Development of Microsatellite Markers by ISSR-suppression-PCR Method in *Brassica rapa*

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Microsatellites or simple sequence repeats (SSRs) consist of repeated arrays of short nucleotide motifs 1–4 bp in length. These regions are interspersed throughout eukaryotic genomes (Hamada et al. 1982, Tautz and Renz 1984, Tautz 1989), and are generally embedded in unique DNA sequences. Microsatellites are characterized by a high degree of polymorphism, most of which results from the variation in the repeat number (Weber and May 1989, Beckman and Soller 1990). Because of their hypervariability, reproducibility, easy PCR detection, Mendelian inheritance, codominant nature and abundance in the genomes, microsatellites can be used as DNA markers for the analysis of genetic polymorphisms in the fields of population genetics (Jarne and Lagoda 1996) and breeding (Weising et al. 1998). Moreover, since microsatellites have unique flanking sequences for locus-specific amplification, protocols and data can be readily exchanged among different laboratories and the results obtained can be easily compared with published primer pair sequences.

The isolation of microsatellites is usually labor-intensive, time-consuming and expensive. To resolve these problems, several protocols have already been developed, mainly involving sophisticated enrichment techniques such as primer-extension approach (Ostrander et al. 1992), the capture techniques including streptavidin-coated magnetic beads (Kijas et al. 1994) and triplex affinity capture (Nishikawa et al. 1995). However, these protocols still require the construction of a genomic library and a rather long step. On the other hand, Lian et al. (2001, 2003) proposed the ISSR-suppression-PCR method that is relatively rapid and simple without enrichment and screening. This method requires only basic skill in molecular biology and the time for procedures has been shortened.

*Brassica* species are used as oilseed crops (*B. napus* and *B. juncea*), leafy vegetables and turnip (*B. oleracea* and *B. rapa*), and are cultivated worldwide. Especially, in East Asia, many varieties of *B. rapa* are used as agronomically important vegetables. For *B. rapa*, several molecular linkage maps have been constructed (Song et al. 1991, Chyi et al. 1992, Ajisaka et al. 2001, Nishioka et al. 2005) and mapping of loci controlling traits of interest has been reported (Teutonico and Osborn 1995, Kole et al. 1996, Ignatov et al. 2000).

The abundance, characterization and the usefulness of the microsatellites of *Brassica* species have been previously reported (Lagercrantz et al. 1993, Kresovich et al. 1995, Uzunova and Ecke 1999). However, only a limited number of microsatellite markers has been developed from *Brassica* species (Szewe-McFadden et al. 1996, Westman and Kresovich 1999, Plieske and Struss 2001, Saal et al. 2001, Suwabe et al. 2002, 2004), compared with that from other agronomically important crops such as maize (Sharopova et al. 2002) and wheat (Khlestkina et al. 2002). The development and sharing of microsatellite markers which involve a comparison of different linkage maps, thus facilitate future marker-assisted selection (MAS) for the breeding of oilseed and vegetable crops. Therefore, we attempted to isolate microsatellites from *B. rapa* using the ISSR-suppression-PCR method.

**Plant materials**

*B. rapa* lines var. *pekinesis*; A9709, A9408 and Homei P09 were used. They were developed at the National Institute of Vegetable and Tea Science. Osaka shirona, Saiou and Maikaze are commercial cultivars of *B. rapa* var. *pekinesis* and Ai-kang that was developed at Nanjing Agricultural University is a cultivar of *B. rapa* var. *chinensis*. They were used for polymorphic analysis. Total DNA was isolated from leaves by the modified CTAB method (Murray...
and Thompson 1980). To integrate the isolated microsatellite markers into the genetic linkage map of *B. rapa* (Nishioka et al. 2005), 200 doubled haploid (DH) lines derived from A9408 × Homei P09 were used.

**Isolation of microsatellites**

Isolation of TG-motif microsatellites was performed by ISSR-suppression-PCR, as outlined by Lian et al. (2001, 2003). *B. rapa* line, A9709 and a cultivar, Ai-kang were used for the isolation of microsatellites. Total DNA was separately digested with *Eco* RV, *Ssp* I, *Alu* I, *Acc* II or *Hae* III restriction enzymes. The fragments were then ligated to a specific adapter (consisting of a 48-mer: 5′ GTAATACGAC TCATAAGGGCCAGCCTGACGGGCGGGCT GGTT 3′ and an 8-mer with the 3′-end capped by an amino residue: 5′ ACCAGCCC-NH₂ 3′). To completely block the polymerase-catalyzed extension of the 8-mer adaptor strand, the ligated fragments were further treated with ddGTP using the Ampli Taq Gold Kit (Perkin Elmer, Branchburg, NJ). This treatment was critical to avoid the amplification of fragments between adapter primers.

