hexane-extraction, grinding 10 weighed F3 seeds of each line in 650 µL of hexane, shaking the mix for 2 min followed by 1 min of centrifugation at 14,000 rpm; 600 µL was transferred to a new tube and left in the fume hood overnight to evaporate the hexane and then the remaining oil was weighed (Goossens *et al*., 1999). The oil content was determined in mg oil per mg seed. The seed debris remaining after oil extraction was used for total protein measurement, using the Bradford essay as described by Goossens *et al*. (1999). Statistical analysis for distribution and correlation were performed in SPSS 11.0.

Genotyping

Total DNA was extracted from frozen leaves and the amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) procedure was performed as described by Choi *et al*. (2007). For AFLP selective amplification, seven combinations of *Eco*RI/*Mse*I (E34M15, E34M16, E37M32, E37M49, E37M56, E40M38, E40M51) and four combinations of *Pst*I/*Mse*I (P23M48, P23M50, P21M47, P23M47) primers were used. The AFLP bands were scored as 1 or 0 for presence or absence of the band respectively. All weak and ambiguous bands were scored as unknown. In addition, 36 public SSR primer pairs (Choi *et al*., 2007) were used to screen for polymorphisms using the LI-COR system (Lincoln, NE, USA) 4200 DNA sequencer.

Construction of a linkage map and QTL analysis

Linkage analysis and map construction were carried out using JoinMap4 (http://www.kyazma.nl). The regression mapping algorithm was used for linkage analysis. Recombination frequencies were converted to centimorgan distances using the Kosambi mapping function. MAPQTL 5.0 (http://www.kyazma.nl) was used for QTL analysis. For each trait a 1000X permutation test was performed to calculate the log of odds (LOD) threshold corresponding to a genome-wide false discovery rate of 5% ($p < 0.05$). A LOD of 2.6 was used as a significance threshold for the presence of a candidate QTL. Multiple-QTL model mapping was performed to locate QTL after the selection of cofactors at initially detected QTL positions.

Statistical analyses

To compare mean values of genotypic classes the *t*-test was used. Also Pearson correlation analysis was performed to identify significant correlations (at $p \leq 0.05$) of seed-related traits.

Results

Construction of a linkage map

A linkage map was constructed using 97 AFLPs and 21 SSRs. The map covered a genetic distance of 757 cM with an average distance of 6.4 cM between markers (Fig. 1). Comparing with the reference linkage map of *B. rapa*, which is 1234 cM long; this map covers 61% of the total map. A9 is the largest chromosome in the A genome (Choi *et al*., 2007) which in the map presented here is 87 cM long and includes more markers than other linkage groups.

In total, 300 AFLP bands were produced. Of these, 158 were polymorphic. Thirty-six AFLPs were excluded from the analysis because they could not be scored for the majority of F2 plants or they showed an identical segregation. Furthermore, 25 AFLPs could not be assigned to a linkage group. Twenty-three out of 36 SSRs tested showed polymorphisms for the parents and 21 of them were mapped, allowing the assignment of linkage groups to their respective chromosomes. Six SSRs with their closely linked AFLP on A1, A2 and A5 (Fig. 1) showed a segregation distortion at $p < 0.005$.

Phenotyping

Seed-related traits and few morphological traits were recorded in the population (Fig. 2). The size and colour of F1 seeds of the reciprocal crosses corresponded to that of the female parents, indicating that these phenotypes reflected the genotype of the mother plant. Transgression beyond the parental values was observed for most of the traits except for seed colour.

There was no significant correlation between seed colour and seed oil content when data for all lines were examined. When only the light-coloured (clusters 1 to 6) lines were analysed, however, a highly significant positive correlation ($r = +0.62$) was observed. Genotypes with reddish-brown seed coat had higher oil content than the yellow-seeded genotypes. Silique number and seed number per silique were positively correlated ($r = +0.34$), but negatively correlated with seed weight ($r = –0.37$ and, $–0.31$ respectively). Seed oil content was positively correlated with the number of siliques ($r = +0.42$). Seed weight and seed size were highly positively correlated ($r = +0.95$), and they were negatively correlated to seed oil content ($r = –0.70$) and the number of siliques ($r = –0.40$ and, 0.50 respectively). In general, plants with more siliques had more, but smaller seeds and higher seed oil content (per mg).

