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ORIGINAL PAPER

Development of SRAP, SNP and Multiplexed SCAR molecular markers for the major seed coat color gene in *Brassica rapa L.*

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Abstract Seed coat color inheritance in *B. rapa* was studied in F_1, F_2, F_3, and BC_1 progenies from a cross of a Canadian brown-seeded variety 'SPAN' and a Bangladeshi yellow sarson variety 'BARI-6'. A pollen effect was found when the yellow sarson line was used as the maternal parent. Seed coat color segregated into brown, yellow-brown and bright yellow classes. Segregation was under digenic control where the brown or yellow-brown color was dominant over bright yellow seed coat color. A sequence related amplified polymorphism (SRAP) marker linked closely to a major seed coat color gene (*Br1/br1*) was developed. This dominant SRAP molecular marker was successfully converted into single nucleotide polymorphism (SNP) markers and sequence characterized amplification region (SCAR) markers after the extended flanking sequence of the SRAP was obtained with chromosome walking. In total, 24 SNPs were identified with more than 2-kb sequence. A 12-bp deletion allowed the development of a SCAR marker linked closely to the *Br1* gene. Using the five-fluorescence dye set supplied by ABI, four labeled M13 primers were integrated with different SCAR primers to increase the throughput of SCAR marker detection. Using multiplexed SCAR markers targeting insertions and deletions in a genome shows great potential for marker assisted selection in plant breeding.

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Introduction

Brassica rapa L. is a major oilseed and vegetable species throughout the world as well as being one of the parent species of *B. napus*. Yellow seed coat color is desirable in any oilseed *Brassica* species because it has been reported that yellow-seeded varieties have a thinner seed coat than black seeded varieties resulting in comparatively larger endosperm which contributes 5–7% more oil in the seed (Liu et al. 1991). The seed meal from yellow seeded varieties also contains higher protein and lower fiber content, which improves the meal quality for poultry and livestock (Shirzadegan and Röbellen 1985).

Early genetic studies by Mohammad et al. (1942) and Jönsson (1975) indicated that three genes are responsible for seed coat color segregation in *B. rapa.* Later, Stringam (1980) reported that two independent loci controlled seed color and proposed a model for seed coat color genes *Br1* and *Br3*. According to Stringam's model, presence of dominant alleles at both loci (*Br1* and *Br3*) or presence of dominant alleles only at the first locus (*Br1*) produce brown seed color, while presence of dominant alleles at a second locus (*Br3*) and homozygous recessive alleles at the first locus (*br1br1*) produce yellow-brown seeds. Yellow seeds are produced only when both loci present are in homozygous recessive condition (*br1/br1,br3/br3*). Schwetka (1982), Zaman (1989) and Rahman (2001) have confirmed the seed coat color inheritance pattern in *B. rapa* as proposed by Stringam (1980).

Traditionally, selection of plant materials with desirable traits is carried out using phenotypic selection and the phenotypic variation is often influenced by environments. In contrast, the development of molecular markers linked to a trait of interest using different molecular marker techniques, enables marker assisted selection (MAS) for the monitoring

of similarly/dissimilarly among different genotypes at the very early stages of plant development, independent of environmental effects. This can significantly reduce the cost of producing breeding lines and can accelerate the breeding program dramatically. Moreover, using MAS makes it possible to transfer a trait from one line to another one with high efficiency, even the traits which are highly influenced by the environment. There are several molecular markers technologies available for MAS in plant breeding including restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) (Williams et al. 1990; Karp et al. 1997), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), and sequence related amplified polymorphism (SRAP) (Li and Quiros 2001). The principles of these marker techniques vary and they generate different amounts of information. The SRAP technique is simple and easy to perform, preferentially amplifies ORF or ORF related sequences and selected SRAP PCR products separated on a polyacrylamide gel are easy to sequence (Li and Quiros 2001). Therefore, the SRAP marker technique was used in this study for the identification of molecular markers linked to seed coat color genes in *B. rapa*.

