

Quantitative Trait Loci Mapping in *Brassica rapa* Revealed the Structural and Functional Conservation of Genetic Loci Governing Morphological and Yield Component Traits in the A, B, and C Subgenomes of *Brassica* Species

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Abstract

***Brassica rapa* is an important crop species that produces vegetables, oilseed, and fodder. Although many studies reported quantitative trait loci (QTL) mapping, the genes governing most of its economically important traits are still unknown. In this study, we report QTL mapping for morphological and yield component traits in *B. rapa* and comparative map alignment between *B. rapa*, *B. napus*, *B. juncea*, and *Arabidopsis thaliana* to identify candidate genes and conserved QTL blocks between them. A total of 95 QTL were identified in different crucifer blocks of the *B. rapa* genome. Through synteny analysis with *A. thaliana*, *B. rapa* candidate genes and intronic and exonic single nucleotide polymorphisms in the parental lines were detected from whole genome resequenced data, a few of which were validated by mapping them to the QTL regions. Semi-quantitative reverse transcriptase PCR analysis showed differences in the expression levels of a few genes in parental lines. Comparative mapping identified five key major evolutionarily conserved crucifer blocks (R, J, F, E, and W) harbouring QTL for morphological and yield components traits between the A, B, and C subgenomes of *B. rapa*, *B. juncea*, and *B. napus*. The information of the identified candidate genes could be used for breeding *B. rapa* and other related *Brassica* species.**

Key words: *Brassica rapa*; quantitative trait loci (QTL); morphological traits; single nucleotide polymorphism (SNP); conserved genome blocks

1. Introduction

Brassica rapa ($2n = 20$, AA) is an important *Brassica* species that is grown widely to produce leafy vegetables in Korea, China, and Japan, for vegetable oil in

India, China, and Canada, and as a fodder crop in Europe. Chinese cabbage, pak choi, sarson, and turnips are distinct morphotypes of *B. rapa* belonging to different subspecies that are adapted to different geographical regions and climatic conditions. During the last two decades, studies by different laboratories using diverse germplasm led the development of several molecular markers and genetic linkage

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maps^{1,2} besides mapping quantitative trait loci (QTL) for erucic acid,³ glucosinolates,^{4,5} disease resistance,^{6,7} plant morphology, flowering time, and yield component traits.^{8,9} Furthermore, comparative mapping between different cultivated *Brassica* species revealed the structural conservation between the homoeologous chromosomes of the A, B, and C subgenomes that were originally derived from three diploid *Brassica* species, namely, the *Brassica rapa*, *Brassica nigra* (BB, $n = 8$), and *Brassica oleracea* (CC, $n = 9$) genomes, and their polyploid derivatives, i.e. the important oilseed crops *Brassica juncea* (AABB, $n = 18$) and *Brassica napus* (AACC, $n = 19$), respectively.^{5,10,11} Furthermore, several comparative mapping studies between *Brassica* and *Arabidopsis thaliana* revealed the triplicate nature of the *Brassica* genome, with an average of three copies of each chromosomal segment of *A. thaliana*, which resulted from the triplication of the whole *Brassica* genome at ~ 11 – 12 MYA,¹² although segmental conservation between *Brassica* and *A. thaliana* chromosomes has been observed at the gross level.^{10,11,13,14}

Therefore, to decode the whole genome sequences of the complicated *Brassica* genomes to be used in breeding programmes and to study the divergence of gene function and genome evolution associated with polyploidy and extensive duplications, the ‘Multinational *Brassica* Genome Sequencing Project Consortium’ was initiated in 2003. The first *Brassica* genome to be sequenced among the six cultivated *Brassica* species was the A genome of the *B. rapa* Chinese cabbage Chiifu-401 cultivar that has a comparatively small genome (529 Mb) among the cultivated *Brassica* species,¹⁵ and the draft genome sequence was published in 2011.¹⁶

With the availability of recently developed advanced next-generation sequencing (NGS) technology to sequence the whole genome of crop plants in a short time span, the emphasis is being shifted to genomics-assisted breeding from traditional crop breeding using conventional molecular markers. The combined use of QTL mapping, which detects functional loci for traits of interest, and whole genome sequence information to identify candidate genes and their variation between the parental lines of *B. rapa* will greatly supplement the development of gene-specific molecular markers for breeding this crop with desired plant architecture and quality. Many oleifera and sarson types were exploited for breeding high seed yield component traits in *B. rapa*, but the vegetable types were not exploited for this purpose. Although several QTL have been mapped for leaf morphology, yield components, and other quality traits, most of the genes underlying trait variation have not been identified.^{9,17}

Therefore, in the present study, we used chromosome-specific bacterial artificial chromosome-derived simple sequence repeats (BAC-SSRs) and gene specific markers, e.g. intron polymorphisms (IPs) and expressed sequenced tag-derived SSRs (EST-SSRs) to map QTL governing morphological and yield component traits. Using whole genome next-generation sequence information data of the parental lines in combination with comparative alignment with the *A. thaliana* genome, potential candidate genes and single nucleotide variations within some of the potential candidate genes were identified. Furthermore, the chromosomal regions of *B. rapa* containing clusters of QTL were aligned with the QTL regions of *B. juncea* and *B. napus* to identify structural and functional conservation between the A, B, and C subgenomes, so that the candidate gene information of *B. rapa* could be used for breeding these crops.

2. Materials and methods

2.1. Plant materials, growth conditions, and trait measurement

The genetic map developed earlier by us^{2,18} using a CRF₂ mapping population that was derived by crossing the diverse parental lines ‘Chiifu 401–42,’ a vegetable-type Chinese cabbage, and ‘rapid cycling *B. rapa*’ (hereafter referred to as ‘RCBr’), was used for QTL mapping in this study. For phenotypic investigation, 12 F₃ plants derived by selfing each of the 190 F₂ plants were planted per replication in 3 replications in 2008, 2009, and 2010 from March to July. The seeds were germinated in cell trays in a greenhouse for 1 month. In 2009 and 2010, two sets were grown, one set was vernalized after 20 days of germination for 1 month and the other set was grown without vernalization. All the plants were transplanted to the open field of Chungnam National University, Daejeon, Korea. Fourteen morphological and yield component traits (Table 1) were recorded in the CRF₃ mapping population and parental lines. Four plants from the middle of each row from each replication were used for phenotypic data measurement for each family, and the average value of three replications was taken as trait data. Flowering time was the only trait that was recorded in the vernalized plants.

2.2. Statistical analysis and QTL mapping

The SAS 9.0 program (SAS institute, Inc., Cary, NC, USA) was used for correlation coefficient analysis. The previously described genetic map¹⁸ was used for QTL mapping. WinQTL cartographer version 2.5¹⁹ was used to perform QTL analysis using the composite interval mapping function as described

Table 1. Details of traits measurement in CRF₃ population

Trait name	ABS	Trait description	Scale	Year
Flowering time	FT	Days from sowing to opening of the first flower	Days	2008/ 2009/ 2010
Bolting time	DB	Days from sowing to the emergence of bud	Days	2009/ 2010
Flowering time after vernalization	FT*	Days from sowing to opening of the first flower after vernalization for 1 month	Days	2009/ 2010
Plant height	PH	Height from ground to the stem top when first flower opens	cm	2008/ 2009/ 2010
Leaf length	LL	From base of petiole to the tip of leaf	cm	2008/ 2009
Leaf width	LW	Leaf width at the widest point	cm	2008/ 2009
Midrib length	MRL	Length from the bottom to the apex of midrib	cm	2008/ 2009
Midrib width	MRW	Width of the bottom of midrib	cm	2008/ 2009
Petiole length	PL	From base of petiole to the bottom of lamina	cm	2009
Siliqua length	SQL	Length from pedicel of siliqua to the top of seed beak	cm	2008/ 2009/ 2010
Siliqua width	SQW	Width at the lengthwise midpoint of each siliqua	cm	2008/ 2009/ 2010
Siliqua beak length	SBL	Length from the top of siliqua to the top of beak	cm	2009/ 2010
Seeds per siliqua	SSQ	Number of seed per siliqua		2009/ 2010
Seed weight	SW	Seed weight of 100 seeds each line	mg	2009/ 2010

* Represent the vernalization treatment.

previously.²⁰ Tests for the presence of QTL were performed at 2-cM intervals using a 10-cM window. Significant QTL-defining logarithm of odds (LOD) values were calculated by 1000 permutations for phenotypic traits derived from each year.

