GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Identification of sequence-related amplified polymorphism and insertion-deletion markers linked to the male fertility restorer gene of *pol*-like CMS06J45 in heading Chinese cabbage (*Brassica rapa* subsp *pekinensis*)

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Genet. Mol. Res. 13 (4): 9606-9614 (2014)
Received September 30, 2013
Accepted September 10, 2014
Published November 14, 2014
DOI http://dx.doi.org/10.4238/2014.November.14.4

ABSTRACT. In order to map the restorer gene *BrRfp* of the *polima* (*pol*)-like cytoplasmic male sterility (CMS) 06J45 line in heading Chinese cabbage, an F_2 segregating population with 258 individuals of CMS06J45 and the restorer line 01S325 were tested by sequence-related amplified polymorphism (SRAP) and insertion-deletion (InDel) technologies combined with the bulked segregant analysis method. As a result, two SRAP markers, me3em3.366 and pm88bg5.263, that were linked with the *BrRfp* gene were identified from 463 SRAP primer pairs. By cloning, sequencing, and basic local alignment search tool analysis, the two markers were targeted to the BGIScaffold000053 of *Brassica rapa* in the *Brassica* database. Using the BGIScaffold000053 sequence, four InDel primer pairs were designed and identified to be linked with

the *BrRfp* gene in this population. Linkage analysis showed that these markers were distributed on both sides of the *BrRfp* gene, the linkage distances of two nearest markers InDel878.1125 and InDel920.713 were 0.82 and 0.46 cM, respectively, and the *BrRfp* gene was restricted to a 243-kb genomic region of *B. rapa*. These specific markers provided basic information for map-based cloning of the *BrRfp* gene and will be very valuable for the marker-assisted selection of a new restorer line in heading Chinese cabbage.

Key words: *BrRfp*; Heading Chinese cabbage; Insertion-deletion; Sequence-related amplified polymorphism; CMS06J4

INTRODUCTION

The cytoplasmic male sterility (CMS) and *Rf* systems have been widely used in commercial hybrid breeding. A chimeric open reading frame (ORF) in the mitochondrial genome was supposed to be responsible for CMS in different plant species, which can be restored specifically by nuclear fertility restorer genes (*Rf*) (Hanson and Bentolila, 2004).

The *pol* cytoplasm from the Polima oilseed rape cultivar contains the *ORF224* gene in the upstream region of the ATP synthase F0 subunit 6 (*atp6*) (Singh and Brown, 1993). In our lab, the *pol* cytoplasms of oilseed rape (*Brassica napus*) and *pol*-like cytoplasms of oilseed rape shaan77A were successfully transferred into the heading Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), and two sterile lines, CMS3411 and CMS7311, respectively, were bred by repeated backcrossing (Ke et al., 1992; Zhang and Hao, 2001). Genetic analyses have shown that this *pol*-like CMS could be restored by the expression of a dominant *BrRfp* gene in heading Chinese cabbage (Zhang and Ke, 1994). The sterility of *pol*-like CMS is temperature sensitive, and can be converted by exposing the plant with the flower bud to a low temperature for three days or more (Zhang and Hao, 2001). An isoform of *β*-esterase was believed to be related to this fertility conversion (Zhang et al., 2008). After sequencing and translated basic local alignment search tool (BLASTx) analysis, the isoenzyme of β-esterase was identified as P^{27}(SJ) protein (Zhang et al., 2010). However, the mechanism of fertility conversion remains enigmatic. We hypothesized that the *BrRfp* gene might be implicated in this process. To expound this proposal, we need to map and subsequently clone the *BrRfp* gene.

In this report, the sequence-related amplified polymorphism (SRAP) and insertion-deletion (InDel) technologies were combined with the bulked segregant analysis (BSA) method to map the restorer gene (*BrRfp*) in an F_2 separation population composed of 258 individuals in heading Chinese cabbage. The objective of this study was to identify molecular markers linked to *BrRfp* that would provide basic information for marker-assisted selection (MAS) and map-based cloning of *BrRfp* in heading Chinese cabbage.

MATERIAL AND METHODS

Plant material and fertility identification

The *pol*-like CMS line 06J45, derived from CMS7311 (Zhang and Hao, 2001), and its restore line 01S325 were crossed, and a single hybrid F_1 plant was self-pollinated to generate an F_2 generation. A total of 258 individuals of the F_2 population were used to map the *BrRfp*

gene. Each male fertile individual of the F_2 generation was self-crossed at the bud stage to produce its progeny, F_3, and almost 30 of these individuals were used to test the homozygous or heterozygous nature of their corresponding F_2 individuals. The male fertility of each individual was checked by observing each plant three times at 10-day intervals during the flowering stage.

