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Development of the SCAR marker for genetic purity testing of cotton hybrid

P.Y. SHINDE* AND MALAVIKA DADLANI
Division of Seed Science and Technology, IARI, New Delhi - 110012
DRPYSSST@GMAIL.COM

ABSTRACT One of the most important attribute of seed quality i.e. genetic purity was evaluated with the study of genetic purity testing of one intra-hirsutum hybrid (Nathbaba). The electrophoresis profiles of tris soluble proteins, globulins and methanol precipitated fractions failed to differentiate the parents from the hybrid in the Nathbaba hybrid and hence not useful for genetic purity testing. The RAPD analysis was found useful in differentiating the two parents, as well as the female parents from the hybrid. Primer OPA-07 was selected for genetic purity testing of Nathbaba hybrid. The sequence tagged sites (STMS) markers were unable to differentiate the female parents from the hybrid. The development of SCAR marker from the RAPD polymorphic primer was successfully accomplished in the present study and this approach is suggested to ensure higher reproducibility in testing the genetic purity in cotton hybrid.

Key words: Cotton, genetic purity, RAPD primer, SCAR primer

Cotton is a major fiber crop of global importance with high commercial value, which is important for the sustainable economy of India and livelihood of the Indian farming community. Maintenance of high level of genetic purity of hybrid is essential to exploit the level of heterosis observed in cotton crop. It is estimated that for every 1% impurity in the hybrid seed of rice, the reduction is 100 kg per hectare [1]. The genetic purity testing is more important in cotton hybrids, which are mostly converted as Bt cotton hybrids by the respective seed companies. These hybrids were released as Bt cotton hybrids after ensuring that their genetic background was the most desirable one for the introduction of Bt gene. A variation in toxin expression of Bt gene due to the change in parental background of the hybrids was reported [2]. This demands a strict vigil on the genetic purity standards of a commercial hybrid. With this background, the following study was conducted to identify a marker suitable for establishing hybridity and testing genetic purity in intraspecific *Gossypium hirsutum* hybrid of cotton.

MATERIALS AND METHODS

Plant Materials

Seed of a commercial hybrid cultivar of cotton, produced by hand emasculation and pollination system and their parental lines, designated P1 (female parent) and P2 (male parent), were obtained from, M/s Nath Seeds, Aurangabad.

Electrophoretic analysis of seed proteins

Electrophoretic protein profiles of hybrid and parental lines were developed for total tris-soluble seed proteins [3-4] and methanol precipitated protein fraction [5] using bulk sample comprising of fifteen seeds. Parental lines and hybrids were identified based on presence or absence of respective bands that were recognized based on their relative mobility values (Rm).

* Corresponding Author
Presently working at Agricultural Research Station, Dhule (MPKV, Rahuri)

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DNA extraction

DNA samples were extracted from pooled leaf samples of 15 day old seedlings of the hybrid and parental lines following a by modified CTAB method [6]. Quantified DNA sample was either immediately used or stored at -20°C till further use.

RAPD analysis

Fifteen random decamer primers of Operon series were purchased from Genuine Chemical Corporation, New Delhi, India and used for RAPD analysis (Table 1). PCR reactions were performed in Biotron thermal cycler QB-96. The PCR procedure [7-8] was followed with slight modifications. PCR reaction mixtures (25 µl) consisted of 25 ng of template DNA, 0.2 µM primer, 400 µm dNTP mix (MBI Fermentas) 1.5 mM/µl $MgCl_2$, 1 X PCR assay buffer (Bangalore Genei, India) and 1 unit Taq DNA polymerase (Bangalore Genei, India). The amplification program was as follows: initial denaturation at 94°C for 1 min, followed by 45 cycles of 1 min at 94°C, 1 min at 35°C and 1.5 min at 72°C, with a final extension step at 72°C for 5 min. The PCR products were stored at 4°C until fractionated by electrophoresis on 1.5% agarose gels. Each RAPD analysis was performed at least three times.

