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ORIGINAL PAPER

Tetralocular ovary and high silique width in yellow sarson lines of *Brassica rapa* (subspecies *trilocularis*) are due to a mutation in Bra034340 gene, a homologue of *CLAVATA3* in Arabidopsis

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Abstract

Key message **Genetic locus for tetralocular ovary (*tet-o*) in *Brassica rapa* was identified and it was shown that the number of locules and width of silique are associated.**

Abstract *Brassica rapa* is a highly polymorphic species containing many vegetables and oleiferous types. An interesting group of oleiferous types is the yellow sarson group (subspecies *trilocularis*) grown mostly in eastern India. This group contains lines that have bilocular ovaries, a defining trait of Brassicaceae, but also lines that have tetralocular ovaries. Yellow sarson lines commonly have high silique width which is further enhanced in the tetralocular types. We mapped the locus influencing tetralocular ovary in *B. rapa* using three mapping populations (F_2, F_6 and F_7) derived from a cross between Chiifu (subspecies *pekinensis*, having bilocular ovary) and Tetralocular (having tetralocular ovary). QTL mapping of silique width was undertaken using the three mapping populations and a F_2 population derived from a cross between Chiifu and YSPB-24 (a bilocular line belonging to yellow sarson group). Qualitative mapping of the trait governing locule number (*tet-o*) in *B. rapa* mapped the locus to linkage group A4. QTL mapping for silique width detected a major QTL on LG A4, co-mapping with the *tet-o* locus in bilocular/tetralocular cross. This QTL was not detected in the bilocular/bilocular cross. Saturation mapping of the *tet-o* region with SNP markers identified Bra034340, a homologue of *CLAVATA3* of *Arabidopsis thaliana,* as the candidate gene for locule number. A C → T transition at position 176 of the coding sequence of Bra034340 revealed co-segregation with the tetralocular phenotype. The study of silique related traits is of interest both for understanding evolution under artificial selection and for breeding of cultivated *Brassica* species.

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Introduction

Brassica rapa is an agronomically important species of Brassicaceae with many vegetable, oilseed, fodder and condiment types. Diederichsen (2001) has identified ten subspecies (ssp.) in *B. rapa* with all the oil yielding types identified under ssp. *oleifera* except the yellow sarson types which have been assigned to ssp. *trilocularis*. The yellow sarson types have distinct morphology, are self-compatible, yellow seeded and contain lines with a bilocular ovary (a typical character of the Brassicaceae) and variant lines with

a very unique character of tetralocular ovary. An extensive study of phylogenetic relationships of *B. rapa* types, using 161 accessions belonging to different subspecies with AFLP markers has confirmed yellow sarson types, both bilocular and tetralocular, as a separate group (Zhao et al. 2005). The yellow sarson group, in all probability, was domesticated in eastern India (Gomez-Campo and Prakash 1999).

The basic floral structure in Brassicaceae is well conserved and in general consists of four sepals, four petals, six stamens and two carpels which are congenitally fused (Roeder and Yanofsky 2006; Bowman 2006). At the time of fertilization the ovary has two locules separated by a septum which connects the two replums. The ovules arise from the placentae, which lie along the inner side of each replum. There are variations encountered in the basic floral structure within the family; however, bilocular ovary is a constant feature of all the members of Brassicaceae including members of the U's triangle (U 1935), namely, *B. rapa* (AA, $n = 10$), *B. nigra* (BB, $n = 8$), *B. oleracea* (CC, $n = 9$) and their allopolyploids, *B. juncea* (AABB, $n = 18$), *B. napus* (AACC, $n = 19$) and *B. carinata* (BBCC, $n = 17$). The only exception is *B. rapa* ssp. *trilocularis* lines which have a tetralocular ovary.

Earlier studies have shown that 'tetralocular ovary' (*tet-o*) is controlled by a locus which is recessive in nature (Varshney 1987; Salava et al. 1996). A more recent study on crosses between the bi- and tetralocular types of yellow sarson has confirmed the earlier results and shown that tetralocular siliques harbor more seeds and possess a higher number of siliques on the main raceme compared to the bilocular types (Roy and Sinhamahapatra 2011). The study also revealed that width of siliques is positively correlated with seeds per silique and thousand seed weight. Previous reports have shown that transfer of tetralocular trait to bilocular *B. juncea* lines led to an increase in the number of seeds, but it was associated with a decrease in seed weight (Katiyar et al. 1998).

Chiifu, a vegetable type belonging to *B. rapa* ssp. *pekinensis* has been extensively used for developing molecular maps in *B. rapa* (Kim et al. 2009; Jiang et al. 2011; Ramchiary et al. 2011). This line has also been used for BAC and NGS based genome sequencing (Mun et al. 2010; Wang et al. 2011) making it an ideal parent in crosses with other *B. rapa* types that contain interesting morphological and agronomic traits for QTL mapping. A major objective of the present study was to map the tetralocular ovary (*tet-o*) character using F_2 population and recombinant inbred lines (RILs) developed from a cross between Chiifu and a tetralocular type of yellow sarson (Tetralocular). The width of tetralocular siliques is significantly more than that of the bilocular siliques. Hence, another objective of the present study was to understand whether the *tet-o* trait governing locus contributed towards increased silique width or whether the two traits are independent. We show that the *tet-o* trait is controlled by a single recessive gene which has a major pleiotropic effect on silique width also. Further, saturation mapping with SNP markers (Paritosh et al. 2013) around the *tet-o* locus revealed that the trait in all probability is due to a mutation in Bra034340 gene, which is a homologue of *CLAVATA3* of *Arabidopsis thaliana*.

