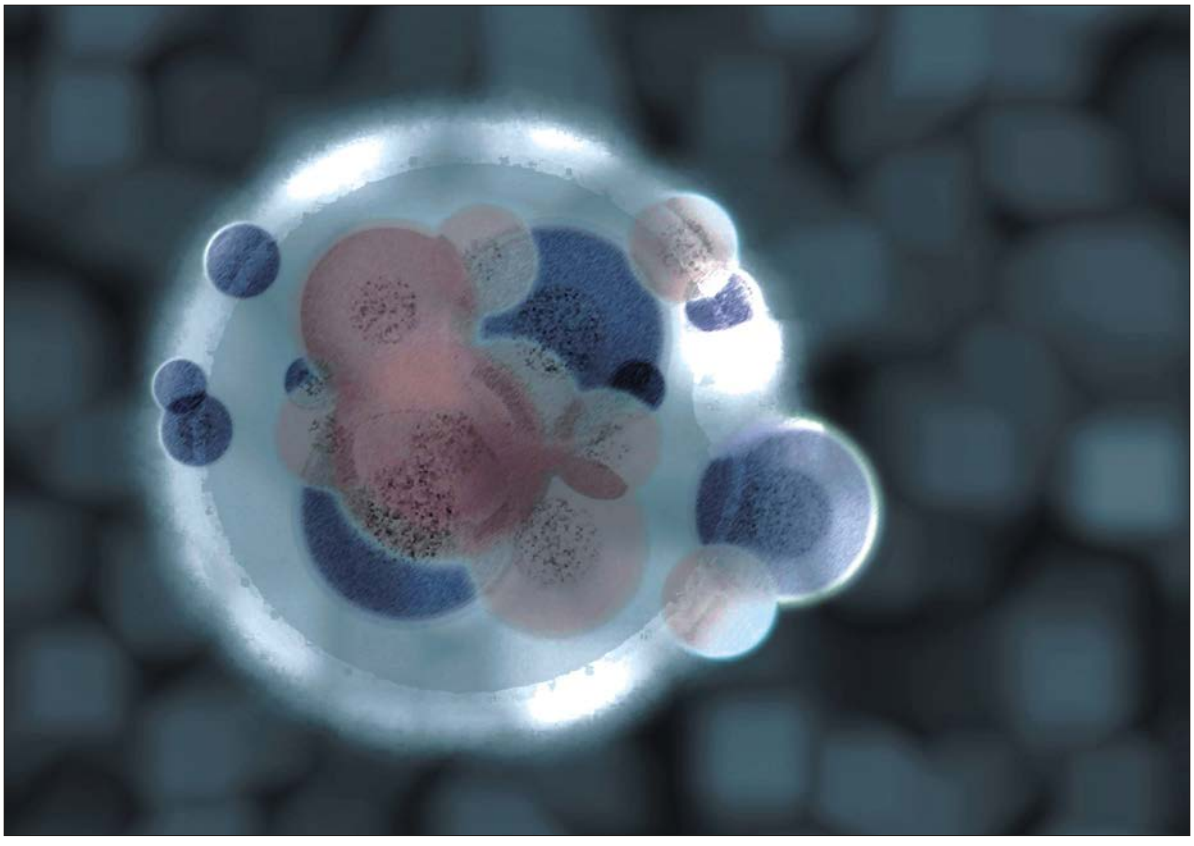

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Homology of Seed Coat Color Specific Marker of *B. juncea* with Brown Seeded Cultivar of *B. rapa*

