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RESEARCH ARTICLE

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Inheritance and molecular markers for the seed coat color in *Brassica juncea*

Abstract To elucidate the inheritance of seed coat color in *Brassica juncea*, Sichuan Yellow inbred (PY) was crossed with the Ziyejie inbred, and their F_1, F_2 and BC_1 and BC_2 progenies, derived from backcrossing to PY, were phenotyped for seed coat color. Results showed that the yellow seed coat was controlled by two independent recessive loci. Seven brown-seeded near-isogenic lines were developed by successive backcrosses to PY and by selfing. One of the BC_6F_2 populations segregated for a single locus controlling seed coat color was used for mapping. Using the 88 primer pairs from sequence-related amplified polymorphism and the 500 random primers, two markers were found to be linked to the gene for brown seed coat, which were designated as SCM57 and SCM1078. The crossover between these markers and the brown seed coat loci was 2.35% and 7.06%, respectively. A sequence characterized amplified region (SCAR) marker according to Negi et al. (2000), designated as SZ1-331, was found to be linked to the gene for brown seed coat, with a crossover estimate of 2.35%. The markers were located on the same side of the brown seed coat loci and 2.41, 7.51 and 2.41 cM away from the brown seed coat locus. The seven brown-seeded near-isogenic lines were classified into two groups by three DNA markers. They were located at the same linkage group of the marker RA2-A11 previously published by Padmaja et al. (2005).

Keywords inheritance, molecular markers, seed coat color, *Brassica juncea*

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1 Introduction

Improving the quality of oil and meal of oilseed is one of the important breeding objectives for rapeseed breeders. Compared with black seeds, yellow seeds of *Brassica* have a significantly thinner seed coat, thereby leading to a lower hull proportion in the seed and, consequently, higher oil content. Some other advantages of yellow seeds include more transparent oil and higher protein and lower fiber content of the meal. *B. napus* is the most important oilseed crop species worldwide. However, no yellow-seeded forms have been discovered in the natural germplasm of *B. napus*. The development of yellow-seeded cultivars for *B. napus* is the main aim of rapeseed breeders. A majority of the Chinese *B. juncea* accessions are yellow-seeded, although there are brown-seeded *B. juncea* accessions. Some studies on inheritance of seed coat color in *Brassica juncea* carried out in Europe and in India revealed that the brown seed coat in *B. juncea* was controlled by two independent segregating dominant genes with duplicate effects (Vera et al., 1979; Vera and Woods, 1982; Liu, 2000) and the yellow seeds could be produced when both loci were in a homozygous recessive condition, and the maternal genotype influenced the expression of the trait.

Some molecular markers of seed coat color traits have been established in *B. juncea*. Negi et al. (2000) found that 3 amplified fragment length polymorphism (AFLP) markers were linked to the seed coat color, converting the dominant AFLP marker (AFLP8) into a simple codominant sequence characterized amplified region (SCAR). Association mapping of the seed-coat color with AFLP markers carried out in 39 *B. juncea* lines showed that 15 AFLP markers were linked to seed coat color (Sabharwal et al., 2004). A RFLP map was used to map QTLs associated with seed color in *B. juncea* using a doubled-haploid population derived from a cross between a black/brown-seeded cultivar and a yellow-seeded breeding line. Segregation analysis suggested that seed coat color was under the control of 2 unlinked loci with duplicate gene actions (Mahmood et al., 2005). Three

microsatellite markers (Ra2-A11, Na10-A08 and Ni4-F11) showing the association with seed coat color were identified through bulk segregant analysis (BSA). Subsequent mapping located the markers Ra2-A11 and Na10–A08 on linkage group (LG) 1 and the marker Ni4–F11 on LG 2 of the linkage map of *B. juncea* published previously (Padmaja et al., 2005).

The objectives of our study were to further elucidate the inheritance of seed coat color and to tag the genes for seed coat color in *Brassica juncea*.