Materials and methods

Plant materials and field experiments

Three *B. rapa* lines—Chiifu (ssp. *pekinensis*), Tetralocular and YSPB-24 (both belonging to ssp. *trilocularis*) were used as parents for the development of four different mapping populations. Chiifu and YSPB-24 both have bilocular ovaries, while Tetralocular has ovaries with four locules. The first three mapping populations, CTF_2 (Chiifu × Tetralocular F_2) consisted of 93 F_2 individuals and the corresponding RILs referred to as CTF_6 and CTF_7 consisted of 94 and 93 lines in the F_6 and F_7 generations, respectively. RILs were developed by single seed descent from a population of 569 F_2 plants of Chiifu/Tetralocular cross. Each CTF_2 plant was self-pollinated by bagging or by bud pollination for the development of RILs. The fourth mapping population CYF_2 (Chiifu × YSPB-24 F_2) consisted of 94 F_2 plants. All the populations were grown in winter (rabi) season which starts from October and ends in April. CTF_2 lines were grown in 2006–07, CTF_6 in 2010–11, CTF_7 in 2011–12 and CYF_2 lines in 2010–11 at a field station located in Delhi. RILs were planted in a row in single replication with a row to row distance of 50 cm and a plant to plant distance of 15 cm. Genomic DNAs were isolated from fully expanded leaves of field grown plants of the parental lines and mapping populations following the method described by Rogers and Bendich (1994).

Linkage mapping and construction of integrated map

Linkage maps for CTF_2, CTF_6, CTF_7 and CYF_2 were constructed with a combination of IP, SSR and SNP markers as reported earlier by Paritosh et al. (2013) (Supplementary Figs. S1–S4). PCR reactions were carried out following Panjabi et al. (2008) for IP markers, Kim et al. (2009), Li et al. (2010), Xu et al. (2010) for SSR markers and Paritosh et al. (2013) for SNP markers. Total genetic lengths spanned by the maps ranged from 679.7 to 869.8 cM. The average interval between two consecutive markers varied from 1.1 to 4.4 cM (Supplementary Table S1). An integrated map of *B. rapa* was constructed using marker data of

CTF_2, CTF_6, CTF_7 and CYF_2. The integrated map consists of 1,037 markers (Supplementary Table S2; Fig. S5) distributed over 918 genetic intervals spanning a total genomic length of 831.4 cM. The average length of the interval covered by two consecutive markers was 0.9 cM.

Trait measurements, statistical analyses and QTL mapping

Segregation of the *tet-o* trait was studied in CTF_2, CTF_6 and CTF_7 mapping populations derived from Chiifu/Tetralocular cross. F_1 plants of this cross were all bilocular indicating that the bilocular silique phenotype is dominant over the tetralocular phenotype as has been reported earlier (Varshney 1987; Salava et al. 1996; Roy and Sinhamahapatra 2011). Anatomy of the tetralocular ovary was studied by microtomy. Sections were prepared and stained following Ruzin (1999). Locule numbers of the siliques in the mapping population were observed by free hand transverse sections and longitudinal splitting. Ten siliques from main shoot of each of the F_2 plant and a single plant from RILs were used for analyzing locule number. Since the locule number varied from two to four with different types of siliques (bi-, tri- and tetralocular) on a single plant, average of ten siliques was used for phenotyping. Plants with silique locule number of two were scored in the bilocular class and those showing values greater than two were scored as multilocular type. Based on this classification, *tet-o* locus was qualitatively mapped to CTF_2, CTF_6 and CTF_7 maps following JoinMap 4.0 format (Van Ooijen 2006). Quantitative data on silique width were recorded as the diameter of the siliques (in millimeters) at approximately half the length of the siliques with a pair of Vernier calipers (Series 530, Mitutoyo Corporation, Japan). The data were collected from ten siliques on the main shoot when they were still green but had started showing signs of maturity, i.e. they were hard to touch and the yellowing of the siliques had set in. Mean of ten observations was taken as the trait value. QTL analysis was performed with Kruskal–Wallis test, interval mapping and Multiple QTL Mapping (MQM) functionalities of MapQTL 6.0 (Van Ooijen 2009). LOD thresholds for QTL significance were determined for each linkage group by permutation tests with 1,000 iterations with a significance level of 0.05. A LOD value of 2.5 was used for indicating the presence of a QTL. Prior to MQM mapping, nonparametric mapping via the rank sum test of Kruskal–Wallis and interval mapping were performed to identify closely linked markers. Using the results of both Kruskal–Wallis test and interval mapping, markers close to the QTL were identified and these markers were selected as cofactors. MQM mapping was performed using the cofactor markers on each of the ten linkage groups. Multiple analyses were performed for MQM mapping to identify the markers showing the highest LOD. QTL analyses combined over the populations were also conducted on the integrated map. Firstly, common QTL was identified among the populations based on their marker intervals in the genome and the trait data were then combined and analyzed by MQM mapping over the selected linkage groups of the integrated map harboring the common QTL. In the combined analyses the LODs of the individual analysis is added and hence has been described as 'combined LOD'.

Saturation mapping of target region and identification, cloning and mapping of the candidate gene

SNP markers were developed as described in our earlier paper (Paritosh et al. 2013). For saturation mapping of the target region, one SNP from each of the 60 gene models that included SNPs from 42 single copy gene models and 18 multi-copy genes were developed. For the multi-copy gene models, SNPs as well as paralog specific variations (PSVs) were marked. For genotyping of SNPs, KASPar technology based on FRET quencher system was used. Primer design and assay development was undertaken by KBiosciences (http://www.kbioscience.co.uk/). Nucleotide sequences of putative candidate gene and its related gene sequences from *B. rapa* and *A. thaliana* were downloaded from Brassica Database (BRAD; Cheng et al. 2011; http://brassicadb.org/brad/) and NCBI GenBank, respectively. A Neighbor-Joining tree was generated in MEGA 5 (Tamura et al. 2011). A bootstrap analysis with 500 replicates was performed to assess the statistical reliability of the tree topology.

DNA amplification of candidate gene(s) was performed from different *B. rapa* lines using gene-specific primers. The amplified fragments were cloned in pGEM-T easy vector (Promega, USA) and sequenced. Gene sequences were assembled from three clones from each of the three independent PCRs to exclude any Taq polymerase-based mutation. SNP genotyping of the candidate gene was performed by SNaPshot method. Reactions were carried out using a SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA) following a protocol recommended by the manufacturer. Amplified product was analyzed by capillary electrophoresis on the ABI Prism 310 Genetic analyzer *(*Applied Biosystems).

