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RAPD and SCAR Marker Linked to the Sterility Mosaic Disease Resistance Gene in Pigeon Pea (*Cajanus cajan* L. Millsp.)

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ABSTRACT

The present study was undertaken to identify random amplified polymorphic DNA (RAPD) primers and to develop a sequence characterized amplified region (SCAR) marker associated with pigeon pea sterility mosaic disease (PPSMD) resistance in pigeonpea cross ICPL-7035 x ICPL-8863. Bulked segregant analysis was employed to identify the RAPD primer linked to PPSMD and to develop a SCAR for the PPSMD resistant gene of ICPL-7035, a known resistance source to different isolates of PPSMV and vector. A total of 200 random decamers were surveyed for identification of polymorphic markers between the DNA of resistant and susceptible parents. Amplification of individual DNA samples out of the bulk with putative marker, OPA18800 only revealed polymorphism in resistant and susceptible lines, indicating that the marker OPA18 was associated with PPSMD resistance in ICPL-7035. Their end sequences were used to design allele-specific sequence characterized amplified region (SCAR) marker SCAR 816(16f/r), which was present in all generations (parents, F_1 and F_2) and would help to identify the transfer of the SMD resistance gene to susceptible lines.

Keywords: bulked segregant analysis, dominant, polymorphism, recessive, selection

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Millsp.; family Fabaceae) is a major grain legume crop, whose seed supplies dietary protein requirements to large numbers of people living in the semi-arid tropics of the Indian subcontinent (Saxena *et al*. 2002). Sterility mosaic disease (SMD), a major constraint to pigeon pea production, was first described in 1931 from Bihar State, India (Mitra 1931) and subsequently from several states of India and from Bangladesh, Nepal, Thailand, Myanmar, and Sri Lanka. SMD, caused by an eriophyid mite-transmitted *Pigeon pea sterility mosaic virus* (PPSMV), is the major disease of pigeon pea in the Indian subcontinent. SMD is responsible for yield loss of worth >US $300 million (Jones *et al*. 2004). In 1984 yield losses in India due to SMD were over 205,000 tons of grain per annum (Kannaiyan *et al*. 1984). Plants infected at an early stage of the crop growth result in 0-10% flowering; late infection results in 40-70% flowering, and poor quality seed (Kumar *et al*. 2001).

In screening trials initiated in 1975, ICRISAT and the Indian Council of Agricultural Research stations identified few resistant lines but their resistance elsewhere in Indian subcontinent was less effective (Reddy *et al*. 1993). Analysis of mite population and vector isolates obtained from India, Nepal and Myamnar indicated that the breakdown in SMD resistance in pigeon pea genotypes is not influenced by variation in the mite population but mainly due to virus variation. Based on the symptoms on these genotypes, the PPSMV isolates were grouped as, 'B' 'C' and 'P' types from Bangalore (Karnataka), Coimbatore (Tamilnadu) and Patancheru (Andhra Pradesh) respectively (Kumar *et al*. 2000). Among these isolates of PPSMV occur in India, the B-types are highly virulent and can overcome host-plant resistance selected against P-types. The B-types occur in northern and southern regions, and P-types occur in central regions of India. Host-plant resistance to B-type isolates is scarce and very few pigeon pea genotypes withstand infection. The majority of lines resistant to PPSMV following inoculations with viruliferous mites were susceptible by graft inoculation, suggesting that vector resistance is conferring resistance to infection with PPSMV (Lava Kumar *et al*. 2003). To assess the possibility of virus variability as the cause of break down, a set of different genotypes were planted at different locations and found no SMD symptoms (severe mosaic and sterility, mild mosaic with partial sterility and chlorotic ring spots) in ICPL-7035 to three isolates of PPSMV, which is a landrace in Madhya Pradesh, collected under germplasm by ICRISAT, Andhra Pradesh in 1973 (Kulakarni *et al*. 2002). SMD resistance in ICPL-7035 is highly stable because it is resistant to three isolates of PPSMD as well as its vector, eriophyid mite *Aceria cajani* confirmed through ELISA (Jones *et al*. 2004). This mite is highly host specific and is restricted to pigeon pea and a few of its wild relatives.