2 Materials and methods

2.1 Plant materials and mapping populations

The inbred line S9 of Sichuan Yellow, a landrace from Sichuan Province, China was used as a yellow-seeded parent, while the inbred line S6 of Ziyejie, a landrace from Hunan Province, China for the brown-seeded one. Both parents were crossed reciprocally and the resultant F_1 and BC_1F_1 plants were backcrossed to the yellow-seeded parent. The brown-seeded plants were selected for successive backcrossing from the BC_2F_1 progenies, segregating for seed coat color using the ratio 1 brown- to 1 yellow-seeded plant. The brown-seeded plants selected from the BC_6F_1 progenies were selfed by bagging to develop the homozygous brown-seeded near-isogenic lines (NILs) with a Sichuan Yellow background. Seven NILs were obtained (Fig. 1). One of the BC_6F_2 progenies consisting of 85 plants, designated as 6-BC_6F_2, was used for initial mapping of the seed coat color in *Brassica juncea*. The seven brown-seeded near-isogenic lines were classified into two groups by DNA markers, one group with 1-BC_6F_2, 2-BC_6F_2, 5-BC_6F_2 and 6-BC_6F_2, and the other group with 3-BC_6F_2, 3-BC_6F_2 and 7-BC_6F_2. In order to testify to the difference of locus control of the seed coat color in the two groups, 6-BC_6F_3 whose putative genotype was AAbb, was selected to cross with 3-BC_6F_3, whose putative genotype was aaBB.

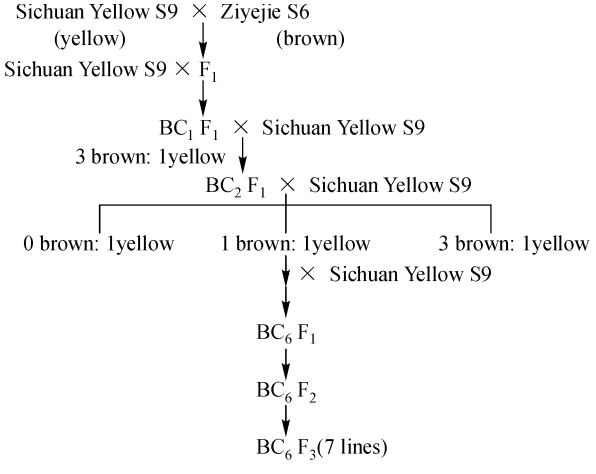

Fig. 1 Pedigree of the genetic stocks used for mapping of the seed coat color in *Brassica juncea*

2.2 DNA amplification and linkage analysis

The 10-mer random amplified polymorphic DNA (RAPD) primers were bought from Sangon (Shanghai), while the sequence-related amplified polymorphism (SRAP) primers (Liu, 2004) were synthesized by Aguct, Beijing. Genetic segregation data of the identified markers showing the association with seed coat color were tested for goodness of fit (χ^2 test) according to the expected Mendelian inheritance. To carry out the bulked segregant analysis (BSA) (Michelmore et al., 1991), equal amounts of DNA from five brown-seeded and five yellow-seeded 6-BC_6F_2 plants were pooled to constitute the brown-seeded (B) and yellow-seeded (Y) bulks, respectively.

