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GENOTYPING ANALYSIS OF SIX MAIZE (*ZEA MAYS* L.) HYBRIDS USING DNA FINGERPRINTING TECHNOLOGY

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Abstract

The objective of the present study was to conduct DNA based genotyping analysis for hybrid identification in maize (*Zea mays* L.) using DNA fingerprinting technology. To achieve this random amplified polymorphic DNA (RAPD) analysis was employed. A total of 40 random ten-mer primers were surveyed. Of these, OPR-03, OPR-11 and OPT-06 were polymorphic which not only verified the purity among three lots of the same maize hybrid type, but also distinguished FM2 maize hybrid type from FM3. These three polymorphic primers produced unique banding patterns that clearly detect the purity of the hybrid lots. Our results revealed that RAPD is a powerful tool for purity detection. This assay will allow screening of seed lots for verification of purity and would improve the efficiency of our breeding programmes.

Introduction

Maize (*Zea mays* L., $2n = 2x = 20$) is the second most important cereal crop in the world after wheat. The leading maize producing countries of the world are USA, Brazil, South Africa, India, The Philippines and Indonesia. In Pakistan, it can be planted successfully throughout the country; however, its cultivation is concentrated in Punjab and NWFP that together contribute 98% of the total production. During 2003-04, maize area and production were 0.94 million hectares and 1.77 million tons, respectively (Anonymous, 2004).

Corn like wheat, has been subjected to intensive genetic and cytogenetic studies. A quantum jump in its yield was achieved with the introduction of hybrid corn, a first generation progeny from a cross involving inbred lines. In Pakistan, although efforts on the development of hybrid corn are underway at public level, it is not yet possible to introduce hybrids in farmers community due to poor yield performance compared to the hybrids launched by multinational private seed companies. Recently, national seed companies are also struggling to introduce hybrids developed by different foreign seed companies. Firstly, hybrid performance is evaluated at different locations followed by the purchase of bulk quantity of better hybrid seed. Before disposing the seed to the farmers, the companies need an accurate hybrid identification to ensure their trueness with the control hybrid samples for which they place the orders.

Traditionally, morphological characteristics of crop plants have been used for varietal and clone identification. However, this method of cultivar identification is difficult, time consuming and ambiguous. From the late 1980s, different electrophoresis (Zillman & Bushuk, 1979; Tkachuk & Mellish, 1980) and reversed-phase high–performance liquid chromatography (RP-HPLC) (Marchylo *et al*., 1988; Scanlon *et al*., 1989) of seed storage proteins are well-established and often considered effective

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methods of cereal cultivars identification. But the ability of such biochemical analyses to distinguish among the cultivars is poor. Alternatively, DNA-based molecular markers are one of those new genomic tools, which offer high resolving power (Perry, 2004). Moreover, these markers are not stage and tissue specific and are not affected by the environment.

Restriction fragment length polymorphism (RFLP) has been proposed for cultivar identification (Pagnotta *et al*., 1996), however, polymerase chain reaction (PCR) based markers have received more attention (Soleimani *et al*., 2002). The PCR-based markers require small amount of DNA, permitting to conduct many reactions from a single kernel. In maize, numerous studies yielded a significant correlation between genetic distances obtained by molecular markers and the coefficient of coancestry (Smith *et al*., 1997; Lubberstedt *et al*., 2000). For this reason, DNA markers, particularly random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs), are proposed to be an appropriate tool for identification of clones (Devarumath *et al*., 2002), somaclonal variations (Rahman & Rajora 2001), breeding lines and hybrids (Bastia *et al*., 2001), and cultivars (Mohanty *et al*., 2001) but also facilitate the monitoring of introgression, mapping of QTLs (Paterson *et al*., 2003) and the assessment of genetic diversity (Rahman *et al*., 2002; 2005; Asif *et al*., 2005) in different crops like maize (Kantety *et al*., 1995; Lubberstedt *et al*., 2000). RAPD markers (Williams *et al*., 1990; Welsh & McClelland, 1990) have been widely used for genotype identification in plants (Yamagishi, 1995; Rahman & Zafar, 2001) and gained importance due to its simplicity, efficiency and non-requirement of sequence information (Karp *et al*., 1997).

The primary objective of this study was to establish a more rapid and efficient method of hybrid identification using RAPD analysis. The efficiency of the RAPD methodology in maize for seed purity testing was evaluated successfully, which would be beneficial for our maize breeding programmes and seed industry.

Materials and Methods

Seed material: A total of six hybrid corn samples were received from Engro Chemicals Pakistan Ltd. Three lots each for FM2 and FM3 type of maize hybrids were designated as FM2a, FM2b, FM2c, and FM3a, FM3b, FM3c, so the total samples were six for genotyping analysis.