Several molecular markers linked to seed coat color in Brassica species have been reported. Van Deynze et al. (1995) identified RFLP markers linked to a seed coat color gene in *B. napus*. Similarly, Somers et al. (2001) developed a RAPD marker for a single major gene (*pigment1*) controlling seed coat color in *B. napus*. Liu et al. (2005) reported that yellow seed color was partially dominant over black seed color and developed 2 RAPD and 8 AFLP markers for the seed coat color gene in *B. napus*. The RAPD and AFLP markers developed by Liu et al. (2005) were not suitable for large scale MAS. Therefore, they converted these markers into reliable sequenced characterized amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers for seed coat color breeding in *B. napus*. Negi et al. (2000) identified an AFLP marker for seed coat color gene in *B. juncea* and converted the marker into a SCAR marker. In another study, SSR markers were developed for mapping and tagging the two independent loci controlling the seed coat color in *B. juncea* (Padmaja et al. 2005). Mahmood et al. (2005) identified QTLs associated with the seed coat color in *B. juncea* from an RFLP map using a doubled-haploid population. Chen et al. (1997) identified a RAPD marker linked to a seed coat color gene in a C genome chromosome of a *B. campestris–B. alboglabra* additional line. Heneen and Jørgensen (2001) identified a RAPD marker on chromosome 4 for brown seed color in *B. alboglabra* using *B. rapa–B. alboglabra* monosomic addition lines. To date, no seed coat color gene in *B. rapa* has been identified. In this study, the inheritance of seed coat color in *B. rapa* was analyzed using cross progeny from a cross of the self-incompatible variety 'SPAN' and the self-compatible yellow sarson variety 'BARI-6'. SRAP, SNP and multiplexed SCAR molecular markers closely linked to a seed coat color gene were developed. These molecular markers will be used for MAS in *Brassica* breeding and map-based cloning of this seed coat color gene.

Materials and methods

Plant materials

The pure breeding brown-seeded self-incompatible Canadian *B. rapa* variety 'SPAN' was crossed with the pure breeding yellow sarson self-compatible Bangladeshi *B. rapa* variety 'BARI-6' and the F_1 was backcrossed with 'BARI-6'. The F_1, F_2, F_3 and BC_1 were grown in a greenhouse at the University of Manitoba. Segregation for seed coat color was studied in F_2 and backcross populations in the greenhouse. Plants from the segregating populations were grouped into brown, yellow-brown and yellow color seed produces. A χ^2 test was performed on the grouped data to check the goodness of fit of the segregating populations to the expected Mendelian phenotypic segregation ratio. A total of 224 F_2 and 197 BC_1 plants were used for seed coat color segregation analysis and molecular marker development for the seed coat color trait.

DNA extraction and SRAP molecular marker development

DNA was extracted using a modified CTAB method according to Li and Quiros (2001) from the flower buds of parental lines and their segregating populations. SRAP PCR amplification was the same as that of Li and Quiros (2001). Instead of autoradiography for signal detection, a five fluorescent dye set including, 6-FAM (blue), VIC (green), NET (yellow), PET (red), and LIZ (orange) supplied by Applied Biosystems (ABI), was used to separate SRAP PCR products with an ABI 3100 Genetic Analyzer (ABI, California).

Chromosome walking and sequencing

The chromosome walking method is commonly used to determine genomic sequence flanking the known sequence of molecular markers. Siebert et al. (1995) described a chromosome walking method on uncloned human genomic DNA, which was commercialized by Clontech Laboratories (Clontech, Mountain View, California). The Genome Walker™ Universal Kit was used to obtain flanking chromosome sequence of the molecular marker linked to seed coat color. The procedure was performed according to the

protocol provided in the Clontech kit. Genomic DNA of 'SPAN' (brown seeded parent) was digested with restriction enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. Sharp and strong bands were obtained after a second PCR amplification. These bands were excised from an Agarose gel and DNA was extracted using a Qiagen Gel Extraction kit. All the DNA fragments were sequenced using a BigDye®Terminator v1.1 Cycle Sequencing Kit.

SNP detection with SNaPshot

SNP primer (GTGGTTGAGCGCTCAGTTGCA) and SCAR primers (MR13, TGCTCGTTCTTGACAACAC; MR54, GAGAATTGAGAGACAAAGC) used in this study were designed using the Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) software. SNPs were detected with an ABI SNaPshot kit (ABI, Toronto). Genomic DNA was amplified first with specific primers targeting the corresponding SNP mutations. The PCR reaction was set up in 10 µl of reaction mix containing 60 ng of genomic DNA, 0.375 µM dNTP, 0.15 µM of each primer, 1× PCR buffer, 1.5 mM $MgCl_2$ and 1 unit *Taq* polymerase. The PCR running program was 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The amplified fragments were further analyzed with SNP detection primers and SNaPshot was performed according to the protocol in the ABI kit. The final products were separated with an ABI 3100 Genetic Analyzer. All four ddNTPs were fluorescently labeled with a different color dye. The alleles of a single marker were identified by the peaks and their colors after the data was analyzed with ABI GeneScan software. The nucleotide 'C' was assigned black; 'T', red; 'G', blue and 'A', green color in the ABI GeneScan software.

