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Research Paper

Identification and validation of major and minor QTLs controlling seed coat color in *Brassica rapa* L.

Yinghuan Zhang[†1)], Yunxia Sun[†1)], Junpeng Sun[2)], Hui Feng[1)] and Yugang Wang*[1)]

[1)] *College of Horticulture, Shenyang Agricultural University*, Shenyang 110866, China
[2)] *Liaoing Dongya Seed Limited Company*, Shenyang 110164, China

Seed coat color is an important agronomic trait in *Brassica rapa*. Yellow seeds are a desirable trait for breeding oilseed *Brassica* crops. To identify quantitative trait loci (QTLs) that condition seed coat color in *B. rapa*, we used a population of recombinant inbred lines (RILs) derived from crossing 09A001, a standard rapid-cycling (RcBr) inbred line of *B. rapa* L. ssp. *dichotoma* with yellow seeds, with 08A061, an inbred line of heading Chinese cabbage with dark brown seeds. Using two phenotypic scoring methods, we detected a total of nine QTLs distributed on four chromosomes (Chrs.), A03, A06, A08, and A09, that explained 3.17 to 55.73% of the phenotypic variation for seed color. To validate the effects of the identified QTLs in the RIL population, chromosome segment substitution lines (CSSLs) harboring the chromosomal segment carrying the candidate QTL region from 08A061 were selected, and two co-localized major QTLs, *qSC9.1* and *qSCb9.1*, and one minor QTL, *qSC3.1*, were successfully validated. The validated QTL located on Chr. A03 appears to be a new locus underlying seed coat color in *B. rapa*. These findings provide additional insight that will help explain the complex genetic mechanisms underlying the seed coat color trait in *B. rapa*.

Key Words: *Brassica rapa*, seed coat color, QTL, recombinant inbred line, chromosome segment substitution lines.

Introduction

Brassica rapa is one of major oilseed crops, along with turnip rape and yellow sarson, that is distributed worldwide with a large ecological range due to its early maturity and cold tolerance (Xiao *et al.* 2012). Yellow seed coats are desirable in any oilseed *Brassica* species because it has been reported that yellow-seeded varieties have advantages in both oil and meal quality over their brown- or black-seeded counterparts (Rahman *et al.* 2014). *B. rapa* is also one of the parent species of *Brassica napus*, the most important oilseed crop in the world that is superior to other *Brassica* oilseed crops with respect to yield and seed quality (Zou *et al.* 2010). The seeds of *B. napus* are black, and no naturally occurring yellow-seeded mutants have been found in this species. Thus, yellow-seeded *B. napus* can be developed through the interspecific transfer of genes for yellow seed coats from related species, such as *B. rapa*, in which yellow-seeded forms do exist naturally (Rahman 2001). Thus, the development of *Brassica* species with a yellow seed coat is an important breeding objective.

Numerous reports have been published over the last four decades describing the inheritance of seed coat color in *B. rapa*. Different genetic models involving one gene, two genes, or multiple genes have been proposed for the inheritance of seed coat color in *B. rapa*. The traditional genetic model for seed coat color in *B. rapa* mostly favors two loci controlling this trait, where yellow seed color is due to a homozygous recessive condition in both loci, and epistatic effects, pollen effects, and a maternal effect are also observed under this genetic model (Rahman *et al.* 2007, 2014, Schwetka 1982, Stringam 1980, Zaman 1989). However, several studies have reported that a single recessive gene is responsible for the yellow-seeded trait in *B. rapa* (Hawk 1982, Li *et al.* 2012, Ren *et al.* 2017b, Xiao *et al.* 2012, Zhang *et al.* 2009) and some of the genes have been successfully cloned (Li *et al.* 2012, Zhang *et al.* 2009). Genetic models in which the yellow-seeded trait is controlled by multiple loci (QTL) have also been reported (Kebede *et al.* 2012, Lou *et al.* 2007, Rahman *et al.* 2014, Wang *et al.* 2013), and all of these studies found that only one or two major QTLs (PVE > 10%) (Collard *et al.* 2005) play important roles in controling this trait. In general, the seed coat color trait is mainly controlled by few genes or major QTLs in

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*Corresponding author (e-mail: lnrc7864@163.com)
[†] These authors contributed equally to this work