2.3. Comparative map alignment between the A, B, and C subgenomes

To identify the functionally conserved loci between *B. rapa* with *B. juncea* and *B. napus* for morphological and yield component traits, comparative alignment of QTL maps was performed between these three species. *B. juncea* contains the A and B subgenomes while *B. napus* contains the A and C subgenomes. For comparison, the *B. juncea* QTL map of

Ramchiary *et al.*²⁰ and the *B. napus* QTL maps of Quijada *et al.*²¹ and Udall *et al.*²² were used. The marker sequences from the respective maps^{21,22} were downloaded from the National Center for Biotechnology Information and aligned with the *A. thaliana* genome using BLAST analysis, and crucifer building blocks¹⁴ containing important trait QTL were defined. The updated map of *B. juncea* based on IP markers¹¹ was used to redraw the QTL map of Ramchiary *et al.*²⁰ For QTL map alignment, the homoeologous A, B, and C subgenome groups of *Brassica* species defined by Panjabi *et al.*¹¹ were used.

2.4. Whole genome resequencing, identification of SNPs in candidate genes, and semi-quantitative RT-PCR analysis

Whole genome resequencing of parental line RCB_r was performed using an Illumina GAII next-generation sequencer. Sequence assembly and single nucleotide polymorphism (SNP) identification were performed in a stepwise manner: (i) the scaffold sequences from each linkage group (LG) of the *B. rapa* Chiifu 401–42 cultivar genome (<http://brassicadb.org/brad/>) were used as reference sequence, (ii) Augustus program²³ was used to predict candidate genes in QTL regions, (iii) short-read sequences generated from RCB_r were aligned to the reference *B. rapa* genome using Bowtie (<http://bowtie.cbcb.umd.edu>)²⁴ and mapped onto each reference LG, (iv) alignments from Bowtie were transformed to MAQ²⁵ to produce consensus short-read sequences and identify SNPs.

Because we previously demarcated 24 crucifer building blocks in the *B. rapa* genome by comparing it with *A. thaliana*,¹⁸ we selected already characterized candidate genes from *A. thaliana* and looked for orthologous genes in the *B. rapa* genome in those blocks harbouring important trait QTL using a homology search approach and synteny analysis. SNPs were identified between the start and stop codons through comparative alignment between the sequences of the parental lines. Gene-specific primer pairs were designed for SNP validation, with an amplicon size of not more than 300 bp. DNA extraction, PCR using 1 × LC Green Plus (Idaho Technologies), and mapping functions were performed as described previously.¹⁸ The PCR conditions were 4 min at 94°C, 45 cycles of 20 s at 94°C, 20 s at 55–60°C, and 20 s at 72°C, with a final extension step of 7 min at 72°C. For the detection of SNPs between the parental lines and the F₂ mapping population, we used a Light Scanner System (Idaho Technologies), as described previously.²⁶ RNA was extracted from 20-day-old leaf samples, and semi-quantitative RT-PCR analysis was performed following Lee *et al.*²⁷ using gene-specific primer pairs listed in Supplementary Table S4.

3. Results

3.1. Variation of phenotypic traits in the parental lines and segregating populations

The parental line Chiifu is a heading-type Chinese cabbage, whereas RCB_r is a short life cycle *Brassica* plant, also known as the Wisconsin fast plant. The two parental lines showed significant differences for flowering, leaf, siliqua, and seed traits (Table 2). Chiifu showed higher phenotypic values for all the traits studied. The CRF₃ population showed transgressive segregation for some of the traits, e.g. plant height in 2008, midrib length and width, siliqua length and width, siliqua beak length, seeds per siliqua, and seed weight. For flowering and bolting time, the transgression phenomenon trend was towards RCB_r because Chiifu did not flower until seed harvesting time. As the bolting and flowering time were influenced by temperature and photoperiod, the mean value of flowering time measured in spring (2009 and 2010) was ~30 days shorter than in winter (2008), even when they were grown in a heated greenhouse. The mean values of the F₃ lines for the leaf traits and plant height evaluated in spring 2009 and 2010 were also lower than those of the F₃ lines grown in 2008. Distribution analysis of the phenotypic values of 14 traits in the mapping population showed a normal distribution for all the traits, suggesting that each trait was governed by many genes. Pearson's correlation coefficient analysis showed moderate to strong positive correlation among leaf traits (LL, LW, MRL, and MRW, correlation coefficient $r = 0.53-0.92$). Siliqua length, width, and beak length were significantly positively correlated with each other, but seed weight was not correlated with other siliqua traits (Supplementary Table S1). In addition, we found a positive correlation between bolting time, flowering time, and four leaf traits (Supplementary Table S1).

3.2. QTL mapping and identification of crucifer building blocks in the *B. rapa* genome

QTL mapping identified a total of 95 QTL for 14 traits in the CRF₃ mapping population. It was observed that almost all the trait-enhancing alleles, except for flowering time, were contributed by the Chiifu parental line. The number of QTL detected ranged from 4 QTL for petiole length, seed number per siliqua and seed weight to 14 QTL for flowering traits, and the confidence interval ranged from 1 to 25 cM (Fig. 1 and Supplementary Table S2).

Plant height A total of seven QTL were detected for plant height, one in each LG, i.e. A2 and A3 (R block), A3 and A5 (J block), A7 (E block), A9 (H block), and

A10 (A block). The QTL on A2 (*qPH1*) and A7 (*qPH4*) showed higher LOD values and phenotypic explanation and were detected both in 2009 and 2010 (Fig. 1 and Supplementary Table S2). The percentage of phenotypic variation explained by individual QTL ranged from 5 to 19%.

Leaf traits Five leaf traits, i.e. leaf length and width, midrib length and width, and petiole length, were used for QTL analysis (Fig. 1 and Supplementary Table S2). A total of six QTL for leaf length were detected in four LGs. *qLL1* and *qLL5* in the R blocks of A3 and A10, respectively, were consistently detected in two consecutive years (2008 and 2009). The percentage of phenotypic variation explained by individual QTL ranged from 7 to 49%. For leaf width, 10 QTL were detected, of which *qLW1* in the U block of A1, *qLW2* in the R block of A2, and *qLW3* in the J block of A3 were consistently found in 2008 and 2009 (Supplementary Table S2). A total of 10 QTL were detected for midrib length, of which QTL in the J (*qMRL3*) and T (*qMRL4*) blocks of A3 and *qMRL6* in the A block of A6 showed comparatively higher LOD values, but none of them were detected consecutively in different years. For midrib width, five QTL were detected, of which QTL in A2 (*qMRW2*) and A10 (*qMRW5*) were detected in the R blocks of both LGs. *qMRW3* in T/U block of A3 and *qMRW4* in E block of A7 were detected both in 2008 and 2009. Four QTL, two on A2 (*qPL1* in R block and *qPL2* in W block) and one each on LG A6 (*qPL3*) and A10 (*qPL4* in R block) were detected for petiole length. The QTL on *qPL3* was major QTL showing the highest LOD value of 11.8. The phenotypic variation explained by individual QTL ranged from 6 to 13%.

Bolting and flowering traits A total of 10 QTL were detected for bolting time, of which *qDB2* in the R block of A2, *qDB4* in the J block of A3, *qDB7* in the E block of A7, *qDB8* in the A block of A8, and *qDB10* in the R block of A10 were detected in 2008 and 2009 (Fig. 1 and Supplementary Table S2). The QTL on A2, A7, and A10 were major loci, and the percentage of phenotypic variation explained by individual QTL ranged from 4 to 29%.