Inheritance of resistance to SMD indicates dominance of susceptibility over resistance (Singh *et al*. 1983; Sharma *et al.* 1984 and Nagaraj *et al.* 2004). Monogenic inheritance of resistance was observed in some crosses (Sharma *et al.* 1984; Srinivas *et al.* 1997a). Resistance was recessive and appeared to be governed by two independent non-allelic recessive genes exhibiting complementary epistasis and the presence of at least one gene conferring resistance to the disease, in homozygous recessive condition was found to be necessary to express the resistance phenotype (Srinivas *et al.* 1997b; Nagaraj *et al.* 2004) Development of high-yielding SMD grain type of different groups is important to stabilize pigeon pea yields in areas of the country where this disease is endemic.

In breeding for SMD resistance in pigeon pea traditional methods are based on crosses between susceptible and resistant genotypes followed by phenotypic selection for resistant genotypes by artificial screening (stapling or grafting methods). The out-crossing nature, long life cycle, difficulty in accurate phenotyping and linkage drag are some of the problems faced in traditional breeding (Saxena 2008). Molecular markers linked to resistance are useful in marker-assisted selection (MAS) breeding. By means of

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molecular markers linked to a trait of interest, selection can be performed at early seedling stages of development, and true breeding genotypes identified with relative ease (Kotresh *et al*. 2006). Hence, identification of molecular markers closely linked to SMD resistance facilitates the identification and breeding of new lines by reducing the time required.

RAPD are generated by amplification of genomic DNA using a single primer of arbitrary nucleotide sequence to drive the amplification reaction. The most useful application of RAPD marker is, however, to quickly generate markers within a genomic region of interest using near isogenic lines (NILs) (Penner *et al.* 1993). However, several generations of back crosses are required to create NILs and several regions of the donor genome can be co-introgressed into the NILs. An alternative method called bulked segregant analysis (BSA) was proposed by Michelmore *et al*. (1991). It aims to replace the NILs by two bulked samples collected from individuals identical for alleles at a specific locus in a single population, each bulk being homozygous for one or the other allele of the gene of interest. The advantage of this technology is that markers are targeted to a smaller region within the genome and likelihood of identifying false positive markers is small using NIL or BSA strategies a number of RFLP or RAPD markers linked to dominant gene have been identified in rice, rape seed, sunflower and rye (Manivannan and Muralidharan 2003). Molecular markers closely linked to major resistance gene will facilitate the breeding of new resistant lines in pigeonpea. To overcome the problems associated with RAPD analysis and to improve their utility in MAS application, RAPD markers can be converted into SCAR markers (Paran and Michelmore 1993). The SCAR markers are generally allele specific and their amplifications is much less sensitive to reaction conditions. A SCAR marker is developed by cloning and sequencing the ends of the amplified RAPD product, generating extended primers specific to the targeted sequences and amplifying DNA samples under higher stringency conditions. They can be developed into dominant markers that generate a single condition of presence or absence, or as potentially co-dominant markers. This strategy has been widely and successfully used to develop markers for various traits in wheat (Myburg *et al*. 1998) and common bean (Melatto *et al.* 1996). Development of resistant varieties against the SMD is complicated due to presence of PPSMV strains of varying virulence. For major diseases like Fusarium wilt and SMD in pigeon pea development of dominant SCARs will be affectively used in a breeding programme as it gives a quick plus or minus assay to identify a locus. Since only a single fragment is amplified, post amplification electrophoresis can be eliminated because the PCR products can be detected directly by staining with ethidium bromide in a microtiter plate or by measuring DNA concentration with a spectrophotometer. This would decrease the cost and increase the speed of the analysis.

