Development and characterization of SCAR markers associated with a dominant genic male sterility in rapeseed (*Brassica napus*. L)

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Abstract

Rs1046AB is a dominant genic male sterility (DGMS) line in rapeseed, of which the sterility was ever regarded to be conditioned by the epistatic interaction of two individual genes, i.e. the male sterility gene (*Ms*) and its suppressor gene (*Rf*). This system not only provides a tool for assisting in recurrent selection but also a promising alternative to the cytoplasmic male sterility (CMS) and self-incompatibility (SI) systems for hybrid production. Based on previous studies, we converted two AFLP markers linked with the *Ms* gene into a dominant and a co-dominant SCAR marker, respectively. The putative linear order relationship of three dominant SCAR markers with the same genetic distance from the *Rf* gene, was also determined by an examination of whether the homologues of these markers are present or not in different lines carrying the *Rf* gene. Interestingly, a bigger fragment generated by the closest marker linked to the *Rf* gene was observed in all lines carrying recessive allele *rf*, suggesting this marker is a co-dominant marker. This finding was incompatible with our previous conclusion; however, it presented strong evidence that the sterility of this dominant GMS should be explained as an inheritance model of one gene with multiple alleles. These SCAR markers developed here specific for *Ms* and its alleles will be especially valuable in marker-assisted DGMS three-line breeding.

Key words: *Brassica napus*, Sequence characterized amplified region (SCAR), marker assisted selection (MAS), dominant genic male sterility (DGMS), inheritance model

Introduction

In the genic improvement of rapeseed, a common strategy for improving the yield potential is the utilization of heterosis by developing hybrid varieties (Fu 2000). Male sterility system including cytoplasmic male sterility (CMS) and genic male sterility (GMS) has been adopted as an effective pollination control approach for commercial hybrid seed production. Though hybrids produced by CMS system are still the main approach for heterosis utilization (Fu 2000), some limitations inherent with this system can never be ignored, i.e. the instability of sterility, the limited number and deleterious agronomic characters of restorers and the potential negative influence of the cytoplasm (Yang and Fu, 1987; Banga et al., 1988; Pellan-Delourme and Renard 1988; Delourme et al 1995). In contrast, GMS materials generally have complete and stable sterility, normal cytoplasm, and almost no restorer limitation. Therefore, they are regarded as a promising alternative to CMS system in the production of hybrid seed. Otherwise, GMS can be extensively used in recurrent selection and construction of special germplasm pool (Li et al. 1990; Zhou and Wu 1995).

The inheritance of genic male sterility in *Brassica napus* has been studied intensively according to different mutants (Takagi 1970; Mathias 1985; Hou et al. 1990; Li et al. 1988; Chen et al. 1998; Hu et al. 2000; Wang et al. 2001; Song et al. 2005). Among them, a natural dominant GMS (DGMS) mutant Yi3A was systematically characterized and a double dominant gene inheritance model was then proposed by Li et al. (1985, 1988, 1990). This model suggests that the expression of a dominant genic male sterility (*Ms*) gene alone would cause sterility, but an inhibitory gene (*Rf* or *Mf* in recent report) gene could suppress its expression, resulting in restoration of fertility. According to this model, a DGMS three-line system (i.e. DGMS two-type line homozygous at the *Ms* locus: 1/2 *MsMsrfrf* + 1/2 *MsMsRfrf*, temporary maintainer line: *msmsrfrf*, and restorer line: *__RfRf*) was subsequently raised (Li et al. 1995). With the registration of some elite hybrid cultivars (Zhou et al. 2003; Shi and Dong 2004), this system has been demonstrated to be feasible in seed production. Nevertheless, DGMS still has a relatively narrow window of application. One of its limitations is the low efficiency of identifying complicated genotypes with desired agronomic traits in traditional breeding process. Therefore, the development of genic markers linked to the *Ms* and *Rf* loci are required to facilitate the genotype identification of GMS three-line breeding in a timely manner.