Genomic DNAs were extracted with cetyl trimethyl ammonium bromide (CTAB) from young leaves of the 6-BC_6F_2 plants, of which 64 plants were brown-seeded and 21 were yellow-seeded, and both parents were used as templates for amplification (Yan, 2004). PCR was carried out in a final reaction volume of 25 μL in a reaction mixture containing 50 ng genomic DNA, 1U *Taq* polymerase, 2.5 μL of 10×buffer, 2.5 mmol·L^{-1} $MgCl_2$, 100 μmol·L^{-1} per dNTP (Beijing TIANGEN), 10 pmol primers (RAPD: 10 pmol, SRAP: 5 pmol forward primer and 5 pmol reverse primer). The PCR analyses were run in a T-Gradient Thermoblock thermocycler (Biometra). The DNA amplification protocol for RAPD was denatured at 94°C for 240 s, followed by 40 cycles at 94°C for 45 s, 37°C for 45 s, and 72°C for 90 s, with a final extension at 72°C for 480 s. The DNA amplification protocol for SRAP was denatured at 94°C for 240 s, followed by 5 cycles at 94°C for 50 s, 36°C for 50 s, 72°C for 60 s, 35 cycles at 94°C for 50 s, 50°C for 50 s, and 72°C for 60 s, with a final extension at 72°C for 480 s. Gels were stained in ethidium bromide and photographed on a digital Gel Doc 100 gel documentation system (Bio-Rad).

To improve the specificity and reproducibility of RAPD and SRAP markers, the polymorphic fragments were sequenced and converted into SCAR markers. The fragments of interest were ligated to the pMD18–T vector following the manufacturer's instruction. Competent cells of *E. coli* strain Top10 were prepared by traditional $CaCl_2$ double suspension method for the transformation of recombinant T-vector under ampicillin selection and IPTG/X-gal blue-white screening (Sambrook et al., 2001). White colonies were cultured and subjected to PCR checking. Positive clones were sequenced in double direction by Invitrogen (Shanghai).

The seed coat color was scored visually from mature seeds. Map distance was estimated by Mapmaker 3.0.

3 Results

3.1 Inheritance of seed coat color in *Brassica juncea*

The F_1 plants from reciprocal crosses between Sichuan Yellow and Ziyejie produced brown seeds. The observation of 15 brown:1 yellow segregation in the F_2, 3 brown:1 yellow segregation in the BC_1F_1, 3 brown:1 yellow segregation or 1 brown:1 yellow segregation in the BC_2F_1, 15 brown:1 yellow segregation in the F_2 population of 6-BC_6F_3×3-BC_6F_3 (Table 1) showed that the seed coat color was controlled by two independent recessive loci, with the brown seed coat being controlled by two independent dominant genes with duplicate effects in *B. juncea.*

3.2 Screening for polymorphism and SCAR development

A total of 500 random 10-mer primers and 88 SRAP primer combinations were screened for polymorphisms against the parental lines Sichuan Yellow and Ziyejie. One SRAP primer pair (me5 + em7, me5:5′-TGAGTCCAAACCGGAAG-3′ and em7:5′-GACTGCGTACGAATTCAA-3′) and one random primer (S1078:5′-ACCCGGAAAC-3′) exhibited polymorphism among the parental lines and the two bulks.

The polymorphic band amplified from Ziyejie using the primer pair me5 + em7 was cloned and sequenced; the band was made up of 418 bp (Fig. 2). A sequence characterized amplified region (SCAR) primer was developed based on the sequence (Table 2). A 383-bp band was amplified from Ziyejie and brown-seeded plants, while a band at a size of about 500 bp was amplified from Sichuan Yellow, the yellow-seeded plants and a few brown-seeded ones (Fig. 3). These polymorphic bands formed a co-dominant marker SCM57.

The 854-bp polymorphic band amplified from Ziyejie using the random primer S1078 was also sequenced and a SCAR primer was correspondingly developed (Fig. 4, Table 2). Using this new primer pair, a 625-bp band was amplified from Ziyejie and the brown-seeded plants. However, no band was amplified from Sichuan Yellow and yellow-seeded plants (Fig. 5). The polymorphic band was a dominant marker SCM1078.

The third SCAR primer SZ1 was developed according to a reference (Negi et al., 2000), and a 331-bp band was amplified from Ziyejie; no band was amplified from Sichuan Yellow and yellow-seeded plants (Fig. 6). No matches were found between the sequences of these markers and any known sequence (BLASTN search of the National Center for Biotechnology Information (NCBI) online database http://www.ncbi.nlm.nih.gov).