In the present study a dominant RAPD marker, $OPA18_{800}$, linked to resistance to SMD was identified by means of BSA of an intraspecific F_2 population developed from a cross, ICPL-7035 x ICPL-8863. Cloning and characterization of this RAPD marker and development of an allele-specific SCAR, which can be used to quickly and accurately identify resistant genotypes for SMD resistance, was performed.

MATERIALS AND METHODS

Plant materials

ICPL-7035 was crossed as a female parent with ICPL-8863 and a segregating population was obtained by the selfing a single resistant F_1 plant identified through marker. 179 segregating F_2 plants were obtained and were grown in a greenhouse in pots along with the parents. Each pot consisted of 20 plants. Normal cultural practices were followed, except that no insecticides were sprayed. ICPL-8863 and LRG-41 highly susceptible cultivars to PPSMD were maintained as sources for mites and disease. F_2 plants were inoculated at the two-leaf stage with viruliferous mites by stapling SMD-affected pigeon pea leaves containing mites onto leaves of test plants (Nene and Reddy 1976). After 3 weeks, plants were assessed for disease symptoms (mild mosaic and small leaves). Asymptomatic plants were inoculated again by petiole grafting with SMD infected material collected from source plants (Reddy *et al*. 2002). Under grafting conditions, resistant plants did not show any pale green leaves while susceptible plants showed critical symptoms after 20-25 days (**Fig. 1**). The plants were classified as resistant (no symptoms) or susceptible (severe mosaic symptoms) by 7-8 weeks. The goodness of fit to Mendelian segregation of resistant and susceptible plants in the segregation population was tested by Chi-square test (Panse and Sukhatme 1961).

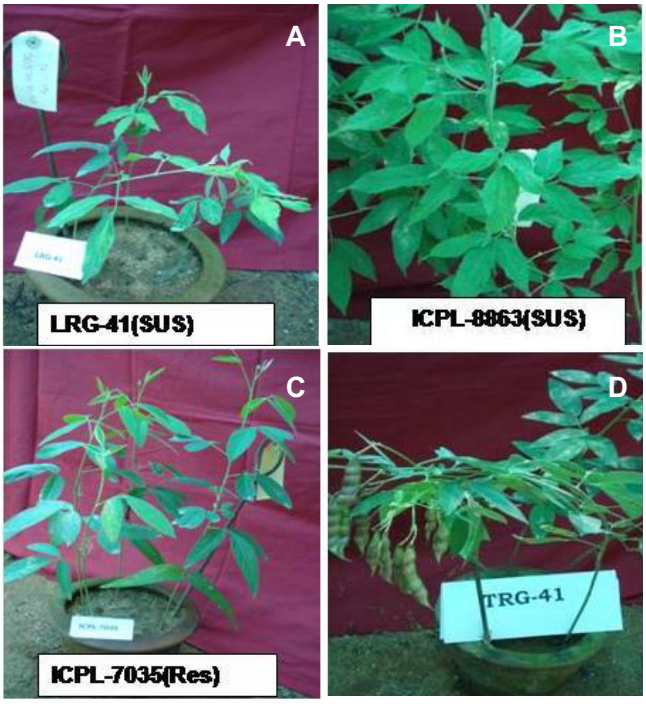

Fig. 1 Sterility mosaic disease transmitted through grafting technique. Clear symptoms in susceptible lines LRG-41 (**A**) and ICPL-8863 (**B**). ICPl-7035 (**C**) and TRG-41 (**D**) showing resistant reaction after grafting.

DNA extraction and amplification conditions

Genomic DNA was extracted from the leaves of parents, F_1 and F_2 plants at first tri-foliate leaf stage (15-20 days old) using the CTAB method (Murray and Thompson 1986) and this DNA was further purified from carbohydrates, proteins and RNA before actual use (Sambrook *et al*. 1989). Quantification and quality checking were done on a 0.8% agarose gel.