As a first step, fragments flanked by (AC), microsatellites at one end were amplified from each of the adapter-ligated, restricted DNA fragment pools by the (AC)₉₀ primer and the adapter primer (AP₂ shown below) designed from the longer strand of the adapter. The conditions for the PCR amplification were as follows: 39 cycles of 30 sec at 94°C, 30 sec at the annealing temperature of 60°C and 1 min at 72°C, followed by 1 cycle of 30 sec at 94°C, 30 sec at 60°C and 5 min at 72°C in a 100 µl reaction solution containing 10 ng of adapter-ligated, restricted DNA, 0.5 µM of the primer, 0.2 mM of each dNTP, 1 × PCR buffer with 2.5 mM Mg²⁺ and 0.5 U of LA Taq DNA polymerase (TaKaRa, Otsu, Japan). Two amplified products from the fragment pools digested with *Eco* RV and *Ssp* I exhibited smeared banding patterns in agarose gel electrophoresis (data not shown), indicating that many kinds of fragments were amplified from the two fragment pools. Therefore, the amplified fragments from these fragment pools were directly ligated into pT7 Blue vectors (Novagen, WI, USA), according to the manufacturer’s instructions and the plasmids were transformed into *E. coli* strain XL1-Blue MRF. To select the optimum size of the positive clones, 730 clones were randomly chosen and colony PCR was performed using M13 forward and reverse primers. One hundred and thirty-nine plasmid DNAs with sizes ranging from 300 bp to 700 bp were selected and sequenced. All the sequenced fragments contained microsatellite sequences at their end. Among them, 131 out of 139 clones (94%) had unique microsatellite sequences. The other eight pairs of clones displayed the same sequences. However, primer design became impossible for 45 clones since the flanking regions fully contained microsatellite-like sequences and/or AT-enriched fragments.

The second step was performed to determine the sequence of the other flanking regions of each microsatellite. A total of 86 microsatellite sequences were chosen to design nested PCR primers (IP₁ and IP₂) for the determination of the unknown flanking regions. A primer IP₁ was designed from the sequenced region flanking the microsatellite and, for nested PCR, another primer IP₂ was prepared based on the sequence between IP₁ and the microsatellite. As adapter-primers for nested PCR, AP₁ (5′CCATCGTAATACGACT CACTATAGGCC) and AP₂ (5′CTATAGGGCCACCGCT GGT3′) were also prepared. The primary PCR reaction was conducted with each constructed DNA fragment pool using IP₁ and AP₁ primers. The PCR conditions were as follows: 34 cycles of 30 sec at 94°C, 30 sec at the annealing temperature of 62°C and 2 min at 72°C, followed by 1 cycle of 30 sec at 94°C, 30 sec at 62°C and 5 min at 72°C in a 20 µl reaction solution containing 0.25 ng of adapter-ligated, restricted DNA, 0.5 µM of the primer, 0.2 mM of each dNTP, 1 × PCR buffer with 2.5 mM Mg²⁺ and 0.5 U of LA Taq DNA polymerase (TaKaRa). The secondary PCR reaction was conducted with a 100-fold dilution of the primary PCR products using IP₂ and AP₂ primers. The same conditions as those in the primary nested PCR were used, except that the annealing temperature and cycle number were reduced to 60°C and 29 cycles, respectively. In the secondary step, nested PCRs for 86 microsatellite sequences produced 55 single bands from some restricted DNA pools. Single-banded fragments were cloned and sequenced. All of them were found to contain microsatellites by sequence analysis. A primer from the newly defined flanking sequence was designed for amplification of the region containing a microsatellite in combination with IP₂.

Out of 55 primer pairs used, 51 primer pairs amplified single PCR products using the genomic DNA of A9709 or Ai-kang as a template. The microsatellites isolated in the present study are listed in Table 1. Four primer pairs for which PCR products could not be obtained were eliminated. The repeated number of TG motifs ranged from 4 to 21, with an average of 8.9 repeats. According to the Weber’s criterion (1990) of repeated structure, 43 microsatellites were classified into the perfect type with a continuous run of TG repeats, three belonged to the imperfect type which consisted of a TG repeat run interrupted by other sequences and five belonged to the compound type, harbouring a continuous run of another repeat adjacent to the TG repeat.

The results indicated that the ISSR-suppression-PCR method (Lian et al. 2001, 2003) enabled to develop easily microsatellite markers from the *B. rapa* genome. The major advantage of this method is that library construction and screening can be avoided. Since it has been reported that this method could also be successfully applied to trees and ectomycorrhizal fungi (Lian et al. 2001, 2003), this approach could be easily used for developing microsatellite markers from many other species. Additionally, microsatellites with other motifs, such as (CT/GA) which is more abundant than (AC/TG) in the *Brassica rapa* genome (Suwabe et al. 2002), could be rapidly isolated by only changing a microsatellite primer in the first step of the same procedure.
SSR markers in *Brassica rapa*