B. rapa and brown seed coat color is dominant to yellow seed coats. In recent years, several important genes underlying seed coat color, such as *BrTTG1* (Ren *et al.* 2017a, Zhang *et al.* 2009) on chromosome (Chr.) A06, and *BrTT8* (Li *et al.* 2012) and *BrTT1* (Wang *et al.* 2017), both on Chr. A09, have been cloned and analyzed functionally in *B. rapa*. On Chr. A09, a major quantitative trait locus (QTL) controlling seed coat color has also been detected in several studies (Kebede *et al.* 2012, Lou *et al.* 2007, Rahman *et al.* 2014). In addition to the few important genes or major QTL that have been detected on Chr. A06 and Chr. A09, major or minor QTLs (PVE < 10%) (Collard *et al.* 2005) have also been detected on Chrs. A08, A03, A05, and A06 in *B. rapa* (Kebede *et al.* 2012, Rahman *et al.* 2014), depending on the different segregating populations used in the mapping studies.

Near-isogenic lines (NILs) have proven to be an effective resource for QTL validation, and the development of NIL populations is considered to be a logical starting point for the creation of fine-mapping populations (Fletcher *et al.* 2013), which have been widely used in diverse crop plants such as tomato, pea, maize, wheat, and rice (Brauner *et al.* 2017, Dao *et al.* 2017, Kinkade and Foolad 2013, Lavaud *et al.* 2015, Miyahara *et al.* 2017, Zhao *et al.* 2017) for verifying the QTLs identified in the primary populations. In this study, we performed QTL analyses for seed coat color in a recombinant inbred line (RIL) population derived from a cross between a standard rapid-cycling (RcBr) *B. rapa* line with yellow seeds, and an inbred line of Chinese cabbage with dark brown seeds. In our previous study, a set of chromosome segment substitution lines (CSSLs) were developed using the yellow-seeded RcBr as the recurrent parent and the dark brown-seeded Chinese cabbage inbred line as the donor parent (Wang *et al.* 2018). Each CSSL was nearly isogenic compared with the recurrent parent, RcBr. We then validated two co-localized major QTLs and one minor QTL identified in the RILs by selecting CSSLs that harbor the candidate genomic region carrying the QTLs of interest.

Materials and Methods

Plant materials

The segregating population consisting of 124 RILs developed in our previous study (Liu *et al.* 2016) was used to identify QTLs controlling seed coat color in this study. The experiment were performed at the Experiment Station of Shenyang Agricultural University, Shenyang, China (41.8°N, 123.4°E) in the spring of 2015 (from Feburary to June). The RIL ($F_{2:6}$) population was derived by a single-seed descentfrom a cross between two parental lines: 08A061, the male parent, is an inbred line of heading Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) with dark brown seed coat color (**Fig. 1A**), and 09A001, the maternal parent, is a RcBr inbred line of *B. rapa* L. ssp. *dichotoma* with yellow seed coat color (**Fig. 1B**). The two parental inbred lines used in the present study, 09A001 and 08A061, have uniform yellow and dark brown seeds, respectively (Wang *et al.* 2013).

Evaluation of seed coat color and QTL detection in the RILs

The seeds of the two parental lines and the 124 RILs were used to evaluate seed coat color. The seeds from two replicated plots of each line grown in the E4 environment, a glass greenhouse plus open field, natural vernalization and day length (Liu *et al.* 2016), were mixed together to determine seed coat color by visual observation and also with a chroma meter (model CR-400; Konica Minolta, Japan). The seed coat colors in the segregating populations were divided into five classes by visual observation that are similar to those of Lou *et al.* (2007); Level 1: yellow; 2 yellow brown; 3: light brown; 4: brown; 5: dark brown (**Fig. 1C**). The