For flowering time in the non-vernalized plants, a total of 12 QTL were identified in 8 LGs (Supplementary Table S2 and Fig. 1). Of these, QTL mapped to the R (*qFT2* and *qFT3*) and W (*qFT4*) blocks of A2, R block of A3 (*qFT5*), E block of A7 (*qFT10*), N block of A9 (*qFT13*), and R block of A10 (*qFT15*) were major loci and were detected consecutively for 3 yrs. A total of nine QTL, including two new QTL, i.e. *qFT1* in the U block of A1 and *qFT13* in the A block of A10, were detected in vernalized population

Table 2. Phenotypic trait values of parental lines and the CRF₃ mapping population of *B. rapa* observed in different years

Traits	Mean trait value of parent Chiifu			Mean trait value of parent RCB			Range in F ₃ population			Mean value in F ₃ population		
	2008	2009	2010	2008	2009	2010	2008	2009	2010	2008	2009	2010
Flowering time	– ^a	– ^a	– ^a	39.0	45.0	45.0	33.0–139.0	30.0–59.0	45.2–64.0	70.6	41.9	53.4
Bolting time	–	90.0	95.0	–	29.0	29.0	–	11.0–44.7	34.0–65.0	–	26.5	42.3
Flowering time after vernalization	–	135.0	145.0	–	30.0	30.0	–	54.2–68.8	66.0–83.5	–	57.9	73.0
Plant height	65.0	100.0	98.0	17.0	20.0	18.5	5.0–98.0	3.6–34.5	4.0–24.8	43.0	13.6	13.1
Leaf length	28.0	20.0	–	9.0	6.5	–	14.0–49.0	2.8–8.0	–	30.2	5.0	–
Leaf width	14.0	16.0	–	3.8	3.0	–	4.5–19.0	2.2–5.8	–	11.2	3.5	–
Midrib length	14.0	7.7	–	7.4	4.5	–	9.0–36.0	3.3–10.0	–	20.8	5.8	–
Midrib width	3.0	2.5	–	0.5	0.3	–	0.5–3.5	0.3–1.5	–	1.7	0.6	–
Petiole length	–	0.0	–	–	6.5	–	–	0.0–6.3	–	–	3.3	–
Siliqua length	3.0	3.8	3.5	2.8	3.2	3.0	1.5–6.2	2.0–6.8	2.2–6.7	3.7	4.2	4.1
Siliqua width	0.5	0.5	0.6	0.2	0.3	0.2	0.2–0.7	0.3–0.8	0.2–0.8	0.5	0.5	0.4
Siliqua beak length	–	1.0	1.0	–	0.7	0.5	–	0.3–1.6	0.2–1.5	–	0.8	0.7
Seeds per siliqua	–	20.0	25.0	–	11.0	15.0	–	4.1–27.1	2.0–24.9	–	14.3	12.6
Seed weight	–	320.0	345.0	–	105.0	20.0	–	115.0–337.0	30.0–257.0	–	211.0	141.4

^a‘Chiifu’ did not flower until populations seed harvest.

‘–’, Not measured in the corresponding year.

in addition to the already detected QTL in the non-vernalized population (marked by asterisks, Supplementary Table S2 and Fig. 1). *qFT2* and *qFT3*, both on A2, *qFT10* on A7, *qFT13* on A9, and *qFT14* on A10, were major flowering time QTL detected in the vernalized and non-vernalized populations.

Siliqua traits A total of nine QTL were detected for siliqua length, two QTL on A2 (*qSQL1* in the R/W block and *qSQL2* in E block), three QTL on A3 (*qSQL3* in R block, *qSQL4* in J block, and *qSQL5* in T/U block), and one each on A7 (*qSQL6* in the E block), A8 (*qSQL7* in the B block), and A9 (*qSQL8* in B block), and A10 (*qSQL9* in the W block) (Fig. 1 and Supplementary Table S2). *qSQL3*, *qSQL5*, and *qSQL9* were detected in two consecutive years while *qSQL6* on A7 was detected in three consecutive years. The QTL on A3 (*qSQL5*), A7 (*qSQL6*), and A10 (*qSQL9*) were major loci, contributing to phenotypic variation ranging from 7 to 19% in different years.

Seven QTL, one each on LGs A1 (*qSQW1* in the F block), A2 (*qSQW2* in the K Block), A8 (*qSQW6* in the C block), and A9 (*qSQW7* in the N block) and three on A3 (*qSQW3* in the R, *qSQW4* in the J, and *qSQW5* in the T/U blocks, respectively), were detected for siliqua width. *qSQW1* on A1 and *qSQW3* and *qSQW5* on A3 were major loci that were detected in two consecutive years and explained 3–16% of the phenotypic variation.

For siliqua beak length, five QTL were detected, one each on A3 (*qSBL1* in the J block), A4 (*qSBL2* in the N Block), A5 (*qSBL3* in the C block), A7 (*qSBL4* in the E block), and A9 (*qSBL5* in H block). The QTL on A3 (*qSBL1*) and A5 (*qSBL3*) were comparatively major loci with high LOD values and were detected in two different years (2009 and 2010).

Seed traits The seed numbers per siliqua and seed weight are important traits for increasing yield (Fig. 1 and Supplementary Table S2). For the number of seeds per siliqua, one QTL each on A2 (*qSSQ1* in the R/W block) and A10 (*qSSQ4* in the W block) and two QTL on A3 (*qSSQ2* in the J block and *qSSQ3* in the T block) were detected. Only *qSSQ4* on A10 was detected in two years, although the other three QTL also showed significantly high LOD values. For seed weight, one QTL each was detected on A2 (*qSW1* in the R block), A3 (*qSW2* in the J block), A6 (*qSW3* in the X block), and A10 (*qSW4* in the R block). Of these, only *qSW1* and *qSW4* were detected in more than 1 yr (2009 and 2010). The phenotypic variation explained by individual QTL ranged from 6 to 17%.

3.3. Identification of potential candidate genes in the QTL blocks

As synteny between our *B. rapa* map and *A. thaliana* was established,²¹ we searched for orthologs of

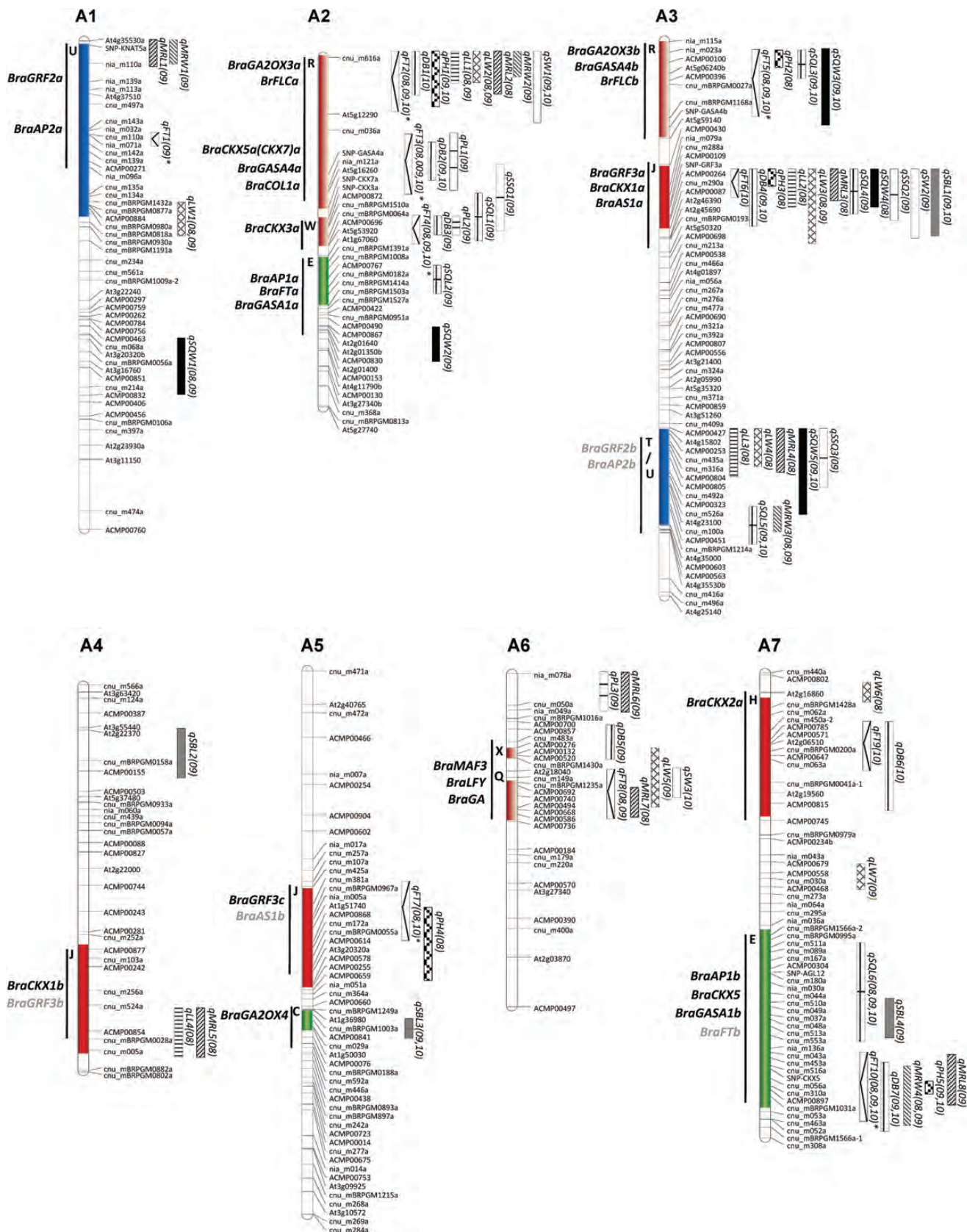


Figure 1. Distribution of QTL for morphological and yield component traits in *B. rapa* genome. QTL names are indicated by abbreviations of trait names as shown in Table 1. The numbers in parenthesis indicate the year of QTL detection. The crucifer building blocks in each LGs of *B. rapa*, which are homologous to five chromosomes of *A. thaliana* At C1–At C5, are indicated by different colours. Putative candidate genes identified within the QTL blocks are shown in bold black letters on the left of each LG, and those outside QTL intervals are shown in bold grey letters.