QTL analysis

Thirteen QTL were mapped for nine different traits (Table 1; Fig. 1). The major QTL was SC on A9 for seed colour. It was co-located with other seed-related QTL for seed size, seed weight, and number of siliques. Correlation analysis already showed significant correlation between these traits, except for seed colour. Fig. 3 shows the box plots of phenotypic values for each of the three genotypic classes at this QTL region. In case of seed colour, genotypes homozygous for the L58 allele and heterozygotes did not differ significantly in trait values, and many lines classified in classes 7 to 13 were actually heterozygous for the QTL on A9 (Fig. 4). The L58 homozygotes and heterozygotes differed significantly from R-o-18 homozygous, though indicating that the L58 allele was fully dominant over the R-o-18 allele. Genotypes homozygous for the L58 allele were significantly different from heterozygotes and from genotypes homozygous for the R-o-18 allele in case of seed weight, seed oil content,

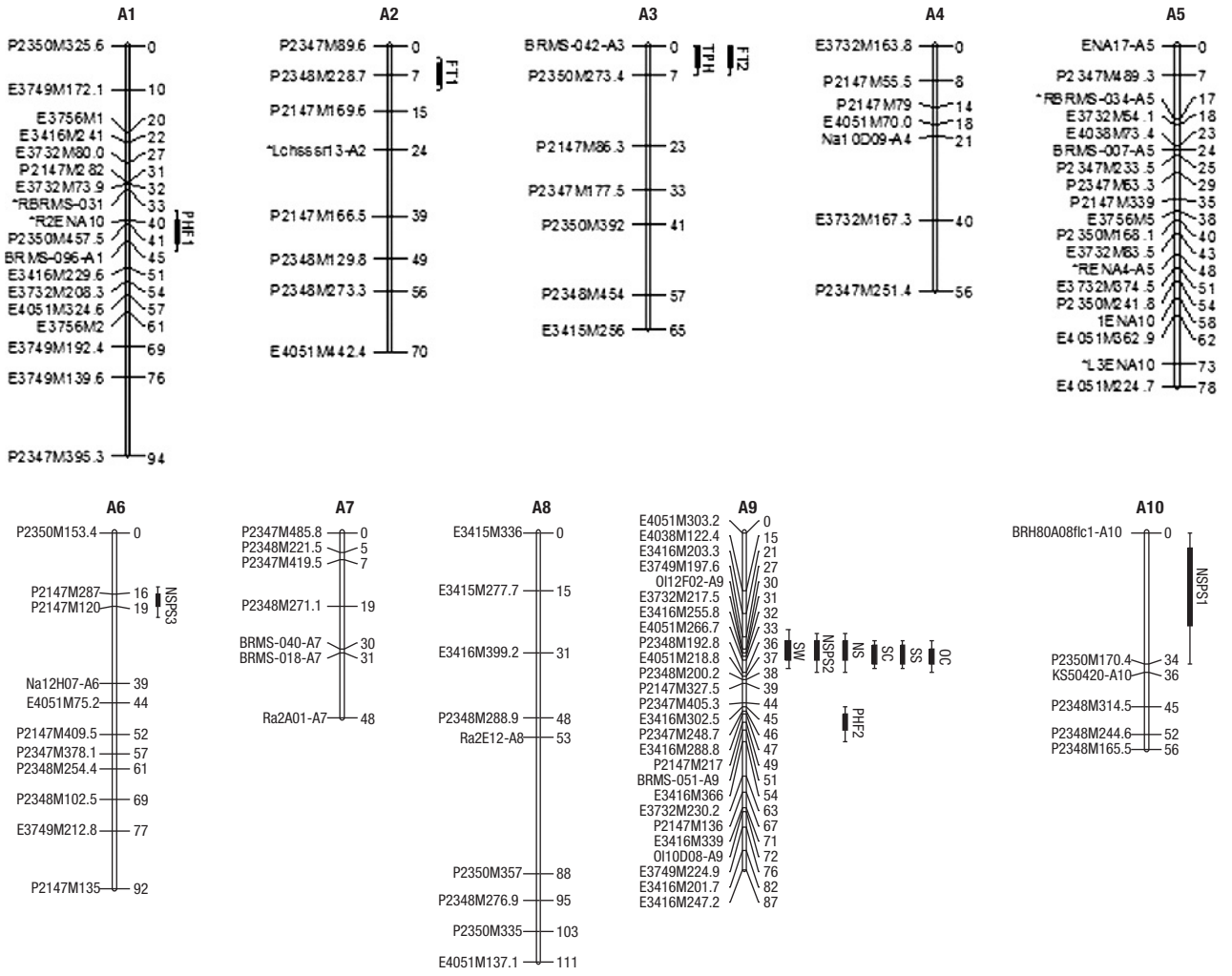

Figure 1. Linkage map of the L58 × R-o-18 F2 population including ten linkage groups (A1-A10), showing the positions of 97 AFLPs, starting with P(*Pst*I) or E (*Eco*RI), and 21 SSRs. QTL are indicated with boxes and whiskers representing 1- and 2-LOD confidence intervals (95%) respectively. Skewed SSR markers in A1, A2 and A5 are indicated with * and letters L or R to show if these markers are coming from L58 or R-o-18 respectively.

and number of seeds per silique. This means that for these traits the parental alleles were co-dominant, resulting in additive effects on the phenotype.

Discussion

A segregating F2 population of *B. rapa* was generated. The limitation of using an F2 population is that the phenotyping is based on a single plant. Thus for most seed traits the average of F3 seeds were used to obtain trait data.

Two QTL for plant height below the first open flower (PHF1, PHF2) were mapped to A1 and A9, respectively. There was not a significant correlation between PHF and TPH and there was no common QTL for both traits, which means that PHF is a poor predictor of TPH in this population. Lou *et al*. (2007) previously mapped plant height QTL in F2/F3 populations of *B. rapa*, and these do not correspond to any of the PHF QTL we found. However, the TPH QTL, which we mapped to the top of A3, may correspond to one of the plant height QTL mapped by Lou *et al*. (2007).

Although both parents are early flowering, two QTL were identified for flowering time. There are four known flowering time genes in *B. rapa; BrFLC1*, *BrFLC2*, *BrFLC3,* and *BrFLC5*, all corresponding to an orthologous copy of the *A. thaliana FLC* gene (Koornneef *et al*., 1994). The *BrFLC* genes are assigned to linkage groups A10, A2, and A3 respectively