Recently, through classical genetic analysis of a new DGMS mutant 609A, Song et al. (2005) questioned this inheritance model and stated that one gene with multiple alleles model is more appropriate to explain the genic phenomena of this kind DGMS. Further proof based on molecular markers was presented recently (Song et al., 2006). However, it is not certain that the mutant genes responsible for the sterility of 609A (Song et al. 2005) and Yi3A (Li et al.1985) are allelic. If we can show some molecular evidences directly based on Yi3A, the more appropriate inheritance model may be fundamentally verified.

We reported that the *Ms* gene for DGMS from Yi3A was bracketed by two tightly linked AFLP markers previously (Lu et al. 2004a, b). Recently, the *Rf* gene was also tagged with six AFLP polymorphic fragments and three nearest ones of them were converted to SCAR markers (Hong et al. 2006). For the practical use of molecular markers in MAS, however, it is crucial to convert the two closet markers linked to *Ms* into SCAR markers; as for the SCAR markers linked to *Rf*, it is desirable to determine which one is closest.

In this paper, we attempted to develop SCAR markers tightly linked to *Ms* and characterize SCAR markers associated with *Rf* for marker-assisted selection (MAS). The interesting PCR amplification result generated by the nearest SCAR marker linked to *Rf* was carefully described, which led us to conclude that the Rf gene and Ms gene is likely to be allelic. How to applying these integrated SCAR markers in new DGMS three-line breeding was also discussed.

Materials and Methods

Plant materials and DNA preparation

Rs1046AB, a GMS two-type line homozygous at Ms locus, was derived by recurrent crossing to Yi3A that was a natural dominant GMS mutant found in *Brassica napus* (Li et al., 1985). In Rs1046AB, the genotypes of sterile and fertile individuals are generally regarded as *MsMsrfrf* and *MsMsRfrf*, respectively. The same BC1 population (Lu et al. 2004) derived from the cross between Rs1046A (MsMsrfrf) and Samourai (msmsrfrf), a European winter canola cultivar, was used to test the SCAR markers linked to *Ms*. Some breeding lines, i.e. 195A (*Rf*/*Rf*), L2085 (*Rf*/*Rf*), Yu125 (*Rf*/*Rf*), Yu136 (*Rf*/*Rf*), L8089 (*Rf*/*rf*), L8096 (*Rf*/*rf*), L8084 (*rf*/*rf*), L6275 (*rf*/*rf*) and Samourai (*rf*/*rf*), were used to examine the closest SCAR markers that were tightly linked to *Rf*. The genotypes in *Rf* locus among these materials have been identified by test cross with Rs1046AB. All the plant materials involved in this study were grown in the research farm at the Huazhong Agricultural University. Several young leaves from each plant of different lines were collected for DNA extraction. Plant DNA was isolated using the sodium dodecyl sulphate (SDS) method described by Li et al. (1994) with minor modification.

SCAR marker conversion and detection

The isolation of polymorphic AFLP fragments from the denatured PAGE and re-amplification of them were carried out as described by Ke et al. (2004). A UNIQ-10 EZ Spin Column DNA Gel Extraction Kit (Sangon, Shanghai, China) and a pGEM-T Easy Vector (Promega, Madison, USA) were used to purify and clone the polymorphic fragments. The recombinant white colonies containing the target fragments were screened on Amp^+/X-gal/IPTG LB plates and cultured overnight in LB liquid media for plasmid DNA extracting. The cloned DNA fragments were sequenced with an ABI Prism3730 DNA sequencer (Applied Biosystems, Foster City, USA) in Beijing Sunbiotech Biotechnology Co., Ltd.

SCAR markers were subsequently designed based on 5′-end most sequences of the cloned AFLP fragments using software Primer3 (Steve & Skaletsky, 1996). PCR amplification from the genomic DNA with SCAR primers was the same as described before (Hong et al. 2006). PCR products were then resolved on 1.5% agarose gels or 6% denaturing polyacrylamide gels to confirm whether the SCAR primers were converted successfully.