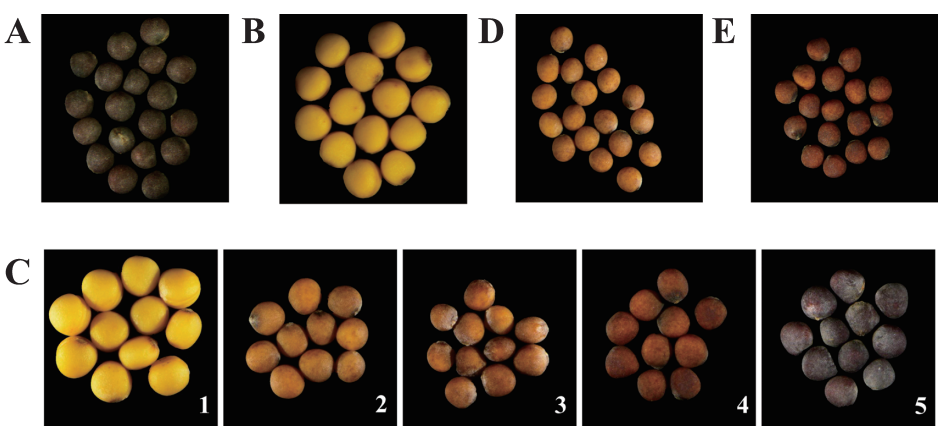

Fig. 1. Seed coat color in the two parental inbred lines, two selected chromosome segment substitution lines (CSSLs), and seed coat color segregation in the recombinant inbred lines (RILs). A: parental line 08A061 (dark brown). B: parental line 09A001 (yellow). C: Seed coat color segregation in the RILs. Level 1: yellow; level 2: yellow brown; level 3: light brown; level 4: brown; level 5: dark brown. D: seed coat color (light brown, level 3) in CSSL-38 harboring the *qSC3.1* candidate region. E: seed coat color (brown, level 4) in CSSL-111 harboring the candidate region for two major co-localized QTLs, *qSC9.1 and qSCb9.1*.

chroma meter measures color by decomposing it into the L*, a*, and b* color space values, where "L*" represents the brightness (0 for black, 100 for white), "a*" represents the color red when positive and the color green when negative, and "b*" represents the color yellow when positive and the color blue when negative (Wang *et al.* 2016). In our previous study, the two parental lines were shown to have significant differences in the seed coat color trait by both visual observation and chroma meter measurement, except for the "a*" color space (Wang *et al.* 2013), which was excluded from the subsequent phenotypic determination and QTL analysis in the RILs.

We previously constructed a molecular genetic linkage map for *B. rapa* based on 88 SSR and 31 InDel marker loci that were evenly distributed on the ten Chrs. in the RIL population (Liu *et al.* 2016). QTL analysis was performed using composite interval mapping with Windows QTL Cartographer v2.5 (Wang *et al.* 2012). The empirical significance threshold was determined by the 1,000 permutation test with a walk speed of 2 cM and a significance level of 0.05 based on our previous studies (Liu *et al.* 2016). The final QTL results were graphically displayed using MapChart 2.1 (Voorrips 2002). QTL nomenclature follows the scheme suggested by McCouch and CGSNL (2008). We consider QTLs to co-localize when their 2-LOD support intervals overlap (Lou *et al.* 2011). Statistical analysis was performed using SPSS 17.0 software for Windows.

Validation of the QTLs identified in the RIL population

In our previous study, we developed a set of CSSLs (Wang *et al.* 2018) using the yellow-seeded RcBr, 09A001, as the recipient (or recurrent) parent and the dark brown-seeded Chinese cabbage variety, 08A061, as the donor parent; these are the same parental lines used in the present study. In order to improve the possibility of identifying donor segments in the 09A001 genetic background, we added 47 SSR and InDel markers (for a total of 166 markers) in the process of developing the CSSLs. To validate the effects of the identified QTLs in the RILs, we used GGT software (van Berloo 1999; http://www.dpw.wau.nl/pv/pub/ggt/) to select CSSLs harboring the Chinese cabbage (08A061) chromosomal segment carrying the candidate QTL region identified in the RILs, but with the highest background recovery of the recurrent parent (09A001). Compared with the recurrent parent, the CSSLs which showed significant differences for seed coat color were further selected for phenotypic validation of the allele effect of the QTLs identified in the RILs.

Results

Phenotypic variation

Seeds of the F_1 plants have dark brown seed coats, similar to the male parent, 08A061, which shows that brown seed coat color is dominant over yellow seed coat color (Wang *et al.* 2013). Therefore, in the RILs, the seed coat color as determined by visual observation indicated that the majority of lines had dark brown seeds, similar to the male parental line 08A061, followed by plants with brown seeds. A smaller number of lines (~20%) had yellow seeds, similar to the female parent, 09A001, and only a very few RILs had seeds with either yellow brown or light brown seed coats. In addition, seed coat color in the RILs measured with the chroma meter showed a similar distribution to the visual scoring; the majority of the RILs had seed colors similar to the male parental line 08A061, and a few lines had seeds similar in color to the female parental line 09A001 (**Fig. 2**).