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Abstract: Polymerase Chain Reaction (PCR) was carried out with seed coat color specific SCAR marker of *B. juncea*. The yellow-seeded cultivars were collected from Japan and Denmark, where the brown-seeded cultivars were collected from Bangladesh and Japan. PCR products were visualized in 1% agarose gel, where the yellow-seeded cultivars produced a strong band at 0.5 kb and weak band at 1.2 kb. In addition of these two specific bands, the Japanese yellow-seeded cultivars expressed two more week bands at 1.1 and 1.0 kb. The brown-seeded cultivars generated a single strong band at 1.1 kb. In segregating population of a cross of yellow-seeded and brown seeded cultivars, the yellow-seed coat color marker segregated at a ratio 15 (brown): 1 (yellow), indicating the digenic inheritance pattern of the traits. PCR was also carried out with the same primers to the yellow-seeded and brown-seeded cultivars of *B. rapa*. It was interesting to note that the yellow-seeded cultivar of *B. rapa* did not show any band, where the brown-seeded cultivar of *B. rapa* showed bands at 1.1 kb and 0.5 kb. The result indicates that the seed coat color specific primer of *B. juncea* has also ability to differentiate the yellow-seeded and brown-seeded cultivars of *B. rapa*. PCR-Southern hybridization was carried out with 0.5 and 1.1 kb fragments as prove to check the homology of the other amplified fragments. The prove DNA fragments (0.5 or 1.1 kb) hybridized all other DNA fragments at 1.2, 1.1, 1.0 and 0.5 kb of *B. juncea* and brown-seeded *B. rapa*. The result indicating that all the mentioned fragments bearing a common homology region of DNA of yellow-seeded *B. juncea* and brown-seeded cultivars of *B. rapa*.

Key words: *Brassica*, brown seed, yellow seed, PCR, southern blotting

INTRODUCTION

Molecular marker has been widely used to map agronomically important gene(s) in *Brassica* genomes and to assist *Brassica* breeding and selection procedures. The majority of the work utilizing molecular marker in *Brassica* oilseed breeding has to date been based on genetic mapping using various DNA marker systems, in segregating populations generated by individual users for investigation of particular traits of interest. *Brassica juncea* is an important oilseed crop in Bangladesh, India and going importance in Canada and the USA as an alternative to rapeseed. It has a number of valuable agronomic characteristics, which include tolerance to heat and drought and resistance to black leg disease. Quality and quantity of oil can be improved by the development of yellow-seeded cultivars in this species. The yellow seed color trait is of particular interest of oilseed breeding not only due to its association with increase oil and protein content, but also because a reduced crude fiber content after oil extraction, improves the feed quality of seed meal[1-3].

Negi *et al.*[4] identified AFLP markers linked to the seed coat color trait of *B. juncea* and converted into a Sequence-characterized Amplified Region (SCAR) marker. In this experiment we tested the yellow-seeded and brown-seeded cultivars of *B. juncea* collected from diverged regions using the mentioned seed coat color marker. The study was also further extended to check the yellow seeded and brown-seeded cultivars of *B. rapa* to find out the homology region of DNA. Consequently, southern hybridization was performed with the seed coat color marker as proves in the yellow-seeded and brown-seeded cultivars of *B. juncea* and *B. rapa*.

MATERIALS AND METHODS

Plant materials: Three yellow-seeded and four brown-seeded cultivars of *B. juncea* and one yellow and one brown-seeded cultivars of *B. rapa* were collected from

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Bangladesh, Japan and Denmark. The F_1 and F_2 were produced from a cross between Danish yellow-seeded (Jun-83) and Japanese brown-seeded (Jun-32) cultivars of *B. juncea* to study the nature of inheritance of seed coat color gene. The experiment was conducted at the field and laboratory of Plant Genetics and Breeding of Tokyo University of Agriculture and Technology, Tokyo, Japan in the year of 2001-2002 and 2002-2003.

DNA extraction and amplification: The DNA was extracted from young leaf by SDS methods according to Dellporta *et al.*[5] and was used as template in amplification. PCR was carried out in a final reaction volume of 25 μL in reaction buffer containing 60 ng total genomic DNA, 0.5U *Taq* polymerase (Takara), 2.5 μL of 10x buffer, 2.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 μM primers (SCM08 and Walk8F) and 15.9 μL autoclaved IEW (Ion Exchange Water). The reaction buffer was covered with 20 μL liquid paraffin and put into a thermo circler. PCR conditions for amplification were as follows: denaturation at 94°C for 3 min, followed by 40 cycles of 45 sec denaturation at 94°C; annealing for 45 sec at 69°C; extension for 1.5 min at 71°C. The PCR products were visualized on 1.0% Agarose gel in 1xTAE (Tris Acetic acid EDTA) buffer. Electrophoresis was carried out at 50v for 5 min and 100v for 20 min. Staining was performed with Ethidium Bromide (5 μL EtBr/200 mL 1xTAE buffer) for 20 min and bands were visualized and photographed with UV light. The following seed coat color specific primer combinations were used in this experiment:

SCM08 5'-GAGCATCTAAACCGTCGTGCTTCC-3' (24 mer)
Walk8F 5'-GCGCGTCCCCTTCCAGAAGTGAAC-3' (24 mer)

PCR-Southern blot analysis: Fifteen micro-liter of PCR products were separated on 1.0% agarose gel and stained with EtBr soln. The gel containing DNA were denatured by acid denaturizing buffer (0.25 M HCl) for 5 min followed by 1 min wash with autoclaved IEW and denatured again by alkaline denaturizing solution (2% NaOH, 10% NaCl) for 15 min. Finally neutralization was done by neutral solution (0.5 M Tris-HCl, 1.5M NaCl, 1mM EDTA, pH=7.0) for 15 min. The gel was blotted onto Hybond™-N+nylon membranes by overnight capillary blotting with 20x SSC. For prove preparation and labeling, 20 μL of PCR product of prove DNA was separated from 1% agarose gel (SEAKEM GTG) and excised the specific bands (0.5 and 1.1 kb). The prove DNA were purified using the Geneclone II kit (BIO 101, INC.). The purified DNA was denatured at 94°C for 10 min followed by 5 min in ice. The prove DNA were leveled with DIG-dUPT kit (2 μL Hexanucleotide Mix 10x conc., 2 μL DIG DNA labeling mix 10x conc., 1 μL Klenow enzyme) for 20 h at 37°C. The blots (membrane) were hybridized with DIG-labeled probes in 20x SSC containing 1% (w/v) blocking reagent, 0.1% (w/v) Sodium N-Lauroryl sarcosinate, 0.02% (w/v) SDS at 37°C for 16 hrs. After hybridization, filters were washed under a stringent condition with twice in 20x SSC, 0.1% SDS (w/v) for 5 min at room temperature, then twice in 1x SSC, 0.1% SDS (w/v) for 15 min at 37°C.

RESULTS AND DISCUSSION

DNA assay: In this experiment, yellow-seeded and brown-seeded specific SCAR marker (SCM08 and Walk8F) were used to check three yellow-seeded cultivars namely Jun-20, Jun-83 and Jun-33 and three brown-seeded cultivars viz., Jun-31, Jun-32 and Jun-34 of *B. juncea*. F_1 and 30 F_2 progenies of a cross of Jun-83 x Jun-32 were also studied here. All three yellow-seeded cultivars produced a common a strong band at 0.5 kb and a weak band at 1.2 kb. In addition of these two specific bands, the Japanese yellow-seeded cultivar had two more weak bands at 1.1 and 1.0 kb. (Fig. 1). The brown-seeded cultivars produced a single intense band at 1.1 kb (Fig. 1). All the yellow-seeded individuals in the segregating populations showed amplification pattern similar to that of yellow-seeded parents (Fig. 2), where as difference were observed in the banding pattern among the brown-seeded individuals (Fig. 2). This is due to the fact that the brown-seeded lines may be either homozygous (BB) or heterozygous (Bb). The homozygous brown-seeded individuals showed a pattern similar to that of the brown-seeded parents, where as the heterozygous (Bb) brown individuals produced 3 bands corresponding to the banding pattern of both the yellow seeded and brown-seeded parents. A total 30 segregating (F_2) individuals were screened with the primers of which only 3 plants showed yellow-seeded specific marker. The Chi square (X^2= 0.72, p= 0.42) test showed that the seed coat color marker segregated in the ratio 15(brown):1(yellow), confirming the digenic inheritance pattern with duplicate gene interaction of the traits.