PCR Walking

PCR Walking (Siebert et al. 1995) was adopted to isolate the adjacent genomic regions of AFLP markers that could not be converted directly from the original sequence. GenomeWalker™ Kits (Clonetech, Madison, USA) was applied to facilitate PCR Walking. The following protocol was followed according to user's manual: first, genomic libraries of Rs1046A were prepared by individually digesting the high quality genomic DNA with blunt-end restriction enzymes *Eco*RV, *Dra*I, *Pvu*II and *Stu*I, and then ligated to the adapter. Primary PCR/secondary PCR were performed using primer combination of adapter primer 1 (AP1)/adapter primer 2 (AP2) and the corresponding nested gene-specific primers (GSPs) respectively, with optimized cycle parameters. At last, the amplified products after the secondary PCR were separated by electrophoresis through 1.5% agarose gel. The single and specific band of each lane was then reclaimed, purified, cloned and sequenced. Based on these sequences, SCAR primers were expected to re-amplify the corresponding polymorphism of AFLP primers.

Differentiation of the relative order between SCAR markers and the Rf gene

To differentiate these three SCAR markers with respect to genic distance from the *Rf* gene, we examined whether the corresponding polymorphic fragments of the SCAR markers were present or not in each of the breeding lines described above. If the corresponding polymorphic fragments were amplified in most or all DGMS restorer lines by one SCAR marker, but not by the other markers, it would indicate that the marker is linked closer to the *Rf* gene than others.

Result

Conversion of AFLP fragments linked to Ms into SCAR markers

The mapping of the *Ms* gene was reported previously (Lu et al. 2004). Five AFLP markers ($P10M13_{350}$, $P13M8_{400}$, $P6M6_{410}$, $E7M1_{230}$ and $E3M15_{100}$), spanning a genic region of 20.7 cM, were confirmed to be tightly linked to the *Ms* gene in coupling phase. $E3M15_{100}$ and $P6M6_{410}$ were the closest AFLP markers of *Ms* at either side with a genic distance of 3.7 and 5.9 cM, respectively. Therefore, the polymorphic fragments of $E3M15_{100}$ and $P6M6_{410}$ were chosen for SCAR conversion. After subtracting adapters from the sequences, the exact size of $E3M15_{100}$ and $P6M6_{410}$ was 77 bp and 381 bp respectively. A pair of the oligonucleotide primers designated as SCP6 (Table 1) were designed based on the sequences of cloned $P6M6_{410}$ fragment. As shown in Fig.1 A, from the genomic DNA of two parents, two bands differing in size on a 6% denatured polyacrylamide gel were amplified with SCP6, indicating that this SCAR primer is a co-dominant marker for differentiating *Ms* and the allele ms. This result was further confirmed when examining SCP6 in 20 individuals randomly selected from the BC1 population (Fig.1 A).

Further analysis showed that SCP6 has the same recombinant individuals as $P6M6_{410}$ and was mapped in the same position on the linkage group as $P6M6_{410}$. However, amplification with the SCAR primer that was derived from the sequence

of $E3M15_{100}$ fragment exhibited a monomorphic band in both parents, indicating the loss of polymorphism corresponding to $E3M15_{100}$. Therefore, the sequences flanking to the polymorphic fragment of $E3M15_{100}$ were expected to raise the probability of successful SCAR conversion.

PCR Walking of marker$E3M15_{100}$ and its SCAR conversion

PCR Walking approach was carried out to obtain the flanking sequences of marker $E3M15_{100}$. Two nested gene-specific primers E3W1 and E3W2 toward upstream direction were designed and synthesized (Sangon, Shanghai, China). Sequences of E3W1 and E3W2 were 5′-CCGTTCTTGGTGAAGTTGATTTTACTCGGT-3′ and

5′-CTCGGTAGTTCTTTCGAGAGATCA-3′, respectively. After two rounds of successive amplification, clear and major bands were observed in libraries corresponding to *Eco*RV (*lane* 1) and *Pvu*II (*lane* 2) (Fig. 2). The band in *lane* 2 was bigger in molecular weight than the one in *lane* 1; however, the band in lane 1 was more specific and legible than that in lane 2. Consequently, only the band in *lane* 1 was excised, cloned and sequenced. Sequence comparison confirmed that the isolated fragment by PCR Walking is indeed the upstream sequence of marker $E3M15_{100}$. Plus the original sequences, a genomic contig of exact 1.282 kb around marker $E3M15_{100}$was constructed.