SCAR detection

The forward primer MR13 (TGCTCGTTCTTGACAACAC) and the reverse primer MR54 (GAGAATTGAGAGACAA AGC) were designed to target a deletion mutation that occurred in the black-seeded lines. To detect this deletion with the ABI 3100 Genetic Analyzer, an M13-tailed primer method (Boutin-Ganache et al. 2001) was applied where the M13 primer sequence (CACGACGTTGTAAAAC-GAC) was added to the 5′ primer end of MR13 to create a primer MR1313 (CACGACGTTGTAAAACGACTGCT CGTTCTTGACAACAC). The M13 primer was labeled with four fluorescence dyes, 6-FAM, VIC, NED, and PET supplied by the ABI Company. In the PCR amplification, four different PCR reactions were set by four fluorescently labeled primers with separately unlabeled MR1313 and MR54 primers. The PCR reactions were mixed together in a 10 µl volume containing 60 ng of genomic DNA, 0.375 µM dNTP, 0.10 µM of M13 primer, 0.05 µM of MR1313 primer, 0.10 µM of MR54 primer, 1× PCR buffer, 1.5 mM $MgCl_2$ and 1 Unit *Taq* polymerase. PCR was performed at 94°C for 3 min, six cycles at 94°C for 50 s, 60°C for 1 min with a 0.7°C decrease of annealing temperature at each cycle, 72°C for 1 min, and then 20 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 10 min for denaturing, annealing and extension, respectively. The PCR amplification products from different dye colors were pooled together so that each well contained four different fluorescently labeled DNA fragments which were detected in ABI 3100 Genetic Analyzer.

Results

Seed coat color inheritance in *B. rapa*

Inheritance of seed coat color was analyzed using 224 F_2 individuals from a cross of "SPAN × BARI-6". It was found that seed coat color was mainly controlled by the genotypes of plants bearing seeds; therefore brown F_1 seeds were produced when a brown-seeded variety was used as female parent. A pollen effect was observed when yellow sarson was used as the female parent so the F_1 seeds were dark yellow instead of bright yellow. Seeds on F_2 plants segregated into brown, yellow-brown and bright yellow color groups (Fig. 1), indicating incomplete dominance of the brown color as described by Shirzadegan (1986). Of 224 F_2 plants, 164 had brown seed color, 48 had yellow-brown seed color and 12 had bright yellow seed color. The Chi-square test showed that seed coat color segregated in a ratio of 12:3:1 ($\chi^2 = 1.238$, $P = 0.5$–0.7), confirming digenic inheritance of the trait. However, when the 164 brown seeded plants were placed in one group and the 60 yellow-brown and bright yellow seeded plants were placed in another group, seed coat color segregated in a monogenic inheritance pattern ($\chi^2 = 0.381$, $P = 0.5$–0.7).

Self-pollinated seeds of 197 BC_1 plants from the [(SPAN × BARI-6) × BARI-6] cross were also used for seed coat color segregation analysis. The seed coat colors in BC_1 also segregated into brown, yellow-brown and bright yellow classes. Of the 197 BC_1 plants, 95 had brown seed color, 55 had yellow-brown seed color and 47 had bright yellow seed color. Chi-square tests showed that the progenies fit a digenic (2:1:1, $\chi^2 = 0.898$, $P = 0.5$–0.7) segregation ratio for seed coat color. However, when the 95 brown seeded plants were placed in one group and the 102 brown yellow and bright yellow seeded plants were placed in another group, seed coat color segregated in a monogenic manner (1:1, $\chi^2 = 0.248$, $P = 0.5$–0.7).