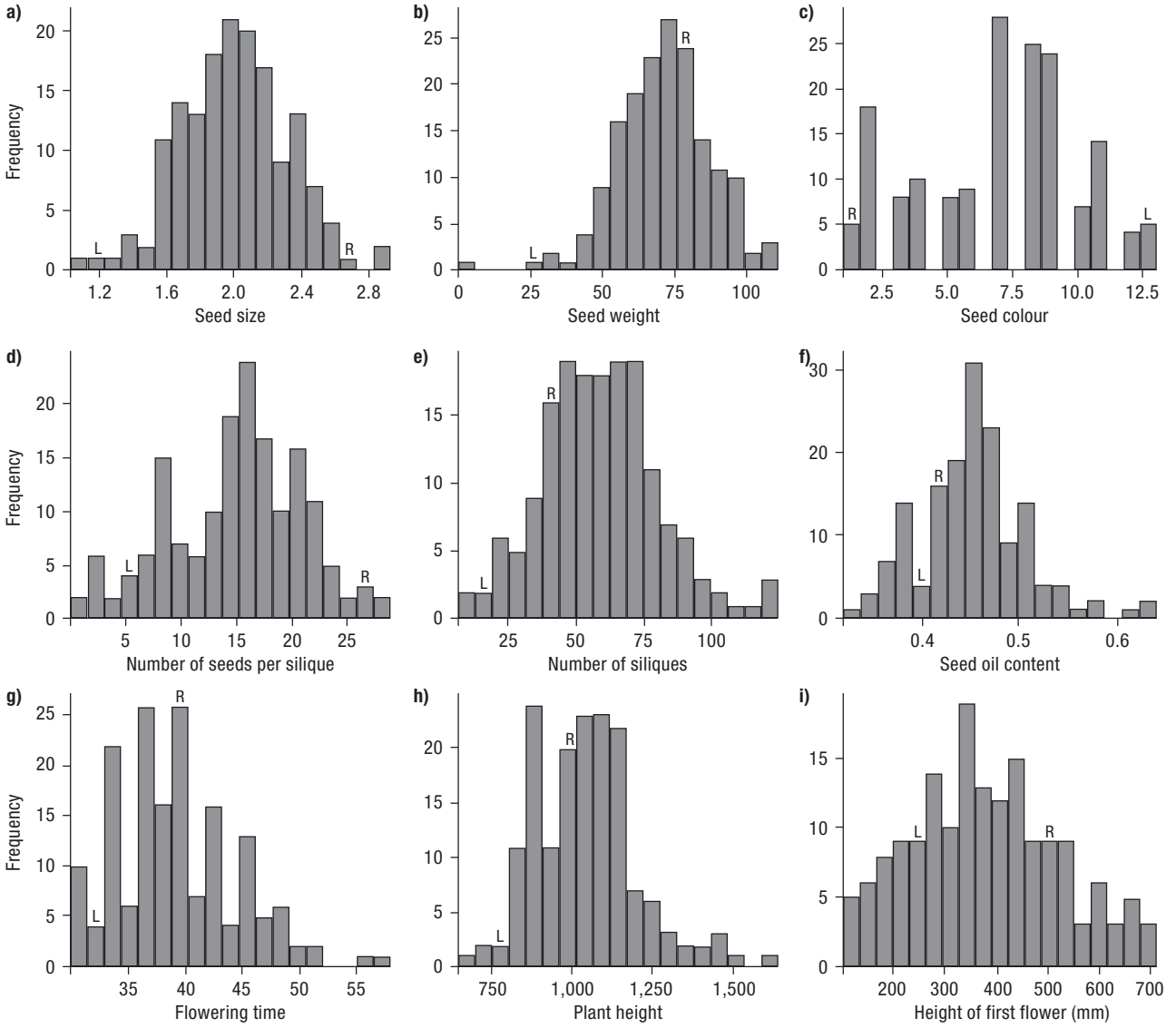

Figure 2. Frequency distributions of non-normalized data of traits in the F2. X axis: number of lines per each trait value class. Y axis: different trait value classes including seed size, mm (a); seed weight, mg (b); seed colour (c); number of seeds per silique (d); number of siliques (e); seed oil content, mg (f); flowering time, in days after sowing (g); plant height, mm (h); and plant height until first open flower, mm (i). Parental values: L as L58 and R as R-o-18.

(Schranz *et al*., 2002). In our population, FT2 co-localizes with TPH on the top of A3 and FT1 maps to A2 and they may correspond to the FLC loci previously mapped to these chromosomes. Lou *et al*. (2007) identified eight QTL for flowering time in four different populations of *B. rapa.* Of these, three were in an F2/3 population and mapped on A2, A3, and A7. FT1 and FT2 can be the same QTL mapped on A2 and A3 by Lou *et al*. (2007).

In this population, seed weight, seed size, number of siliques, number of seeds per silique and seed oil content were highly correlated, and shared one co-locating QTL on A9. Four seed weight QTL have been reported in *B. rapa* on A2, A3, A6 and A10 (Li *et al*., 2013). Therefore, this QTL on A9 is a new one. This is the only QTL we detected for the number of siliques; however, there were two additional QTL for the number of seeds per silique, on A6 and A10. The one on A10 has also been reported by Li *et al*. (2013). Both traits are positively correlated with seed oil content, but they are negatively correlated with seed size and seed weight. This finding is in agreement with that found by Zhang *et al*. (2011) in *B. napus*, who suggested that this negative correlation is due to the competition

Table 1. QTLs detected in the F2 population for nine different traits.

