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Full Length Research Paper

Identification of a microsatellite marker linked to the fertility-restoring gene for a polima cytoplasmic male-sterile line in *Brassica napus* L.

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Polima cytoplasmic male sterility (pol CMS) has been widely used for exploiting heterosis in rapeseed breeding. The dominant restorer gene of pol CMS (*Rfp*) is found in the nucleus and is a key component of hybrid production by achieving F1 progeny with complete fertility restoration. To identify the molecular markers associated with the *Rfp* gene, a near isogenic line (NIL) population of 2,000 individuals segregating for the *Rfp* locus was generated by crossing and backcrossing for 12 times. This NIL population was used to screen *Rfp* markers by AFLP technique. Based on the sequence information of AFLP markers that have been identified in previous research, we identified a homologous region of *Rfp* locus in chromosome 1 of *Arabidopsis*. Then, six sequenced *Brassica rapa* BAC clones corresponding to this target region were chosen to design microsatellite (SSR) primer pairs. Twenty-two SSR markers were designed and one of them, KBrDP1, was verified in the 2, 000 NILs population and proved to be strongly linked to *Rfp* locus. The genetic distance between KBrDP1 and *Rfp* was 0.2 cM. KBrDP1 marker was found located on linkage group N9 of a published DH mapping population. This SSR marker was useful in marker assisted selection breeding of the elite pol CMS restorer lines in rapeseed.

Key words: *Brassica napus*, polima cytoplasmic male sterility, fertility restorer gene, microsatellite.

INTRODUCTION

Heterosis plays an important role in breeding programs to increase crop yield, especially in maize, rice and rapeseed (Obolensky, 1958; Virmani, 1994; Brandle and McVetty, 1990). There are several efficient methods for utilizing heterosis in rapeseed breeding, example, genic male sterile (GMS), cytoplasmic male sterile (CMS), transgenic male sterile (TMS), self-incompatibility (SI) and chemical hybridizing agents (CHA) (Fu, 2000; Daneill, 2002). Among these heterosis utilization methods, CMS has been widely used for rapeseed hybrid production in China (Fu, 1981).

CMS is a maternally transmitted trait that results in the failure of pollen formation, while nuclear genes called fertility restorers (*Rf*) have the ability to suppress the male-sterile phenotype. Therefore, the CMS/Rf system can greatly facilitate hybrid production by eliminating the need for tedious hand emasculation to ensure that each seed is the result of cross-pollination (Hanson, 1991). Polima CMS (pol CMS) has been successfully utilized for rapeseed commercial hybrid seed production since spontaneous male-sterile plants were found in the variety "Polima" in 1972 (Fu, 1981). The pol CMS system can be used in both a three-line system (pol CMS sterile line, maintainer line and restorer line) and a two-line system (pol temperature-sensitive CMS sterile line and restorer line) in commercial hybrid seed production (Fu et al., 1990; Yang et al., 1995). The restorer line plays an

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Abbreviations: pol CMS, Polima cytoplasmic male sterility; **NIL**, near isogenic line; **GMS,** genic male sterile; **CMS,** cytoplasmic male sterile; **TMS,** transgenic male sterile; **SI,** self-incompatibility; **CHA,** chemical hybridizing agents.

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important role in both pollination control systems and this is the reason why breeders pay particular attention to the restorer line during the application of pol CMS.

However, the most time consuming step is identifying the restore gene of pol CMS (*Rfp*) while developing elite restorer lines. The presence of the restore gene can only be identified at flowering and at times only by observing fertility of F1 progeny of sterile line and restorer line. Since the advent of polymerase chain reaction (PCR), various DNA markers (RAPD, SCAR, SSR and AFLP), markers linked to the genes can be used to select plants possessing the target traits efficiently, any time during plant growth. This process is known as marker-aided-selection (MAS) and has encouraged the need for developing a marker for the fertility restorer (*Rf*) gene. To date, studies have shown that pol CMS fertility restorer gene (*Rfp*) is dominant and monogenic, and a number of molecular markers linked to *Rfp* have been reported. Jean et al. (1997, 1998) identified 10 RFLP and one RAPD molecular markers linked to the *Rfp* gene. Wang et al. (2000) identified two RAPD markers and converted these two markers into SCAR markers. Based on one RFLP marker from Jean et al. (1997), Formanova et al. (2006) mapped the *Rfp* gene in *Brassica rapa* and identified a syntenic interval of 4.3 Mb on chromosome 1 of the *Arabidopsis* genome. Liu et al. (2007) identified an AFLP marker and a RAPD marker, Zhao et al. (2008) identified 23 AFLP markers and two out of these markers (SA04TG08, SA07TG01) had been successfully converted into SCAR markers. Additionally, Zeng et al. (2009) identified 13 AFLP markers and five out of these markers have been converted into SCAR markers.