Fig. 1 Seed coat color segregation in the progenies of a cross of yellow-seeded 'BARI-6' and 'brown-seeded 'SPAN'. Seeds in the *left* Petri dish representing 'SPAN' and *brown*-seeded progeny; in the *middle* Petri dish, *yellow-brown* progeny; in the *right* Petri dish, 'BARI-6' and *bright-yellow* progeny

SRAP molecular markers for seed coat color

Forty-eight different SRAP primer pairs were used for the development of molecular markers for the seed coat color trait in *B. rapa.* Initially, sixteen brown-seeded lines and sixteen bright yellow-seeded lines from the BC_1 population were used for the identification of molecular markers using all 48 primer combinations. The markers SA7BG29-245, ME2FC1-266, FC1BG69-530, PM88PM78-435, SA12BG 18-244 and SA12BG38-306 were found to be linked to the seed coat color with few recombinants. After testing these markers using the F_2 and BC_1 generations, the marker SA7BG29-245 was found to be closely linked to seed coat color. There were two recombinant alleles in a total of 448 alleles in the 224 F_2 plants, equal to a genetic distance of 0.47 cM between the molecular marker SA7BG29-245 and the seed coat color gene. Similarly, in the BC_1 generation, there were two recombinant alleles in a total of 197 F_1-derived alleles used to produce this generation, equal to a genetic distance of 1.02 cM between the molecular marker SA7BG29-245 and the seed coat color gene.

Chromosome walking and SNP development

The SRAP molecular marker SA7BG29-245 was sequenced and its flanking sequences were obtained by chromosome walking. Two-step PCR reactions were performed. The first PCR amplification using the left side marker specific primer MWalk27 and adaptor specific primer AP1 produced a smear in all lanes (Fig. 2a). The second PCR amplification using the adaptor specific primer AP2 and marker specific primer MWalk28 produced a single strong band with *Eco*RV and *Pvu*II (Fig. 2b). Similarly, the first PCR amplification using the adaptor specific primer AP1 and marker specific primer MWalk24 from the right end generated a smear in all lanes (Fig. 2c); and the second PCR amplification using the adaptor specific primer AP2 and marker specific primer MWalk25 generated two strong bands with *DraI* and *StuI* (Fig. 2d). A total of 529 bp was extended from left end and 427 bp from right end and in total an 1,170 bp fragment was obtained from brown

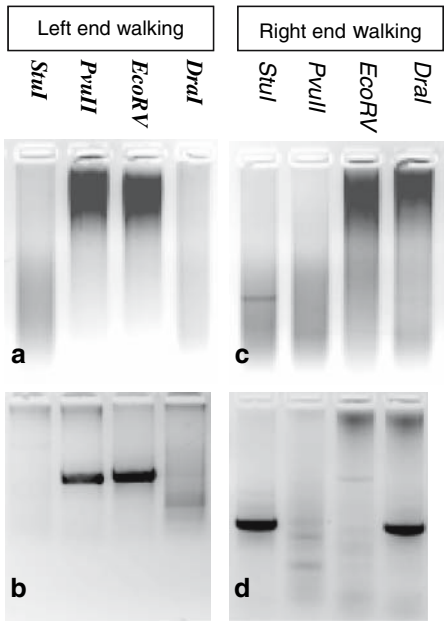

Fig. 2 PCR walking from left end and right end of the marker (SA7BG29-245) sequence. Two-step PCR using primer combination AP1/MWalk27 and AP2/MWalk28 from the left end; and another two-step PCR from the right border with the primer combinations AP1/MWalk24 and AP2/MWalk25 were performed. The DNAs were taken for first PCR and second PCR from four different genomic libraries constructed by *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. **a** AP1+MWalk27, first round PCR; **b** AP2+MWalk28, second round PCR; **c** AP1+MWalk24, first round PCR; **d** AP2+MWalk25, second round PCR

seeded variety "SPAN" (GenBank Accession Number EF488953, EF488954). Unfortunately, the sequence did not match any gene in *Arabidopsis* after BLAST analysis against the *Arabidopsis* database (http://www.arabidopsis.org). After sequencing the corresponding region in the yellow-seeded parent, 24 SNPs were found between the brown-seeded and yellow-seeded parent lines (S1 in supplementary material). The SNPs were detected with an ABI SNaPshot Multiplex kit. For example, one SNP position (at 1,041 bp position of 'SPAN') for homozygous brown seed color was 'C' and generated a black peak, heterozygous plants, 'C/T', generated both a black peak and a red peak,

and homozygous yellow-brown or bright yellow seed coat color, 'T', generated a red peak (Fig. 3). Since the marker was closely linked to the major seed coat color gene *Br1/br1*, the black peak identified homozygous brown seed color Br1Br1 genotypes; the dual black and red peaks identified heterozygous brown seed color Br1br1 genotypes; while the red peak identified homozygous bright yellow or yellow-brown seed color br1br1 genotypes. The SNP markers were tested using both the F_2 and BC_1 generations, and were found to be at the same genetic distance from the seed coat color gene as the SRAP molecular marker SA7BG29-245.