Trait	QTL	Linkage group	LOD[1]	Position of peak LOD (cM)	% Expl. var.[2]	Effect[3]
Flowering time (days)	FT1	A2	4.2	6.8	10.1	3.8
	FT2	A3	4.1	4.2	9.5	–5.2
Plant height until first open flower (mm)	PHF1	A1	4.7	41.3	11.3	–131.4
	PHF2	A9	3.4	45.2	8.0	–110.7
Total plant height (mm)	TPH	A3	4.9	4.2	13.6	–137.3
No. seeds/silique	NSPS1	A10	3.1	15.0	8.5	–3.0
	NSPS2	A9	2.9	30.1	3.8	4.6
	NSPS3	A6	2.6	17.2	20.7	5.0
Seed oil content (mg/seed)	OC	A9	6.6	30.1	20.0	0.1
Seed size (mm)	SS	A9	7.0	30.1	19.0	–0.4
Seed colour	SC	A9	26.3	30.1	52.6	5.4
No. siliques	NS	A9	3.1	30.1	8.5	16
Seed weight (mg)	SW	A9	4.6	30.1	12.0	–0.6

[1] LOD: log of odds (the genome-wide significant threshold for suggestive QTL). [2] % Expl. var. is the percentage of total phenotypic variance explained by individual QTL. [3] Effect: for each QTL the allelic effect is indicated. This is calculated as μA-μB, where A and B are F2 carrying L58 and R-o-18 genotypes at the QTL positions, respectively; μA and μB are estimated by MapQTL.

among the sinks for assimilates. This negative correlation between the number of seeds per silique and seed size was previously also observed for *A. thaliana* (Alonso-Blanco *et al*., 1999). When selecting lines carrying the L58 allele(s) at this locus, one will select for plants with more siliques and more, but smaller,