STMS (Sequence Tagged Microsattelite Site) analysis

Ten STMS primers spread across different chromosomes as per [9] were used to detect the polymorphism between the parental lines and hybrid. The details of the STMS primers along with their sequences are given in the table 2. The STMS primers were locally synthesized and PCR amplification was performed in a 0.2 ml reaction tube with total volume of 10 µl containing 30 ng of template DNA, 0.5 µM of forward and reverse primers, 2 mM dNTP mix (MBI Fermentas) 1.5 mM/µl $MgCl_2$, 1 X PCR assay buffer (Bangalore Genei, India) and 0.6 unit of Taq DNA polymerase (Bangalore Genei, India). The amplification profile consisted of following steps: initial denaturation at 95°C for 5 min, followed by 40 cycles of 1 min at 94°C, 30s at 55-62°C (temperatures varied depending on primers) and 1 min at 72°C, with a final extension step at 72°C for 8 min. The PCR products were electrophoresed on a 3 % SFR agarose gel (Superfine resolution, Amresco Inc, OH, USA)

Table 1. List of RAPD primers used for genetic purity studies.

Sr. No.	Name of Primer	Sequence (5'.3')
1.	OPA-07	GAAACGGGTG
2.	OPA-08	GTGACGTAGG
3	OPA 09	GGGTAACGCC
4	OPA-10	GTGATCGCAG
5.	OPA 11	CAATCGCCGT
6	OPA 12	TCGGCGATAG
7	OPA 13	CAGCACCCAC
8.	OPB-04	GGACTGGAGT
9.	OPB-05	GGTGACGCAG
10	OPB 06	TGCTCTGCCC
11	OPB 07	GGTGACGCAG
12	OPB 18	CCACAGCAGT
13	OPB 20	GGACCCTTAC
14	OPC 04	CCGCATCTAC
15	OPC 05	GATGACCGCC

Table 2. List of STMS primers used for the genetic purity studies.

Sr. No.	Name of Primer	Sequence (5′.3′)
1.	BNL-1721 F	TGTCGGAATCTTAAGACCGG
	BNL-1721 R	GCGCAGATCCTCTTACCAAA
2.	BNL-1053 F	AGGGTCTGTCATGGTTGGAG
	BNL-1053 R	CATGCATGCGTACGTGTGTA
3.	BNL-3255 F	GACAGTCAAACAGAACAGATATGC
	BNL-3255 R	TTACACGACTTGTTCCCACG
4.	BNL-3408 F	ATCCAAACCATTGCACCACT
	BNL-3408 R	GTGTACGTTGAGAAGTCATCTGC
5.	BNL-3034 F	AAAGGAAATGGTCATTGGCA
	BNL-3034 R	AGTACCCGCCATTTCAAGTG
6.	CM-43F	GCGCAGATATTATTATCACAGC
	CM-43R	TATATAAATTTGCATCAGTTGGC
7.	BNL-2590F	GAAAAACCAAAAAGGAAAATCG
	BNL-2590R	CTCCCTCTCTCTAACCGGCT
8	BNL-3590 F	TTTAGCCCCAGTAACATGCC
	BNL-3590 R	ACTGCAAGCTCTGCCCTAAA
9.	BNL-786 F	CTTTCCACGTGTAATTTGTTGATA
	BNL-786 R	GATCTTAACTCTTGCTCTCTCTCTCTC
10.	BNL-3452 F	TGTAACTGAGCAGCCGTACG
	BNL-3452 R	GCCAAAGCAGAGTGAGATCC

Table 3. List of SCAR primers used for genetic purity testing.

Sr. No.	Name of Primer	Sequence (5′.3′)
1.	MD-1 F	TTTTGAAACGGGTGCAATTCAG
	MD-1 R	GAGTATTCCGGGTCCCCTGT
2.	MD-2 F	CCCGAATTGAAATACCGATG
	MD-2 R	AGTATTCCGGGTCCCCTGT
3.	MD-3 F	AATTCAGGACCATCTGAGGCTA
	MD-3 R	ACAACGACAAGCAACAGACACT
4.	MD-4 F	GAAACGGGTGCAATTCAGGACC
	MD-4 R	GAGTATTCCGGGTCCCCTGT

Cloning and sequencing of RAPD markers

Polymorphic male parent specific band (~1kb) of RAPD marker OPA_07 was excised from the agarose gel and ligated into PGMET vector (Promega USA). After confirming that transformation of *E. coli* DH-5a yielded recombinant clones containing the RAPD markers, the cloned fragment was sequenced and pairs of SCAR primers (Table 3) were constructed using primer 3 software.