Based on this longer sequence, SCAR primer SCE3 (sequences shown in Table 1) was designed to amplify the relative region of genomic DNA. The polymorphism was found in band pattern between the parents and the same 20 plants used above (Fig.1, B), A clear band was present in Rs1046A and all 10 sterile plants but absent in Samourai and all fertile plants, indicating that SCE3 is a dominant marker linked to the *Ms* gene.

Putative linear order relationship of certain SCAR markers linked to the Rf gene

In previous study, we have identified six AFLP markers specific for the *Rf* locus and converted all of them into dominant SCAR markers. Linkage analysis showed that three markers, designed as SCHDA, SCHDC and SCHDF, were co-segregant with each others. They all had the same genic distance of 0.7 cM from *Rf* (Hong et al. 2006). Considering that all these molecular markers are distributed on the same side of the targeted gene and only the closest marker is desired for MAS, it is necessary to understand the linear order relationship between these three markers.

Eight inbred lines with known genotypes in *Rf* locus were selected to differentiate these markers with respect to distance to the *Rf* locus. We examined whether the fragments of the same size from three of the SCAR markers were present or not in the eight inbred lines. Amplification results showed that the fragment of the same size corresponding to SCHDA was detected only in Rs1046B but not in other lines (Fig.3, A). For marker SCHDC, no fragment of expected size was yielded in all lines except in Rs1046B and L8096 that carry heterozygous gene in *Rf* locus (Fig.3, B).

Distinctly, SCAR primer of SCHDF could amplify product in all the inbred lines examined except for Rs1046A. As shown in Fig.3 (C), a single product with the same size of the fragment (SCF_{420}) from Rs1046B was amplified from each line carrying *Rf* gene (L3, L4, L5 and L6); a larger fragment (SCF_{550}) was obtained from lines homozygous in recessive rf locus (L9, L10 and L11) and both fragments were amplified in two lines heterozygous in *Rf* locus (L7 and L8). This result seemed to suggest that marker SCHDF was a co-dominant marker for *Rf* locus. However, the fact that only one band was obtained in Rs1046B (*MsMsRfrf*) and no product in Rs1046A (*MsMsrfrf*) was incompatible with this assumption, even though it was clearly demonstrated that marker SCHDF was closer to the *Rf* locus than the other two markers examined. The putative linear order relationship between these markers to *Rf* gene is SCHDF, SCHDC and SCHDF in turn.

Sequence comparisons of fragments corresponding to SCHDF

Genetically, SCF_{420} and SCF_{550} appeared to be allelic since the former one was specific to *Rf* and the latter one to *rf*. In order to confirm this conclusion, we cloned and sequenced SCF_{420} and SCF_{550} from all the tested lines. After alignment and comparison, the numbers of the nucleotide residues of these SCAR markers and percentage of homologies between their nucleotide sequences are shown in Tables 2. It was clearly demonstrated that the fragments SCF_{420} obtained from different lines shared more than 98% nucleotide identity (deep gray part of Table 2), and 97% for SCF_{550} (light gray in Table 2). Although the size of fragment SCF_{420} was about 70 bp smaller than that of SCF_{550}, the nucleotide identity between them still reached a high value of 93.7% to 96.2%. Further investigation showed that the difference between SCF_{420} and SCF_{550} was mainly caused by an approximate 70 bp fragment insertion into SCF_{420}, suggesting that the fragment SCF_{420} and SCF_{550} are likely allelic.