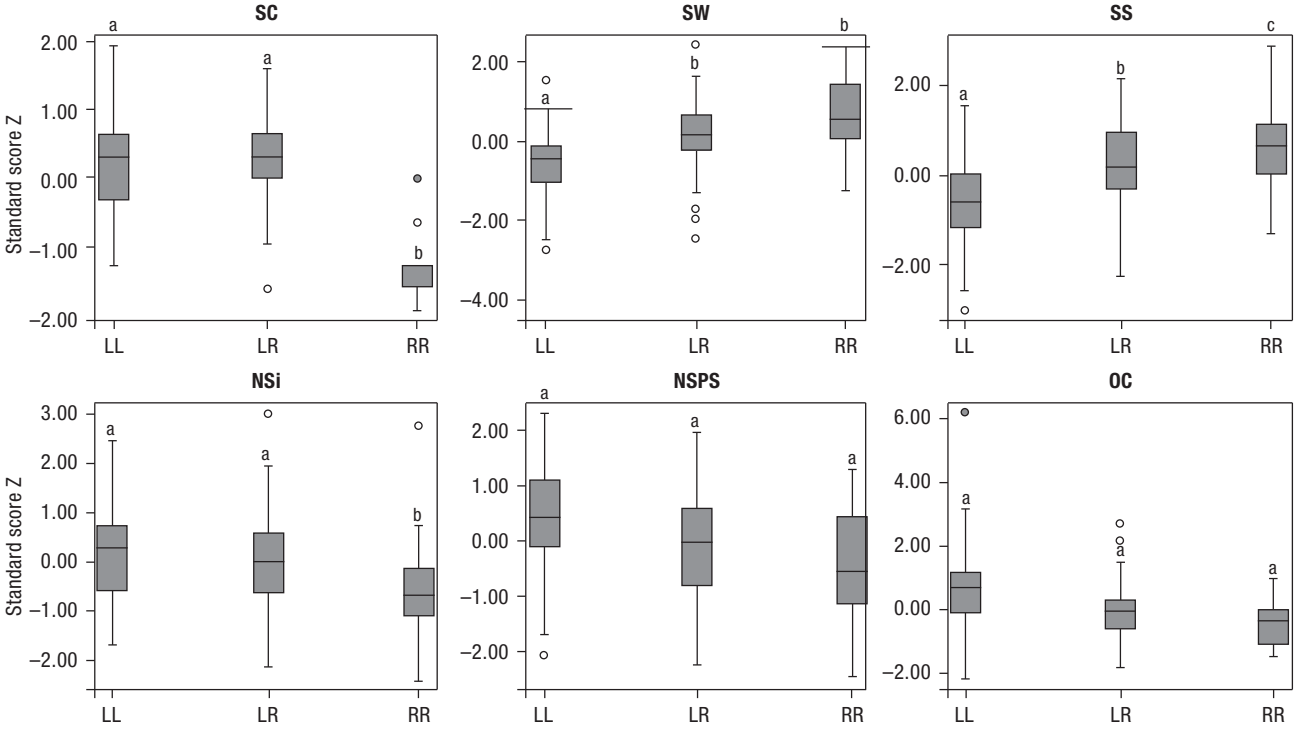

Figure 3. Box plots of the phenotypic values of the three genotypic classes for six seed traits for which one co-localizing QTL was found on A9. X-axis: genotypic class (LL: homozygote for L58 allele, LR: heterozygote, RR: homozygote for R-o-18 allele). Y-axis: standard Z score, SC: seed colour; SW: seed weight; SS: seed size; NS: number of silique; NSPS: number of seeds per silique; OC: seed oil content. Box plots show the median, interquartile range, outliers (○) and extreme cases (◉). a, b and c are significance classes at $p < 0.05$ in a *t*-test comparing mean values of genotypic classes.

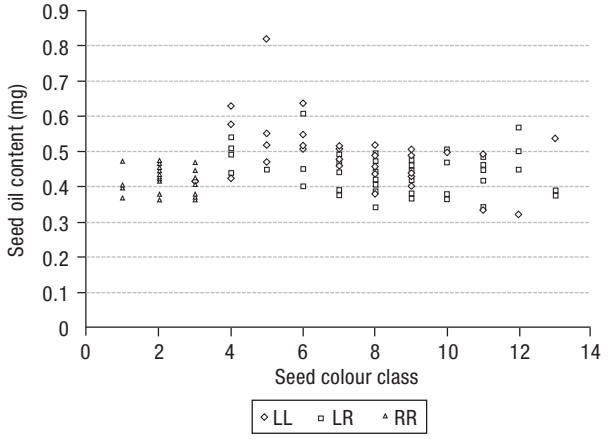

Figure 4. Frequency of genotypic classes at the SC QTL in each of the different seed colour classes. LL: homozygote for L58 allele, LR: heterozygote and RR: homozygote for R-o-18 allele.

seeds per silique, which will have a higher oil content, than when lines are selected that are homozygous for the R-o-18 allele. Breeding for lines with more siliques is one approach to increase seed and oil yield per plant.

One major QTL on A9 (SC) was detected explaining 52 % of the seed coat colour variation (Table 1). Previously Lou *et al*. (2007) had mapped a major locus for seed coat colour to A9. Since yellow-seeded 'Yellow Sarson' line was included as one of the parents of the population, this is most likely