Analysis of the sequence characterized amplified regions (SCAR)

After getting the sequence of the insert, four pairs of forward and reverse primers were designed by using the primer-3 programme. These primers were named as MD-1, MD-2, MD-3 and MD-4. The developed primers were used for the PCR analysis with the template DNA of parental lines and hybrid. The SCAR primers were used to detect the polymorphism between the parental lines and hybrids seeds. Total of 4 SCAR primers were used for a STMS analysis. The details of the SCAR primers along with their sequences are given in the table 3. The PCR reaction components contained following 10ng of the template DNA, 5 µm of each of the reverse and forward primers, 2 mM of dNTPs, 10 x pcr buffer and 0.6 units of Taq polymerase. The amplification profile consisted of following steps "Initial denaturation step at 95^{0}C for 5 min followed by 40 cycles of 94^{0}C for 1 min, annealing step at 55-62^{0}C (different temperature for different primers) for 30 seconds and final extension step 72^{0}C for 1 min which was followed by one step of 72^{0}C for 8 min.

RESULTS AND DISCUSSION

Protein profiling

In the present study, three different protein fractions i.e. tris soluble protein, salt soluble globulin and methanol precipitated proteins were analyzed, following SDS polyacrylamide gel electrophoresis (SDS-PAGE) technique.

Total Tris-soluble proteins

Total of 15 bands were observed in hybrid, having Rm values from 0.21 to 0.96. The protein profile of the hybrid was found similar to that of female parent where as the male parent was clearly identified by the absence of polypeptide band of Rm value 0.69 and presence of additional band of the Rm value 0.78 (Fig. 1).Thus, though the total soluble protein profile could differentiate the two parents, it was not sufficient to distinguish the hybrid from the female parent.

Salt soluble globulins

The salt soluble globulin profile of the hybrid showed very little polymorphism from its parental lines, except for the absence of two bands at Rm value 0.41 and 0.47 in the male parent, which were recorded in the female parent and the hybrids. There was a total of 12 bands observed ranging from Rm value 0.21 to 0.80. (Fig 1).

While in case of *hirsutum* cotton hybrid Nathbaba, the two parental lines were distinguishable from each other with respect to the tris soluble protein profile, the hybrid was identical to its female parent and hence it was not of much relevance for testing hybrid purity. Tris soluble protein profiles have been also reported to be useful in differentiating intra *hirsutum* and inter-specific (*hirsutum* x *barbadense*) cotton hybrids from their parents, though to a limited level [10-12]. A better polymorphism among cotton genotypes in globulin fraction than the total soluble protein profile was observed by [10]. A clear-cut

observed by [13] following SDS-PAGE of salt soluble globulins. Fair level of polymorphism between some of the hybrids and parents of AAH-1 was reported by [14] in which globulin electrophoresis was found to be a reliable technique for testing genetic purity of hybrid AAH-1. However, the salt soluble globulins were not found much useful in the present study as both the hybrids recorded globulin profiles very similar to their respective females though it clearly differentiated from their male parents.

Methanol precipitable fraction

A total of 12 bands having different Rm value were observed in the methanol precipitated fractions of hybrid and its parental lines. Similar to the tris-soluble protein fraction, the methanol protein profile of the male parent was distinct from the hybrids with respect to the band of Rm value 0.48. The profile of hybrids was similar to that of its female parent. (Fig 1). Following a different protocol, [5] Vasudevan *et al* (2004) observed higher degree of polymorphism between four cotton hybrids and their parental lines with respect to methanol precipitate protein profile. Contrary to their report, in the present study, SDS-PAGE of methanol precipitated fraction of the hybrid seeds were also quite similar to their respective parental lines and hence was not found suitable for assessment of genetic purity of hybrid cotton seed. This supports the limited polymorphism in *Gossypium* species reported earlier by [15].

Molecular markers

In the present study, 15 random primers (which were selected from the preliminary studies done in the same laboratory) used, could generate 70 and 81 amplified fragments in the hybrids i.e. Nathbaba.

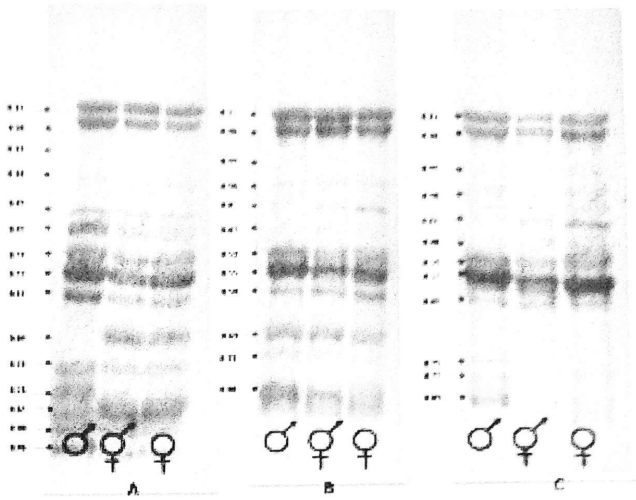

Fig 1 : Tris solubule protein (A), Salt Soluble Globulins (B) and Methanol Precipitated protein (C) of Nathbaba Hybrid

