Molecular markers for the seed coat color in *Brassica juncea*[§](B3)

YAN Mingli, LIU Zhongsong*, GUAN Chunyun, CHEN Sheyuan, YUAN Mouzhi, LIU Xianjun

Oilseed Crop Research Institute, Hunan Agricultural University, 410128 Changsha, Hunan, China
Email:ymljack@163.com; zsliu48@sohu.com

Abstract

The yellow-seeded rapeseed has many advantages over the brown-seeded one, such as higher oil content, higher feeding value, and better market value. To elucidate inheritance of seed coat color in *Brassica juncea,* the 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. The seven brown-seeded near-isogenic lines were developed by successive backcrosses to PY and selfing. One of the BC_5F_2 populations segregating for a single locus controlling seed coat color was used for mapping. Using the 88 primer pairs of sequence-related amplified polymorphism and the 500 random primers, two markers were found to be linked to the gene for brown seed coat, which designated as SCM57 and SCM1078. The crossover between these markers and the brown seed coat locus was 2.35% and 7.06%, respectively. The markers were located at the same side of the brown seed coat locus, and 2.41 and 7.51cM away from the locus. They are located at the same linkage group of the marker RA2-A11 previously published by Padmaja et al. (2005).

Key words: Molecular markers, seed coat color, *Brassica juncea*

Introduction:

Improvement in 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, higher protein content and lower fiber content of the meal. *B. napus* is one of the most important oilseed crop species in the world. However, no yellow-seeded forms were discovered in natural germplasm of *B. napus*. To develop yellow-seeded cultivars for *B. napus* is a main aim for rapeseed breeders. Majority of the Chinese *B. juncea* accessions are yellow-seeded while 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* is controlled by two independent segregating dominant genes with duplicate effect (Vera et al. 1979,1982; Liu,2000) and the yellow seeds will be produced when both the loci are in a homozygous recessive condition, and the maternal genotype influences the expression of the trait.

Some molecular markers of the seed coat color trait have been established in *B.juncea.* Negi et al. (2000) found 3 amplified fragment length polymorphism (AFLP) markers linked to the seed coat color, and converted the dominant AFLP marker (AFLP8) into a simple codominant SCAR. Association mapping of the seed-coat color with AFLP markers carried out in 39 *Brassica 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 *Brassica 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 action(Mahmood et al., 2005). Three microsatellite markers (Ra2-A11, Na10-A08 and Ni4-F11) showing association with seed coat color were identified through bulk segregant analysis (BSA). Subsequent mapping placed 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* (Padmaja, et al., 2005).

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

Materials and methods

Plant material and mapping populations: The inbred line S9(H) of Sichuan Yellow , a landrace from Sichuan, China was used as a yellow-seeded parent While the inbred line S6(Z) of Ziyejie, a landrace from Hunan, China was used as the brown-seeded parent. 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 with the ratio of 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 Sichuan Yellow background. Seven NILs have been obtained (Fig. 1). One of the BC_6F_2 progenies consisting of 85 plants, designating

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* Corresponding author LIU Zhong-Song, E-mail: zsliu48@sohu.com

as 6-BC_6F_2, was used for initial mapping of the seed coat color in *Brassica juncea*.

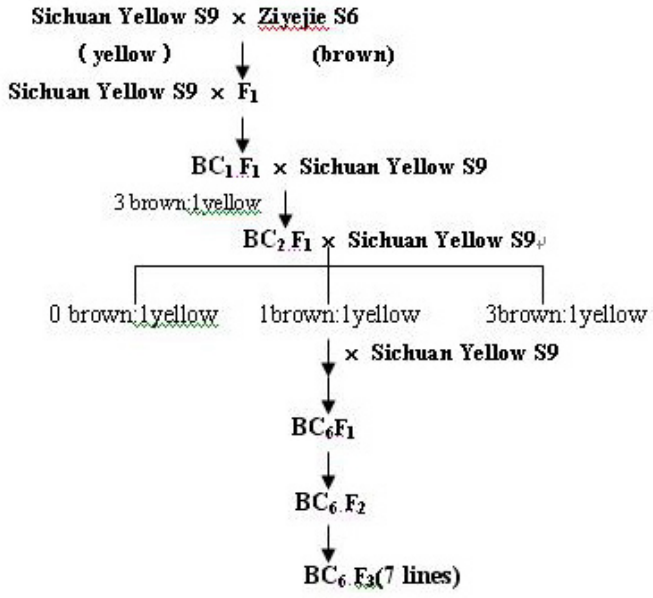

Fig. 1 Pedigree of the genetic stocks used for mapping of the seed coat color in *Brassica juncea*