Discussion

SCAR marker development

Previously, we reported that the *Ms* gene has been mapped in a linkage group with two nearest AFLP markers at a distance of 3.7 cM and 5.4 cM, respectively (Lu et al. 2004a, b). Now, these two AFLP markers were converted to locus-specific, stable and convenient SCAR markers, which are much more valuable for targeting the *Ms* gene in breeding for new DGMS lines with MAS Though, two dominant SCAR markers developed by Song et al (2006) have much nearer distance to the *Ms* gene, both lost their polymorphisms in our mapping populations. Especially, SCAR marker SCP6 is a co-dominant marker, which can distinguish the homozygous genotype in *Ms* locus from heterozygous ones in segregation generations. This is more valuable than just dominant SCAR markers when being applied in MAS breeding. Otherwise, the wide occurrence of these two SCAR markers ensure the efficient targeting the *Ms* gene in backcross breeding program (data not shown).

Molecular characterization of SCAR markers linked to Rf gene

Theoretically, it is difficult to predict how many NIL plants should be analyzed to find recombinants between tightly linked markers. Estimating a rough marker order to the target locus is an alternative approach by examining whether the homologues of these markers are present or not in different lines carrying the target genes (Sobir et al., 2000). In this study, a total of eleven lines including Rs1046A and Rs1046B were chosen to determine the putative linear order relationship between three markers tightly linked to *Rf* locus. This method is generally efficient to estimate the relative genic distance between the target gene and its flanking molecular markers, especially when the mapping populations are not big enough.

SCHDF is a dominant marker between the two mapping parents Rs1046B and Rs1046A, due to the specific fragment only amplified in Rs1046B. Interestingly, beside the same fragment corresponding to the one obtained from Rs1046B, a bigger fragment was detected in all lines carrying recessive allelism *rf*. Nucleotide identity analysis confirmed these fragments obtained from different lines are likely amplified from the same position of genomic DNA. This has suggested that marker SCHDF is a co-dominant marker for *Rf* locus and these two polymorphic fragments are allelic to each other.

Obviously, these two conclusions are in contrast to each other, which can be hardly explained by two genes inheritance pattern developed by Li et al (1988). Song et al. (2005) presented a model of multiple alleles for one gene to explain the fertility inheritance of a newly bred sterile line 609AB, i.e. the dominant male sterility gene (*Ms*), the allelic restorer gene (*Rf* or *Mf*) and the recessive allele (*ms*) for normal fertility. Similarly, the restorer gene Rf is dominant over Ms and Ms over ms. Here, three polymorphic positions can be detected by one SCAR primer in different breeding lines, showing that the genomic region associated with the *Rf*/*rf* locus has three different genotypes. This conclusion is completely consistent with the model of multiple alleles. Consequently, we regarded the fertility inheritance of Rs1046AB should also be explained by this model rather than that of two genes with epistatic interaction.

It must be mentioned that the genotypes of Rs1046A, Rs1046B and Samourai were completely different when DGMS line Rs1046AB was inherited by multiple allele or inherited by double unlinked genes. However, that the essential function of the two populations used in this study was to map *Ms* and *Rf* genes individually was affirmatory, not influenced by the inheritance model.

Application of these SCAR markers in DGMS breeding

Large-scale commercial production of DGMS hybrid mainly depends on three-line approach in rapeseed, with which a population of 100% sterile individuals will be generated, thus greatly reducing the labour of eliminating all fertile plants in the sterile line when adopting a two-line approach. The key step of breeding DGMS three lines is to culture new homozygous sterile lines homologous to the maintainers or new maintainers homologous to homozygous sterile lines. In this study, molecular characterization of SCAR marker SCHDF confirmed the multiple alleles responsible for the fertility of Rs1046AB. This makes genotype identification in the segregation populations much easier, because only six genotypes will probably appear according to multiple allele inheritance, while nine genotypes exist if explained by previous digenic inheritance. Furthermore, applying these two co-dominant and one dominant SCAR markers will permit us to efficiently distinguish the six different genotypes in the process of breeding homozygous sterile lines or maintainers. Similarly, backcrossing for transferring *Rf* (*Mf*) gene from restorer lines can also be accelerated by crossing restorer lines identified by marker SCHDF with other lines, without need of testing for restoration ability in each generation.