Results

Phenotypic variation and mapping of tetralocular ovary (*tet-o*) trait

A normal bilocular ovary in *B. rapa* (Fig. 1a) contains two well-defined locules which are separated by a septum that

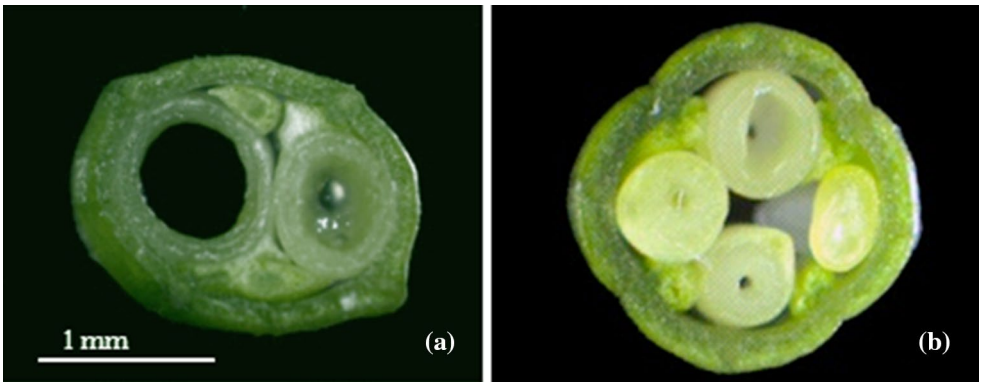

Fig. 1 Transverse hand sections of (**a**) a bilocular silique of yellow sarson type (YSPB-24) (**b**) a tetralocular silique in the variant Tetralocular yellow sarson

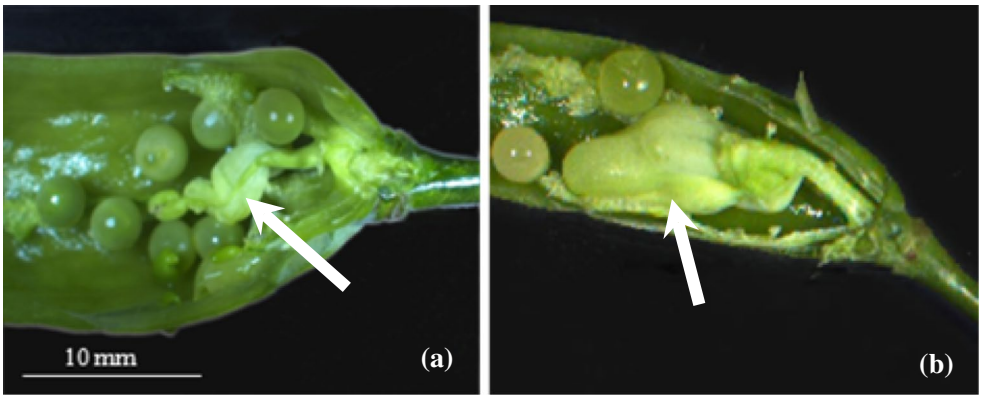

Fig. 2 Siliques cut along their length to show the presence of an extra gynoecium within the primary gynoecium in (**a**) Tetralocular yellow sarson (**b**) a RIL of the cross Chiifu/Tetralocular. The extra gynoecium (shown with *white arrows*) remains highly suppressed in the parental line Tetralocular but is more pronounced in the RILs of the cross Chiifu/Tetralocular

connects the two replums. There are two rows of ovules which differentiate from placentae and lie along the inner sides of each of the replum. The bilocular ovary of YSPB-24 develops along the lines described in detail for *A. thaliana* by Roeder and Yanofsky (2006). In comparison, a section of the tetralocular ovary shows four replums, four placentae and four rows of ovules which after fertilization and development are present as four rows of seeds in the mature fruit (Fig. 1b). The developing silique does not show septa connecting the four replums. Another important characteristic of the tetralocular ovary is the presence of a degenerated ovary within the functional ovary (Fig. 2). The secondary ovary remains highly suppressed in the line Tetralocular and does not contain seeds at maturity. However, in the Chiifu/Tetralocular RILs (CTF_6 and CTF_7), many lines were observed to have tetralocular ovaries which had a very pronounced secondary ovary (Fig. 2b) that contained developing seeds. Detailed anatomical structure of a tetralocular silique is shown in Supplementary Fig. S6.

The CTF_2 mapping population of 93 individuals was used for genetic mapping of the tetralocular phenotype consisting of 69 bilocular and 24 multilocular plants. RILs of F_6 generation were also used for genetic mapping of the tetralocular phenotype and consisted of 94 lines of which 66 were scored as bilocular and 27 as multilocular, while of the 93 RILs of F_7 generation, 52 were bilocular and 35 were multilocular. One RIL in CTF_6 and six RILs in CTF_7 could not be phenotyped for locule number. Qualitative mapping of the tetralocular phenotype (*tet-o*) on CTF_2, CTF_6 and CTF_7 mapped the locus to linkage group A4 (Supplementary Figs. S1, S2 and S3).

QTL mapping for silique width

Significant variation was observed in the size of the siliques of the three *B. rapa* lines—Tetralocular, YSPB-24 and Chiifu (Fig. 3). The silique width of Tetralocular is approximately twice the silique width of Chiifu (Table 1). The silique width of YSPB-24 is higher than that of Chiifu but is less than the silique width of Tetralocular. The distribution of silique width in CTF_2, CTF_6 and CTF_7 showed a skewed distribution towards Chiifu (Fig. 4). In all the three populations, no transgressive segregants were observed with higher silique width than that recorded for the parental line Tetralocular. Silique width was more equally distributed in