DNA amplification and linkage analysis

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

The random 10-mer primers were bought from Sangon(Shanghai), while the SRAP primers (Liu, 2004) synthesized by Aguct (Beijing). Genetic segregation data of the identified markers showing association with seed coat color were tested for goodness of fit (χ^2 test) according to the expected Mendelian inheritance. To carry out 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.

To improve specificity and reproducibility of RAPD and SRAP markers, the polymorphic fragments were sequenced and converted into SCAR markers. The fragments of interests were ligated to the pMD18-T vector according to 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 directions by Invitrogen (Shanghai).

Seed coat color was scored visually from mature seeds.

Map distance was estimated according to the formula $RF=(1-e^{-2x})/2$(RF:Recombination fraction; x: Genetic distance; e: natural logarithm).

Results

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_2 F_1 (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 effect in *B. juncea*.

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′; em7: 5′-GACTGCGTACGAATTCAA-3′) and one random primer (S1078: 5′-ACCCGGAAAC-3′) exhibited polymorphism

among the parental lines and the two bulks.

Table1 Segregation of the seed coat color of the parental lines, F_1, F_2, BC_1 and BC_2 in *Brassica juncea*

Materials and their generation	Seed coat color (brown:yellow)	Expected ratio	χ^2	*p*
Sichuan Yellow	0: 22			
Ziyejie	25: 0			
Ziyejie×Sichuan Yellow F_1	70: 0			
Sichuan Yellow×Ziyejie F_1	62: 0			
(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	*>0.85*

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

The 854-bp polymorphic band amplified from Ziyejie using the random primer S1078 was also sequenced and a second new primer pair 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 is a dominant marker SCM1078.

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 2 Names and primer sequence 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

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1     TGAGTCCAAA CCGGAAGAAA ACGTCCCCTT AACGTACCGT AGTATCTGTT TGAGAGCTGC
61    GTAGTGTGAT TCTTTGGGTT CATGCGTGTA ACGGCTCAGT ACTCCAACAG AGTAAGCTAG
121   ATCAGGCCGG GTGTGTAGCA AGTAGCGTAA ACACCCGATA ACTCTCCTAA AATCTTTCTC
181   TTCTACTCCT TGTTCTTCTT GAGCCTTTGA TAGCTTTAAA CCAGCATCCA TCGGGATCTG
241   AGTTGCATTG CAGTCAGTCA TGCTTGTCTC TTCCAAAATC TTCTTTGCAT AACTCTCCTG
301   CCTTATCGAA ATTCCATCAT CATATTGGTA AACTTCAATA CCAAGATAGT ATGTCAGTAA
361   ACCTAGATCA GTCATCTCGA ACTTTGTCGC CATATTCTTT TTGAATTCGT ACGCAGTC
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Fig. 2 The 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 underlined

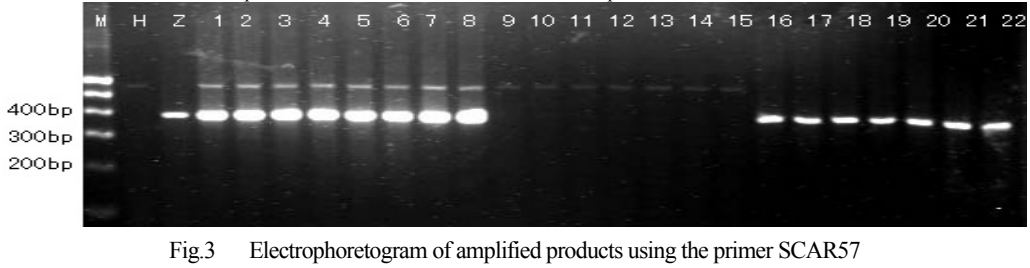

Fig.3 Electrophoretogram of amplified products using the primer SCAR57

Notes:Lane M:100bp ladder; Lane H: Sichuan yellow; Lane Z: Ziyejie; Lane 1-8 and16-22: the brown-seeded plants of 6-BC_2F_2 and Lane 9-15: the yellow-seeded plants of 6-BC_2F_2.