Table 1 Segregation of the seed coat color of *Brassica juncea*

materials and their generation	seed coat color (brown:yellow)	expected ratio	χ^2	P
Ziyejie×Sichuan Yellow F_1	70:0	1:0	0.000	1.000
Sichuan Yellow×Ziyejie F_1	62:0	1:0	0.000	1.000
(Sichuan Yellow×Ziyejie) F_2	122:6	15:1	0.267	> 0.50
(Ziyejie×Sichuan Yellow) F_2	130:8	15:1	0.024	> 0.80
Sichuan Yellow×(Ziyejie×Sichuan Yellow) BC_1 F_1	102:36	3:1	0.043	> 0.80
Sichuan Yellow×(Sichuan Yellow×Ziyejie) BC_1 F_1	44:12	3:1	0.191	> 0.70
(BC_1×Sichuan Yellow) BC_2 F_1	0:50	0:1	0.000	1.00
(BC_1×Sichuan Yellow) BC_2 F_1	28:30	1:1	0.035	> 0.80
(BC_1×Sichuan Yellow) BC_2 F_1	44:46	1:1	0.022	> 0.75
(BC_1×Sichuan Yellow) BC_2 F_1	46:46	1:1	0.000	1.00
(BC_1×Sichuan Yellow) BC_2 F_1	42:38	1:1	0.100	> 0.70
(BC_1×Sichuan Yellow) BC_2 F_1	20:26	1:1	0.763	> 0.30
(BC_1×Sichuan Yellow) BC_2 F_1	16:18	1:1	0.118	> 0.75
(BC_1×Sichuan Yellow) BC_2 F_1	12:15	1:1	0.346	> 0.5
(BC_1×Sichuan Yellow) BC_2 F_1	70:26	3:1	0.111	> 0.75
(BC_1×Sichuan Yellow) BC_2 F_1	48:14	3:1	0.097	> 0.80
(BC_1×Sichuan Yellow) BC_2 F_1	64:26	3:1	0.363	> 0.50
(BC_1×Sichuan Yellow) BC_2 F_1	52:14	3:1	0.253	> 0.55
(BC_1×Sichuan Yellow) BC_2 F_1	48:20	3:1	0.353	> 0.50
(BC_1×Sichuan Yellow) BC_2 F_1	70:22	3:1	0.028	> 0.80
(6-BC_6F_3×3-BC_6F_3) F_2	162:11	15:1	0.004	> 0.95
(6-BC_6F_3×3-BC_6F_3) F_2	85:5	15:1	0.009	> 0.95
(6-BC_6F_3×3-BC_6F_3) F_2	114:9	15:1	0.028	> 0.80

Table 2 Names and primers of the SCAR markers, recombination fraction and map distances between the markers and the gene for seed coat color

marker	primer name	primer sequence (5′→3′)	recombination fraction/%	genetic distance/cM
SCM57	SCAR57	F: AACCGGAAGAAAACGTCCCC R: GGCGACAAAGTTCGAGATGA	2.35	2.41
SCM1078	SCAR1078	F: AAACCCAACAGATCCACA R: AGCCCAATAACCAACTCA	7.06	7.51
SZ1-331	SZ1	F:ACGATCCATTCCTCGACCAC R:CGTGAAACCTAATCAGCTGGC	2.35	2.41

1 **TGAGTCCAAACCGGAAG**AAAACGTCCCCTTAACGTACCGTAGTATCTGTTTGAGAGCTGC
61 GTAGTGTGATTCTTTGGGTTCATGCGTGTAACGGCTCAGTACTCCAACAGAGTAAGCTAG
121 ATCAGGCCGGGTGTGTAGCAAGTAGCGTAAACACCCGATAACTCTCCTAAAATCTTTCTC
181 TTCTACTCCTTGTTCTTCTTGAGCCTTTGATAGCTTTAAACCAGCATCCATCGGGATCTG
241 AGTTGCATTGCAGTCAGTCATGCTTGTCTCTTCCAAAATCTTCTTTGCATAACTCTCCTG
301 CCTTATCGAAATTCCATCATCATATTGGTAAACTTCAATACCAAGATAGTATGTCAGTAA
361 ACCTAGATCAGTCATCTCGAACTTTGTCGCCATATTCTTT**TTGAATTCGTACGCAGTC**