Polymerase chain reaction

The PCR reactions were performed in a 25 µl volume in 0.2 ml PCR tubes (Axygen Pvt, Ltd., India). The reaction mixture contained 25 ng of template DNA, 1X amplification buffer (10 mM of Tris-HCL pH 8 (Fermentas), 2.5 mM of $MgCl_2$, 0.2 mM of DNTPs, 0.5 µM primers (Operon) and 0.5 U *Taq* DNA polymerase (Fermentas). The reactions were performed in a Master Cycler Gradient 5331 (Eppendorf version, Germany). The reaction had an initial denaturation step at 94°C for 2 min, followed by 45 cycles of denaturation at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min. The final extension step was at 72°C for 7 min. The PCR products were separated on 1% agarose gels in TBE (89 mM Tris-HCL, 89 mM boric acid and 2 mM EDTA, pH 8.0) buffer stained with ethidium bromide (0.75 µg/ml) and visualized under UV light using an Alpha Innotech Corporation gel doc system and the results were documented.

A total of 200 random oligonucleotide primers were surveyed for potential polymorphism between the DNA bulks of resistant

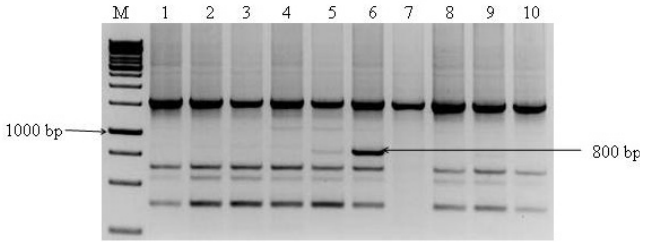

Fig. 2 Amplification profile of the RAPD primer OPA18 in resistant and susceptible genotypes. Lane M, 1 KB marker; 1) LRG-41; 2) LRG-38; 3) TRG-7; 4) TRG-2; 5) WRG-65; 6) ICPL 7035; 7) ICPL 8863; 8) ICPL 87119; 9) TRG-2; 10) BCMR 736.

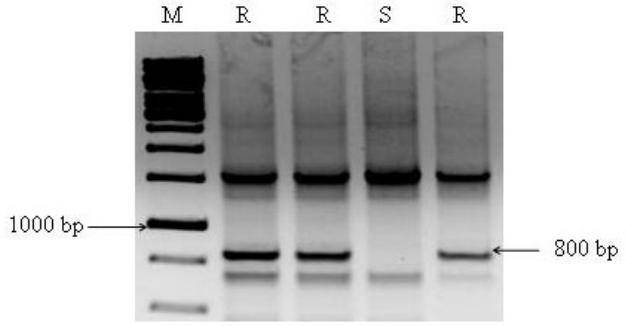

Fig. 3 Amplification profile of the marker SCAR 816 in pigeon pea genotypes. M, Marker; Lane 1, ICPL 7035; Lane 2, ICP 6974; Lane3, ICPL8863; Lane 4, ICP 15225.

Table 1 Ratio of F2 plants for SMD reaction and marker status in cross ICPL-7035x ICPL-8863.

Marker	№ of plants			Ratio	χ^2 probability
	Present	Absent	Total	R:S	
OPA18 observed	65	110	179	7:9	
SCAR 16f/r observed	69	110			1.198 (0.35-0.25)
Expected	78.3	100.6	179		

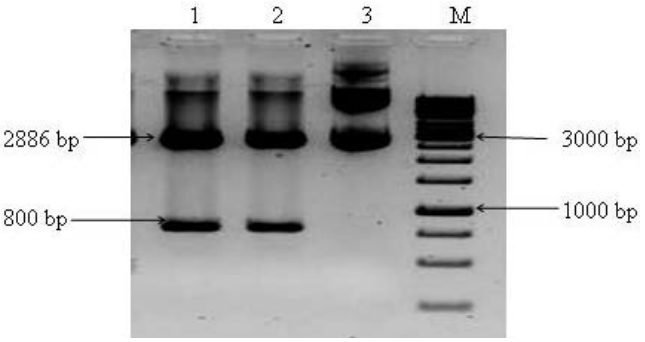

Fig. 4 Confirmation of clones by Restriction digestion. Lanes 1 and 2, positive clones; Lane 3, negative clone; M, 1 Kb marker.

and susceptible parents. Primer OPA18 was found to produce a putative marker which differentiated resistant parent and resistant bulk from susceptible parent and susceptible bulk (**Figs. 2, 3**). The same was confirmed through grafting. Co-segregation analysis of the putative marker in the F_2 population confirmed the association of $OPA18_{800}$ primer with the SMD gene (**Table 1**).