Detecting QTLs for seed coat color in the RIL population

QTL analysis indicated that a total of nine QTLs distributed on Chrs. A03, A06, A08, and A09 explained 3.17 to 55.73% of the phenotypic variation for seed coat color in the RIL population identified by visual observation and chroma meter measurement (**Table 1**, **Fig. 3**). There were four QTLs identified in the RILs that condition seed coat color determined by visual observation. Of these four

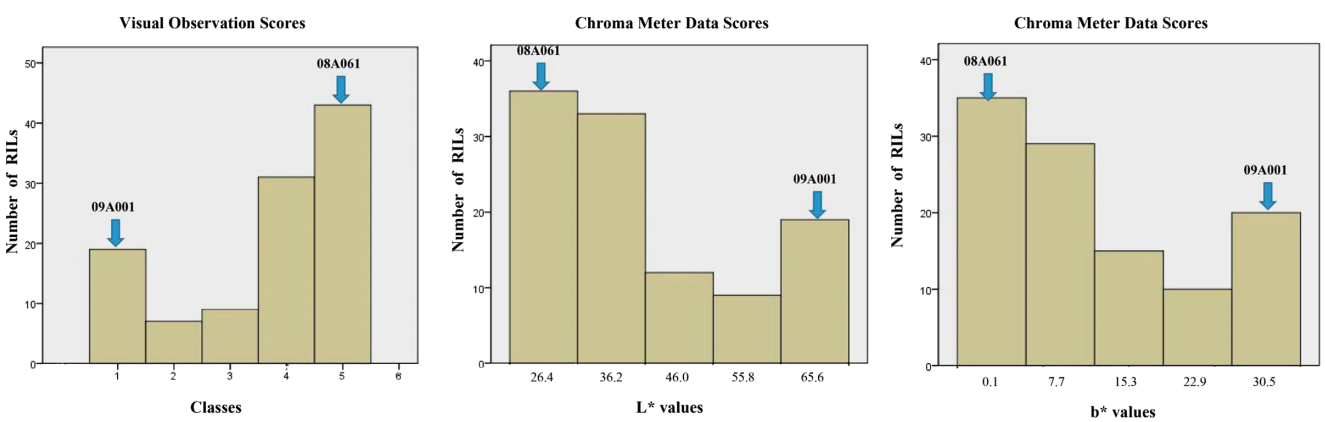

Fig. 2. Frequency distribution of seed coat color segregation in the recombinant inbred lines (RILs) scored by visual observation and the chroma meter measurement data for the two individual parameters L* and b*. The seed coat color scores for the two parental inbred lines 09A001 (yellow) and 08A061 (dark brown) are indicated by arrows.

QTLs, only one major QTL, *qSC*9.1, was detected on Chr. A09 (peak position at 57.3 cM), with a LOD score of 16.6, and accounted for 55.73% of the total phenotypic variance. A minor QTL, *qSC*3.1, with a LOD score of 2.1, was detected at position 41.6 cM on Chr. A03, and accounted for 4.28% of the phenotypic variance. Two minor QTLs, *qSC*6.1 and *qSC*6.2, which were detected in adjacent regions on Chr. A06, accounted for 7.45 and 5.21% of the phenotypic variation, respectively. Five QTLs that control seed coat color were identified using the chroma meter phenotypic scores. Among them, only one major QTL, *qSCb9.1*, was detected on Chr. A09 (peak position at 54.9 cM), with a LOD score of 15.1, and it explained 12.46% of the total phenotypic variance. In addition, four minor QTLs, *qSCb6.1*, *qSCb8.1*, *qSCb8.2*, and *qSCL3.1* detected on Chrs. A03, A06, and A08 explained 6.14, 6.71, and 6.15% of the b* phenotypic variation, and 3.17% of the L* phenotypic variation, respectively. Interestingly, phenotypic variations explained by the QTLs that were detected by visual observation were larger than those detected by the chroma meter measurement, no matter whether they were major or minor QTLs. Except for the three QTLs *qSC6.1*, *qSC6.2*, and *qSCb8.2*, the positive alleles of the other additive QTLs from 08A061 contributed to the formation of dark brown or brown seed coat color in *B. rapa*.