Genomic DNA extraction and bulk DNA construction

Young leaves from individuals of the F_2 generation were used to extract genomic DNA by the cetyltrimethylammonium bromide method (Porebski et al., 1997). The DNA quality was checked using 0.8% agarose gel electrophoresis and a spectrophotometer. The genomic DNA was diluted to 50 ng/μL. Equal quantities of genomic DNA of flower buds from 12 male fertile and 12 male sterile individuals that were randomly selected were used to construct fertile (Mf) bulks and sterile (Ms) bulks, respectively, for further screening.

SRAP marker screening and sequence analysis

The SRAP technique was performed following the protocol that was first developed by Li and Quiros (2001). The first five cycles were denaturing at 94°C for 1 min, annealed at 35°C for 1 min, and extended at 72°C for 1 min. Then, the annealing temperature was raised to 50°C for another 35 cycles. Furthermore, a new polymerase chain reaction (PCR) procedure was also employed, in which the annealing temperature was changed to 45°C for the first five cycles. The primer sequences were adapted from Li and Quiros (2001) and Sun et al. (2007) (Table 1). PCR amplification was carried out with two DNA bulks in a 25-μL volume containing 50 ng template DNA, 2.5 μL 10X PCR buffer, 2.0 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTPs, 1.0 U Taq (Takara Biotechnology (DaLin) company limited, China), and 1.0 μL 10 μM of each primer. PCR products were separated on a 6% polyacrylamide gel and visualized with silver staining.

The linked bands were cloned and sequenced, the sequences were investigated in the *B. rapa* database (http://Brassicadb.org/brad/) (Cheng et al., 2011) by BLAST analysis, and sequences were compared with the genetic linkage map of *B. rapa* (Sun et al., 2007; Li et al., 2011) to search for the nearest markers.

Table 1. Nucleotide sequences of the related sequence-related amplified polymorphism (SRAP) and insertion-deletion (InDel) primers.

Primer pair	Forward/reverse primer	Product size (bp)[a]
me3em3	Me3: 5'-TGAGTCCAAACCGGAAT-3' Em3: 5'-GTCTGCGTACGAATTGAC-3'	366/372
pm88bg5	Pm88: 5'-CGAAACCTCACCTCTCTCA-3' Bg5: 5'-GTCAAAGAAGGCTCAAGGCA-3'	262/-
InDel837	5'-ATGCTGGATAAGTATGGTGACA-3' 5'-CAACACACTAATCCGAGCACAT-3'	768/720[b]
InDel878	5'-ATCATATCCTGTCTATTTCGTGCT-3' 5'-GTTATAACGAGGCACATAGGTTTC-3'	1125/936
InDel920	5'-GTCTCTGATGTATGTTTGCGTTG-3' 5'-ACAGATTCCACATTCTTCCTCAC-3'	713/-
InDel980	5'-GATGCGGAGAAATGTATTGAG-3' 5'-GAATCTTGCTCTGAATCACTGG-3'	680/673

[a]The product size was shown as fertile/sterile male individuals. [b]The product size was predicted without sequencing; (-) = no amplification.

Designing and screening of InDel markers

InDel primer pairs were developed based on the sequence of *B. rapa* restricted by SRAP markers. PCR amplification was carried out using two DNA bulks in a 25-μL volume like the SRAP system and amplified with the following cycling parameters: an initial denaturing at 94°C for 4 min; 35 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining. The related InDel primer sequences are listed in Table 1.

Linkage analysis and map construction

The linkage markers were screened with the F_2 population, and the molecular marker data were combined with the phenotypic data describing the fertility for linkage analysis using the Joinmap 3.0 software (Van Ooijen and Voorrips, 2001). A logarithm of the odds value of 3.0 was set as the lowest limit for map construction. The recombination values were converted into a genetic map distance (cM) using the Kosambi mapping function (Kosambi, 1943; Huehn, 2010).

RESULTS

Genetic analysis of the *BrRfp* gene

An F_2 population containing 258 individuals was used for the genetic analysis of the restorer gene. A phenotypic investigation showed that 186 individuals were fertile male plants and 72 were sterile male plants. The ratio of fertile male plants to sterile male plants was consistent with the ratio of 3:1 ($\chi^2 = 0$, $P > 0.05$), which further confirmed dominant single-gene to control the male fertility in CMS06J45.