Constitution of DNA bulk and evaluation of polymorphism

Equal quantities of DNA were bulked from 10 resistant and susceptible F_2 plants. These bulks along with parents were screened with primer to identify the polymorphic marker which is present in resistant parent as well as resistant bulk and not in the susceptible parent and susceptible bulk. The linkage of the polymorphic marker was confirmed by using an individual segregating population from which the bulks were generated.

Cloning and sequencing of RAPD markers

RAPD primer OPA18 was used to amplify genomic DNA of the resistant parent ICPL-7035. Amplified fragments were separated on a 1% (w/v) low melting point agarose gel before being excised and purified by means of the QIAquick Gel Extration Kit (Qiagen, Valencia, CA). The purified DNA fragments were ligated and transformed with the pTZ57R/T Vector System. Cloned RAPD fragments were identified via Colony PCR with RAPD primer of OPA18 and confirmed by restriction digestion with *Eco*RI (**Fig. 4**).

SCAR primer design and amplification

On the basis of the sequence of cloned RAPD product (GenBank Accession No. FJ215867), oligonucleotide primer pairs of 14 to 18 bases were designed using Oligo Explorer 2.0 software for specific amplification of the loci identified by selected RAPD marker (**Table 2**). Each primer contained the original 10 bases of the RAPD primer sequence plus the next 4, 6 and 8 internal bases. Care was taken to avoid possible primer dimer or secondary structure formation. Primers were synthesized by Operon Technologies, Inc. SCAR amplification of ICPL-7035, ICPL-8863 and LRG-41 genomic DNA was performed in 25 µL reaction volumes containing the same components used for RAPD analysis except for the concentration of $MgCl_2$ (2.0 mM) and SCAR primers (1 µM). PCR amplification consisted of 35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C followed by a final extension of 10 min at 72°C. The amplified products were resolved by electrophoresis on 1% (w/v) agarose gels in 0.5% (v/v) TBE buffer and stained with ethidium bromide.

RESULTS AND DISCUSSION

In this study a total of 200 random oligonucleotide primers were surveyed for potential polymorphism between the DNA bulks of resistant and susceptible parents. Primer OPA18 was found to produce putative markers which differentiated resistant parent and bulk from susceptible parent and bulk (**Figs. 2, 3**). Absence of the marker in F_1s indicating the susceptibility to the disease. The inheritance pattern of resistance to SMD was studied in the F_2 population based on a segregation ratio of alternative phenotypes (resistant and susceptible). A set of 179 F_2 plants of ICPL-7035 X ICPL-8863 were individually evaluated for the incidence of SMD. Of the 179 F_2 plants of cross 69 were resistant to SMD disease and the remaining was susceptible (**Table 3**). The F_2 segregation pattern of the resistant x susceptible cross revealed a digenic ratio of 7 resistant and 9 susceptible and thus confirms that the disease was controlled by two genes. Similar observations on susceptibility being under the influence of dominant genes over resistance in different cross combinations involving a resistant and a susceptible parent was reported and the F_2 segregation pat-

Table 2 SCAR primers designed based on the sequence of RAPD marker and the size of amplicon observed.

Primer	Primer sequence (5′ – 3′)	Length (mer)	Annealing temp. (°C)	Size (bp)
SCAR1-F	AGG TGA CCG TGC TAG A	16	56	816
SCAR1-R	AGG TGA CCG TCC GAG T	16		
SCAR2-F	AGG TGA CCG TGC TA	14	54	750
SCAR2-R	AGG TGA CCT TCC GA	14		
SCAR3-F	AGG TGA CCG TGC TAG AAC	18	55	700
SCAR3-R	AGG TGA CCG TCC GAG TAG	18		

Table 3 Phenotypic segregation ratio for reaction to SMD in F2 of ICPL-7035 x ICPL-8863 through grafting technique.