PCR-Southern blot assay: PCR was carried out through with SCM08 and Walk8F primer to the cultivars of yellow-seeded and brown-seeded *B. juncea,* F_1 of the cross of yellow-seeded and brown-seeded *B. juncea,* as well as yellow-seeded and brown-seeded cultivars of *B. rapa.* After gel electrophoresis in 1% agarose gel, the yellow-seeded and brown-seeded cultivars of *B. juncea* and its progenies amplified the expected bands at 1.2, 1.1, 1.0 and 0.5 kb and the prove DNA showed band 0.5 kb (when 0.5 kb band was previously excised for prove preparation, transferred to TA plasmid vector, multiply the

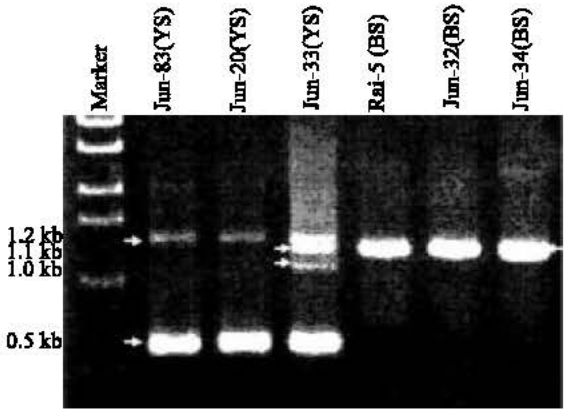

Fig. 1: Gel amplification using specific marker for the classification of yellow-seeded (Jun-83, Jun-20, Jun-33) and brown-seeded (Jun-31, Jun-32, Jun-34) cultivars of *B. juncea*

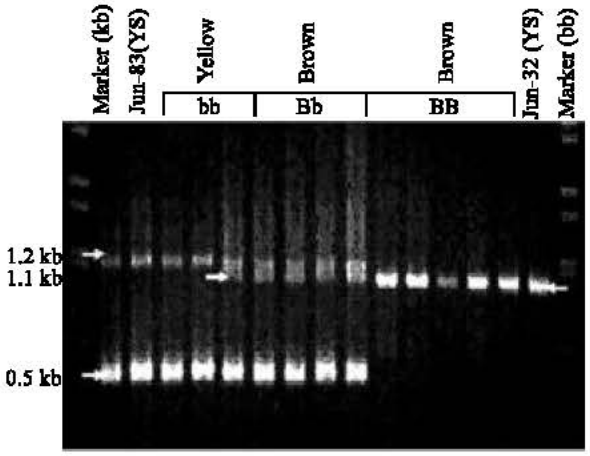

Fig. 2: Analysis of PCR products obtained using the SCAR primer on individual plants. Jun-83 is the yellow-seeded parents and Jun-32 is the brown seeded parent. F_2 showed 15:1 segregation ratio between brown-seeded and yellow-seeded specific markers

vector in *E.* coli, extracted plasmid DNA and amplified by the primers). But in the case of *B. rapa,* the yellow-seeded cultivar did not amplify any band where as the brown-seeded cultivar produced bands at 1.1 and 0.5 kb (Fig. 3). Southern blotting was carried out to testify the homology region of all amplified bands. The amplified DNA was run in 1% agarose gel and blotted onto Hybond™-N+nylon cellulose membrane. The blotted DNA were hybridized with 0.5 kb DNA fragment of yellow-seeded *B. juncea* as prove and it was found that the prove DNA hybridized the 1.2, 1.1, 1.0 and 0.5 kb fragment of *B. juncea* and 1.1 and 0.5 kb fragments of

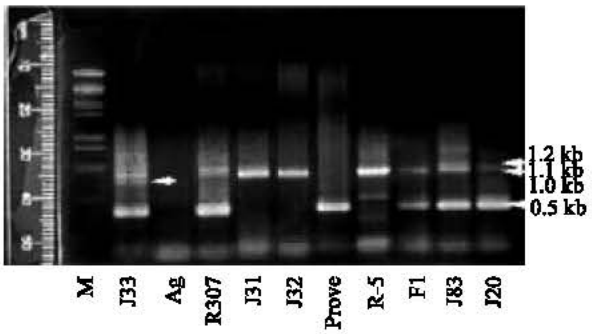

Fig. 3: PCR of the yellow-seeded (YS) and brown-seeded (BS) cultivars of *B. juncea* and *B. rapa* using seed coat color specific primer. M-molecular marker, J33, J83, J20 are YS *B. juncea*; J31, J32, Rai-5 are BS *B. juncea*; F_1 is the cross between J83 and J32; Ag is the YS *B. rapa*; R307 is the BS *B. rapa,* Prove is 0.5 kb fragment of YS *B. juncea*

