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IDENTIFICATION OF DIFFERENT WHEAT GENOTYPES THROUGH POLYMORPHISM BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

S. FAROOQ, T.M. SHAH, E. ASKARI, A.A. ZAIDI, M. ARIF AND N. IQBAL

Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Abstract

Polymerase chain reaction was used to detect polymorphism in 10 different salt-tolerant wheat introgression lines produced through wide hybridization, 2 cultivated hexaploid wheat varieties, a tetraploid wheat variety and salt-tolerant accession D of *Aegilops cylindrica*. DNAs extracted from fresh leaves by the CTAB method were amplified using 25 μL reaction volume in a Perkin Elmer Thermal Cycler and randomly sequenced 10 mer synthetic primers. Based on amplification reactions, all the primers were divided into 4 categories. Category A primers reacted with all the wheat lines, category B with none, category C with 10-14, and category D with 3-7 wheat lines. Of the 18 reactive primers, 11(61%) detected polymorphism in all the test material. The level of polymorphism was low and ranged from 1.2 to 22.8%. Most of the primers produced monomorphic bands including very intense, easily visible and less visible bands. Of the 9 salt-tolerant introgression wheat lines, 6 were identified on the basis of polymorphic bands. Our study indicated possibilities for using Randon Amplified Polymorphic DNA (RAPD) markers to detect specific variations in the genome that could be used for varietal finger printing.

Introduction

Identification of different genotypes of crop species and varieties is important particularly when new crop varieties are to be released, newly produced germplasm is to be registered, different accessions of wild species are to be characterized and purity of the germplasm is to be determined. For a long time, such identification has been based on phenotypic differentiation or morphological traits such as ear morphology, awning, and hairiness of neck or peduncle (Worland *et al.*, 1984). However, since there were many varieties with awns and hairy peduncle, identification of two similar varieties was often difficult. Similarly, the introgression lines which are sometimes different from the parent by only few base pair sequences are difficult to differentiate from each other on morphological or phenotypic bases. To overcome such difficulties, biochemical markers, such as isozymes, were developed and have been used successfully for the last two decades (Arus *et al.*, 1982, Gupta & Robbelen, 1986). Since the frequency of polymorphism detected by biochemical markers in different crop species was low, molecular markers such as Restriction Fragment Length Polymorphism (RFLP) were developed, and are being used widely for the mapping of plant genomes such as maize (Weber & Helentjaris, 1989), tomato (Tanksley *et al.*, 1992), rice (McCouch *et al.*, 1988), potato (Gebhardt *et al.*, 1989), arabidopsis (Reiter

Table 1. Germplasm used in the study.

S.No.	Plant designation number	Percentage
01	41	LU-26 / *Ae. cylindrica* D. // Pak-81
02	359	LU-26 / *Ae. cylindrica* D. // Pak-81
03	879	LU-26 / *Ae. cylindrica* D. // LU-26
04	880	LU-26 / *Ae. cylindrica* D. // LU-26
05	83	Durum / *Ae. cylindrica* D. // Pak-81
06	885	LU-26 / *Ae. cylindrica* D. // Pak-81
07	886	LU-26 / *Ae. cylindrica* D. // Pak-81
08	1073	LU-26 / *Ae. cylindrica* D. // LU-26
09	1076	LU-26 / *Ae. cylindrica* D. // Pak-81
10	LU-26	(salt-tolerant wheat parent)
11	Durum	(salt-sensitive tetraploid wheat)
12	Pasban	(salt-tolerant cultivated wheat)
13	*Ae. cylindrica*	Accession D
14	Pak-81	(salt-sensitive cultivated wheat)
15	751	LU-26 / *Ae. cylindrica* D. // Pak. 81

Note: Further detail on germplasm is given in Farooq *et al.*, (1992).

et al., 1992), barley (Graner *et al.*, 1991) wheat (Anderson *et al.*, 1992), peanut (Kochert *et al.*, 1991) and *Brassica rapa* (Song *et al.*, 1991). Recently, a new class of molecular markers (William *et al.*, 1990) known as Random Amplified Polymorphic DNA (RAPD) was developed. Unlike RFLP markers, RAPD markers do not require large amounts of DNA, restriction digestion, southern bloting and radioactive labelling.