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Table 1 Sequences and parameters of SCAR primers linked to *Ms* and *Rf*

Marker designation	Specific for:	Direction	Sequence (5′→3′)	Optimal annealing Temp. (°C)
SCP6	*Ms*	Forward	TTAACTTCAGGGGGCAAC	67.0
		Reverse	CTGCAGCTGACACACCGT	
SCE3	*Ms*	Forward	ATCTTCTCCAgTTgAAAACA	61.0
		Reverse	GAATTCAACAAGTGAAGACT	
SCHDA	*Rf*	Forward	GCTCAGCATGACATCGGTTAG	60.0
		Reverse	TGGTCAAGAGGAGGTACATCG	
SCHDC	*Rf*	Forward	TTAAGAGGAAACCGGGAC	58.0
		Reverse	GAATTCCCTCAGAGTGGAGC	
SCHDF	*Rf*	Forward	GCTTTCAAGGAAGTTTGAAGC	62.7
		Reverse	TCACGAAAGAGTACATTGTGG	

Table 2 Nucleotide identity (percentage) and number (bp) of the fragments corresponding to SCHDF in different breeding lines

Line	Rs1046B 426 bp	195B 426 bp	L2085 426 bp	Yu125 426 bp	Yu136 426 bp	L8089[a] 426 bp	8096[a] 426 bp	L8089[b] 550 bp	L8096[b] 547 bp	L8084 550 bp	L6275 550 bp	Samourai 550 bp
Rs1046B	100	100	99.1	99.2	99.8	99.3	99.5	96.2	96.0	95.8	95.3	96.2
195B		100	99.1	99.2	99.8	99.3	99.5	96.2	96.0	95.8	95.3	96.2
L2085			100	98.1	98.8	98.4	98.6	95.3	95.1	94.8	94.4	95.3
Yu125				100	98.9	98.6	98.9	94.8	94.5	94.3	93.7	94.8
Yu136					100	99.1	99.3	96.0	95.8	95.5	95.1	96.0
L8089[a]						100	98.8	95.5	95.3	95.1	94.6	95.5
L8096[a]							100	95.8	95.5	95.3	94.8	95.8
L8089[b]								100	98.2	99.5	99.3	100
L8096[b]									100	97.4	97.4	98.2
L8084										100	98.7	99.5
L6275											100	99.3
Samourai												100

[a] the fragment with low molecular weight;
[b] the fragment with high molecular weight