To investigate the level of polymorphism of the developed microsatellite loci, attempts were made to detect SSLPs (simple sequence repeat length polymorphisms) among the four cultivars (Osaka shirona, Saiou, Maikaze and Aikang) and two lines (A9709 and Homei P09) in *B. rapa*. The PCR conditions were as follows: 25 cycles of 45 sec at 94°C, 30 sec at 58°C, 1 min at 72°C. The PCR products were

| Marker name (TG)| Repeat motif Forward/IP2 primer sequence (5'-3') Reverse primer sequence (5'-3') Size (bp) Number of alleles |
|-----------------|-------------------------------------------------|--------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mstg001 (TA) | CATGAGTTTTCATATAAATAAA | TATGCAACTTGTCTTTGATAT | 345 | 2 |
| mstg002 (TA) | AAGTTTTCAGTGAACGCTTG | AGCAGTTTTCATTTGATAT | 364 | 3 |
| mstg003 (TG) | TTCCAATGGCTCAGGTGTGTA | TTCTTGTCGTCCGTGGCTTGT | 610 | 2 |
| mstg004 (TA) | CATATAGCATGAGTGGTGC | CTTAAAGGGCACTCTTTCATG | 364 | 3 |
| mstg005 (TG) | GTGAGTTTTCATATAAATAAA | TATGCAACTTGTCTTTGATAT | 345 | 2 |
| mstg006 (TG) | AGTTTTCAGTGAACGCTTG | AGCAGTTTTCATTTGATAT | 364 | 3 |
| mstg007 (TG) | TTCCAATGGCTCAGGTGTGTA | TTCTTGTCGTCCGTGGCTTGT | 610 | 2 |
| mstg008 (TG) | CATATAGCATGAGTGGTGC | CTTAAAGGGCACTCTTTCATG | 364 | 3 |
| mstg009 (TG) | GTGAGTTTTCATATAAATAAA | TATGCAACTTGTCTTTGATAT | 345 | 2 |
| mstg010 (TG) | AGTTTTCAGTGAACGCTTG | AGCAGTTTTCATTTGATAT | 364 | 3 |

The informativeness of microsatellites loci

To investigate the level of polymorphism of the developed microsatellite loci, attempts were made to detect SSLPs (simple sequence repeat length polymorphisms) among the four cultivars (Osaka shirona, Saiou, Maikaze and Aikang) and two lines (A9709 and Homei P09) in *B. rapa*. The PCR conditions were as follows: 25 cycles of 45 sec at 94°C, 30 sec at 58°C, 1 min at 72°C. The PCR products were
separated into 13% non-denaturing polyacrylamide gels. After the run, the gels were stained by Vistra Green (Amersham Biosciences, Piscataway, NJ, USA) and the electrophoretic patterns were detected with Typhoon 9410 (Amersham Biosciences).

In Table 1, the number of alleles for each of the primer sets is listed. Thirty-eight markers (74%) were found to be polymorphic and the number of alleles ranged from 1 to 3 with an average number of 1.9 alleles per primer set. Although Ai-kang is a chinensis variety, only five primer sets, mstg8, 27, 39, 52 and 55, detected the Ai-kang-specific alleles and polymorphisms among pekinensis varieties were detected with 37 primer sets. Figure 1 shows the polymorphisms among four cultivars and two lines using a microsatellite marker, mstg039.

Mapping of microsatellite markers

Linkage analysis was performed to evaluate the distribution of newly found microsatellite loci on the AFLP genetic map of 13 linkage groups which was constructed using the DH lines derived from A9408 × Homei P09 (Nishioka et al. 2005). Attempts were made to detect SSLPs between the mapping parents using the successfully amplified microsatellite loci. Detected polymorphic markers were integrated in the genetic linkage map using MAPMAKER ver. 2.0 (Lander et al. 1987). The Kosambi function was used for calculating the map distances, and a LOD score of 3.0 was used as the threshold for confirmation of linkage.

Fig. 1. Polymorphisms among four cultivars and two lines revealed by a developed microsatellite marker, mstg039. 1; A9709, 2; Homei P09, 3; Osaka shirona, 4; Maikaze, 5; Saiou, 6; Ai-kang

Fig. 2. Segregation of a microsatellite locus, mstg027, among the lines of the doubled haploid mapping population.

Fig. 3. Integration of microsatellite loci into an AFLP linkage map of Brassica rapa. Bold type letters indicate the microsatellite loci on the linkage groups.
After a preliminary assessment, nine out of 51 primer sets were found to be polymorphic in the parents. Using the same mapping criteria as those for the AFLP map, the segregation of the microsatellite loci was analyzed for the 200 lines of the mapping population (Fig. 2). All the microsatellite loci were positioned on seven of the 13 linkage groups and eight new loci were defined among the AFLP markers (Fig. 3).

Cregan et al. (1999) integrated soybean linkage maps using microsatellite markers based on their correspondence with some known linkage maps. Suwabe et al. (2004) reported the isolation of 228 microsatellite markers in B. rapa and illustrated their potential utilization for analysis of other Brassica species. Sharing of primer pair sequences enables to integrate maps, which were constructed using different populations and to analyze genome collinearity in Brassica species. Therefore, all the microsatellite primers designed here could be useful in map integration and synteny analysis among Brassica species.

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Literature Cited