Crosses	№ of plants			Ratio	χ2 probability
	Susceptible	Resistant	Total	R:S	
Observed	110	69	179	7:9	1.198 0.35-0.25
Expected	100.6	78.3	179		

tern in above findings revealed digenic ratio of 7 resistant: 9 susceptible as resistant parent was differed from susceptible parent in respect of two gene pairs by Singh *et al.* (1983), Sharma *et al*. (1984) and Nagaraj *et al.* (2004).

Validation of RAPD and SCAR markers in parents and segregating population

The presence or absence of the $OPA18_{800}$ and SCAR 16f/r marker with the SCAR1-F and SCAR1-R primers was investigated in parents, F_1 and F_2 plants of the cross ICPL-7035 x ICPL-8863. Absence of the marker in F_1 plants (**Figs. 5, 6**) indicated that these F_1 plants were susceptible. In the F_2 generation the OPA18 marker was present in all SMD-resistant plants and absent in all the susceptible plants. The SCAR marker was specifically present in the resistant plants and resistant bulk and absent in the susceptible plants and susceptible bulk in F_1 and F_2 plants, like the original marker OPGA18 (**Figs. 7, 8**).

Co-segregation analysis of putative markers in the F_2 population confirmed the association of $OPGA18_{800}$ by the primer with the SMD resistance gene. The SCAR marker with 16 base pairs designed from the sequence of resistant RAPD DNA fragment also produced consistent results (**Table 1**). This segregation ratio fits well with the expected ratio of 7 resistant and 9 susceptible and thus confirms that the resistance was controlled by two genes indicating the complementary nature of the two dominant genes for susceptibility. It is therefore postulated that susceptibility is under the control of two independent loci exhibiting complementary gene action. When loci 1 or 2, or both, occur in a homozygous recessive state a resistance reaction occurs, while dominant alleles at both loci would be necessary to result in susceptibility. Accordingly, resistance is expressed in the presence of recessive alleles in homozygous state at least at one locus.

Many commercial pigeon pea varieties are susceptible to PPSMD and there is a need to identify tightly linked markers that could facilitate the transfer of resistant genes to popular cultivars using marker-assisted breeding. The use of molecular markers for resistance genes is particularly powerful as it removes delays in breeding programmes associated with the phenotypic analysis.

The essential requirement for MAS in a plant breeding programme is the identification of a linked marker and an efficient means for screening large populations in a reproducible manner. Marker validation is a process of examining the behavior of markers and the associated polymorphism in different genetic backgrounds (Gupta *et al*. 1999). Identification of flanking DNA markers located within 5-10 cM from a gene of interest has yielded high levels of selection accuracy for resistance (Hittalmani *et al.* 1995). The presence or absence of the RAPD and SCAR marker in diverse pigeonpea genotypes was investigated. The marker was consistently associated with the genotypes resistant to PPSMD but susceptible genotypes without the resistance gene lacked the marker. These results are to be expected because of the linkage of the marker to the resistance gene.

In the present study, the RAPD approach was utilized for identifying markers linked to the PPSMD resistance gene. Viral resistance genes have been tagged with markers in several other crops like soybean (Zheng *et al.* 2003), *Phaseolus* (Urrea *et al*. 1996) and pea (Gao *et al.* 2004).

The RAPD marker in this study converted into a simple SCAR marker SCMR 816(16f/r) for large-scale application in marker-assisted breeding. This involves the characterization of the linked marker and the design of locus-specific primers. The conversion of a linked marker to SCAR has been applied successfully in a number of crops, like common bean (Adam-Blondon *et al.* 1994), rice (Naqvi and Chattoo 1996), tomato (Zhag and Stommet 2001) and soybean (Zheng *et al.* 2003). Work on identifying more markers around the resistance locus is underway and would aid in isolating the PPSMD resistance at the molecular level. The SCAR marker identified will be utilized to develop cleaved amplified polymorphism markers.