Development of multiplexed SCAR markers

On the basis of 1,170 bp for the SRAP marker and its flanking sequences, no deletion or insertion polymorphic region was found between brown and yellow seeded lines. Therefore, chromosome walking was performed again to obtain additional extended flanking sequence from the left side. With the new chromosome walking sequence, a 12-bp deletion in the brown seeded lines or a 12-bp insertion in the yellow-brown or bright yellow seeded lines was identified (S1 in supplementary material), which were used for the development of multiplexed SCAR markers. Primers MR1313 and MR54 were designed to target the 12-bp deletion. Together with the 19-bp M13 sequence, a 388-bp fragment for brown seeded lines and a 400-bp fragment for yellow-brown or bright yellow seeded lines were produced, respectively (Fig. 4). Since the SCAR marker was not far from the SRAP marker and SNPs mentioned previously, the genotyping of the SCAR marker in 224 F_2 plants and 197 BC_1 plants were exactly the same as that of the SRAP and SNP markers.

Discussion

The *B. rapa* yellow sarson parent line variety "BARI-6" was taxonomically different from the Canadian *B. rapa*

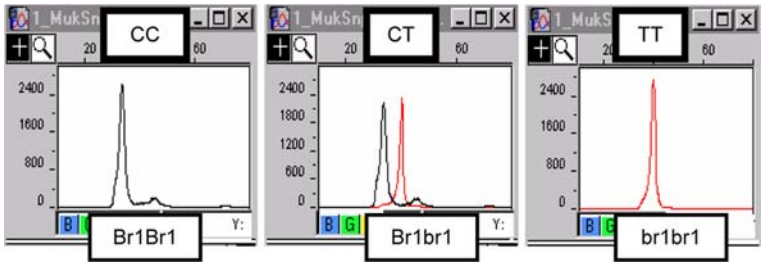

Fig. 3 Figure showing SNP detection by GeneScan software (ABI 3100 genetic analyzer) to analyze the SNaPshot Multiplex kit data. The peak information was transformed manually for each locus (e.g. *black* for 'C' and the genotype *Br1Br1*; *red* for 'T' and genotype *br1/br1*; and *black/red* for 'C/T' and genotype *Br1/br1*)

Fig. 4 Multiplexed SCAR marker linked to seed coat color was detected in ABI 3100 genetic analyzer using four different fluorescently labeled primers M13 with unlabeled MR1313 and MR54. The marker linked to the *brown* seed gene (*Br1Br1*) produced 388 bp, the *yellow* or *yellow-brown* (*br1br1*) gene generated 400 bp and the heterozygotes (*Br1br1*) produced both 388 and 400 bp fragments. **a** SCAR marker segregation in the *brown*-seeded, *yellow*-seeded and F_1 genotypes; **b** SCAR marker segregation in the F_2; **c** SCAR marker segregation in the BC_1

parent line variety "SPAN". Yellow sarson belongs to ssp. *trilocularis* and is self-compatible, while "SPAN" belongs to ssp. *oleifera* and is self-incompatible. Using a self-compatible parent in the cross made it easier to self plants in the greenhouse. A pollen effect was observed when yellow sarson was used as the female parent, resulting in dark yellow F_1 seeds instead of bright yellow F_1 seeds. This pollen effect is known as a Xenia effect in yellow sarson and could be used as an indicator for successful crosses. This phenomenon was also observed by Rahman et al. (2001) who used an open pollinated yellow-seeded *B. napus* line that was derived from yellow sarson, suggesting that yellow sarson contains the gene(s) for Xenia effect. Stefansson and Hougen (1964) reported such effect of pollen (Xenia effect) on the fatty acid profile of the seeds of self-pollinated and reciprocal cross-pollinated *B. napus* plants.