Validation of QTLs identified on chromosomes A03 and A09

The seeds produced by one of the CSSLs, CSSL-38, which harbored the *qSC3.1* candidate region, had light

Table 1. QTLs identified for seed coat color in the recombinant inbred lines

QTL	Chr.	Peak position (cM)	Marker interval	LOD[a]	AE[b]	PVE (%)[c]
qSC3.1	A3	41.6	Cnu-m098a–Cnu-m416a	2.10	0.31	4.28
qSC6.1	A6	34.2	Nia-m133a–BOE600	3.21	–0.42	7.45
qSC6.2	A6	47.5	Nia-m134a–Cnu-m149a	2.53	–0.34	5.21
qSC9.1	A9	57.3	BrID10667–BrID90137	16.6	1.24	55.73
qSCb6.1	A6	107.8	Cnu-m400a–Nia-m041a	4.68	0.65	6.14
qSCb8.1	A8	12.3	Cnu-m090a–BrID10315	3.08	2.21	6.71
qSCb8.2	A8	27.4	Cnu-m490a–Cnu-m432a	3.09	–1.01	6.15
qSCb9.1	A9	54.9	BrID10669–BrID90137	15.14	0.74	12.46
qSCL3.1	A3	20.7	Cnu-m288a–Cnu-m384a	2.65	3.73	3.17

[a] LOD: logarithm of the odds.
[b] AE: additive effect: positive values indicate that the positive allele is derived from the parental line with the higher value, while negative values indicate that the positive allele is derived from the parental line with the lower value.
[c] PVE: percentage of the total phenotypic variation explained by each QTL.

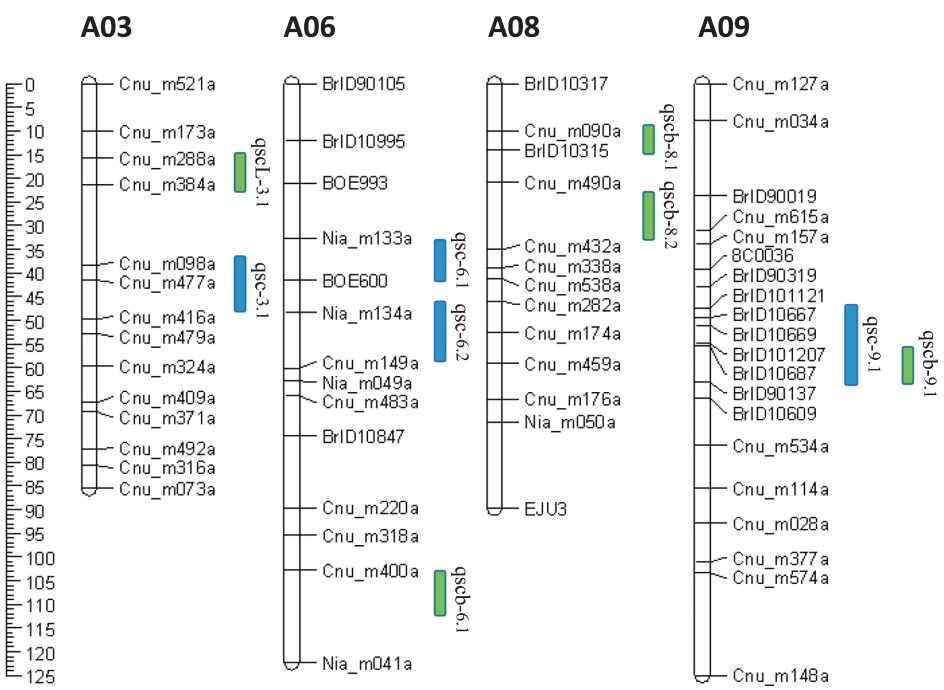

Fig. 3. Map positions on chromosomes A03, A06, A08, and A09 for the nine seed coat color QTLs identified in the recombinant inbred line (RIL) population in this study. The four blue and five green colored regions to the right of the linkage groups represent the positions of the mapped QTLs determined by visual scoring and the chroma meter data, respectively.