Fig. 2 Nucleotide sequence of the band amplified from Ziyejie using the primer pair me5 + em7
Note: The primer combination me5 + em7 is in bold and the primers used for SCAR are underlined.

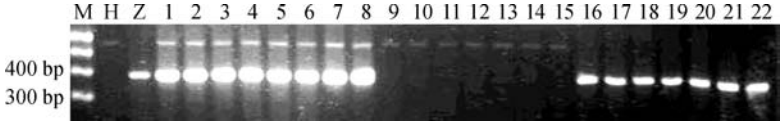

Fig. 3 Electrophoretogram of amplified products using the primer SCAR57
Note: Lanes M, H and Z represent the 100-bp ladder, Sichuan yellow and Ziyejie,respectively. Lanes 1–8 and 16–22 are the brown-seeded plants of 6-BC_2F_2 and Lanes 9–15 are the yellow-seeded plants of 6-BC_2F_2.

1 **ACCCGGAAAC**AGAAACCGGCGAAGAAGATGCTGAGAAAGCCATCACTTCCCGGAACAAAA
61 TCAGACAGCCACCGTAGATAGGTGCGCCGCCTAGACTAAACCCAACAGATCCACAAACTA
121 AAAACCTAAACCCCCGTTTAAAAGAAACTTGAAAAACATGACCGGACCGACGGTGGCTA A
181 AGGAGCCTCGCCGTCGGCCGGGAACCCTCTCTCTCTCTTCTCTCTCTTCTGGCGAGTAAT
241 GAGTATGTAGTAGTTCTTTTTACTGAAAACATTTTCTCGGAACTTTTTTTTTTAATTATG
301 GTAAGGCTTAAGGTACAGTTCTAAAAGTATAAGGACGTTTTCTCACAACTGCCATAAAAT
361 AGCATCTGAAACAAACAAACAAAAATGATAAATAAATCATAGCACATGCTAAACTGCATA
421 TATATTCTAGATGAAACCATAGATTATAATTATTAAAAATGCAATTTATCTTGACCCAGT
481 TAGTAGCAAATATTTAATATATATTTTTAATATGTGTAAAAACATACTCCGCAAAGAGAA
541 ACATTATTAGCGGTAAGGAAACGACAACCATTAATACCTTTCAGATAGCAATAGTGGAAG
604 CCGAGAGCTGGAGAAATAAGGAAGCGAAAGATGAAGAAGAGGAAGAGATGGACCTAGCCA
661 GTTTGATGTGGCCCCATTGAACTCTTTCCAAATTCCAATCTTCTTGAGTTGGTTATTGGG
721 CTTGTGTTATTATGAGAGATGTTATAGGATATGACTTTGGACAAAAAAAAGCTGCTGCTT
781 AAGTTTGTCAGTACTATATTTGGAGATGGACTCATCGGAGCAATGACTTGTCTCCAAGAT
841 AAGG**GTTTCC GGGT**

Fig. 4 Nucleotide sequence of the band from Ziyejie using the random primer S1078
Note: The random primer S1078 is in bold and the primers used for SCAR are underlined.

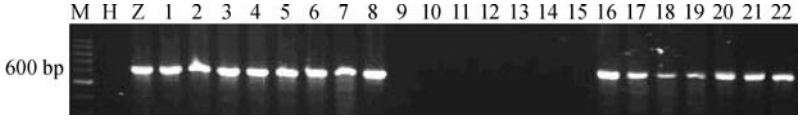

Fig. 5 Electrophoretogram of amplified products using the primer $S1078_{625}$
Note: Lanes M, H and Z represent the 100-bp ladder, Sichuan yellow and Ziyejie, respectively. Lanes 1–8 and 16–22 are the brown-seeded plants of 6-BC_2F_2 and Lanes 9–15 are the yellow-seeded plants of 6-BC_2F_2.