In our wide hybridization program, we crossed wheat cultivar Pak-81 and LU-26 with salt-tolerant accessions of *Aegilops cylindrica* (Farooq *et al.*, 1992) and produced different salt-tolerant introgression wheat lines having the plant height, ear morphology, heading date, and grains per spike of wheat cultivars Pak-81 and LU-26, and the salt tolerance of *Ae. cylindrica*. Our objective was to detect visible differences simultaneously in Pak-81, LU-26, *Ae. cylindrica*, and salt-tolerant introgression lines. In the present study, we have used RAPD markers to differentiate these lines from parents and from each other. The results are reported in this paper.

Materials and Methods

Ten different wheat lines produced through wide hybridization, 2 cultivated wheat varieties (Pak-81 and LU-26) used as female parents for the production of F_1 hybrids and backcross derivatives, accession D of *Ae. cylindrica*, salt-tolerant wheat cultivar "Pasban", and tetraploid wheat cultivar "Durum" were used in the RAPD assay (Table 1). Twenty synthetic 10 mer primers belonging to S-series, obtained from Operon

Technologies (Alameda, California, USA) were used for amplification. Taq Polymerase, together with 10x PCR buffer, $MgCl_2$ and dNTPs, were purchased from Perkin Elmer Cetus. A Perkin Elmer DNA Thermal Cycler was used for DNA amplification.

Healthy seeds were germinated on moist filter papers in Petri-plates. Seedlings were raised in a growth chamber for 2-3 weeks at 20 $\pm$ 2°C and used for DNA extraction following the CTAB method of Rogers & Bendich (1988), replacing dry ice with liquid nitrogen. DNA concentration and quality were determined on a Spectronic-21 spectrophotometer at a wavelength of 260 and 280 nm. Working solution of 5 ng/μL DNA was prepared by diluting the stock DNA. Each dilution was then repeatedly rechecked on the spectrophotometer until the exact concentration (5 ng/μL) had been obtained.

The reaction volume (25 μL), containing 2.5 μL (1x) PCR buffer, 2.5 μL (2.5 mM) $MgCl_2$, 2.5μL (0.2 mM each) dNTPs, 0.2 μL (one unit) Taq Polymerase, 0.5 μL (0.4 μm) 10 mer primer, 5 μL (5 ng/μL) genomic DNA, and 11.8 μL water overlaid with one drop of mineral oil was amplified in a Perkin Elmer DNA Thermal Cycler according to the procedure of Wiliams *et al.*, (1990), using step cycle file programmed for 35 cycles. After amplification, one drop of 5x RAPD dye (bromophenol blue mixed with 10% glycerol, 0.1 M EDTA, and 2% SDS) was added to the reaction mixture. Only 12 μL of the reaction mixture was loaded on 2.0 % agarose gel submerged in TAE buffer. Lambda DNA digested with Hind III was used as a molecular size marker. Samples were electrophoresed for approximately 2 h., at 100 volts. After electrophoresis, the gels were rinsed twice with distilled water and stained in 0.5 g/mL ethidium bromide for 20 min., followed by two rinses in distilled water. The amplified products were viewed under U.V. transilluminator and photographed using the Stratagene Eagle Eye still video system. All the reactions were repeated twice using freshly extracted DNAs. Those reactions where any specific primer failed to produce amplification products were repeated three times, in order to confirm the nonreactivity.

Data were scored from three good-quality photographs produced for three replicates of each amplification reaction. Bands were scored from the top of the gel (band no. 1) to the bottom. The left lane of the gel was considered as Lane-I. The bands produced by a primer in all the wheat lines used in this study were scored under the heading of "total bands produced by that primer." All the characteristic bands produced by a primer that were repeatedly present and/or absent in some of the lines were scored as polymorphic bands. Reproducibility of the reaction was confirmed when a specific band appeared repeatedly in a specific region. Bands of less than 500 bp, except those which were clearly visible, were sometimes difficult to score and were not considered.

Based on the presence and absence of amplification products, all the primers were divided into 4 categories. Category A comprised 7 different primers that produced amplification product in all the wheat lines, while category B comprised 2 primers that failed to produce any amplification product in any of the wheat lines. Category C comprised 5 primers each produced amplification products in 67-93% of the wheat lines, while category D comprised 6 primers each produced amplification products in only 13-60% of the wheat lines.

Table 2. Total bands and polymorphie bands produced by 20 s-series primers and their distribution in salt-tolerant wheat germplasm.