Total genomic DNA extraction: DNA was extracted separately from pre-soaked six hybrid maize seed embryos using modified Hexadecyltrimethyl ammonium bromide (CTAB) procedure (Mehboob-ur-Rahman, personal communication). DNA concentration was determined by DyNA Quant 200. Quality of maize DNA was checked by running on a 0.8% agarose gel and for PCR analysis, DNA was diluted in 0.1X TE buffer to a concentration of 10 ng/ul.

PCR and primers: For RAPD analysis, concentration of DNA of six maize hybrids, 10X PCR buffer with $(NH_4)_2SO_4$, $MgCl_2$, gelatin, dNTPs (dATP, dTTP, dGTP and dCTP), decamer random primer and *Taq* DNA polymerase were optimized, respectively. Random oligonucleotide primers were of Operon Technologies Inc. Alameda, Calif. USA. *Taq* DNA polymerase together with 10X PCR buffer, $MgCl_2$ and dNTPs were from

MBI Fermentas, however gelatin of Sigma, St-Louis, was utilized. A 25 ul reaction volume was used for PCR amplification, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dGTP and dCTP, 30 ng of primer, 0.001% gelatin, 25 ng of maize DNA and 1 unit of *Taq* DNA polymerase. DNA fingerprinting for testing purity of six maize hybrids was performed with a set of 40 RAPD primers (OPR 1-20, OPS 1-10 and OPT 1-10). PCR profile used with a first denaturation step of 5 minutes at 94°C followed by 40 cycles of 94°C for 1 min., 36°C for 1 min. and 72°C for 2 min. After 40 cycles the reactions were incubated at 72°C for 10 min. and then held at 20°C until the PCR tubes were removed. All the PCRs were run with Eppendorf Mastercycler Gradient.

Analysis of RAPD data: After PCR, the products were electrophoresed on 1.2% agarose gels using 0.5X TBE buffer and visualized under UV light by staining with ethidium bromide and photographed with EagleEye, Gel Documentation System. Six maize hybrids were compared with each other using their amplification profiles.

Results and Discussion

Forty random decamer primers consistently amplified 320 RAPD loci. The number of loci resolved by one primer ranged from 4 to 13 with an average of eight loci per primer. Three primers (OPR-03, OPR-11 and OPT-06) produced specific loci to identify and verify the purity of maize hybrids. These three primers produced 20 amplification fragments, while the number of resolvable bands was 8, 5 and 7 for the primers OPR-03, OPR-11 and OPT-06, respectively (Table 1). These primers produced unique banding patterns, allowing the discrimination of FM2 and FM3 maize hybrids and verification of purity among the three lots each of these two types of maize hybrids. RAPD banding patterns generated by primers OPR-03, OPR-11 and OPT-06, of six maize hybrids (three samples each of two types of maize hybrids) are shown in Figs. 1 and 2.

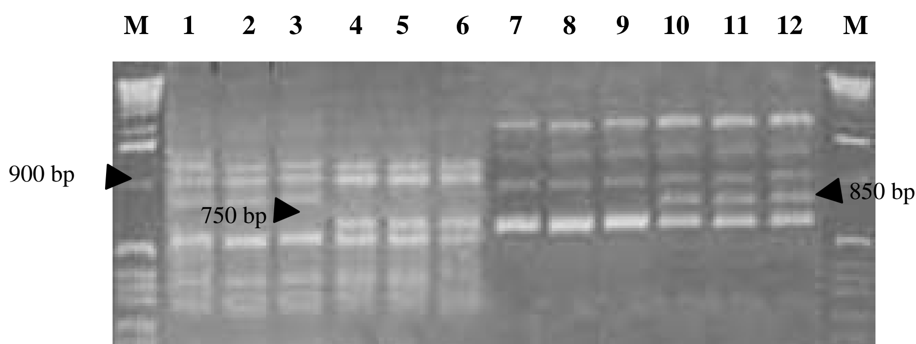

Fig. 1. PCR profile of six maize hybrids with OPR-03 and OPR-11.

M= DNA Ladder, Lane 1 to 6= Primer OPR-03 and Lane 7 to 12= Primer OPR-11
1= FM-2a, 2= FM-2b, 3= FM-2c, 4= FM-3a, 5= FM-3b, 6= FM-3c, 7= FM-2a, 8= FM-2b, 9= FM-2c, 10= FM-3a, 11= FM-3b, 12= FM-3c