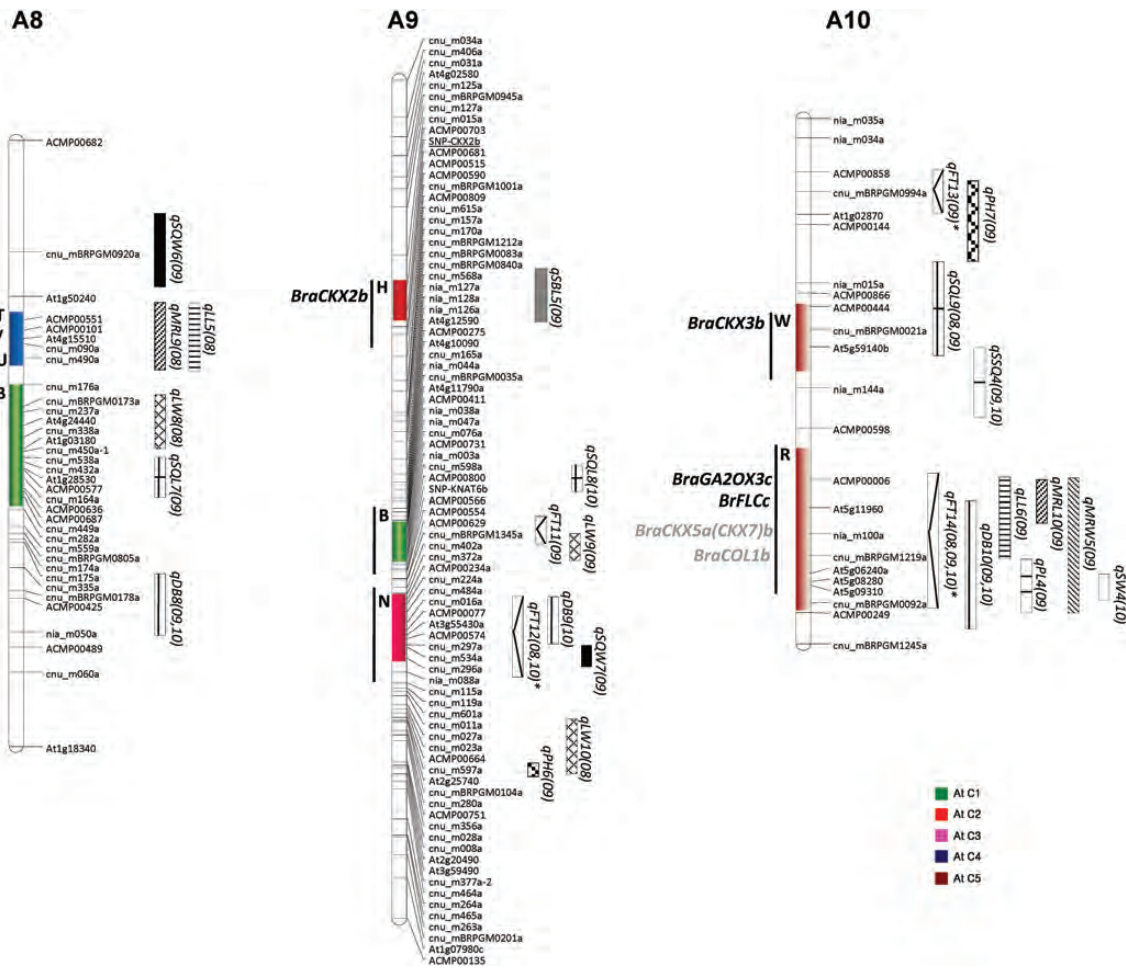


Figure 1. Continued

previously characterized *A. thaliana* genes in the *B. rapa* genome (Fig. 1 and Table 3). The most important candidate genes, identified in the R blocks of A2, A3, and A10 harbouring the major QTL clusters for flowering and bolting, leaf, and silique traits, were orthologous to genes in the gibberellic acid (GA) oxidase 3 (*AtGA2Ox3*),²⁸ cytokinin oxidase/dehydrogenase 5 (*AtCKX5*),^{29,30} flowering locus C (*FLC*),^{8,9} GA-stimulated *Arabidopsis* (*GASA4*),³¹ and constans-like (*COL*) gene families. The *Brassic* homologs *BraFLC* and *BraGA2Ox3* were identified in the R blocks of A2, A3, and A10. However, some paralogs were differentially present on *Brassic* genomes such as *BraCKX5* on A2 and A10, but not on A3, *BraGASA4* only on A2 and A3, *BraCOL1* on A2 and A10, etc. (Fig. 1). These suggest the preferential retention and fractionation of paralogous genes in duplicated segments of the *B. rapa* genome.

We detected two W blocks, one each on A2 and A10, that harboured QTL for petiole length, seeds per silique, leaf traits, days to bolting and flowering, and silique length (Fig. 1). These two blocks harbour

paralogous *BraCKX3* genes (Table 3). The E block on A2 only contained QTL for silique traits, but the paralogous block on A7 contained major QTL clusters for leaf traits, flowering and bolting, plant height, and silique traits. We detected *GASA1*, *flowering locus T* (*BraFT*), and *apetala 1* (*BraAP1*) on both LGs. *FT* and *AP* are components of the flowering gene network. *BraCKX5* was found only on A7 (Fig. 1).

The J block of A3 contained many QTL for leaf, silique, and seed traits, plant height, and days to bolting and flowering, whereas the same blocks in A4 harboured QTL for leaf length and midrib length, and A5 contained QTL for flowering time and plant height (Fig. 1). *A. thaliana* growth-regulating factor³² (*BraGRF3*) was found in all three LGs. However, *BraCKX1* paralogs were found only on A3 and A4, whereas asymmetric leaves 1³³ (*BraAS1*) paralogs were found only on A3 and A5 (Fig. 1).

The candidate gene *BraGA2Ox4*³⁴ was identified in the C block of A5, which contains QTL for silique beak length. The H block of A7 contained QTL for flowering, bolting, and leaf width, whereas the

Table 3. Putative candidate genes identified in the QTL blocks of *B. rapa* genome and SNPs observed between gene sequences of the parental lines, Chiifu and RCBr