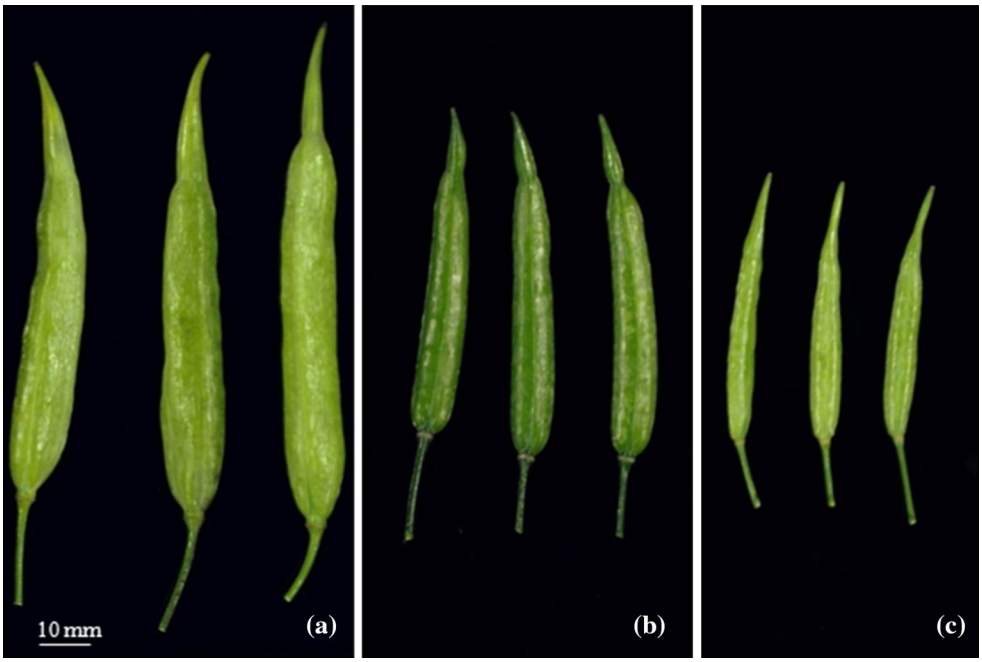

Fig. 3 A comparison of the silique dimensions in *B. rapa* lines used in the mapping studies (**a**) Siliques of tetralocular yellow sarson line 'Tetralocular' (**b**) Siliques of bilocular yellow sarson line 'YSPB-24' (**c**) Siliques of 'Chiifu', a leafy vegetable type

Table 1 Phenotypic values for silique width (mm) among the parental lines used for the development of the mapping populations CTF_2, CTF_6, CTF_7, CYF_2 and the range of variation (± standard deviation) in the F_1, F_2, F_6 and F_7 generations

Mapping population	Parental lines			F_1	Mean ± standard deviation in the mapping population
	Chiifu	Tetralocular	YSPB-24		
CTF_2	5.24 ± 0.52	10.46 ± 0.97	–	6.38 ± 0.73	6.17 ± 1.22
CTF_6	4.94 ± 0.29	12.40 ± 1.30	–	–	7.07 ± 1.54
CTF_7	5.18 ± 0.30	11.88 ± 0.93	–	–	6.69 ± 1.28
CYF_2	5.14 ± 0.09	–	7.66 ± 0.27	–	6.20 ± 0.87

C Chiifu, *T* Tetralocular, *Y* YSPB-24

CYF_2 and transgressive segregation exceeding Chiifu (low parent) and YSPB-24 (high parent) was observed. Silique width was more dispersed in CTF_2, CTF_6 and CTF_7 than in CYF_2 as evidenced by the higher standard deviations in the first three in comparison to the latter (Table 1).

Both nonparametric mapping via Kruskal–Wallis test and interval mapping were carried out as a prelude to Multiple QTL Mapping (MQM) and only the QTL identified by MQM analysis have been described here. A QTL was named according to the map in which it was identified followed by *Sw* for *S*ilique *w*idth and the linkage group number (Table 2). In CTF_2, of the three QTL detected on LGs A1 (*CTF_2-Sw1*), A3 (*CTF_2-Sw3*) and A4 (*CTF_2-Sw4*) (Table 2 and Supplementary Fig. S7), *CTF_2-Sw4* showed the highest LOD (7.52) and was found to co-locate with the *tet-o* locus on the genetic map. *CTF_2-Sw4* also accounted for the largest percentage (41.8 %) of the variation for silique width. While trait enhancing alleles for the QTL *CTF_2-Sw1* and *CTF_2-Sw4* were inherited from the high value parent Tetralocular, the trait enhancing allele for the QTL *CTF_2-Sw3* was inherited from the low value parent Chiifu. QTL *CTF_2-Sw1* exhibited a high and positive dominance effect connoting that the individual plants heterozygous at this locus had higher silique width. In contrast QTL *CTF_2-Sw3* and *CTF_2-Sw4* exhibited negative dominance effects implying that the individuals heterozygous for these loci had decreased silique width. In both CTF_6 and CTF_7, two QTL on LGs A4 and A8 were detected. QTL *CTF_6-Sw4* exhibited the highest LOD of 10.25 and also accounted for 39.8 % of the phenotypic variance, while QTL *CTF_7-Sw4* was detected at a LOD of 4.11 and explained a phenotypic variance of 18.9 %. For these QTL co-locating with the *tet-o* marker, Tetralocular contributed alleles for increased silique width (Table 2; Supplementary Figs. S8–S9).