the same locus. This QTL is probably the same locus mapped by Kebede *et al*. (2012) on A9 (SCA9-2) almost at the same position. The yellow seed coat allele is fully recessive to the black seed coat allele. Seed coat colour is inherited maternally, which is in agreement with the deposition of seed pigments in the testa layers (Vaughan *et al*., 1976). Ahmed & Zuberi (1971) already reported that a single gene is responsible for the dominant reddish brown seed colour in Indian *B. rapa* Toria's lines. Chen & Heneen (1992) also showed that a single maternal gene controls seed colour, with the black allele dominating over the yellow one in *B. rapa*. In the model proposed by Van Deynze & Pauls (1993) for *B. napus*, black seed colour is controlled by dominant alleles at three loci. All known *transparent testa (tt)* mutations in *A. thaliana* are recessive and show maternal inheritance of the seed phenotype. Zhang *et al*. (2009) identified the *Brassica TTG1* (TRANSPARENT TESTA GLABRA 1) ortholog as the first gene controlling hairiness and seed coat colour in *B. rapa*. They suggested that the deletion in the Brassica *TTG1* homolog has led to the yellow seeded natural mutant. Recently through map-based cloning method, the seed coat colour gene located on A9 in *B. rapa* has been identified as a homolog of TRANSPARENT TESTA8 (*TT8*) locus in Arabidopsis (Li *et al*., 2012).