Gene family	Gene name	At gene ID	<i>B. rapa</i> gene ID	Block (LGs)	QTL co-mapping	SNP location (number)
Gibberellin biosynthesis	AtGA2OX4	AT1G47990	Bra032238	C (A5)	<i>qSBL3</i>	Intron & exon (4)
	AtGA2OX3	AT5G07200	Bra028706	R (A2)	<i>qFT2, qDB1, qPH1, qLL1, qLW2, qMRL2, qMRW2, qSW1</i>	–
			Bra005927 Bra009285	R (A3) R (A10)	<i>qFT5, qSQW3</i> <i>qFT14, qDB10, qMRL10, qMRW4, qSW4, qLL5, qPL4</i>	Exon (1) –
Cytokinin oxidase/dehydrogenases	AtCKX5	AT1G75450	Bra015842	E (A7)	<i>qFT10, qDB7, qMRL8, qMRW3, qPH4</i>	Intron & exon (2)
	AtCKX2	AT2G19500	Bra036719	H (A9)	<i>qSBL5</i>	Intron & exon (3)
			Bra040677 Bra000229	H (A7) J (A3)	<i>qLW6, qDB5, qFT9</i> <i>qPH2, qLL2, qLW3, qMRL3, qDB4, qFT6, qSQL3, qSQW4, qSBL1, qSSQ2, qSW2</i>	Intron (1) –
	AtCKX1	AT2G41510	Bra016928	J (A4)	<i>qLW4, qMRL5</i>	Intron & exon (6)
			AtCKX5 (CKX7)	AT5G21482	Bra020157 Bra002371	R (A2) R (A10)
	AtCKX3	AT5G56970	Bra035640 Bra002777	W (A2) W (A10)	<i>qFT4, qDB3, qPL2, qSQL1, qSSQ1</i> <i>qSQL8, qSSQ4</i>	Intron (1) Intron & exon (2)
Gast1 protein homolog	GASA4	AT5G15230	Bra023513	R (A2)	<i>qFT3, qDB2, qPL1</i>	Intron & exon (3)
	GASA1	AT1G75750	Bra006291 Bra008222	R (A3) E (A2)	<i>qFT5, qSQW3</i> <i>qSQL2</i>	Intron (1) –
			Bra015820 Bra003743	E (A7) E (A7)	<i>qFT10, qDB7, qMRL8, qMRW3, qPH4</i> <i>qFT10, qDB7, qMRL8, qMRW3, qPH4</i>	Exon (1) Intron (2)
Growth-regulating factor	AtGRF2	AT4G37740	Bra011781 Bra017851	U (A1) U (A3)	<i>qMRL1, qMRW1</i> –	Exon (3) Exon (4)
	AtGRF3	AT2G36400	Bra023066	J (A3)	<i>qPH2, qLL2, qLW3, qMRL3, qDB4, qFT6, qSQL3, qSQW4, qSBL1, qSSQ2, qSW2</i>	Intron (1)
			Bra017240	J (A4)	–	Intron (4)
			Bra005268	J (A5)	<i>qFT7, qPH3</i>	Intron & exon (3)
Apetala1	AP1	AT1G69120	Bra004007 Bra038326	E (A7) E (A2)	<i>qSQL5, qSBL4</i> <i>qSQL2</i>	Intron (6) –
			Apetala2	AP2	AT4G36920	Bra011741 Bra017809
Asymmetric leaves 1	AS1	AT2G37630	Bra000011	J (A3)	<i>qPH2, qLL2, qLW3, qMRL3, qDB4, qFT6, qSQL3, qSQW4, qSBL1, qSSQ2, qSW2</i>	Exon (1)
			Bra005177	J (A5)	–	–
Constans like	COL1	AT5G15850	Bra023541 Bra008668	R (A2) R (A10)	<i>qFT3, qDB2, qPL1</i> –	Exon (2) Exon (7)
			Flowering locus T	FT	AT1G65480	Bra022475 Bra004117
Leafy	LFY	AT5G61850	Bra019619	X (A6)	<i>qDB5</i>	–
GA-insensitive dwarf 1C	GA	AT5G27320	Bra009970	Q (A6)	<i>qFT8, qMRL7, qLW5, qSW3</i>	Intron & exon (6)
Mads affecting flowering 3	MAF3	AT5G65060	Bra024350	X (A6)	<i>qDB4</i>	Intron & exon (4)
Flowering locus C	FLC	AT5G10140	Bra028599	R (A2)	<i>qFT2, qDB1, qPH1, qLL1, qLW2, qMRL2, qMRW2, qSW1</i>	Intron (1)
			Bra006051 Bra009055	R (A3) R (A10)	<i>qFT5, qSQW3</i> <i>qFT14, qDB10, qMRL10, qMRW4, qSW4, qLL5, qPL4</i>	– Intron (1)

paralogous block of A9 contained QTL for siliqua beak length. *BraCKX2* paralogs were found in the QTL regions of both LGs (Fig. 1). The U block of A1 contained QTL for flowering and leaf width, whereas the paralogous block on A3 (U/T block) contained QTL for leaf width, length, and seeds per siliqua. The candidate genes *BraGRF2* and *BraAP2* were detected in both LGs. The X block in A6 contained QTL for days to bolting and harboured candidate gene *LEAFY* and *mads affecting flowering time 3*. The Q block harboured QTL for seed weight, leaf, and flowering traits and contained the *GA-insensitive 1* gene (*BraGA*) that interacts with GA and is involved in floral transition and GA signalling.

3.4. Identification, validation, and functional significance analysis of SNPs in candidate genes

To develop gene-specific SNP markers for breeding morphological and yield-related traits, we aligned all the identified candidate gene sequences from the

parental lines and identified varying number of SNPs, ranging from one to many, between them (Fig. 2 and Table 3). As expected, we found more SNPs in the intronic regions than in the exonic regions. However, we did not detect any SNPs in a few genes, e.g. *BraGA20OX3* in the R blocks of A2 and A10 and *BraCKX1* in the J block of A03 (Fig. 2 and Table 3).

To validate these SNPs experimentally, we designed a total of 12 primer pairs, 1 each from different genes flanking the SNPs and genotyped the parental lines and mapping population. Of the 12 SNPs, 10 primer pairs revealed clear polymorphisms (Supplementary Table S3). We genotyped using these 10 primer pairs in the mapping population and mapped them precisely in the *B. rapa* genome, which indicated their co-localization with the QTL positions on the respective chromosomes (Figs 1 and 3). Association analysis with the different phenotypes of the F₃ population showed a correlation with the phenotypes and their co-localization in the QTL regions. We sequenced

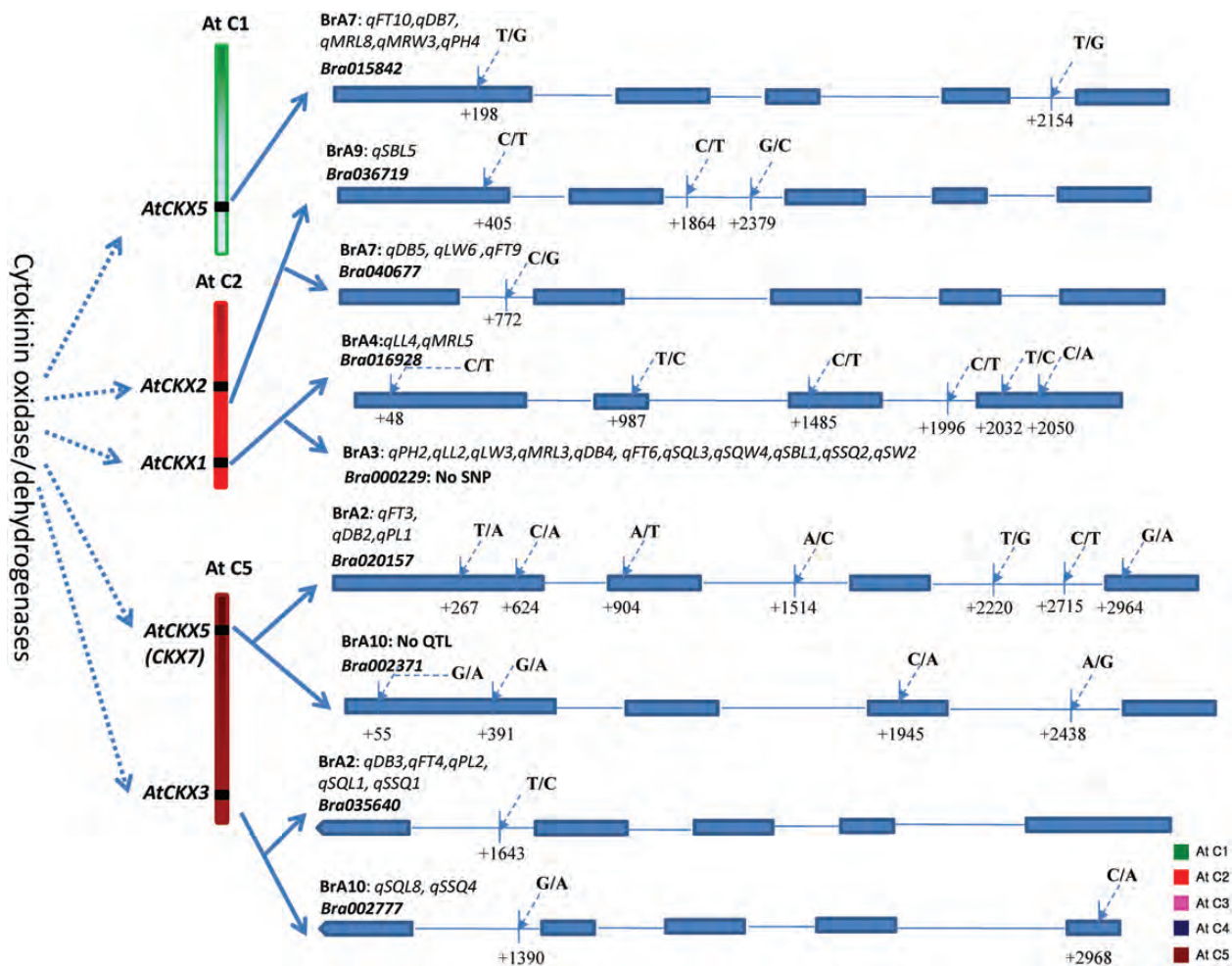


Figure 2. SNPs identified for different cytokinin oxidase/dehydrogenases (*CKX*) paralogs between Chiifu and RCB derived from whole genome resequenced data. Exons and introns are represented by blue rectangular bars and lines, respectively. Numbers indicated by arrows show the SNP positions from the start codon for Chiifu (left nucleotide) and RCB (right nucleotide).