brown (level 3) seed coats (**Fig. 1D**), and also were significantly different from the two parents, 09A001 and 08A061, by color space values for L* (~43) and b*(~17) (**Table 2**). The CSSL-38 line contains three chromosomal segments derived from the dark brown-seeded parent (08A061), and all three segments were found to be homozygous. The estimated length of the substituted chromosomal regions was 22.75 cM, and 97.87% (22.75/1068.56) of the CSSL-38 genome originated from 09A001 (**Fig. 4A**). Another CSSL, CSSL-111, that harbors the candidate region for the two co-localized QTLs, *qSC9.1* and *qSCb9.1* had brown (Level 4) seed coats, and the color space L* (~35) and b* (~8) values were also significantly different from those of the two parental lines (**Table 2**, **Fig. 1E**). The genome of CSSL-111 contains several heterozygous chromosomal segments, including the candidate QTL region, and consists of 94.69% of the recurrent parent, 09A001, genome (**Fig. 4B**). We also identified a CSSL that harbors the candidate QTL region and is homozygous for the chromosomal segments, but have

Table 2. Phenotypic analysis of seed coat color in the two parental lines and two CSSLs

Year	Line	Mean ± SD		
		VO	L*	b*
2016 Spring	09A001	1 ± 0	61.73 ± 1.04 A	31.13 ± 0.75 A
	08A061	5 ± 0	26.24 ± 1.61 D	5.13 ± 0.86 D
	CSSL-38	3 ± 0	43.32 ± 1.06 B	15.34 ± 0.68 B
	CSSL-111	4 ± 0	35.02 ± 0.94 C	7.19 ± 0.65 C
2017 Spring	09A001	1 ± 0	59.01 ± 1.34 A	33.84 ± 0.94 A
	08A061	5 ± 0	25.89 ± 0.91 D	6.13 ± 0.87 D
	CSSL-38	3 ± 0	44.15 ± 0.81 B	19.12 ± 1.05 B
	CSSL-111	4 ± 0	35.96 ± 1.07 C	9.01 ± 1.74 C

SD: standard deviation.
VO: visual observation.
L* and b* represent the parameters in the chroma meter CR-400.
Values with different letters in the same row indicate that the phenotypic values are significantly different in the same environment according to Fisher's protected LSD test.