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1    ACCCGGAAAC AGAAACCGGC GAAGAAGATG CTGAGAAAGC CATCACTTCC CGGAACAAAA
61   TCAGACAGCC ACCGTAGATA GGTGCGCCGC CTAGACTAAA CCCAACAGAT CCACAAACTA
121  AAAACCTAAA CCCCCGTTTA AAAGAAACTT GAAAAACATG ACCGGACCGA CGGTGGCTAA
181  AGGAGCCTCG CCGTCGGCCG GGAACCCTCT CTCTCTCTTC TCTCTCTTCT GGCGAGTAAT
241  GAGTATGTAG TAGTTCTTTT TACTGAAAAC ATTTTCTCGG AACTTTTTTT TTTAATTATG
301  GTAAGGCTTA AGGTACAGTT CTAAAAGTAT AAGGACGTTT TCTCACAACT GCCATAAAAT
361  AGCATCTGAA ACAAACAAAC AAAAATGATA AATAAATCAT AGCACATGCT AAACTGCATA
421  TATATTCTAG ATGAAACCAT AGATTATAAT TATTAAAAAT GCAATTTATC TTGACCCAGT
481  TAGTAGCAAA TATTTAATAT ATATTTTTAA TATGTGTAAA AACATACTCC GCAAAGAGAA
541  ACATTATTAG CGGTAAGGAA ACGACAACCA TTAATACCTT TCAGATAGCA ATAGTGGAAG
601  CCGAGAGCTG GAGAAATAAG GAAGCGAAAG ATGAAGAAGA GGAAGAGATG GACCTAGCCA
661  GTTTGATGTG GCCCCATTGA ACTCTTTCCA AATTCCAATC TTCTTGAGTT GGTTATTGGG
721  CTTGTGTTAT TATGAGAGAT GTTATAGGAT ATGACTTTGG ACAAAAAAAA GCTGCTGCTT
781  AAGTTTGTCA GTACTATATT TGGAGATGGA CTCATCGGAG CAATGACTTG TCTCCAAGAT
841  AAGGGTTTCC GGGT
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Fig. 4 The nucleotide sequence of the band from Ziyejie using the random primer S1078

The random primer S1078 is in bold and the primers used for SCAR are underlined

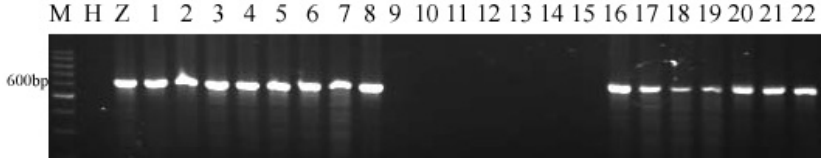

Fig.5 Electrophoretogram of amplified products using the primer primer $S1078_{625}$

Notes: Lane M:100bp ladder; Lane H: Sichuan yellow; Lane Z: Ziyejie Lane 1-8 and16-22: the brown-seeded plants of 6-BC_2F_2 and Lane 9-15: the yellow-seeded plants of 6-BC_2F_2.

Linkage analysis

To confirm linkage of the 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 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 2.41% and 7.51% (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 SCAR marker was designed (Table 2). This primer pair can distinguish the brown-seeded plants from the yellow-seeded plants in 6-BC_6F_2 progeny (Yan et al.2006). 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.

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 are located on the same side of a brown-seeded gene (Yan et al.2006).

Discussion

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 trait do not affect these markers (Negi et al. 2000). We have converted the RAPD (S1078) and SRAP (me5+em7) markers into SCAR markers. The marker SCAR1078 is dominant markers. However, the marker SCAR57 is a co-dominant marker. Scoring of this marker in a segregating population easily distinguished yellow-seeded from brown-seeded *B. juncea* and also differentiated between homozygous (AAbb) and heterozygous (Aabb) brown-seeded individuals in populations. The development of these three new markers further saturated linkage map of the region in which the genes for seed coat color lie (Pradhan et al.,2003; Mahmood et al., 2005). The next aim is to establish the markers flanking the genes for seed coat color and to apply these markers to transfer of the genes controlling seed coat color from *B. juncea* into *B. napus*.

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