Identification of SRAP markers

Initially, 88 SRAP primer pairs were used to screen DNA polymorphism between Mf and Ms bulks. One primer pair, me3em3, amplified a specific band of about 360 bp in only the Mf bulk. This band was further identified to be consistent with male fertile individuals of the Mf bulk. Furthermore, a new, larger band that was identified in male sterile and hybrid plants was also consistent with sterile male individuals and heterozygous individuals (Figure 1A). The exact sizes of these bands were 366 and 372 bp and were therefore named me3em3.366 and me3em3.372, respectively. By sequencing and alignment analysis, we found that the sequences of these two bands were highly similar to each other except two small fragments of insertion and deletion, and both mapped to the same site of BGIScaffold000053 in the *B. rapa* database (Figure 1B). Unfortunately, these markers could not be found in any reported genetic linkage map of *B. rapa* or related species, which obstructed us from further identifying any closer markers based on the previous genetic linkage map.

Next, 234 primer pairs spanning 10 chromosomes of *B. rapa* were selected randomly based on the reported genetic linkage map (Sun et al., 2007) and were further screened between the Mf and Ms bulks. One specific band that was amplified by primer pair pm88bg5 was much

stronger in the Mf bulk than in the Ms bulk, and the same phenomenon was verified in 16 randomly selected individuals from the F_2 population (Figure 2A). To remove the faint band from Ms plants, we tried to raise the original annealing temperature, and we found that an annealing temperature of 45°C during the first five cycles could obviously reduce the quantity of the faint band (Figure 2B). Sequencing showed that the exact size of this band was 263 bp, so this marker was named pm88bg5.263. The sequence of this marker was further used in BLAST analysis of BGIScaffold000053 of *B. rapa* with a physical distance of about 1.1 Mb from the targeted site of the me3em3 marker (Figure 2C). In addition, this marker seemed to correspond to pm88bg5 263 bp in the linkage group N09 of *B. rapa* from Sun et al. (2007). Hence, 141 primer pairs around pm88bg5 263 bp from Sun et al. (2007) were further used to screen the two bulks, but no primer pair displayed any differences in polymorphism. Therefore, in total, 463 SRAP primer pairs were screened and two SRAP markers that were linked to the *BrRfp* gene were identified.