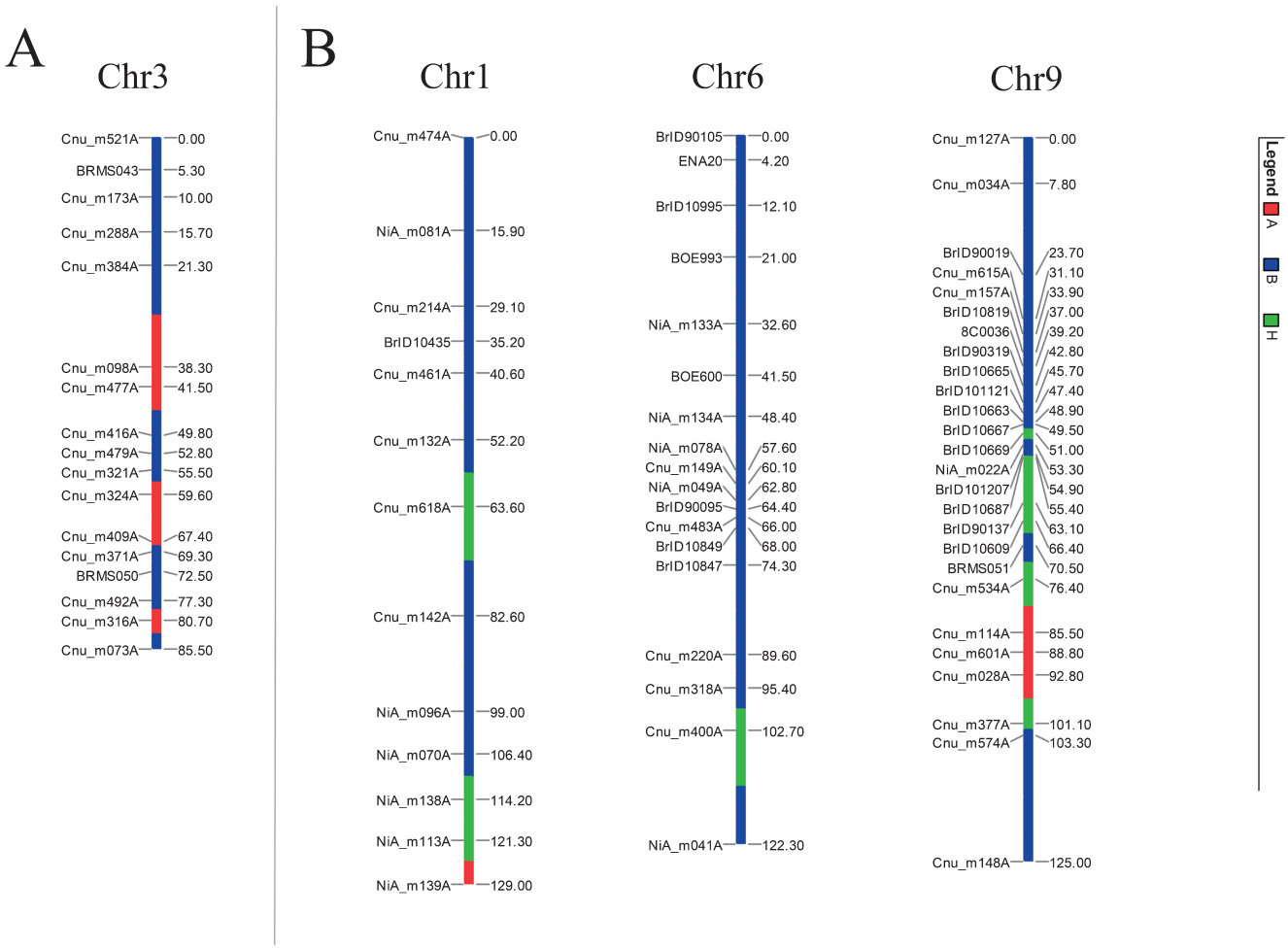

Fig. 4. Graphical genotypes of selected chromosomes harboring 08A061 genome segments in the two chromosome segment substitution lines, CSSL-38 and CSSL-111. Dark blue regions represent homozygous segments from the recurrent parent, RcBr 09A001; red regions indicate homozygous segments from the donor parent, Chinese cabbage inbred line, 08A061; green regions indicate segments that are heterozygous. A: Graphical genotype of CSSL-38 harboring the *qSC3.1* candidate region. B: Graphical genotypes of CSSL-111 that harbors the candidate genomic region for the two co-localized QTLs, *qSC9.1* and *qSCb9.1*.

lower background recovery (87.5%) of the 09A001 genome (see **Supplemental Fig. 1**), and shows the same seed coat color as CSSL-111; this indicates that brown seed coat color is dominant over yellow in *B. rapa*. The seeds of those CSSLs that harbor the other candidate QTL region had coats similar in color (yellow) to the recurrent parent, 09A001, which suggests that QTLs identified in the RIL population may be false positives or are dependent on genetic background.

The two selected CSSLs, CSSL-38 and CSSL-111, have light brown and brown seed coats, respectively, across multiple environments, and both of the CSSLs have been shown to have significant differences in the seed coat color trait compared to the two parental inbred lines 09A001 and 08A061 (**Table 2**). Over all, two co-localized major QTLs, *qSC9.1* and *qSCb9.1*, and one minor QTL, *qSC3.1*, identified in the RILs were successfully validated by selecting the corresponding NILs in the CSSLs.

Discussion

Yellow seed coat color is one of the most valuable traits related to oil seed production in *Brassica* species. In our previous studies, we investigated the inheritance of seed coat color in *B. rapa* in F_1, F_2, and BC_1 populations derived from crosses between 08A061 and 09A001, the same parental inbred lines used in this study. The earlier results showed that seed coat color in *B. rapa* is determined mainly by two major genes, and significant interaction (epistasis) between the two major genes was also detected (Wang *et al.* 2013). This conclusion is supported by the work of Rahman *et al.* (2007, 2014), Schwetka (1982), Stringam (1980) and Zaman (1989). QTL analysis in this study was dependent on the RIL population, and indicated that yellow seed coat color is determined by one major gene (QTL) plus multiple minor genes. These conclusions are similar to those of Kebede *et al.* (2012) and Lou *et al.* (2007), but are inconsistent with the above-mentioned digenic inheritance model. We conclude that the differences are due to the fact that multiple populations derived from the same parents were tested in different environments, indicating that the inheritance of seed coat color in *B. rapa* is complex.