Stringam *et al*. (1974) already reported a direct relationship between seed coat thickness and seed colour in *B. rapa*. However, seed coat pigment biosynthesis is complex. Recently Snowdon *et al*. (2010) found that seed colour alone is not always a proper selection marker for meal digestibility as genotypes with very similar seed colour can show large variation in seed acid detergent lignin. His group (Liu *et al*., 2012) also identified a key phenylpropanoid biosynthesis pathway gene within the major QTL of seed acid detergent lignin in *B. napus,* which is located on A9 as SC locus does.

Seed size is another trait, which is maternally controlled. In *A. thaliana* most seed pigmentation mutants show reduced seed weight and seed size (Debeaujon *et al*., 2001). Nevertheless, in *B. rapa* this is different with the yellow-seeded genotype R-o-18 having the largest seeds.

The same region on A9 to which the SC QTL was mapped, also harboured a QTL explaining 20% of the phenotypic variance for seed oil content (Table 1). Oil content at the QTL level is poorly understood in *B. rapa*. Tanhuanpää *et al*. (2004) mapped an oleic acid QTL to A6 in *B. rapa* ssp. *oleifera*. In *B. napus* six or seven QTL involved in seed oil content have been detected (Zhao *et al*., 2006; Cao *et al*., 2010). Based on previous work in *B. napus* (Badani *et al*., 2006), we expected a negative correlation between seed colour and oil content, with dark-seeded genotypes having lower oil contents. Instead, we found a significant positive correlation in the yellow/brown seed colour genotypic clusters. One reason for this could be that dark-seededness is fully dominant trait and if there is no bias for darker seeds to be homozygous for the L58 allele, which is the case (Fig. 4), while there is such distinction for the co-dominant oil seed content alleles, this will seriously disturb the correlation. Another reason could be the presence of additional, undetected loci with opposite effects that independently control seed oil content and seed coat colour. Since the A9 QTL only explains about 20% of the oil content variance, compared to 52% for seed colour, this is likely to be the next reason. Another observation supporting this, is that even though both parents have very similar seed oil contents (Fig. 2), there is strong transgression for oil content in the F2 progeny, suggesting that loci

that contribute positively and negatively to oil content are present in both parents. It is likely that seed colour and oil content are controlled by two closely linked genes for which favourable alleles happen to be in coupling phase in most yellow-seeded genotypes, but that a recombination in the ancestry of R-o-18 and L58 caused them to be in in repulsion phase in this lineage. Ahmed & Zuberi (1971) also described *B. rapa* varieties with reddish brown seeds that produced more oil than yellow seed coat varieties, suggesting that similar genotypes occur more frequently. Alternatively, L58 contains an allele contributing positively to seed oil content, which has gone undetected so far, since CaiXin vegetable types have not been considered before for oil content.

In this F2 population, oil yield per plant is mostly dependent on seed size, number of siliques and number of seeds per silique. In a DH population of *B. napus*, Zhao *et al*. (2006) found that oil content showed the strongest correlation to seeds per silique, which is correlated with silique number. The possibility remains that seed size, the number of seeds per silique and the number of siliques per plant are not pleiotropic effects of the same genetic locus but the result of two or more closely linked loci.

In conclusion, in this F2 population traits for both vegetable and oil seed types are segregating, therefore it is a useful source for genetic analysis and identification of genetic loci to design markers for marker assisted selection of important traits in *B. rapa*.

Although yellow seededness has often been promoted for oilseed Brassicas, there are no yellow-seeded modern commercial varieties in many parts of the world. There may be some experimental ground for that, as we found. There is a significant positive correlation between seed coat colour and seed oil content and, in general, black seeded genotypes have higher oil seed contents than yellow-seeded genotypes, therefore it may not always be advantageous to select for yellow-seededness when breeding for high seed oil content in Brassicas. Although further detailed genetic analysis will be needed, possibly there are two closely linked genes at the A9 QTL for which favourable alleles for low fibre content due to yellow seededness and for high oil content happen to be in coupling phase in most genotypes, but not in one or both of the parents used to make the population. Our observation suggests that silique number could be a good morphological marker for oil content improvement of *B. rapa* instead of yellow seed coat colour.

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