In our laboratory, a number of AFLP, RAPD and SCAR markers linked with *Rfp* gene have been identified (Wang et al., 2000; Liu et al., 2007; Zhao et al., 2008; Zeng et al., 2009). However, in many cases, the implementation of mapping information for marker-assisted selection has not achieved our expectations. Markers for *Rfp* derived from a given mapping population are not necessarily transferable to other restorer lines. It is not efficient to start a new cycle of identifying new markers with each new genetic segregation population. Considering detailed information published on the *Arabidopsis* genome and its syntenic relationships with the *Brassica* genome, an efficient way to construct new PCR markers for the MAS breeding of elite restorer lines should exist.

Microsatellite, also called simple sequence repeats (SSRs), have been widely used in the genetic map construction, MAS breeding, genetic diversity evaluation and variety identification due to abundant polymorphism, reliability and stability reference. The initial process of developing SSR primer sequence was tedious and costly because it is a DNA sequencing based marker; nonetheless, lots of genome sequences and expressed sequence tags (ESTs) have been released online, which results in a few SSR markers successfully developed and used in rapeseed genetic and breeding research programs (Lowe et al., 2004; Piquemal et al., 2005; Cheng et al., 2009). However, there is no SSR marker reported to associate with *Rfp* gene. The purpose of this study was to: (1) Explore a possible way to identify SSR marker associated with target *Rfp* gene; and (2) Verify the SSR marker in different genetic or breeding population.

MATERIALS AND METHODS

Development of a segregating near isogenic line (NIL)

1141A, a polima cytoplasmic male sterility line, was chosen as female parent to cross with Huayehui, a polima cytoplasmic male sterility restorer. Then, F1 progeny was crossed with Wu108, a polima cytoplasmic male sterility maintainer. Next, the fertile plants among the progeny of F1/BCn population were backcrossed for 12 cycles with Wu108 until a near isogenic line (NIL) population of 2000 segregating plants was obtained. The genotypes of 1141A, Wu108, Huayehui and NIL population are identified as S (*rfprfp*), N (*rfprfp*), N (*RfpRfp*), and ½ S (*Rfprfp*) + ½ S (*rfprfp*), respectively.

Verification of male fertile and sterile phenotypes

The phenotypes S (*Rfprfp*) and S (*rfprfp*) of the NIL population are identified as fertile and sterile, respectively. Individual plants were graded during the flowering period by carefully observing flower development from three flowers per plant. The fertility of each individual was checked again after 10 days. Fertility was classified into six categories using procedures described by Yang and Fu (1991). In this system, the first four classes are considered sterile, while the fifth and sixth levels are considered fertile.

Preparation of genomic DNA and construction of BF and BS DNA bulks

Young leaves from individual plants of each NIL were collected for genomic DNA extraction. Genomic DNA was isolated according to a modified version of the cetyltrimethyl ammonium bromide (CTAB) procedure as described by Doyle and Doyle (1987). The concentration and quality of DNA was determined with a Beckman spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Fertile bulks (BF) and sterile bulks (BS) were constructed by pooling equal quantity of DNA from twelve fertile (*Rfprfp*) and twelve sterile (*rfprfp*) individuals which were randomly selected from fertile and sterile segregants.

Genomic DNA samples of each individual of DH mapping population (Qiu et al, 2006) and its parents, Tapidor and Ningyou 7, were provided by research group of Dr. Meng.