RAPD (Random Amplified Polymorphic DNA) analysis

of the 20 random primers, 15 resulted in amplified products. Each of the individual primers produced 2 to 9 amplified products with the size variation from 300 bp to 2000 bp. However, out of these only 2-5 fragments were of high intensity whereas the intensity of other fragments was low. Altogether, a total of 70 amplification products were generated from 15 primers. Among the 15 primers showing amplification, OPA-07 showed most reproducible polymorphism with respect to the banding pattern. In all, nine amplified fragments were generated with the primer OPA-07 in the hybrid and nine and seven fragments in the male and female parental lines, respectively. The size of these products ranged from 600-1800 bp. (Fig. 3). Thus, a male specific marker fragment of the size of approximately 1000 bp was useful in differentiating it from the female parent as well as to detect the presence of selfed female in the hybrid seeds. The size of the fragments ranged from 300 to 2000 bp, which was comparable to 280-3000 bp fragments

by [16] in eight commercial hybrids amplified with 25 RAPD primers, and also with 400-2300 bp amplified fragments reported by [17]. The source of polymorphism in RAPD assay of hybrids and their parents could be due to base change within the priming site, insertion that renders priming site too distant to support amplification, and deletion or insertion that change the size of DNA fragment without preventing its amplification [7]. The difference in markers between the parents and hybrids may be the result of DNA recombination, mutation or random segregation of chromosome during meiosis following hybridization [18].

Among the other useful primers, OPA-13 could also differentiae the two parental lines as well as female parental lines from the hybrid. OPA-13 could differentiate between male and female parent by the fragment of size of 1000 bp which was present in the male parent only (Fig 3). Primer OPA-08 also could amplify a fragment of 700 bp in the male parent as well as in the hybrids. However, it was not of much practical use because of the presence of another fragment of the size 750 bp (Fig 3) and hence it was difficult to differentiate the two fragments from one another. Among the polymorphic primers, reproducibility of OPA-07 was highest and hence it was selected for detection of selfed seed in the commercial hybrid cotton seed lots of hybrid Nathbaba. Of the 15 primers used, only 4 primers, OPA-07, OPA-10, OPA-13 and OPA-08 resulted in useful polymorphism between the hybrids and their parental lines. For testing the hybridity of Nathbaba ,a male specific fragment of about 1000 bp generated by OPA-07 was found most useful, whereas OPA-10 generated a male specific fragment of a about 700 bp for differentiating the two parents as well as the hybrid PKVDH-1 from its female parent. Therefore, these two primers, respectively, were used for testing genetic purity on the basis of RAPD analysis of hybrid Nathbaba.

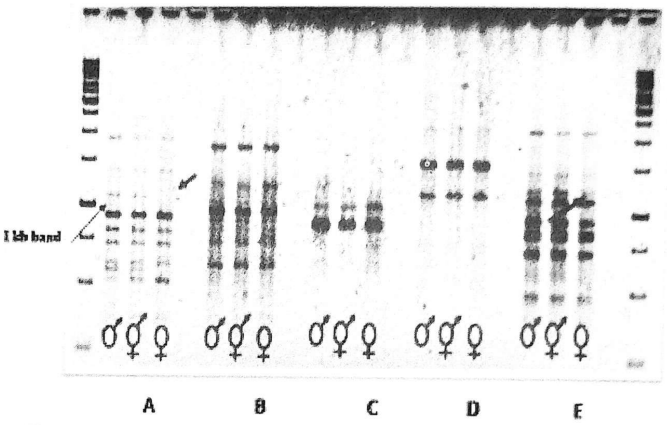

Fig 2. Banding pattern of parental lines of Nathbaba hybrid using OPA-7(A), OPA-9(B), OPA-11(C), OPA-12(D) and OPA-13(E) RAPD primers revealed on 1.4% agarose gel

STMS (Sequence Tagged Microsatellite Site) analysis

The template DNA of hybrid and its parental lines were screened using 10 STMS primers. Out of the 10 STMS markers, of which seven gave scorable banding pattern on 3% SFR gel. All STMS primers produced a single band, except the primer BNL-3255, which amplified 2 bands in the hybrid Nathbaba (Fig 3). The size of the amplified product was between 90 bp to 100 bp. From the banding patterns of STMS primers, it was clear that none of the STMS markers could differentiate between the parental lines of the cotton hybrid on 3% SFR gel. Thus, among the RAPD and STMS analysis, RAPD analysis of the hybrid Nathbaba and parental lines was useful in discriminating the female parental lines from the respective hybrids with a male specific marker. The male specific marker was tested for its reproducibility by repeating the PCR reaction thrice and it was found to be reliable.