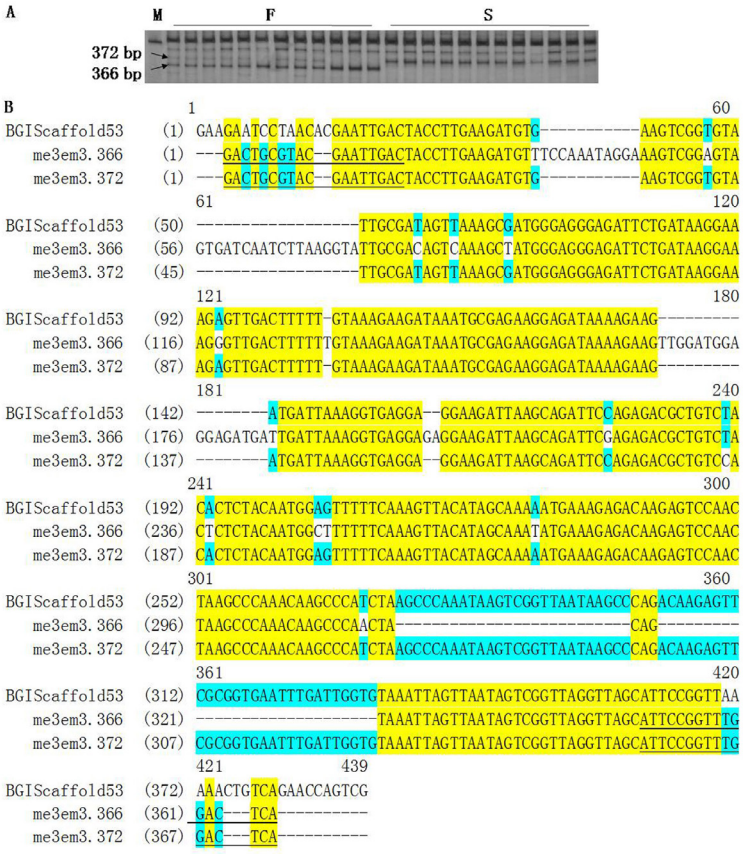

Figure 1. Sequence-related amplified polymorphism (SRAP) marker of primer pairs me3em3 linked to the restorer gene. **A.** Primer pair me3em3 amplified two specific bands of 366 and 372 bp that were linked to *BrRfp* and *Brrfp*, respectively. F = fertile male individuals; S = sterile male individuals; M = DL2000 marker. **B.** Alignment of three sequences: me3em3.366, me3em3.372, and BGIScaffolld00053. me3em3.366 was the marker linked to the male fertility gene *BrRfp*; me3em3.372 was the marker linked to the male sterility gene *Brrfp*; BGIScaffold53 was the part of BGIScaffold00053 sequence from the *Brassica rapa* database. The highlighted nucleotides indicate the same sequence. The underlined nucleotides were the primer sequences.

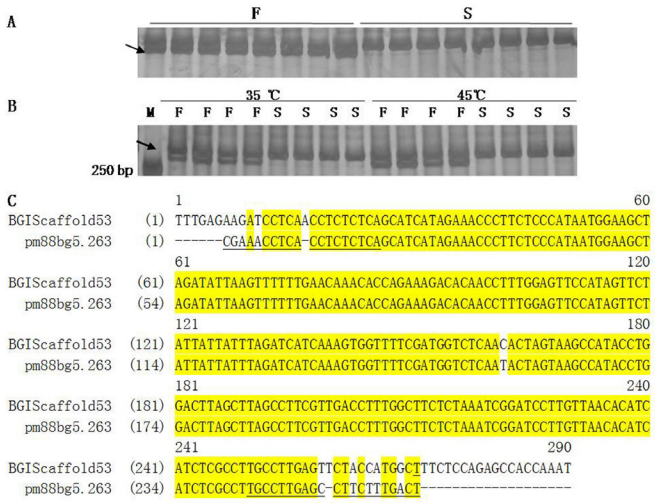

Figure 2. Sequence-related amplified polymorphism (SRAP) marker pm88bg5.263 was linked to the *BrRfp* gene. **A.** The SRAP primer pair pm88bg5 amplified a fragment of 263 bp that produced a stronger band in Mf individuals than in Ms individuals. *lanes F* = fertile male individuals; *lanes S* = sterile male individuals; *lane M* = DL2000 marker. **B.** Fragment amplified by the SRAP primer pair pm88bg5 was much weaker when amplified at the higher annealing temperature of 45ºC than at 35ºC in sterile male individuals, while there was no obvious difference in intensity of the band in fertile male individuals between the two annealing temperatures. **C.** Sequence alignment of the pm88bg5.263 and BGIScaffold00053 sequences. pm88bg5.263 was the marker that was linked to the male fertility gene *BrRfp*; BGIScaffold53 was the BGIScaffold00053 sequence from the *Brassica rapa* database. The highlighted nucleotides indicate the same sequence; the underlined nucleotides indicate the primer sequences.

Identification of InDel markers

Using the sequence of BGIscaffold000053, a set of PCR primer pairs with predicted fragment sizes that ranged from 500 to 1500 bp were designed and screened in the Mf and Ms bulks. Finally, four InDel markers, including InDel878, InDel920, InDel837, and InDel980, amplified obviously different products between the two bulks. Among them, the InDel878 primer amplified two bands, including a larger band of about 1125 bp that only appeared in Mf individuals and a band that was about 189 bp smaller that only appeared in Ms and heterozygous Mf individuals (Figure 3A). Interestingly, InDel920 amplified a single fragment of about 750 bp in Mf individuals, but it did not amplify any fragments in Ms individuals, which was like a sequence-characterized amplified region marker (Figure 3B).

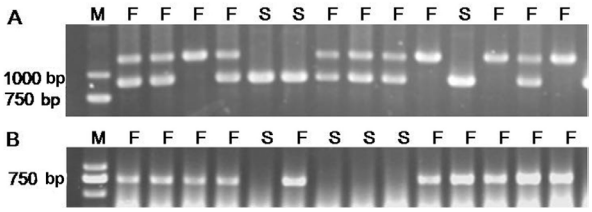

Figure 3. Insertion-deletion (InDel) markers linked to the *BrRfp* gene in heading Chinese cabbage. **A.** InDel878 primer pair amplified two specific bands; the smaller band corresponds to the sterile male individuals and the larger band corresponds to the fertile male individuals. The lanes with two bands indicate heterozygous fertile individuals. **B.** InDel920 primer pair amplified only a specific fragment of with 750 bp from fertile male individuals. *lane F* = fertile male individuals; *lane S* = sterile male individuals; *lane M* = DL2000 marker.