Nested primer design and PCR walking

To identify the flanking sequence of AFLP markers based on the original sequence, PCR walking (Siebert et al. 1995) was adopted to isolate the flanking sequence of AFLP markers. Following manufacture's protocol (GenomeWalker™ Kits, Clonetech, Madison, USA), the following procedure was used : (1) Nested gene-specific primers towards 5'-end and 3'-end were designed based on the original sequence of AFLP markers; (2) high quality genomic DNA of NILF was individually digested by *Eco*RV, *Dra*I, *Pvu*II and *Stu*I to construct four different DNA libraries; (3) DNA libraries were individually ligated with the adapter; and (4) Primary

Figure 1. A: Fertile plant in NIL population; B: Sterile plant in NIL population. Pictures were captured under Leica DC300 microscope at a magnification of 0.8x field lens and 10x eyepiece.

PCR and the following secondary PCR were performed with primer combination of AP1 or AP2 and the corresponding gene-specific primer. The PCR products of the secondary PCR were separated by electrophoresis through 1.2% agarose gel. Bands of interest were then purified, cloned and sequenced. Total size of PCR walking flanking sequences and original AFLP marker sequences were combined with DNAstar 5.0 software for sequence Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis.

Developing SSR analysis

SSR primer pairs were searched and designed by SSR Finder software (University of Missouri, Columbia, USA). The PCR reaction volume was 10 µl containing approximately 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 200 µM each of dATP, dTTP, dGTP and dCTP, 0.3 µM each of forward and reverse primers, and 1 unit of *Taq* DNA polymerase (MBI Fermentas, Ontario, USA). PCR was conducted on PTC200 (Bio-Rad Laboratories, Hercules, USA) following a program of 5 min at 94°C; 10 cycles of 40 s at 94°C, 45 s at 60°C for the first cycle, then decrease to 0.5°C per cycle after 60°C, 45 s at 72°C; 30 cycles of 40 s at 94°C, 45 s at 55°C, 45 s at 72°C; 72°C for 10 min and soaking at 4°C. Amplified PCR products were separated in a 6% denaturing polyacrylamide gel in 0.5 × TBE buffer at a constant power of 80 W and maximum voltage of 2000 V for 30 min. A simple and rapid silver staining method described by Lu et al. (2004) was used for imaging.

Linkage analysis

The molecular marker data and corresponding individual's fertility/sterility trait in NIL were analyzed using MAPMAKER 3.0 software (Lander et al., 1987). A minimum LOD score of 3.0 was used for map construction. The recombination values were converted into centi-Morgans (cM) using the Kosambi mapping function (Kosambi, 1944).

Bioinformatics analysis

Sequences of markers associated with *Rfp* were extended to identify putative homologous sequences within the *Arabidopsis* genome using Blastn searches against The *Arabidopsis* Information Resource (TAIR) genome database (http://www.arabidopsis.org). After identification of a syntenic interval of *Arabidopsis* genome, the published BAC clone sequences of *B. rapa*, which are comparatively located on the Arabidopsis syntenic region, were searched on the website of *B. rapa* Genome Project (http://www.brassica-rapa.org/BRGP/index.jsp) for SSR search and primer design.

RESULTS

Development of a segregating NIL

A NIL population, including 2000 individuals, was successfully constructed after 12 backcrosses to Wu108. Phenotypic evaluation revealed segregation of 997 sterile individuals and 1003 fertile individuals (Figure 1) indicating the expected 1:1 (sterile:fertile) ratio for a single dominant gene. Segregation was confirmed by a chi-square test ($p = 0.89$). We therefore concluded that the parent line carried only one *Rfp* locus.

Identification of an *Arabidopsis* homologous region of *Rfp* locus

The sequences from four polymorphic fragments (SA04TG08, SA07TG013 and EC10/MC10 were obtained from Zhao et al. (2008), while EA02MG03 came from Xu et al. (personal communication). These were submitted to the NCBI website (http://www.ncbi.nlm.nih.gov/) for nucleotide- nucleotide BLAST (Blastn) analysis. The four sequences were found to have highly conserved homologues with *Arabidopsis* genome (Table 1). Four genes, *AT1G12775*, *AT1G14180*, *AT1G13780* and *AT1G12760* were found to be homologues with SA04TG08, SA07TG013, EC10/MC10 and EA02MG03, respectively. These genes are located in three different BAC clones in *Arabidopsis*. All the homologues spanned a relative narrow syntenic region from gene *AT1G12760* to gene *AT1G14180* on *Arabidopsis* genome chromosome 1 (Table 1).