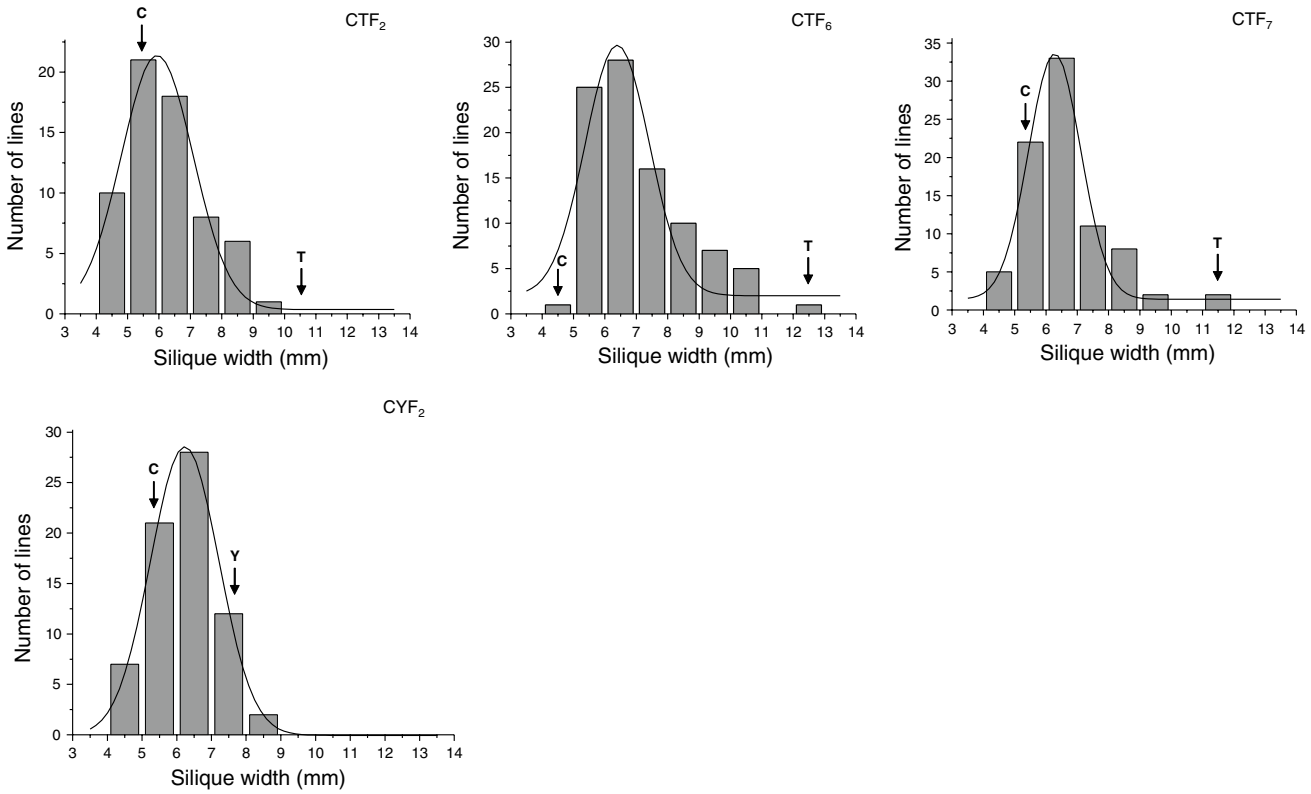

Fig. 4 Distribution of silique width in four mapping populations of *B. rapa* derived from the crosses Chiifu/Tetralocular and Chiifu/YSPB-24. The *arrows* mark the mean values of the parental lines, respectively, in each population. Gaussian fit approximations are shown by the *curves*. CTF_2: Chiifu × Tetralocular F_2; CTF_6: Chiifu × Tetralocular F_6; CTF_7: Chiifu × Tetralocular F_7; CYF_2: Chiifu × YSPB-24 F_2. *C* Chiifu, *T* Tetralocular, *Y* YSPB-24

Table 2 QTL for silique width identified in four mapping populations of *B. rapa* (CTF_2, CTF_6, CTF_7 and CYF_2) with MQM mapping implemented in MapQTL 6.0

QTL[a]	Linkage group	Position	Closest marker	LOD score	$R^{2\,b}$	A^c	D^d	Source of trait enhancing allele
CTF_2-Sw1	A1	47.5	cnu_m462a	3.38	18.1	−0.24	1.35	Tetralocular
CTF_2-Sw3	A3	14.2	BrGMS480	4.24	25.3	1.16	−0.58	Chiifu
CTF_2-Sw4	A4	35.3	*tet-o*	7.52	41.8	−0.93	−0.88	Tetralocular
CTF_6-Sw4	A4	36.0	*tet-o*	10.25	39.8	−1.14	–	Tetralocular
CTF_6-Sw8	A8	45.8	cnu_m239a	3.17	14.5	−0.61	–	Tetralocular
CTF_7-Sw4	A4	34.1	*tet-o*	4.11	18.9	−0.70	–	Tetralocular
CTF_7-Sw8	A8	20.5	cnu_m518a	4.20	20.8	−0.64	–	Tetralocular
CYF_2-Sw2	A2	22.3	At5g15470.2	2.84	17.0	−0.82	0.31	YSPB-24
CYF_2-Sw9	A9	43.7	BrGMS588	5.05	28.3	−0.63	−0.43	YSPB-24

[a] The prefixes CTF_2, CTF_6, CTF_7 and CYF_2 in the QTL name denote the maps in which the respective QTL was detected. CTF_2: Chiifu × Tetralocular F_2; CTF_6: Chiifu × Tetralocular F_6; CTF_7: Chiifu × Tetralocular F_7; CYF_2: Chiifu × YSPB-24

[b] Phenotypic variation explained (%)

[c] Additive effect has been defined as (mu_A − mu_B)/2 where, mu_A and mu_B are the estimated mean of the distribution of the quantitative trait associated with the "a" and "b" genotypes, respectively

[d] Dominance has been defined as mu_H − (mu_A + mu_B)/2 where, mu_A, mu_B and mu_H are the estimated mean of the distribution of the quantitative trait associated with the "a", "b" and "h" genotypes, respectively. A positive dominance effect connotes that heterozygotes have increased silique width than the corresponding homozygote class