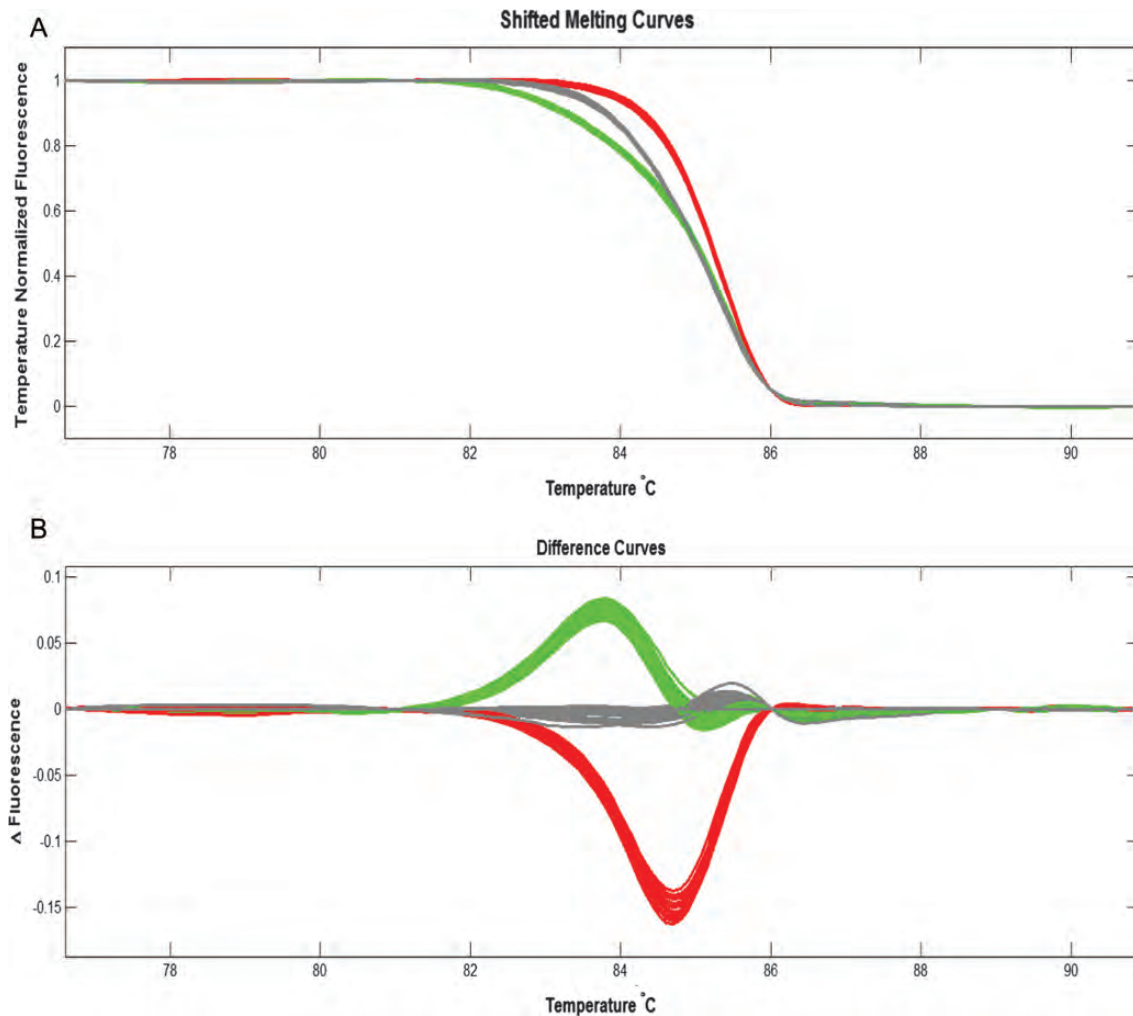


Figure 3. Amplicon scanning analysis of the CRF₂ mapping population for SNP markers designed from candidate gene *BraGASA4b* located in A3 using Light Scanner. Plot A represents the curve-shifted plot, and B represents a difference plot showing 'Chiifu' type in red colour, 'RCBr' type in grey colour, and hetero type in green colour.

some of the genes containing SNPs using the resequenced data to validate whether the NGS data of the parental lines were correct. Resequencing of 10 genes showed the exact sequences as shown by NGS. We observed that the SNPs in coding regions of some potential candidate genes affected amino acid sequence, suggesting possible functional roles in phenotypic differences (Supplementary Fig. S1). The SNP in exon 2 of *BraGA2Ox3b* codes for isoleucine in Chiifu and leucine in RCB, methionine/threonine in exon 2 of *BraCKX1b* while serine was found in Chiifu, but no counterpart amino acid was observed in RCB in exon 5 of *BraCKX3b*. Two amino acid changes were observed in exon 1 of *BraGA2Ox4* (arginine/serine and serine/threonine), one each in exons 2 (aspartate/valine), and four (alanine/threonine) of *BraCKX7a*, two each in exon 1 (valine/isoleucine and glycine/serine) of *BraCKX7b* and exon 4 (isoleucine/serine and threonine/alanine) of *BraGRF2a*, one each in exon 2 (leucine/arginine)

and exon 4 (glycine/serine) of *BraGRF3c*, and two in exon 1 (methionine /valine and threonine/serine) of *BraCOL1* gene.

Semi-quantitative RT-PCR analysis was used to evaluate expression differences of candidate genes between the parental lines. The gibberellin biosynthesis and oxidase, cytokinin oxidases/dehydrogenases and growth-regulating factor gene families that affect leaf, plant, and flower development, showed differential gene expression between Chiifu and RCB lines (Fig. 4). *BraCKX5*, *BraCKX3a*, *BraCKX1a*, and *BraCKX7a* showed higher expression in Chiifu, whereas very little or no expression was seen in RCB. Genes that are involved in flowering pathways, such as *FLC*, *FT*, *COL*, and *GA*, were also analysed for differential expression between the parental lines. Of the three *BraFLC* paralogs, only *BraFLCa* on A2 did not show any expression in RCB while the other two *BraFLCs* showed no difference in expression. *BraMAF3* on A6 showed higher expression level in

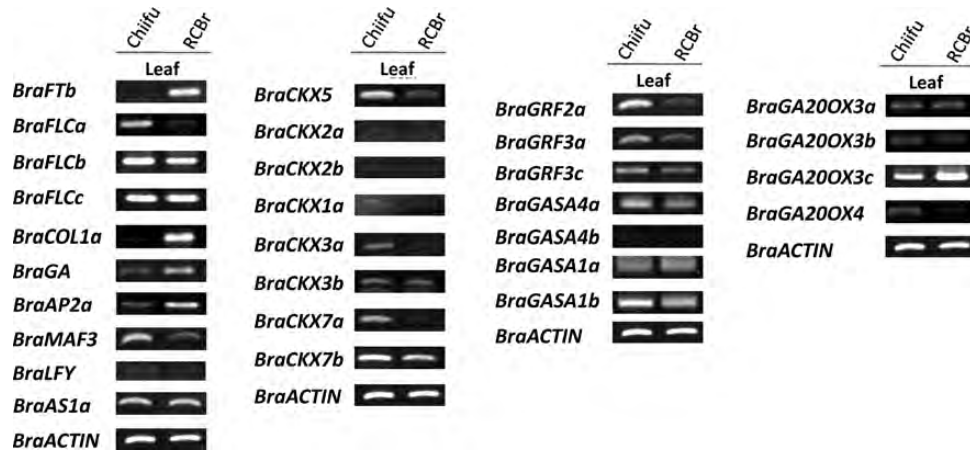


Figure 4. Semi-quantitative RT-PCR analysis of candidate genes using RNA samples extracted from 20-day-old seedlings of Chiifu and RCBr. Actin gene amplicons were used as control for RNA quantity.

Chiifu when compared with RCBr. *BraAP2a* on A1, *BraCOL1a* on A2, *BraGA* on A6 and *BraFTb* on A7 showed strong expression in RCBr, but very little to no expression in Chiifu. *BraGRF2a*, *BraGRF3a*, and *BraGA20OX4* showed comparatively higher expression in Chiifu while *BraGA20OX3c* showed higher expression in RCBr. This differential gene expression and the amino acid changes suggest possible roles in phenotypic differences between the parental lines and in the segregating population.