Linkage analysis and genetic mapping of the *BrRfp* gene

These markers were further screened in 258 individuals of the F_2 population. Linkage analysis of the F_2 population showed that these six markers were located on both sides of the *BrRfp* gene. The nearest markers were InDel878.1125 and InDel920.713, which had distances of about 0.82 and 0.46 cM, respectively (Figure 4). As a result, the *BrRfp* gene was limited to a 243-kb genomic region of *B. rapa* based on the genome sequence of the *Brassica* database (http://Brassicadb.org/brad/) (Cheng et al., 2011).

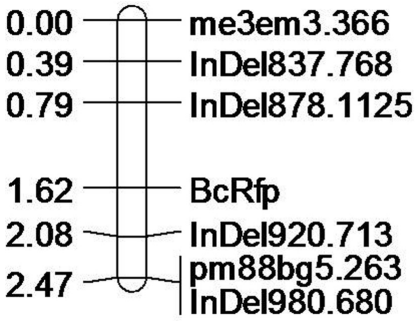

Figure 4. Linkage map of the *BrRfp* gene. Numbers on the left indicate the genetic distance (cM), and labels on the right indicate the names of molecular markers.

DISCUSSION

MAS has been widely used in the process of hybrid breeding (Xiao et al., 2012). Previously, a set of molecular markers linked to multiple characteristics has been developed for MAS (Hagihara et al., 2005; Rahman et al., 2007). Furthermore, some genes, such as the restorer genes *RF1A* and *RF1B* of *Boro* II cytoplasm in rice (Wang et al., 2006), have been successfully cloned by molecular marker-based fine mapping. In our laboratory, 21 tightly linked simple sequence repeat and InDel markers were identified and delimited the *Br-or* locus to a 16.7-kb genomic region of *B. rapa* (Zhang et al., 2013). In a preliminary study, we screened 500 RAPD primers, but we failed to find the linked marker. Here, a set of SRAP and InDel primer pairs were screened with BSA technology, and six markers linked to the *BrRfp* gene were identified.

In PCR, the annealing temperature is one of the important parameters. Both sub- and super-optimal annealing temperatures may form non-specific products or reduce the yield of products (Rychlik et al., 1990). This characteristic has been widely used for PCR-based marker selection and allele-specific PCR (Liu et al., 2012a), where the product yield was usually affected by different annealing temperatures. SRAP is also a PCR-based marker technology. The initial annealing temperature for the first five cycles was set at 35ºC to ensure the binding of both primers to sites with a partial match in the target DNA (Li and Quiros, 2001). However, a specific marker, pm88bg5.263, was identified by modifying the annealing temperature in this study. The 263-bp band of interest was weaker in our Ms individuals than in the Mf individuals when the initial annealing temperature was 35ºC, and it became weaker as the annealing temperature was raised to 45ºC. The BLAST result of pm88bg5.263 in the *B. rapa* database suggested that there was a highly similar sequence that may be amplified with

low PCR efficiency (data not shown). This was the first SRAP marker that was identified by modifying the annealing temperature; this suggested that some amplification products that differ in quantity may change with differences in the PCR quality.

Tetraploid *B. napus* was supposedly derived from *B. rapa* and *Brassica oleracea* through interspecific breeding (Lysak et al., 2007). Two independent groups have independently limited the *Rfp* gene to the BGIScaffold000053 genome region of *B. rapa* (Formanová et al., 2010; Liu et al., 2012b). It is interesting that our results also restricted the *BrRfp* gene to the adjacent locus of *B. rapa* with the two markers InDel878.1125 and InDel920.713. These results suggested that the restorer genes of *pol* CMS and *pol*-like CMS may belong to the same gene family or are arranged in series, which needs to be verified by new markers and a larger population.

In summary, two SRAP and four InDel markers were identified to be linked with the *BrRfp* gene. Of these, the two nearest markers, InDel878.1125 and InDel920.713, restricted the *BrRfp* locus to a 243-kb genomic region of *B. rapa*. These results provide basic information for the map-based cloning of the *BrRfp* gene and will be helpful for the MAS of new restorers in heading Chinese cabbage.

ACKNOWLEDGMENTS

Research was supported by a National Natural Science Foundation of China grant to Lugang Zhang (#30871717) and the start-up Foundation of Shanxi Agricutural University (#2013YJ46).

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