Primer	Total scorable bands produced in the test material	Polymorphic bands	Distribution of Polymorphic bands in the test material														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S-01	22	5(22.8)	5	+	+	+	+	+	+	+	+	0	0	0	0	0	0
S-02	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S-03	04	0	0	0	0	0	0	+	0	0	0	+	0	0	0	0	0
S-04	119	7(5.9)	+	+	+	+	+	2	+	1	+	+	1	1	2	0	0
S-05	33	0	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+
S-06	04	0	0	0	0	0	0	0	0	+	+	+	0	0	0	0	0
S-07	87	1(1.2)	+	+	+	+	+	+	+	+	+	+	+	+	1	+	0
S-08	67	7(6.5)	+	+	+	1	1	1	1	1	+	+	1	+	1	+	+
S-09	62	9(14.5)	+	+	+	+	2	1	1	2	1	1	+	1	+	+	+
S-10	55	2(3.6)	0	0	0	0	0	+	0	1	0	+	0	+	1	0	+
S-11	115	3(2.6)	+	+	+	+	+	+	+	+	+	+	1	1	1	+	+
S-12	65	5(7.7)	+	+	+	1	1	+	+	2	+	+	+	+	1	+	+
S-13	41	0	0	+	+	0	+	+	0	+	0	+	+	0	0	0	0
S-14	62	2(3.2)	+	+	0	0	+	0	0	+	0	+	0	+	2	0	0
S-15	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S-16	30	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S-17	101	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S-18	80	0	+	0	+	+	+	+	+	0	0	+	+	+	+	0	0
S-19	83	1(1.2)	+	+	+	+	+	+	+	+	+	+	+	+	1	+	+
S-20	48	3(6.3)	+	+	+	0	+	+	1	+	0	0	1	1	+	+	+

+ = monomorphic bands 0 = no bands, Figures in parentheses are % Polymorphic bands.

Results

Details of amplification products produced by 20 different primers in all the wheat lines used in the study showed that all the primers used in the study except primer S-02 and S-15, produced amplification products that ranged between 2 and 119 products per primer (Table 2). Among the 18 primers, 7 (08, 09, 11, 12, 16, 17 and 19) were in category A and produced amplification products in all the wheat lines; 6 were (01, 04, 05, 07, 18 and 20) were in category C and produced amplification products in most of the lines while another set of 5 primers (03, 06, 10, 13 and 14) were in category D and produced amplification products in some of the lines. Of the 18 primers 11 (61%) detected polymorphic bands that ranged between 1 (S-07) and 9 (S-09) bands per primer. The level of polymorphism was low and ranged from 1.2% (primer S-07) to 22.8% (S-01). Most of the scorable bands (96%) produced in all the wheat lines were monomorphic, and in all the reactions they appeared in the 2000 to

500 base pair region, except in reaction with primer S-09, where less than 500 base pair fragment was amplified.

The amplification products produced by category A primers were further divided into 4 classes (Table 3). All primers, except S-19, amplified products of varying frequencies which appeared as high-intensity bands, intense bands easily visible bands, and less visible bands. Generally, in all the amplified products, the frequency of high-ntensity and easily visible bands was higher than that of very intense and less visible bands.

The amplification products obtained with some of the primers are shown in Figs. 1, 2, and 3. There is a set of 2 and 3 bands respectively in wheat lines 880 and 883 (lanes 4 and 5) immediately below the very intense band in the middle (Fig. 1). These bands are not present in wheat cultivar LU-26, and hence differentiates wheat line 880 from 883 and both of them from LU-26. Similarly, there is a set of bands located above the very intense band in wheat line 886 which is also present in *Ae. cylindrica* (lane 13) and absent in LU-26, and hence differentiates 886 from the other wheat lines.

Table 3. Different types of bands produced by 18 S-series 10 mer primers in salt-tolerant wheat germplasm.