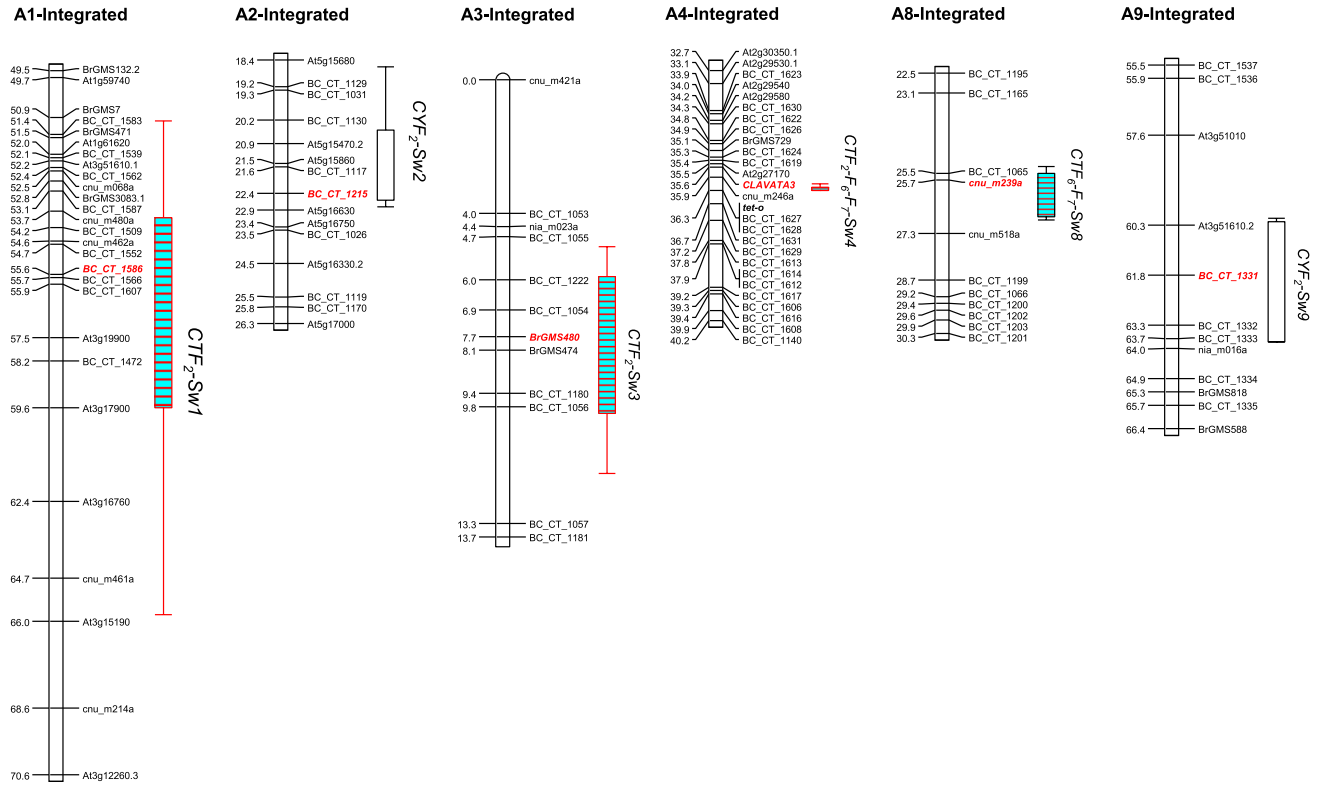

Fig. 5 QTL for silique width on the integrated map of *Brassica rapa* by Multiple QTL Mapping (MQM). Only the regions of linkage groups that harbor a QTL are shown. Marker names are on the *right* of the linkage group *bar* and the positions in centiMorgans (cM) are on the *left*. The position of QTL (one LOD interval) is indicated by *bars*. The two LOD intervals on either side of the *bar* are represented by *whiskers*. QTL identified in Chiifu/Tetralocular mapping populations have been shown by *filled bars* while QTL identified in Chiifu/YSPB-24 F_2 population have been represented by *empty bars*

QTL analysis in CYF_2 revealed two QTL, one each on LGs A2 and A9 (Table 2; Supplementary Fig. S10). The alleles for increasing silique width at these loci were provided by YSPB-24. QTL *CYF_2-Sw9* showed the highest LOD of 5.05 and also explained the largest amount of phenotypic variance (28.3 %). These QTL increased silique width by both additive and dominant modes of gene action. Dominance effects at *CYF_2-Sw2* were positive connoting that the individual plants heterozygous at this locus had higher silique width. No QTL was detected on LG A4 in comparison to the three Chiifu/Tetralocular mapping populations.

Integrated map was also used for MQM using the trait data for silique width and the same set of cofactor markers used in the four bi-parental populations. The QTL for silique width on the integrated map are shown in Fig. 5. Four single population QTL namely, *CTF_2-Sw1*, *CTF_2-Sw3*, *CYF_2-Sw2* and *CYF_2-Sw-9* mapped to similar positions on the integrated map. The combined analysis for silique width on LG A4 of the integrated map showed that the LOD peak for common QTL (*CTF_2-F_6-F_7-Sw4*) was present at the same genomic region of the LG A4 as in the populations: CTF_2 (*CTF_2-Sw4*), CTF_6 (*CTF_6-Sw4*) and CTF_7 (*CTF_7-Sw4*) (Supplementary Fig. S11). The combined LOD (22.3) at this region exceeded the threshold (4.0) by 5.6 folds. QTL *CTF_6-Sw8* and *CTF_7-Sw-8* were also detected as the same QTL detected in the F_6 and F_7 generations of the cross Chiifu/Tetralocular. *CTF_6-F_7-Sw8* showed a combined LOD of 8.84 with a contribution of LOD values equal to 3.16 and 3.46 from *CTF_6-Sw-4* and *CTF_7-Sw4*, respectively.

Saturation of *tet-o* region with SNP markers and identification of the candidate gene

Of the 60 SNP markers used for saturation mapping of the region containing the *tet-o* locus, 52 SNPs were successfully mapped onto the LG A4 of CTF_7 map with a mean marker interval of 0.5 cM (Paritosh et al. 2013). A high correspondence was observed between the order of SNPs on the genetic map and the physical map (Supplementary Fig. S12). The minimum distance of the SNP markers with the morphological marker *tet-o* was observed to be 0.8 cM with Bra034279 (BC_CT_1619) on one side and 0.5 cM with Bra034366 (BC_CT_1627) on the other (Supplementary Fig. S3). Search for the genes present

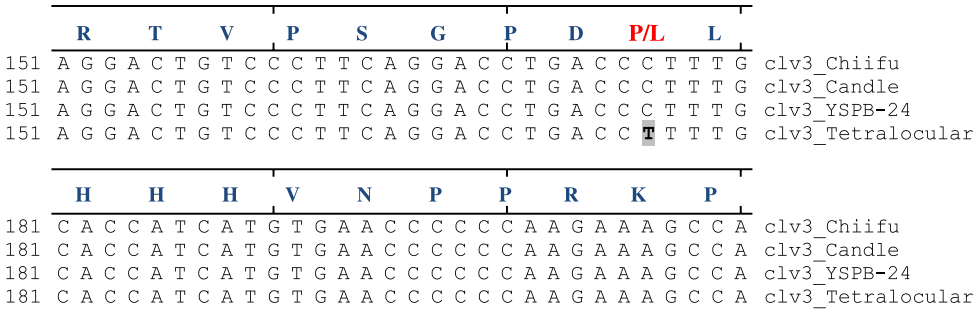

Fig. 6 Sequence comparisons of Bra034340 (a homologue of *CLAVATA3* in *A. thaliana*) showing the SNP (*shaded grey*) between the bilocular (Chiifu, Candle and YSPB-24) and Tetralocular lines of *B. rapa*. Amino acid sequence is on the *top* of the nucleotide sequence alignment

between the two tightly linked flanking genes in *B. rapa* using BRAD genome browser (http://brassicadb.org/brad/) revealed that the *tet-o* locus is present in the I block of LG A4 and there are eighty-seven genes present between the two tightly linked markers. All the 87 genes were found to code for functional proteins; 62 of these genes were found to have homologues in the syntenous region of the I block of chromosome 2 of *A. thaliana* (Supplementary Table S3).