The STMS primers tested, could not differentiate between the parental lines and the hybrids and hence were not found useful for testing genetic purity of the hybrid seeds and hence were not found useful for testing genetic purity of the hybrid seeds.

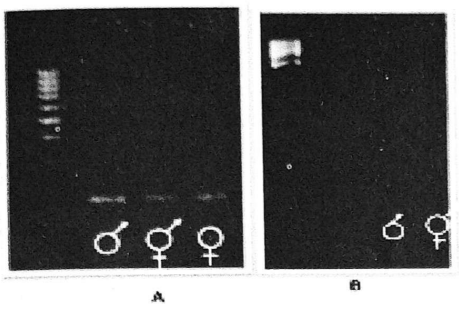

Fig 3. Banding pattern of parental line of Nathbaba and hybrid using BNL3252 (A) and BNL 1053(B) STMS primer revealed on 3% SFR agarose gel

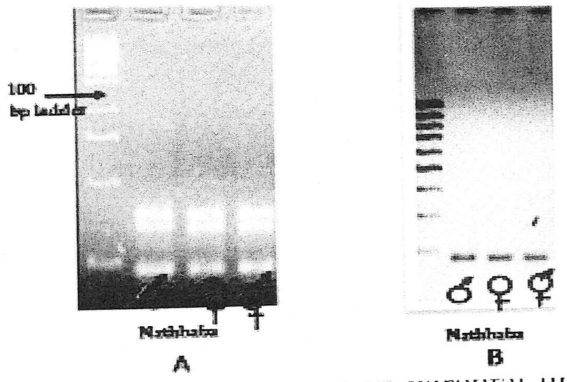

Fig 4. Banding pattern of parental lines of Nathbaba hybrid using BNL3255(A)and BNL-1721(B) STMS primer revealed on 3% SFR agarose gel.

Development of SCAR (Sequence Characterized Amplified Region) marker

In order to improve the reliability of RAPD marker analysis, an attempt was made to produce a SCAR marker from the fragment, polymorphic for female and hybrid in Nathbaba. The results established that SCAR marker, which produces a single clear band, can be developed from the polymorphic band of RAPD marker and it holds potential for testing the genetic purity, particularly where multiplexing of molecular markers is desired, such as testing the purity of Bt hybrid cotton. For validation of results with RAPD markers and obtaining more reproducible results, SCAR marker was developed from the polymorphic fragment amplified with OPA-07.

Testing of the eluted band

The 1000 bp fragment (OPA-07, 1000), which was polymorphic for hybrid and the female parent of hybrid Nathbaba, was eluted from the gel, the DNA was extracted from it and purified. The purified DNA was loaded in a 0.8% gel along with 1 kb DNA ladder. The presence of a single band of the 1000 bp confirmed the presence of the DNA band (which was polymorphic) in the eluted product (Fig. 5).

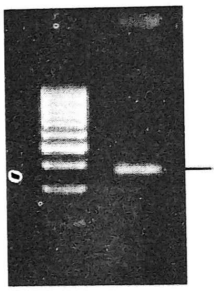

Fig.5. Confirmation of purified eluted product of 1kb for the development of SCAR marker Arrow indicates 1 Kb Fragment.

Confirmation of transformation by blue-white complementation

The DNA liagated with PGEM-T vector was transformed into the electro competent cells by the electroporation method. The confirmation of the transformation was done by plating the transformed cells on the Loria-Agar plates containing ampicillin and Isopropyl b-D-1 thiogalactopyranoside (IPTG). The blue-white complementation test

colonies confirmed the positive transformation. In all 16 positive colonies were picked.