Nested primer design and PCR walking

Although, a homologous region in *Arabidopsis* was

Table 1. *Arabidopsis* conserved homologues genes and BAC clones of *Rfp* associated markers.

AFLP marker	SCAR marker	Size (bp)	Genetic distance	Homologue gene in *Arabidopsis*	BAC clone in *Arabidopsis*
SA07/TG01	SCAP0612ST	167	0.4 cM*	AT1G12775	T12C24
SA04TG08	-	231	2.1 cM*	AT1G14180	F7A19
EC10/MC10	SCAP0612EM2	165	0.4 cM*	AT1G13780	F16A14
EA02/MG03	SCARE2M3	238	0.4 cM**	AT1G12760	T12C24

*NIL population (BC10) derived from (1141A × Huayehui) × Wu108 (Zhao et al., 2008); **F_2 population derived from 501-8S × Yuyou No. 1 (Xu et al., personal communication).

Table 2. The nest primers designed in identifying the flanking sequences of AFLP marker.

Marker	Extend direction	First round primer	Second round primer	Total size after walking (bp)
SA07/TG01	3'	CATCTCAGAGGCAGTGACATAACCAC	GTTCTCAGCAGATGCTTCCACCATAA	167
	5'	TCGAGAAGGACCAGACAGCATATCTA	GAGTTCAACTGAAGTGGTTATGTCAC	
EC10/MC10	3'	GATATCCCATTGAGAAAACCACGGAT	CCCATGGATCAAAAAATCCGTTCAAG	1213
	5'	CATGGGATGCATCAAAGAGGAATGAA	GGTTTTCTCAATGGGATATCGAGTGT	
EA02/MG03	3'	TCTTCTAGACTTCCTCATGATCCAAG	ATTTGTCTACGCACGAGCAGTGGAAA	612
	5'	CTTGGATCATGAGGAAGTCTAGAAGA	TACCTAACTTTGTGTCGACCTCGAAT	

successfully identified, more flankin sequence information of the 4 AFLP markers should be analyzed to verify the homologous region. Three out of four AFLP markers with closer genetic distance were chosen to apply PCR walking techniques to identify the flanking sequences. The sequences of the nest primer of these three AFLP markers were designed. Two markers were successfully extended. Both 5' and 3' direction of EC10/MC10 were successfully extended; the 5' direction of EA02/MG03 was also successfully extended. The total sizes of EC10/MC10 and EA02/MG03 after PCR walking were 1213 and 612 bp, respectively. The two AFLP extended sequences were submitted to the NCBI website for Blastn analysis again and the same homologues genes, *AT1G13780* and *AT1G12760*, were identified. The homologues region in *Arabidopsis* was confirmed to be associated with *Rfp* gene (Table 2).

Developing SSR analysis

B. rapa should be much closer to *B. napus* than *Arabidopsis* because *B. napus* was derived from a natural cross between *Brassica oleracea* and *B. rapa*. The published BAC clone sequences of *B. rapa*, which were comparatively located on the Arabidopsis syntenic region, were searched on the website of *B. rapa* Genome Project (http://www.brassica-rapa.org). There were 14 *B. rapa* BACs located in the *Arabidopsis* homologous region of *Rfp* locus. Six out of 14 were sequenced; these were: KBrB086J10, KBrB017B11, KBrB070M24, KBrB086M23, KBrB077F22 and KBrS004B22. The remaining BACs were only sequenced for the BAC-ends. SSR's were identified based on the sequences of the 6 BACs and 22 SSR primer pairs were designed and listed in Table 3.

All the 22 primer pairs were screened with BF and BS bulks and only SSR primer pair KBrDP1 (also abbreviate as Kbr) expressed polymorphism between the bulks. After screening 2,000 NIL individuals for fertility and marker segregation using KBrDP1 (Figure 2), four individuals, F35, F129, S14 and S24 were found to mismatch with the fertility phenotype. The genetic distance between Kbr and *Rfp* was 0.2 cM.

After checking the KBrDP1 marker genotype of Tapidor and Ningyou 7, the two parents of DH mapping population (Qiu et al, 2006), the map location of *Rfp* gene was determined by the following genotype analysis of each DH individuals. KBrDP1 marker was located on linkage group N9, between markers pW123bE and CNU008 (Figure 3).