As fate of the gynoecium is determined in the shoot apex (Robles and Pelaz 2005), a search was made amongst these 87 genes for those that express specifically in the shoot meristem. Expression profile of all the 87 genes was checked in the transcriptome data obtained earlier from young inflorescence of *B. rapa* lines Chiifu and Tetralocular (Paritosh et al. 2013). This analysis showed that only 17 of the 87 genes expressed in the young inflorescence. Homologues of these 17 genes in *A. thaliana* were also checked for their expression pattern by BAR e-northern analysis (Toufighi et al. 2005) and AtGenExpress Tool (Schmid et al. 2005). Expression and protein function analyses showed that the most likely candidate gene encoding for tetralocular ovary is Bra034340 which is a homologue of *CLAVATA3* (*CLV3*) described in *A. thaliana*. *CLV3* is a very well characterized gene in *A. thaliana* which expresses in the apical meristem and is involved with the control of meristem size and gynoecium development (Roeder and Yanofsky 2006).

CLAVATA genes belong to a large family of genes called CLE or *CLAVATA3*/ESR related genes (Cock and McCormick 2001). The CLE genes are characterized by a conserved 12 amino acid long CLE motif [RLVPSGPNPL(H/N)N] which is located in the C-terminal domain of CLE genes in *A. thaliana* (Ni and Clark 2006). We carried out a phylogenetic analysis of 101 different CLE family genes identified in *B. rapa* (http://brassicadb.org/brad/) and *A. thaliana* (Oelkers et al. 2008) and found that *CLAVATA3* and Bra034340 have the highest level of sequence identity (Supplementary Fig. S13).

Bra034340 in *B. rapa* line Chiifu has a 234 bp coding sequence and an intron of 178 bp length. Bra034340 sequences were amplified from YSPB-24, Candle and Tetralocular using primers based on the sequence of the gene from Chiifu available in BRAD. Forward and reverse primers (Forward-5′ ATGGGAAACAAAGTGGTGCTTATCTA 3′; Reverse-5′ GAATTGTATCGAATGGAAATATAGACAAAG 3′) amplified 500 bp of the promoter along with the full coding region and intron of Bra034340. PCR products from three independent reactions were cloned and sequenced to find the consensus sequence for Bra034340. These sequences have been deposited in GenBank with accession numbers KC914351, KC914352 and KC914353 for Candle, Tetralocular and YSPB-24, respectively. Alignment of Bra034340 sequence of Chiifu, Candle, YSPB-24 and Tetralocular showed that at position 176 of the coding region, base 'T' is present in Tetralocular and base 'C' in the three bilocular lines (Fig. 6). This nucleotide change leads to an amino acid change from Proline to Leucine at position 59 in the mature protein. Further analysis showed that this mutation leads to a change in the CLE domain at position P^9 as a result of which wild type CLE domain RTVPSGPDPLHH in lines containing bilocular type of ovary is changed to RTVPSGPDLLHH in the Tetralocular line. The amino acid Proline is conserved at the 9th position of all the *CLAVATA* or related genes of CLE family in *A. thaliana* and substitution of this Proline to Alanine leads to severe loss of the CLE activity (Kondo et al. 2011).

Genetic mapping with the SNP 'C/T' in Bra034340 placed the marker on linkage group A4 co-mapping with the *tet-o* locus. To confirm whether the SNP (C/T) is linked to *tet-o* encoded trait of tetralocular ovary, simple phenotype-genotype correlations were carried out on 171 RILs in F7 generation. These 171 RILs were genotyped for 'C/T' polymorphism revealing 55 'CC' homozygotes, 63 'CT' heterozygotes and 53 'TT' homozygotes. 'CC' and 'CT' genotypes were all bilocular while all the 'TT' genotypes were multilocular.

On the basis of synteny, expression pattern, nucleotide identity and SNP association we conclude that Bra034340, a homologue of *CLAVATA3* of *A. thaliana*, is the candidate gene where a C → T transition is responsible for the trait of tetralocular ovary.

Discussion

Extensive genomic resources are now available for the line Chiifu—a leafy vegetable type belonging to *B. rapa* ssp. *pekinensis*. These include BAC libraries (Mun et al. 2008), SSR markers (Choi et al. 2007), and more recently sequence of the genome (Wang et al. 2011). However, mapping population resources in *B. rapa* remain limited. Most of the recent mapping work has been carried out on DH lines derived from a cross between two leafy vegetable types Chiifu and Kenshin, both belonging to ssp. *pekinensis* (Kim et al. 2009; Choi et al. 2007; Li et al. 2010) providing insights into the limited variability available within the Chinese cabbage group. We have developed new molecular marker based linkage maps in *B. rapa* for mapping qualitative and quantitative traits for silique related characters. In this study, using different generations of the same cross, QTL for silique width were compared.

A total of nine QTL affecting silique width were identified in the four mapping populations (CTF_2, CTF_6, CTF_7 and CYF_2). The phenotypic variation explained ranged from 14.5 to 41.8 %. All the QTL (except *CTF_2-Sw3*) had trait enhancing alleles from the high value parent (Tetralocular or YSPB-24). In all the three generations of the mapping populations derived from the cross Chiifu/Tetralocular, the major QTL for silique width co-located with the *tet-o* locus on LG A4 of the individual maps and the integrated map. Interestingly, this QTL was not detected in the CYF_2 population which was derived from a bilocular/bilocular cross. Co-location of the major QTL for silique width on LG A4 with *tet-o* locus and Bra034340—a homologue of *CLAVATA3* which is known to result in increased number of floral organs, especially carpels, enlarged floral meristem, and increased width of siliques (Clark et al. 1995) strengthens the assertion that the *tet-o* locus by itself imparts additional width.