Category of Primer	Primer designation and sequence 5' to 3'		Total bands	% Frequency of different bands produced by each primer *	+	(+)	(-)
A	S-08	TTCAGGGTGG	108	1.9	7.4	35.2	55.6
	S-09	TCCTGGTCCC	62	1.6	24.2	38.7	35.5
	S-11	AGTCGGGTGG	115	0.9	2.6	61.7	34.8
	S-12	CTGGGTGAGT	65	13.9	4.6	40.0	41.5
	S-16	AGGGGGTTCC	30	7.0	10.0	23.0	40.0
	S-17	TGGGGACCAC	11	1.0	3.0	67.0	19.0
	S-19	GAGTCAGCAG	83	-	36.0	17.0	47.0
C	S-01	CTACTGCGCT	22	-	4.5	59.1	36.4
	S-04	CACCCCCTTG	119	2.6	15.1	47.1	25.2
	S-05	TTTGGGGCCT	33	-	-	100.0	-
	S-07	TCCGATGCTG	87	-	-	65.5	34.5
	S-18	CTGGCGAACT	80	7.0	5.0	45.0	43.0
	S-20	TCTGGACGGA	48	-	4.2	60.4	35.4
D	S-03	CAGAGGTCCC	4	50.0	-	-	50.0
	S-06	GATACCTCGG	4	-	-	50.0	50.0
	S-10	ACCGTTCCAG	55	3.6	12.7	43.6	40.1
	S-13	GTCGTTCCTG	41	-	4.9	39.0	56.1
	S-14	AAAGGGGTCC	62	-	-	45.1	54.9

* = very intense bands + = intense bands (+) = visible bands (-) = less visible bands.

The amplification products obtained with primer S-09 are different from one another (Fig. 2). Wheat lines 41 and 359 produced identical amplification profiles which differs from the profile of wheat line 880 by the presence of band no. 2 in this line. Wheat lines 880 and 883 can be differentiated on the basis of the different sizes of the two bands present in the bottom of both the lines as well as the presence of the first band in line 883 which is missing in line 880. In wheat lines 885 and 886, the difference in the size of the very intense band in the middle is evident. Similarly, the presence of two bands at the top of lines 885 and 886 differentiates both of them from LU-26. In wheat lines 1073 and 1076, variation in size of the last two bands is clear. In wheat cultivar Durum and acc., D of *Ae. cylindrica*, the last band is missing, while in Pasban, the very intense band in the bottom is missing. All the lines can therefore, be easily differentiated from each other and from the parents. S-09 is the only primer that produced at least one polymorphic fragment (lane 12) which is less than 500 base pair in size.

In the profile on the amplification products obtained with primer S-12., wheat line 41 can be differentiated from wheat lines 359 and 879 on the basis of the absence of a fragment in line 359 (Fig. 3). Likewise, wheat line 880 can be differentiated from 883 by the presence of fragment no. 1, which is also present in wheat lines 885 and 886, but missing in wheat lines 883, 1073, 1076 and LU-26. Similary, fragment no. 1 in line 883 is also present in wheat lines 41, 1073, 1076 and LU-26 while those present in 879, 880, 885 and 886 are different and separate these lines from the others.

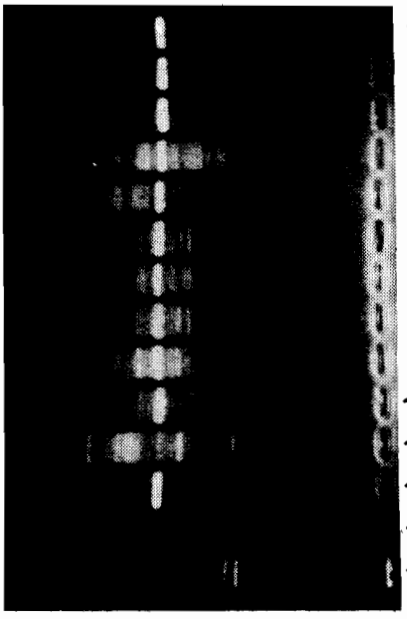

Fig.1. Amplification products produced by primer S-08. Wheat lines 880, 883 (lanes 4 and 5), 886 (lane 7) and LU-26 (lane 10) are clearly distinguishable.

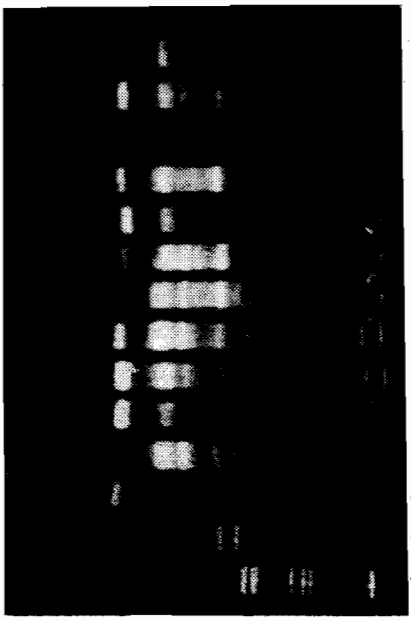

Fig.2. Polymorphism produced by primer S-09. Most of the lines can easily be distinguished from each other and from the parents.