Digenic inheritance with dominant epistasis was observed for seed coat color segregation in *B. rapa.* The dominant epistatic gene was responsible for brown color and the hypostatic gene was responsible for yellow-brown seed color, and yellow seed color was observed when both the genes were in homozygous recessive condition. These results confirm the seed coat color segregation results reported by Stringam (1980) and by Rahman (2001).

A dominant SRAP marker is less convenient than a co-dominant marker for large scale MAS in plant breeding. Consequently, the dominant SRAP marker developed in this study was converted to co-dominant SNP and SCAR markers, following the lead of several researchers who converted their dominant markers into co-dominant markers, such as SCAR marker from RAPD markers (Naqvi and Chattoo 1996; Lahogue et al. 1998; Barret et al. 1998) and AFLP markers (Negi et al. 2000; Adam-Blondon et al. 1998; Bradeen and Simon 1998), and SCAR and CAPS markers from RAPD and AFLP markers (Liu et al. 2006). There was no difference between brown-seeded and yellow-seeded lines in the 214 bp sequence of the SRAP marker. A single nucleotide polymorphic position is required for the development of co-dominant SNP markers. Co-dominant SCAR markers are developed from insertion or deletion fragment positions in any of the two sequences. Development of CAPS markers remains DNA fragment in the size range 500–1,500 bp (Barret et al. 1998). Therefore, a 214 bp SRAP sequence limits the development of any co-dominant SNP, SCAR or CAPS markers. However, the extended flanking sequence from the SRAP marker allowed the development of SCAR or SNP co-dominant markers. A chromosome walking approach was used to obtain the flanking sequence adjacent to the SRAP marker. It has been proven that chromosome walking is one of the best methods for determining the flanking sequence adjacent to a sequence of interest (Devic et al. 1997, Negi et al. 2000). Negi et al. (2000) successfully converted AFLP markers to SCAR markers using the chromosome walking method and isolated the large-sized fragments adjacent to the AFLP markers, which did not require any optimization for different walking. We obtained more than 1.8 kb flanking sequences from the SRAP markers that showed 24 SNPs and a 12 bp deletion or a 12 bp insertion site, which allowed development of SNP markers and SCAR markers, respectively.

The SNaPshot method used in this study is simple, requires very little optimization and is high throughput using an ABI 3100 genetic analyzer (Pati et al. 2004). SNP markers are co-dominant, they are found more abundantly in genomic sequences and therefore can potentially be used for MAS. The SNP markers developed in this study used to screen the F_2 and BC_1 generations showed the same pattern as the SRAP marker, indicating that the SRAP marker was successfully converted into SNP markers that were closely linked to the *Br1* seed coat color gene. The major shortcoming of the SNP marker approach is cost.

A cost effective alternative to SNP markers are SCAR markers, most especially multiplexed SCAR markers. In this study, a 12-bp deletion in the brown seeded lines allowed the development of multiplexed co-dominant SCAR markers. Here we used four fluorescently labeled M13 primers with single unlabeled primer that allowed pooling four PCR products for the detection in an ABI 3100 genetic analyzer (four fluorescently labeled M13 primers were universally used to combine with any co-dominant multiplexing SCAR markers in our laboratory). However, in principle, any primers covering this 12 bp deletion region would produce two bands with a 12 bp sequence difference. Using the M13 primer labeled with four fluorescent dye colors and a series of primers that produced fragments 12-bp different in length permitted the pooling of several hundred amplified DNA samples for signal detection using the ABI Genetic Analyzer. Multiplexed SCAR markers can reduce the running cost of the ABI DNA Genetic Analyzer dramatically and significantly increase the efficiency of MAS in a breeding program compared to the high cost of SNP detection. For example, we designed 20 unlabeled primers to target a two-base deletion position in the *Bn-FAE1-2* gene of the C genome of *B. napus* and combine with a genome-specific primer that was labeled with four fluorescent colors to form 80 primer pairs in total, and each primer pair was used to amplify different DNA samples. After PCR, 80 samples were pooled and 1,280 (16×80) samples were analyzed with an ABI 3100 Genetic Analyzer in 40 min (unpublished data). The running cost was reduced by 80 times compared with that of SNP detection with the ABI SNaPShot detection kit. Actually more unlabeled primers could be designed to increase the pooled samples to reduce the cost further. Therefore, multiplexing any co-dominant SCAR markers targeting

deletions or insertions (INDELs) has great potential for MAS in plant breeding if a sample pooling strategy as described in this report is implemented.

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