3.5. Functional conservation of crucifer building blocks between the A, B, and C subgenomes of *Brassica* species as revealed by comparative QTL mapping

We aligned the QTL map of *B. rapa* from this study with the *B. juncea* map of Ramchiary *et al.*²⁰ and *B. napus* QTL maps of Quijada *et al.*²¹ and Udall *et al.*²² to identify the structural and functional conservation of QTL blocks for important morphological and yield component traits. Comparative alignment of the QTL maps between these three species identified a total of four homologous/homoeologous groups (HGs) containing QTL for agronomic and yield component traits (Fig. 5). The first group was the R block of A^r2, Aⁱ2, Aⁿ2, Cⁿ2, and B^j2 (r, j, and n stand for *B. rapa*, *B. juncea*, and *B. napus*, respectively). Although many major QTL for different traits were detected in the *B. rapa* A subgenome, in comparison with *B. napus*, three traits, namely, plant height, flowering time, and seed yield, were commonly detected in the A subgenome of the two species and the homoeologous region of the same block in the C subgenome of *B. napus*. We did not detect any QTL in the Aⁱ2 or B^j2 subgenomes of *B. juncea* (Fig. 3). The second HG was the A3, B3, and C3 chromosomes of the A, B, and C subgenomes of *Brassica* species.

Three blocks, namely, R, J, and F, harboured common QTL between the three subgenomes. The R block of this HG contained common QTL for seed weight, yield, and test weight (hereafter referred to collectively as SW) and flowering time QTL between the A and C subgenomes of *B. napus* and the A subgenome of *B. rapa*. In addition, the homoeologous region of this block contained QTL for a number of branches of the B subgenome of *B. juncea*. The J block of this group contained many QTL in A^r3, but few in Aⁿ3, Aⁱ3, and B^j3. The common loci were for SW and flowering time in addition to siliqua traits, e.g. siliqua length, density, and number, and plant height. The last common block, i.e. the F block of this group, harboured common trait QTL mainly for SW and plant height between Aⁿ3 and Cⁿ3. The most important common block of HG7 was the E block. This block contained common QTL for plant height, SW, flowering time, siliqua number and density, and seed number per siliqua in A^r7, Aⁱ7, Cⁿ7, and B^j7. In addition, some leaf traits were detected in A^r7. The last common group of the A, B, and C *Brassica* subgenomes was HG10 that contained the W and R blocks. The W block contained common QTL for siliqua length, seed number per siliqua, and SW between A^r10, Aⁿ10, and Aⁱ10. In addition, QTL for flowering, plant height, and leaf traits were detected in A^r10 and Aⁿ10, respectively. However, in this block, no single or corresponding common QTL were detected in B^j10 and Cⁿ10. The most important block of HG10 was the R block. This block harboured common major QTL for plant height, branch number, flowering time, siliqua traits, and SW in A^r10, Aⁱ10, Aⁿ10, B^j10, and Cⁿ10, respectively. Similarly, the middle region of A^r6 (X and Q block) in the present study detected QTL for SW, leaf, and flowering traits while the same chromosomal region in *B. juncea*

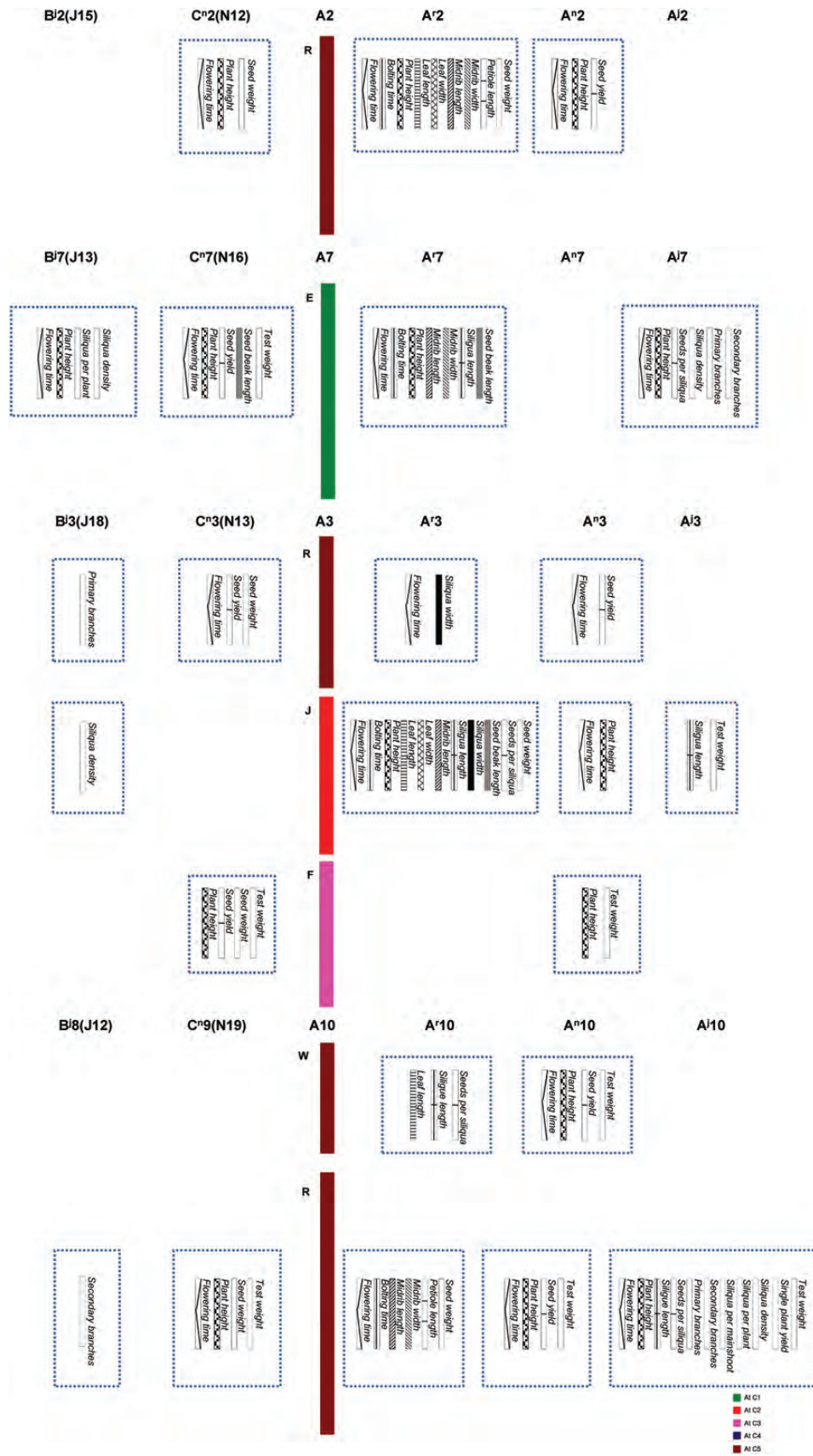


Figure 5. Comparative QTL mapping between *B. rapa*, *B. juncea*, and *B. napus* revealed conservation of crucifer building blocks governing morphological and yield component traits. Superscripts ‘r’, ‘n’, and ‘j’ after each A, B, and C LGs denote chromosomes that were derived from *B. rapa*, *B. napus*, and *B. juncea*, respectively. QTL in B genome of *B. juncea* were taken from Ramchiary et al.,²⁰ and the C genome of *B. napus* from Quijada et al.,²¹ and Udall et al.²²

detected major QTL for siliqua length and density.²⁰ We also detected common QTL for flowering and SW, both in A^r6 and Cⁿ6 subgenomes.²¹

4. Discussion

4.1. QTL mapping revealed some crucifer blocks that were functionally more active than duplicated paralogous blocks in the *B. rapa* genome