DISCUSSION

When compared with the traditional breeding methods, marker-aided-selection can improve the progress of crop breeding program by selecting the target gene with more efficiency and accuracy. However, the molecular marker identified in one genetic/breeding population frequently cannot be necessarily transferable and used in the other

Table 3. SSR primer pairs designed based on the sequences of the six *B. rapa* BACs.

Name	Forward primer	Reverse primer	*B. rapa* BAC
KBrBP1	acccactttgactccgtttg	acgacctcgtatggttcagg	KBrB086J10
KBrBP2	tgagctcaccaaatgtctgc	gcaagaaccagacatcagca	KBrB086J10
KBrBP3	acgagggttgagctgaaaga	gctgcaaagggaagtcaaag	KBrB086J10
KBrCP1	tgaagctgaggatgtgcaac	acccctcagcaagagactca	KBrB017B11
KBrCP2	gcttctctttgttcgcaacc	acgaaaggttttgtccgatg	KBrB017B11
KBrCP3	tagagcattgcggctgtatg	acgaaattagccgggtttct	KBrB017B11
KBrCP4	gcagccatctgtgacgacta	aacctgcagctgtcgagaat	KBrB017B11
KBrCP5	atggaaccgaggctttaggt	catacagccgcaatgctcta	KBrB017B11
KBrDP1	cactcttttaggagaagcacgaa	cccgcatagtttgtcagtttct	KBrB070M24
KBrEP1	gattaagttcgggcgtgtgt	gcttcaacagctttcggttc	KBrB086M23
KBrEP2	acttcaaaccctctcgctga	tctcatccgtctccatttcc	KBrB086M23
KBrEP3	ctgcacactcttgggggtat	aaggagcacgcacttcatct	KBrB086M23
KBrEP4	tcaccagcgatatgaagcag	tggatcccttggagaagatg	KBrB086M23
KBrEP5	Ttcactgctctggttg atgc	caacaaccacgaaatcatgc	KBrB086M23
KBrFP1	gttcgaaagaggtgcgaaag	aggatacgctgccgtagaga	KBrB077F22
KBrFP2	acctgacaccccagtgactc	tgtcttggacttcgctgatg	KBrB077F22
KBrFP3	gcagagcggagcatttttac	ggtgccttgttgagtttggt	KBrB077F22
KBrFP4	cttcctcggctgtttagtcg	gacttgtccacgccatttttt	KBrB077F22
KBrFP5	gcgagacctaggtgctgttc	tcaacgtactccgcctctct	KBrB077F22
KBrGP1	gacacctgaaagctggaagc	tgcggatgagacttgttcag	KBrS004B22
KBrGP2	tgagcttaggcacatgaacg	ccctctcgctgttctgaaac	KBrS004B22
KBrGP3	tggcctctcagactgtgttg	ctggcagcctggagtcttac	KBrS004B22

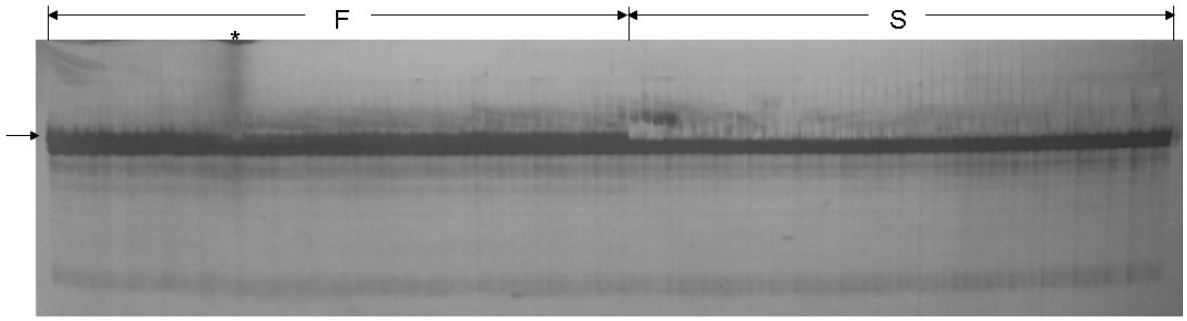

Figure 2. PCR profiles in partial NIL individuals with SSR primer pair KBrDP1. F, fertile individuals; S, sterile individuals; the arrow indicate the bands specific for fertile individuals; the asterisk indicated the recombinants.