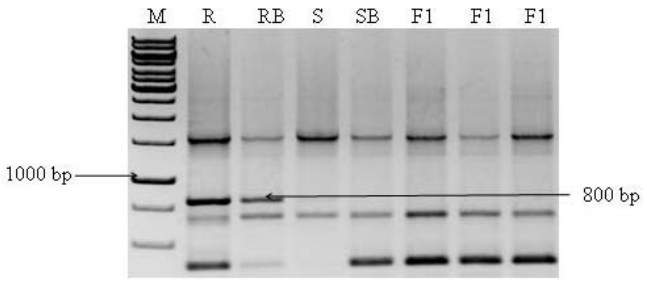

Fig. 5 Amplification products following PCR directed by primer OPA18 in parents and F1s. M, 1 KB marker; Lane R, ICPL 7035; Lane RB, ICPL 7035 bulk; Lane S ICPL 8863; Lane SB, ICPL 8863 bulk; Last 3 lanes, 3 F_1 individual plants.

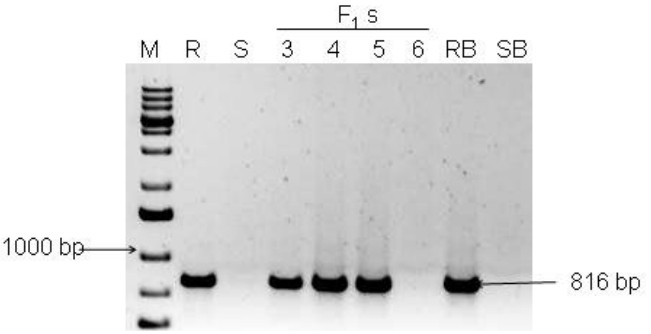

Fig. 6 Amplification profile of the marker SCAR 816 in parents and F_1s. M, marker; Lane 1, ICPL7035 (R); Lane 2, ICPL 8863 (S); Lanes 3-6, F1s of cross ICPL7035 x ICPL 8863; Lane 7, ICPL 7035 (Res. bulk); Lane 8, ICPL 8863 (Sus. bulk).

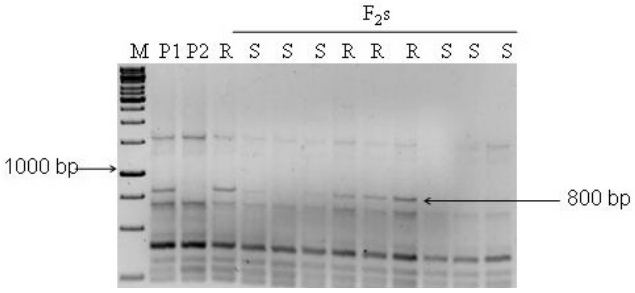

Fig. 7 Amplification profile of the primer OPA18 in parents and F_2 individual plants. M, marker; Lane P1, ICPL7035; Lane P2, ICPL 8863; Lane 3-12 F_2 individual plants.

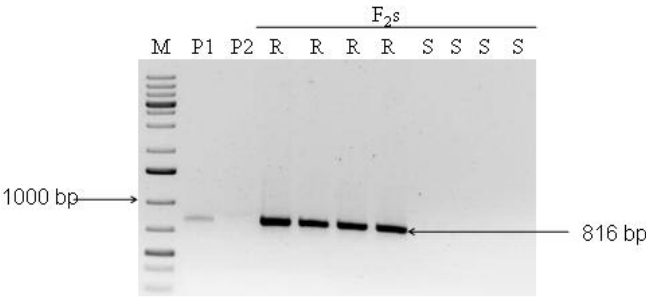

Fig. 8 Amplification profile of the SCAR 816 marker in parents and F_2 individuals.

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