Although in many studies, immortal mapping populations such as doubled haploid and recombinant inbred lines are used, in our present study, we used F₃ families for three consecutive years to detect QTL, as F₃ families represent the comprehensive genetic information of heterozygous and homozygous plants and their interacting effects on phenotype. As the parental lines Chiifu and RCBr were very different with respect to all the traits under study, it was easy to detect many QTL in *B. rapa* using the segregating F₃ population. Correlation coefficient analysis revealed significant correlations between leaf and yield-related traits that were further supported by the co-mapping of several QTL governing different traits in a few chromosomal blocks of the *B. rapa* genome. The co-localization of QTL governing different traits in the same genetic interval has been suggested to be due to either pleiotropy exhibited by a single gene or tight linkage of genetic loci governing different traits in the same genetic interval.^{8,20} The R block of A2 contained QTL for eight traits while the same paralogous blocks on A10 and A3 contained QTL for five and four traits, respectively (Fig. 1). Prominent QTL clusters were observed for seven traits related to leaf, flowering, bolting, and siliqua traits on the E block of A7 while the same block on A2 contained only one QTL for siliqua length. A similar case was observed for the J blocks, where the same block on A3 harboured QTL for 10 traits, but only harboured QTL for 2 traits on A4 and A5. These findings suggest that the R block of A2, E block of A7, and J block of A3 contain more active genes that govern leaf traits, plant morphology, and seed traits. Lou *et al.*⁸ and Li *et al.*⁹ also observed QTL clusters in these LGs for days to flowering, bolting, and leaf traits. We presumed that the chromosomal blocks of Lou *et al.*⁸ were the R block on A2 and E block on A7. The W block on A2 contained QTL clusters for days to flowering and bolting and seeds per siliqua, whereas A10 contained QTL for siliqua and leaf traits. This kind of observation suggests that only those crucifer blocks harbouring QTL might possess functionally active genes for the traits under study, as reported by Wang *et al.*¹⁶ and Tang *et al.*³⁵ for the *B. rapa* genome. They reported that, of the triplicate duplicated subgenomes, fractionation

and deletion of genes were more frequent in some subgenomes when compared with other paralogs. We also observed that some candidate genes were missing in major QTL blocks, such as *BraGASA4* from A10 and *BraCKX5* from A3, which both belong to the R block that is homologous to *A. thaliana* chromosome 5. Other reasons for not finding equal QTL/genes in all paralogous blocks may be that the genes are not functionally active or the absence of polymorphisms in the causal genes between the parental lines.

4.2. Gene family members were detected in different QTL blocks of the *B. rapa* genome

Many candidate genes in QTL blocks for flowering, leaf, seed, and siliqua traits were identified through synteny analysis of the *B. rapa* and *A. thaliana* genomes. The R block, homologous to *A. thaliana* chromosome 5, harbours many flowering genes, including *FLC* and *COL1*. We identified *BraFLC* and *BraCOL1* paralogs in those blocks containing QTL for flowering time, as reported by earlier studies.^{8,36} Recent studies have shown that members of cytokinin oxidase/dehydrogenases (CKX), GA-stimulated *Arabidopsis* (*GASA*), and GA oxidase (*GA2OX*, *GA2OXS*, etc.) gene families are involved in the regulation of flower and inflorescence development, thereby increasing the seed size and yield.^{28–31} CKX genes are important for agricultural productivity as mutations in these genes cause accumulation of high levels of cytokinin in the inflorescence meristem and increased reproductive organs and larger flowers and seeds.^{29,30} These genes co-localized in the QTL blocks of *B. rapa* LGs A2, A3, A4, A7, A9, and A10. *GASA* genes are active in the shoot apical meristem, developing flowers, and embryos. *GASA* mutants and *GASA* over-expressing plants demonstrated that *GASA* genes are involved in floral meristem identity and affect seed size and yield in *A. thaliana*.²⁹ We observed two paralogs of *BraGASA4*, one on each R block of A2 and A3, but not on the R block of A10, suggesting that the paralogs on A10 may have been lost over time, although all blocks harboured QTL for flowering, siliqua, and seed traits. GA oxidases were observed in the QTL regions of the R, J, and other blocks containing prominent QTL for leaf and flowering traits, and a possible involvement of this gene family in leaf traits was reported by Li *et al.*⁹ The *BraGRF* members found on A1 and A5 are also candidates for leaf and flowering traits, as supported by their co-localization with these QTL regions. Several members of the *BraCOL* and *BraFT* families were also found in different LGs in the QTL regions, suggesting an involvement in trait variation.

SNPs analysis revealed some amino acid changes between the parental lines in many genes belonging to conserved families like 1(*COL1*), cytokinin oxidases/dehydrogenases, growth-regulating factor, and GA oxidases gene families. Further expression analysis showed higher expression of genes that facilitate early flowering such as *BraCOL1a*, *BraFT*, *BraAP2* in RCB, but minimal expression in Chiifu, whereas the flowering repressor gene *BraFLCa* was highly expressed in Chiifu suggesting an association between flowering trait and expression of these genes. The co-mapping of these genes with flowering QTL in different LGs further supported this notion. Members of growth-regulating factor, GA oxidase, and cytokinin oxidase/dehydrogenases gene families that co-localized within the QTL region showed higher expression in Chiifu, suggesting their role in better growth and development of leaf, plants, and reproductive organs when compared with RCB. Either non-synonymous SNPs or differential genes expression or a combination of these factors may underlie phenotypic differences between the parental lines and in the segregating populations. However, this should be further assessed in different plant tissues.

4.3. QTL blocks are evolutionarily conserved between the A, B, and C subgenomes of *Brassica* species

Our comparative QTL map alignment between *B. rapa*, *B. napus*, and *B. juncea* revealed conservation of QTL blocks that contain several genetic loci influencing plant height, flowering, silique, and seed traits between the A, B, and C subgenomes of *Brassica* species (Fig. 5). Although many earlier studies reported the conservation of chromosomal blocks at the gross level,^{10,11,14} a comparison of QTL location has not been performed for yield component traits between different *Brassica* subgenomes. In our present study, we identified conserved QTL blocks in 4 of the 10 HGs of the A, B, and C subgenomes reported by Panjabi et al.¹¹ As plant height, flowering time, silique, and seed traits were the only common traits studied in the three species, we could study the conservation of these traits in the four HG QTL blocks. The R blocks of HG2, HG3, and HG10, E block of HG7, W block of HG10, and J and F blocks of HG2 were major QTL blocks harbouring common QTL for SW, plant height, flowering, and silique traits between the A, B, and C subgenomes of these three species. This suggests that the genes governing these traits are structurally and functionally conserved not only between the A, B, and C subgenomes of *Brassica* species but also between *A. thaliana* and *Brassica* genomes, even though they diverged from their common ancestor a long time ago.¹² We previously found the conservation of QTL for seed oil,

total seed glucosinolates, and seed protein content between the A subgenomes of *B. rapa* and *B. napus*.⁵ Studies on *B. napus*^{21,22} and *B. juncea*²⁰ detected QTL in homoeologous LGs of the A and C and A and B subgenomes, respectively, but detailed information with respect to specific crucifer blocks was not provided. We found more common QTL between the A and C subgenomes when compared with the B subgenome with either the A or C subgenomes. Although we believe that more comparisons using QTL maps from different mapping populations are needed, the present study suggests that A and C (diverged 8 MYA) are more close than the A/B and C subgenomes, as reported earlier.¹² We also observed many QTL in one species that had no counterparts controlling the same traits in the corresponding locations of other species, which might have been the result of a lack of genetic polymorphisms between the parental lines or diversified molecular polymorphisms between the species due to the presence of genomic rearrangements/structural changes causing differences in functional expression between different *Brassica* species. We believe that adding more markers to generate a high-density map and a detailed comparison of QTL for common traits using different mapping populations of more *Brassica* species would give more detailed information about the conservation and diversification of genetic loci governing morphological and yield component traits.

4.4. Breeding opportunities in *Brassica* species using candidate gene information from *B. rapa*

The present QTL mapping study identified some interesting findings regarding the presence of candidate genes for important traits in a few specific crucifer building blocks of the *B. rapa* genome (Fig. 1). Although, the crucifer blocks demonstrated the co-localization of important QTL and candidate genes, association analysis using large segregating population with different trait phenotypes and functional validation of candidate genes by transformation should be done because each QTL block contains several genes governing overlapping traits. We observed few gene paralogs within the QTL interval in one duplicated block but not in others, suggesting the need for further validation. However, we believe that some of the SNPs identified in candidate genes such as *BraCKXs*, *BraGASA4*, *BraGA2OxS*, *BraGRFs*, *BraFLC*, *BraCOLs*, *BraMAF*, and *BraAP2* would be helpful in breeding leaf, flowering, bolting, silique, and seed traits of *B. rapa*, as their differential expressions in parental lines and previous studies suggest their role in creating phenotypic differences.⁹ However, additional expression studies using different tissues are needed. Although genome sequencing of other

cultivated *Brassica* species is ongoing, gene sequence information of *B. rapa* could be used to isolate and develop molecular markers for breeding other *Brassica* species, especially *B. juncea* and *B. napus*, as *B. rapa* is one of the progenitor species of both species and there still exists QTL block conservation for important agronomic and yield component traits between these three species.

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Supplementary data: Supplementary Data are available at www.dnaresearch.oxfordjournals.org.

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