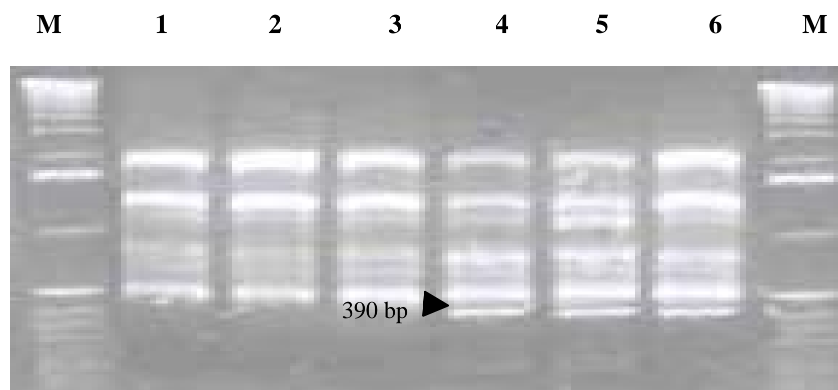

Fig. 2. PCR profile of six maize hybrids with OPT-06.

M= DNA Ladder, 1= FM-2a, 2= FM-2b, 3= FM-2c, 4= FM-3a, 5= FM-3b, 6= FM-3c

In this study, RAPD analysis was used to verify the purity of six different lots of two types of maize hybrids. The use of three selective primers allowed the discrimination of two types of hybrids analyzed and purity was confirmed. RAPD analysis has been successfully used for hybrid and parentage verification of other crop plants. Yamagishi (1995) developed RAPD markers that were used for *Lilium* species characterisation and hybrid detection. In cherokee rose, *Rosa laevigata,* RAPD analysis allowed the identification of the erroneous classification of the hybrid 'Silver Moon' (Walker & Werner, 1997). RAPD markers were also used to verify interspecific hybridisation (Benedetti *et al*., 2000; Mei *et al*., 2004).

Table 1. Polymorphic primers and presence of DNA markers in maize hybrids.

S. No.	Random Primer	Total Bands	Polymorphic Loci		DNA Marker Name & Presence	
			No.	Size in bp	FM2 Hybrids	FM3 Hybrids
1	OPR-03	8	2	750 & 900	$OPR03_{900}$	$OPR03_{750}$
2	OPR-11	5	1	850	---	$OPR11_{850}$
3	OPT-06	7	1	390	---	$OPT06_{390}$

Thirty seven out of 40 primers showed totally monomorphic banding pattern, while three primers exhibited polymorphic as well as monomorphic bands and these three primers amplified polymorphic bands to verify and confirm the purity of maize hybrids. Primer OPR-03 amplified two polymorphic loci of 750 bp and 900 bp size, which have been designated as $OPR03_{750}$ and $OPR03_{900}$. While primers OPR-11 and OPT-06 produced polymorphic fragments of 850 bp and 390 bp length respectively, which have been named as $OPR11_{850}$ and $OPT06_{390}$ respectively. DNA marker $OPR03_{900}$ is present only in three lots of FM2 type maize hybrids and absent in three lots of FM3 type of maize hybrids. However, DNA markers $OPR03_{750}$, $OPR11_{850}$ and $OPT06_{390}$ have been found in all the three lots of FM3 type of maize hybrids and absent in three lots of FM2 hybrid. Hence, the DNA markers found in this study have not only distinguished the two types of maize hybrids, but also tested and verified genetic purity of three lots of each hybrid type. Moreover, monomorphic bands among all the maize hybrids expressed the common blood relation among *Z. mays* species.

The data obtained in this experiment confirmed the efficiency of RAPD technique for verification of hybridity and purity identification. Because of the simple experimental procedures, the requirement of minimal amount of plant tissue and the possibility of

automation (Terzi, 1997; Rahman *et al.,* 2004), RAPD analysis would be very useful in breeding for rapid and early verification of hybridity in large seedling populations and even purity testing of different seed lots, allowing the detection of true hybrids and verification of parentage of the hybrids and lines/cultivars.

The three lots of the same hybrid type of maize were pure while the two maize hybrid types were found genetically different. Among the major causes of variation among different genotypes are mutations within the primer binding regions that may yield null alleles, whereas a mutation between the primer regions may result in new alleles. The natural mutation rate for genomic DNA is estimated to range from 10^{-8} to 10^{-6} per locus and generation (Drake *et al*., 1998) and these mutations are mostly insertions and deletions (Twerdi *et al*., 1999). Unequal crossover in amplicons is another genetic reason for the unexpected variation as reported in wheat (Plaschke *et al*., 1995).

Our results illustrate the crucial importance of increased level of homogeneity and purity within different lots of the same hybrids or crop varieties before applying for plant breeder's rights. If lines are heterogeneous, further selfing generation should be performed. A more rapid procedure would be the prescreening of newly developed lines with molecular markers. This will result in a higher accuracy of genetic relationship and genetic diversity estimates, a prerequisite for essentially derived varieties (EDV) and hybrids identification (Heckenberger *et al*., 2002).

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