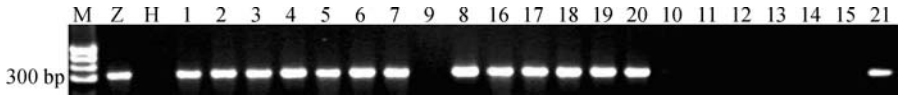

Fig. 6 Electrophoretogram of amplified products using the primer SZ1

Note: Lanes M, H and Z represent the 100-bp ladder, Sichuan yellow and Ziyejie, respectively. lanes 1–8 and 6–22 are the brown-seeded plants of 6-BC_2F_2 and lanes 9–15 are the yellow-seeded plants of 6-BC_2F_2.

3.3 Linkage analysis

To confirm the linkage of seed coat color to these markers, the 6-BC_6F_2 population consisting of 64 brown-seeded plants and 21 yellow-seeded ones was used for segregation analysis of these three markers. The three markers all were segregated in accordance with the expected Mendelian ratio of 3∶1. Tight linkage was indeed observed between the seed coat color gene and all these markers, with recombination fractions of 2.35% and 7.06% (Table 2).

Based on the primer pair of the AFLP marker developed by Negi et al. (2000), a polymorphic fragment was sequenced and the third primer pair for the SCAR marker was designed (Table 2). This primer pair could distinguish the brown-seeded plants from the yellow-seeded plants in the 6-BC_6F_2 progeny. A 331-bp band was amplified from Ziyejie and the brown-seeded plants, although no band was amplified from Sichuan Yellow and the yellow-seeded ones (Fig. 6).

The markers were mapped to the existing *B. juncea* map using the 6-BC_6F_2 population following the mapping criteria described by Padmaja et al. (2005). The three markers and Ra2-A11 were located on the same side of a brown-seeded gene.

4 Discussion

The two-gene inheritance of seed coat color and the subsequent segregation of two genes to two different genetic stocks indicated that the two genes for seed coat color are either present on two different chromosomes or quite far from each other on the same chromosome. Among the brown-seeded lines, the intensity of brownness was so similar that it was difficult to distinguish by the naked eye genotypes with two dominant genes from those with one dominant gene. So it is necessary to use molecular markers to select the seed coat color.

Markers linked to agronomic traits have the potential to be employed in map-based cloning and marker-assisted selection (MAS) programs. Maternal inheritance, environmental effects and the recessive character of the yellow seed coat color traits do not affect these markers (Negi et al., 2000). We have converted the RAPD (S1078) and SRAP (me5 + em7) markers into SCAR markers. The markers SCAR1078 and SZ1-331 are dominant markers. However, the marker SCAR57 is a co-dominant marker. Scoring of this marker in a segregating population is easy in order to distinguish yellow-seeded from brown-seeded *B. juncea* and also to differentiate between homozygous (AAbb) and heterozygous (Aabb) brown-seeded individuals in populations. The development of these three new markers further saturates the linkage map of the region where the genes for seed coat color lie (Pradhan et al., 2003; Mahmood et al., 2005). The application of these markers to diverse germplasms of *B. juncea* with respect to the seed coat color indicated that the brown-seeded cultivars contained brown-specific marker alleles, and yellow-seeded lines contained yellow-specific marker alleles at both loci. These markers will provide the foundation for uncovering the genetic regulation of seed coat color formation and for cloning the genes for seed coat color in *B. juncea*. These markers will be helpful in establishing the markers flanking the genes for seed coat color and applying these markers to transfer the genes controlling seed coat color from *B. juncea* into *B. napus*.

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