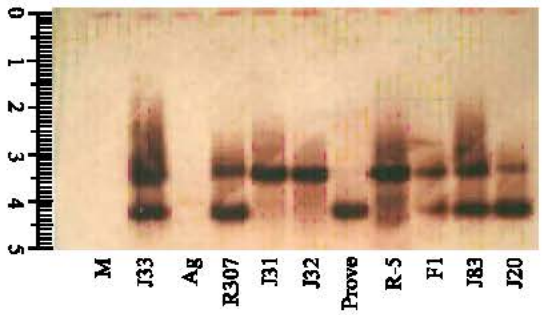

Fig. 4: PCR-Southern blot analysis of YS and BS cultivars of *B. juncea* and *B. rapa* with the YS specific (0.5 kb) DNA fragment as prove. M-molecular marker, J33, J83, J20 are YS *B. juncea*; J31, J32, Rai-5 are BS *B. juncea*; F_1 is the cross between J83 and J32; Ag is the YS *B. rapa*; R307 is the BS *B. rapa*. The result indicates that all the amplified fragments at 1.2, 1.1, 1.0 and 0.5 kb have common homology region

brown-seeded cultivar of *B. rapa* (Fig. 4). Similar result was obtained when 1.1 kb fragment of brown-seeded *B. juncea* was used as prove (result has not showed here).

Yellow-seeded and brown seeded cultivars of *B. juncea* and their progenies were tested by seed coat color specific SCAR marker. The three yellow-seeded cultivars produced two common bands and the Japanese yellow-seeded cultivar generated two additional bands, where the brown-seeded cultivars produced single band. The result showed almost full agreement with Negi *et al.*[4], except the two additional bands of Japanese

yellow-seeded cultivar. Negi *et al.*[4] collected the yellow-seeded cultivar from Poland and here Japanese and Danish yellow-seeded cultivars were used. The Japanese yellow-seeded cultivar exhibited two seed color related specific bands with additional two bands. Therefore, the current result gives a further authentication of seed coat color specific markers in diversified origin of cultivars. These results also extended the confirmation of the tight linkage of the SCAR marker to seed coat color and indicated that this marker will be useful for marker assisted breeding. The markers were extended to analyze the segregating population of *B. juncea.* The PCR assay using the SCAR primers were useful as all the individuals could be classified as bb, Bb or BB. Thus, the SCAR markers has an advantage of being co-dominant and useful for screening heterozygous. In the case of *B. napus,* a SCAR marker linked to the dwarf locus was co-dominant in nature and was able to easily distinguish the heterozygous plants[5].

The primers were used to amplify the genomic DNA of yellow-seeded and brown-seeded *B. rapa* with same PCR condition. The brown-seeded *B. rapa* produced bands at 1.1 and 0.5 kb, where as the yellow-seeded didn't produced any band. This is may be due to different genomic constitution of yellow-seeded and brown-seeded *B. rapa.* Rahman and Hirata[6] observed a clear differentiation between yellow-seeded and brown-seeded cultivars of *B. rapa* using SDS-PAGE for seed protein and esterase isozyme. The result also indicated that the brown-seeded *B. rapa* might contain a DNA sequence which are similar to *B. juncea* specific 1.1 and 0.5 kb fragments. PCR-Southern blotting showed the confirmation of the said hypothesis, where yellow-seeded and brown-seeded *B. juncea* specific (0.5 and 1.1 kb) DNA fragment as prove, hybridized all other fragments of *B. rapa* and *B. juncea.* The result strongly supported that the yellow-seeded and brown-seeded *B. juncea* have a common homology region with brown-seeded *B. rapa.* This is also indicating that all four (1.2, 1.1, 1.0, 0.5 kb) amplified fragments have a common DNA region.

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