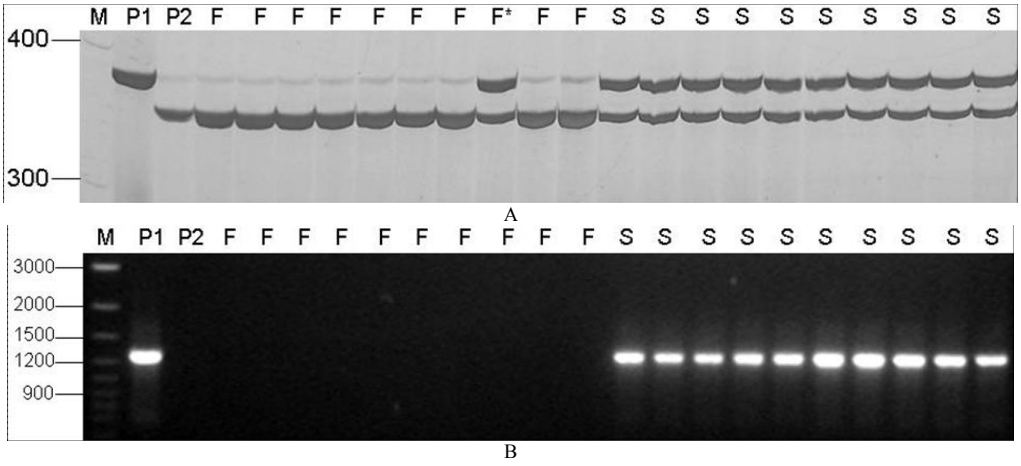

Fig. 1 (A) PCR patterns of SCAR marker SCP6 linked to the *Ms* gene. **(B)** PCR patterns of SCAR marker SCE3 linked to the *Ms* gene. *P1*: Rs1046A, *P2*: Samourai, *F*: fertile individual, *S*: sterile individual, *F**: a recombinant individual, *M* (**A**): GeneRuler^{TM} 100bp DNA Ladder (MBI), *M* (**B**): GeneRuler^{TM} 100bp DNA Ladder Plus (MBI).

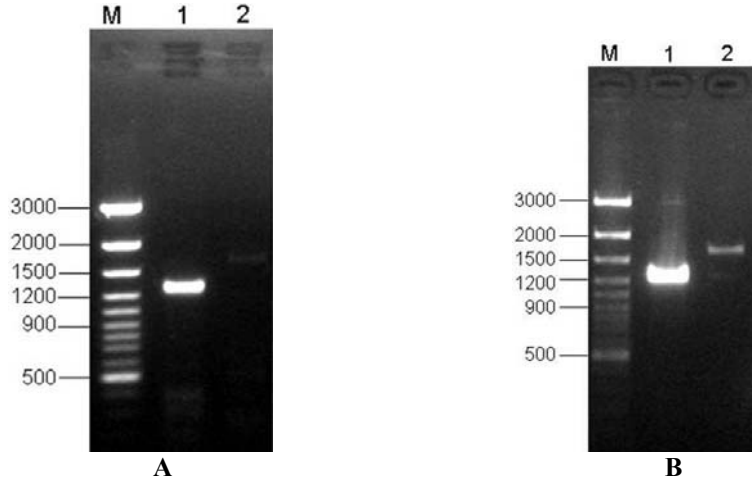

Fig. 2 Agarose gel electrophoresis of PCR products obtained from PCR Walking of E3M15$_{100}$. *Lane 1* and *lane 2*, the genomic libraries constructed by *Eco*RV, *Pvu*II. *M* represented GeneRuler^{TM} 100bp DNA Ladder Plus (MBI). (**A**), the first PCR products using primer GSP-E3W1/AP1. (**B**), the secondary PCR products using primer GSP-E3W2/AP2

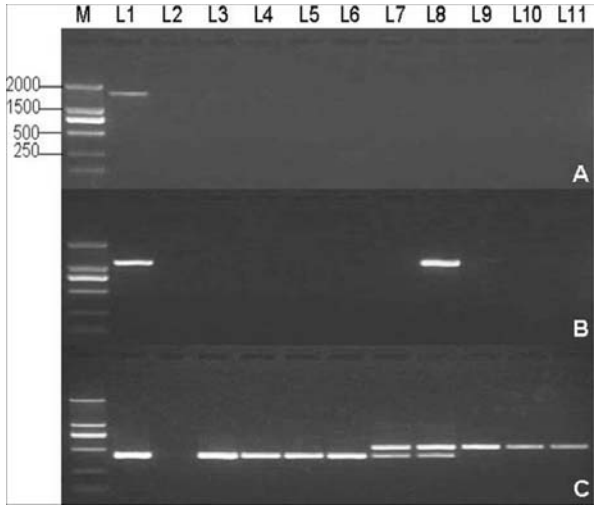

Fig.3 Electrophoretic patterns of PCR products from genomic DNA of different lines using SCAR primers tightly linked to *Rf* gene. (**A**) SCHDA. (**B**) SCHDC. (**C**) SCHDF. *L1*: Rs1046B, L2: Rs1046A, L3: 195A, L4: L2085, L5: Yu125, L6: Yu136, L7: L8089, L8: L8096, L9: L8084, L10: L6275, L11: Samourai. *M*, D2000 marker (Tiangene)