Discussion

Since the RAPD markers were discovered (Williams *et al.*, 1990) they have been used extensively to accelerate the construction of linkage maps. Their large-scale use in tomato (Martin *et al.*, 1991; Klein-Lankhorst *et al.*, 1991), lettuce (Michelmore *et al.*, 1991), brassica, broccoli and cauliflower (Quirose *et al.*, 1991; Hu & Quirose, 1991) has successfully demonstrated their effectiveness in the identification of strains and varieties through genomic fingerprinting. In the present study, the technique of Polymerase Chain Reaction was used successfully for the generation of RAPD markers in different introgression lines of wheat produced through wide hybridization. The polymorphism thus detected was based largely on the presence and/or absence of a characteristic band and in some cases variation in the size of the bands, and was used for identification of different genotypes.

In the three earlier attempts to use RAPD markers in wheat, polymorphism was detected either by specifically sequenced primers (D' Ovidio *et al.*, 1990; Weining & Langridge, 1991) or by randomly sequenced 2-operon primers of series A and B (Devos & Gale, 1992). In the present study, 20-operon primers, of S-series were used to detect polymorphism in wheat varieties, as well as in wheat introgression lines. Of the 20 primers, 11 detected polymorphism were based on bands which are either absent in both the parents or polymorphic bands coming from the donor parent (*Ae. cylindrica*). The source of the polymorphism detected in the present study could have

been the changes in or between the priming sites due to (i) the introgression of foreign DNA or (ii) the introgression of a whole segment of *Ae. cylindrica* DNA. Although, category A primers detected polymorphism in all the wheat lines nevertheless, the level of polymorphism was generally low which may be due to the fact that most of the amplified products in wheat originate from repeated DNA sequences which are abundant in most cereals (Devos & Gale, 1992).

In the present study, 18 out of 20 primers amplified DNA fragments in all the wheat genotypes, and only 11 primers detected polymorphism. This differential behavior of the primers indicated that for a meaningful RAPD analysis, selection of a proper primers is very important. The selection could possibly be made either by testing a large number of randomly selected primers or by choosing primers of known sequences.

All the primers, amplified in all the wheat lines, four different types of bands, and among them frequency of visible bands was very high. Such classification in banding profiles has recently been reported by Heun & Helentjaris (1993) in corn. In their study, the frequency of quantitative polymorphism, based on variation in intensity of the bands was very high. We have also observed such variations, which although were scientifically justified, were not the basis for the genotypic identification performed in the present study as most of the lines were differentiated on the basis of presence and/or absence of a characteristic band. Since introgression lines generally look alike and most of the time, it is difficult to differentiate them from each other. The present study showed that RAPD analysis is one of the techniques that has the potential to be used effectively to make such differentiation.

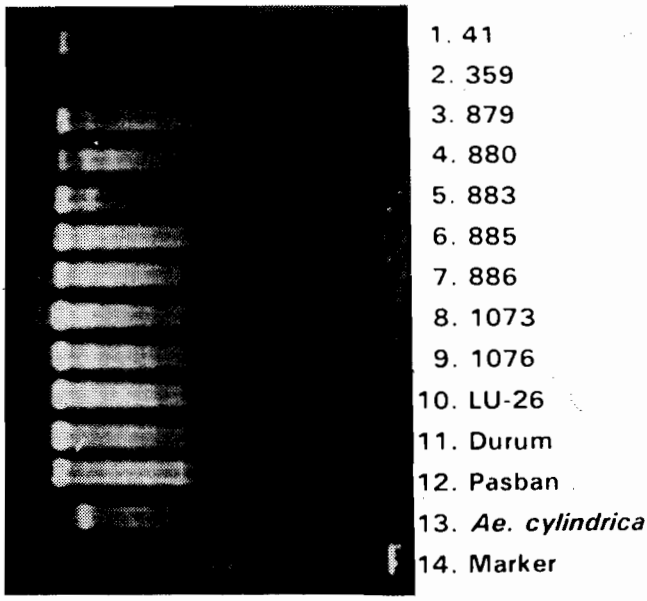

Fig.3. Amplification products obtained with primer S-12. Polymorphism can be detected in wheat lines 41. 359. 880, 1073, 1073, 1076 and LU-26.

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