Accurate phenotypic evaluation is of vital importance in QTL analysis, especially for those traits that appear to be more complex due to the involvement of major and minor QTLs as well as epistatic interactions. Seed coat colors in the RIL population used in this study ranged from yellow to dark brown, and were visually assigned to five classes (yellow, yellow brown, light brown, brown, and dark brown). Some difficulty was experienced in distinguishing the individual color classes, particularly between the yellow brown and light brown seed coat colors; such confusion has also been experienced by other researchers (Kebede *et al.* 2012). Different classification schemes or methods for measuring seed coat color (such as near-infrared spectroscopy, a color optical scanner, and the chroma meter have also been reported (Kebede *et al.* 2012, Rahman *et al.* 2014, Wang *et al.* 2016, Yu *et al.* 2016, Zhang *et al.* 2011). In the present study, two phenotype scoring methods, visual observation and chroma meter measurements, were used to evaluate seed coat color in the RILs, which improved mapping power to identify the potential QTLs.

In the present study, a total of nine QTLs controlling seed coat color were detected in the RIL population, and depended on the method used to score seed coat color. There were no co-localized QTLs detected by the two phenotyping scoring methods except for *qSC9.1* and *qSCb9.1*, which were detected on Chr. A09 and explained 55.73 and 12.46% of the phenotypic variance, respectively. According to the literature, most of the major QTLs (or genes) controlling seed coat color in *B. rapa* have been localized to Chr. A09 (Kebede *et al.* 2012, Li *et al.* 2012, Lou *et al.* 2007, Rahman *et al.* 2014, Wang *et al.* 2017, Xiao *et al.* 2012). Gene annotation and BLAST analyses (http://brassicadb.org/brad/index.php) based on the physical position of the corresponding markers revealed that three candidate genes, *DFR* (*Bra027457*), *TT8* (*Bra037887*), and *TT1* (*Bra028067*) responsible for seed coat color are located in the mapping interval. All three candidate genes showed single nucleotide polymorphisms (SNPs) between CSSL-111 and the recurrent parent, 09A001 (data not shown), and thus, further experimentation is needed to determine the most likely candidate gene. Similar to *B. rapa*, most of the major QTLs or genes underlying seed coat color have also been detected on Chr. A9 in other compound *Brassica* species, such as *B. napus* (Qu *et al.* 2015, Yu *et al.* 2016, Zhang *et al.* 2011) and *B. juncea* (Huang *et al.* 2016, Padmaja *et al.* 2014). Some of these species share the same gene with *B. rapa*, such as *BrTT8* in *B. juncea* (Padmaja *et al.* 2014), and may be syntenic with *B. rapa*, which shows the structural and functional conservation of gene order in *Brassica* species (Li *et al.* 2013). In addition to the two co-localized major QTLs located on Chr. A09, three minor QTLs on Chr. A06 were detected in this study, and they do not co-localize with a gene (*BrTTG1*) that was previously identified as being responsible for yellow seed coat color in *B. rapa* (Ren *et al.* 2017a, Zhang *et al.* 2009). There is also a major QTL, *RSC1-2*, located on Chr. A08 that has been reported to control seed coat color in *B. rapa* (Rahman *et al.* 2014), however we only detected minor QTLs for seed coat color on Chr. A08 in this study, and they do not co-localize with the major QTL, *RSC1-2*. Unlike Chrs. A06, A08, and A09, only minor QTLs controlling seed coat color have been detected on Chr. A03 in *B. rapa* (this study and Kebede *et al.* 2012) and one of the two minor QTLs detected on Chr. A03, *qSC3.1*, was verified. Other candidate genes involved in seed coat color have also been detected in the candidate interval of the verified QTL *qSC3.1*, including *PAP2* (*Bra001917*) and *ANS* (*Bra019350*). We also checked SNPs of the two candidate genes between CSSL-38 and 09A001, the recurrent parent, and the results were similar to those for the above mentioned candidate genes located on Chr. A09.

The development of NILs harboring candidate QTL regions has proven to be an effective and widely accepted method for QTL validation, and an increasing number of NIL populations have been constructed in various crops (Brauner *et al.* 2017, Dao *et al.* 2017, Kinkade and Foolad 2013, Lavaud *et al.* 2015, Miyahara *et al.* 2017, Zhao *et al.* 2017) due to their significant contribution to QTL characterization and subsequent breeding programs. In this study, we detected nine QTLs for seed coat color in the RIL population and successfully validated three of them in the CSSLs; one is the major QTL located on Chr. A09, and the other is a minor QTL located on Chr. A03. The verified QTL on Chr. A03 appears to be a new locus involved in determining seed coat color in *B. rapa*. Our findings will provide further insight into understanding the complex genetics of seed coat color in *B. rapa*, and establish a solid foundation for future fine mapping and cloning of this locus.

Acknowledgments

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