Earlier studies (Varshney 1987; Choudhary and Solanki 2007; Lou et al. 2007; Bagheri et al. 2012; Li et al. 2013; Roy and Sinhamahapatra 2011; Xu et al. 2013; Xiao et al. 2013) either focused on the genetics of number of locules in siliques or width of siliques. Our results indicate that locule number and silique width are strongly correlated and probably the number of seeds formed in a silique is determined by the joint action of these traits. In a recent study carried out for identifying genes responsible for multilocular siliques, Xu et al. (2013) have proposed that the trilocular trait is governed by two independent recessive genes in *B. juncea*. Genetic mapping of one of the two loci placed it on LG A7 flanked by an AFLP marker and a SCAR marker. The authors however did not report the mapping of the other independent locus. In another study Xiao et al. (2013) mapped the gene for locule number in *B. juncea* on LG A7 and Scaffold000019 was found to be common with the location of trilocular gene reported by Xu et al. (2013). The *tet-o* locus in *B. rapa* is present on LG A4 and therefore the locus and the causal mechanism for multilocular ovary in *B. rapa* and *B. juncea* appear to be different.

RILs offer increased power for detecting additive QTL with smaller effects due to their homozygosity. However, in this study not all QTL detected in CTF_2 could be mapped in the RILs (CTF_6 and CTF_7). This could be attributed to the lesser number of markers deployed to genotype CTF_6 as compared with CTF_2. Additionally, multiple cycles of recombination involved in the development of CTF_6 and CTF_7 could have resulted in disrupting the marker-QTL linkages. QTL *CTF_2-Sw1* was detected as a dominant QTL with a small additive component and therefore it could have remained undetected in CTF_6 and CTF_7 wherein QTL with a high additive component could be detected. We were able to detect one minor QTL on LG A8 with a phenotypic variance of 14.5 % in CTF_6 which was not identified in CTF_2. This QTL explained a phenotypic variance of 20.8 % in CTF_7. The smallest phenotypic variance accounted by a QTL (*CTF_2-Sw1*) in CTF_2 was 18.1 % but in F_6, the minor QTL (*CTF_6-Sw8*) exhibited a general contribution of less than 18.1 %. Clearly, this QTL had small effects and consequently remained undetected in CTF_2, whereas it could be identified in CTF_6 and CTF_7 due to high resolution and increased homozygosity.

The bilocular ovary is a typical character of *Brassicaceae* and naturally occurring multilocular ovary has only been reported in some *B. rapa* ssp. *trilocularis* lines. However, multilocular mutants have been described in the model crucifer—*A. thaliana* (Roeder and Yanofsky 2006; Alvarez-Buylla et al. 2010). The structure of the tetralocular variants in yellow sarson types comes closest to the mutants described for the *CLAVATA* genes – *CLV1*, *CLV2* and *CLV3* (Clark et al. 1993, 1995; Kayes and Clark 1998). The major role of the *CLAVATA* genes is to keep in check the size of the apical meristem. *CLV3* is expressed in the outer L1 and middle L2 layer of shoot apical meristem, while *CLV1* is expressed in the central L3 layer of the shoot apical meristem. *CLV1* encodes a Leucine-rich repeat type receptor kinase (LRR-RLK) protein, while *CLV2* encodes a similar protein lacking the kinase domain (Clark et al. 1997; Jeong et al. 1999). It is hypothesized that the *CLV3* gene product is secreted from the meristematic region and acts as a ligand for the *CLV1/CLV2* receptor complex (Trotochaud et al. 2000). Mutant alleles of the gene *CLV3* in *A. thaliana* have been shown to accumulate excess of undifferentiated cells in the shoot and the floral meristem leading to an increase in the number of carpels from two to five and the presence of an additional gynoecium within gynoecium (Clark et al. 1995) resembling the carpel described in *B. rapa* line Tetralocular.

By alanine scanning experiments in *A. thaliana*, it has been shown that the V^3, G^6, N^8, P^9 and N^{12} substitutions could cause a severe loss of the CLE domain activity (Ito et al. 2006; Kondo et al. 2008). Further, substitution P^9 has been shown to play an important role in maintaining the bioactive conformation of the peptide chain (Kondo et al. 2011). In *A. thaliana*, it has been reported that the hydroxylation of P^9 may render the peptide more hydrophilic, facilitating its movement and resistance against proteolysis in the apoplasm (Shimizu et al. 2005; Ito et al. 2006; Kondo et al. 2011). Therefore, due to change of the P^9 of the CLE domain of *CLAVATA*3 protein to L could lead to the manifestation of mutant phenotype in the line Tetralocular. The correlation of the SNP variation to the locule number also indicates that this variation is the causal factor for multilocular ovary.

We propose that under artificial selection no mutant phenotypes with deleterious effects on yield would have been selected. It can therefore be concluded that the *tet-o* locus became fixed in the yellow sarson types because these are self-pollinated but more importantly because the *tet-o* locus did not induce any pronounced pleiotropic abnormalities in the overall background of the bilocular types of yellow sarson. However, the *tet-o* locus when present in Chiifu (which is distantly related) background can cause abnormalities like a pronounced gynoecium within the main gynoecium thereby suppressing the yield. As a consequence, agronomic use of *tet-o* locus even though leading to a greater number of seeds forming in a silique, may be limited in terms of introgressions into other *B. rapa* types and allopolyploid oilseed crops like *B. juncea* and *B. napus*.

Authors' contributions SKY and PP-M carried out the phenotyping and mapping work. VG helped with genotyping and YSS along with SKY produced and maintained the RILs. AC helped with phenotyping and carried out the anatomical work. KP developed the SNP markers and analyzed *CLAVATA* sequences. AKP supervised the genetics and mapping work. DP conceived and supervised the overall study and along with SKY, PP-M and AKP wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no competing interests.

Ethical standards The manuscript entitled "Tetralocular ovary and high silique width in yellow sarson lines of *Brassica rapa* (subspecies *trilocularis*) are due to a mutation in Bra034340 gene, a homologue of *CLAVATA3* in Arabidopsis" being submitted for publication in Theoretical and Applied Genetics complies with the current laws of India for academic and research purposes.

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