Confirmation of the size of the insert

After extracting the plasmid DNA from the positive colonies, the DNA from each of the plasmid colony of the picked colonies was digested with EcoRI enzyme. The colony No. 16 showed two intact bands. The upper band of the size 3 kb confirms the PGEM-T vector and the lower band confirms the insert of size 1000 bp which was exactly of the same size as that of the OPA-07 1000 fragment. (Fig. 6). Polymorphic RAPD marker was converted to SCAR for genetic purity testing in chilli pepper by [19]. The advantage of RAPD is its simplicity, rapidity, requirement for only a small quantity of DNA and the ability to generate numerous polymorphisms [20]. One drawback of RAPD is that they are dominant in nature and one might argue that the heterozygotes cannot be differentiated from the homozygotes. The observed band in F1 lot thus might not be one of the F1 hybrids, but that of one of the parents, and therefore there is always a chance of error. But this type of error would arise only when the RAPD marker is dominant for the female parent in a manually crossed hybrid seed production programme. There will be no problem in identification of hybrid progeny when the marker, dominant for male parent, is used for testing the hybridity. As seeds are harvested from female parents only there is no chance of male parent self seed mixtures in the F1 seed lot, so a dominant band will always indicate a true hybrid.

Sequencing of the insert

The purified plasmid DNA was custom sequenced following the automatic capillary

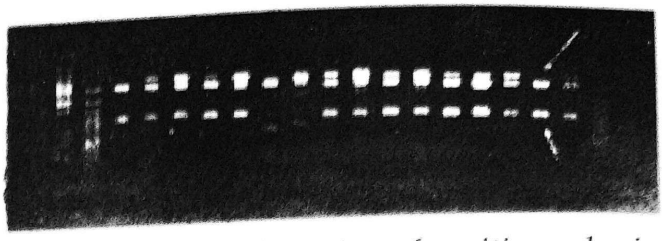

Fig 6. Restriction digestion of positive colonies for the identification of the colony having same size of the insert .Upper arrow denotes the 3 Kb size of the plasmid and lower arrow denote the fragement of 1 kb of the insert DNA

sequencing method.

Development of primers based on the sequence data

The sequence of the insert was processed by primer-3 design and a total of three primer pairs were developed. The sequences of the primers used are given in Table 3. The primers were used for SSR (Simple Sequence Repeats) analysis of the parental lines along with the hybrids Fig. 7 illustrates the banding pattern of the male, female and hybrid of Nathbaba hybrid with different SCAR primers. The forward and reverse combinations of 4 primers were used. In those combinations, there was no polymorphism between the parents and the hybrid. Then the different forward and reverse combinations of three SCAR primers were used for the SSR analysis. One of the combinations, in which SCAR-1 forward and SCAR-3 reverse primer were used, gave a polymorphic banding pattern. (Fig 7). This combination resulted in a fragment of 1000 bp in the male parent and the hybrid, while no amplification was seen in the female parent.

Hybrid cotton is regarded as a high value seed. Ever since the first commercial release of Bt hybrid cotton in 2004, a large number of Bt cotton hybrid varieties have been introduced and adopted by the farmers in cotton growing belt of all agro climatic zones.

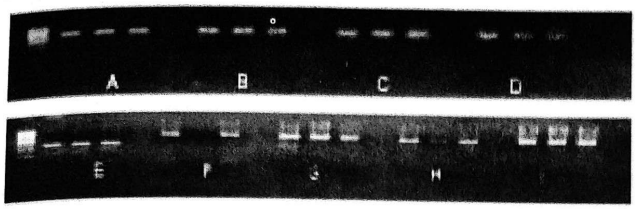

Fig 7 : Different combinations(A to I) of Reverse and Forward primer pairs of MD-1, MD-2, MD-3 & MD-4 SCAR primers. Combination F shows polymorphic banding pattern.

It is important that a reliable methodology of quality assurance is adopted by the seed producing agencies to ensure high quality standards. The grow-out test on the basis of morphological characters can only be useful for testing the genetic purity and this must be supplemented with other tests for checking the Bt-presence. This is going to be a major limiting factor of the grow-out test for testing the hybrid purity in transgenics in future. Since the presence of Bt gene must be reliably established either using a PCR based technique or by Enzyme Linked Immuno Sorbent Assay (ELISA) techniques, it will be desirable that technology such as multiplexing PCR analysis can be adopted both for verification of Bt genes as well as hybrid nature of the tested seed. For such purpose, the SCAR marker developed in the present study, from the polymorphic fragment amplified by RAPD marker such as OPA-07, can be used very effectively for multiplexing so that following one PCR reaction, both trait-specific and male specific markers are amplified and detected. Thus, the polymorphic RAPD marker can be developed into a SCAR marker, which gave a single clear band. The scoring of gel was made easy due to only one band present. This showed that this technique can be used for testing genetic purity of the hybrid as well as for multiplexing to detect other trait specific bands.

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