genetic/breeding populations. That is, the molecular marker may not express polymorphism in a different genetic/breeding population. The up-front investments required to construct a new population and identify molecular markers for MAS breeding and for fine mapping and gene cloning can be large. In this study, we utilized the sequence information of markers from different genetic population to compare with the genome sequence of *Arabidopsis* and identified a homologous region of *Rfp* locus in *Arabidopsis*. This homologous region was used to screen homologous *B. rapa* BAC clones and finally mined the SSR marker KBrDP1. This SSR marker was located on linkage group N9 of DH mapping population (Qiu et al., 2006). The information of each marker located on N9 linkage group can be used in verifying the homologous region in *Arabidopsis* and developing additional markers as well. This is a highly efficient approach to mining a large number of molecular markers for rapeseed breeding program and for fine mapping and cloning of important agronomy traits of *Brassica* species.

Nowadays, the development of SSR markers becomes much easier than the traditional methods after completion of many plant sequencing projects. SSR marker has

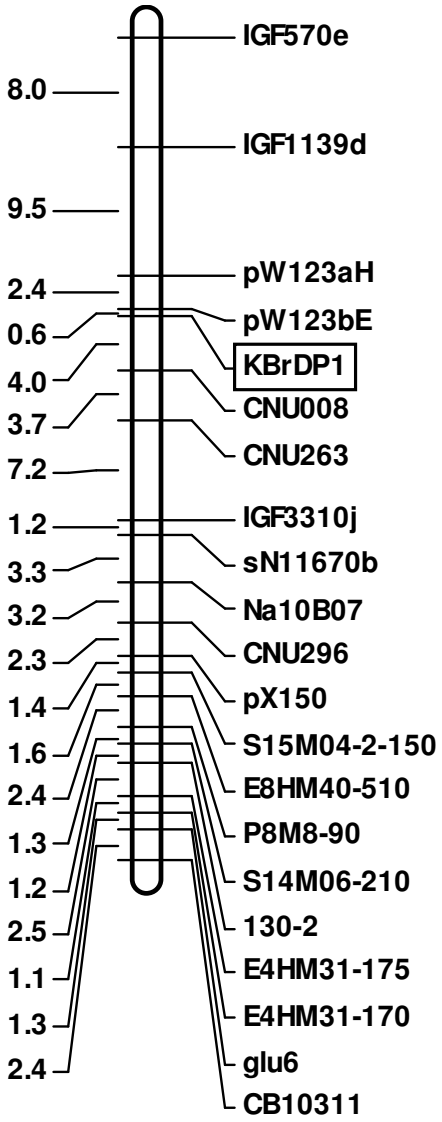

Figure 3. A linkage map of N9 developed from the cross Tapidor × Ningyou7 (Qiu et al., 2006) shows the location of KBrDP1. The map was constructed with a LOD score >4.0 and a recombination frequency <0.4. The Kossambi map distance (cM) and marker names are indicated on the left and right sides, respectively.

been widely used in plant genome analysis because of its highly polymorphic, reliable and stable PCR amplification. It has been used to construct the framework linkage map of a highly dense genetic map, and to compare and combine maps developed in different laboratories (Lowe et al., 2004; Piquemal et al., 2005; Cheng et al., 2009). An SSR marker that is associated with target *Rfp* gene was successfully identified based on the *Arabidopsis* genome information and *B. rapa* BAC clones sequence information in this study. This study provided a potential efficient way to screen for new molecular markers without constructing new genetic/breeding population. The critical step for this purpose is to make sure of the homologous region identified by molecular marker sequence analysis, especially in polyploidy species. In this study, we started with the sequence information of four AFLP molecular markers. Although, a homologous region in the *Arabidopsis* genome were identified, it was still uncertain for the next step research work due to the small size of these four AFLP markers (From 165 to 238 bp) (Table 1). To verify the homologous region, PCR walking technique was performed to extend the flanking sequences of the four markers. Two of them, EC10/MC10 and EA02/MG03, were successfully extended up to 1213 and 612 bp, respectively. Then, the homologues region in *Arabidopsis* was confirmed by the extended sequences (Table 2). Both the homologues region identified in *Arabidopsis* and the SSR marker KBrDP1 will greatly promote the future breeding and genetic mapping program.

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