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Application RAPD Technique for Recognition Genotypes Tolerant to Drought in some of Bread Wheat

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Abstract: The objective of this study was to provide selection criteria for drought tolerance by RAPD technique. Common wheat (*Triticum aestivum* L.) is a grass species, cultivated world wide. Genetic diversity evaluation of germplasm is the basis of improvement in wheat. The complexity of the wheat genome has delayed the development and application of molecular markers to this species and wheat now lies behind barley, maize and rice in marker development. However, improvements in marker detection systems and in the techniques used to identify markers linked to useful traits has allowed considerable advances to be made in recent years. The use of PCR based assays having advantage of being quick, easy to use and refractory to many environmental influences can complement traditional methods of germplasm characterization. The RAPD markers were used to determine the genetic differences between the 30 bread wheat genotypes and to determine the molecular markers associated with tolerance to drought.The present study found that RAPD analysis is a valuable diagnostic tool. Different sets of RAPD primer were used to study the polymorphism at molecular level. Initial screens involved growing 20 genotypes at seedling stage under drought conditions (-5 and -8 bar exerted by PEG 6000 in ahydroponic experiment). These tests confirmed the tolerance of the 6 above mentioned genotypes. Thirty 10-mer RAPD primers were used for fingerprinting of the genotypes of which primers P16 (TCGGCGGTTC) and P17 (CTGCATCGTG) produced, respectively a 900 and a 750 bp band present in drought tolerant (absent in others) genotypes. The results substantiate the use of these DNA markers in germplasm screening for drought tolerance in wheat.

Key words: Bread wheat, drought tolerance, RAPD, PCR

INTRODUCTION

Drought and salinity are the most serious threats to agriculture and are far more important globally (Altman, 2003), water stress is major harmful factor in arid and semi-arid regions worldwide (Roy *et al*., 2006). Breeding for drought tolerance by selecting solely for grain yield is difficult, because the heritability of yield under drought conditions is low, due to small genotypic variance or to large genotype-environment interaction variances (Blum, 1988; Ludlow and Muchow, 1990).

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The genetic structure and phenotypic expression of a quantitative trait are highly influenced by environmental factors, thus, one barrier for understanding the inheritance of a quantitative trait is genotype-environment interactions (Breese, 1969). Among all the factors limiting the wheat productivity, drought remains the single most important factor affecting the world security and sustainability in agricultural production. At least 60 million ha of wheat is grown in marginal rainfed environments in developing countries. For improving yields under dry land conditions, the development of new wheat genotypes with high grain yield potential through identifying drought tolerance mechanism is of great significance (Rajaram and van Ginkel, 1996). Bread wheat is the most important crop in Iran, providing half of the food supply. Of its production area 60% is located in arid and semi-arid regions. Under this condition, wheat is permanently challenged by drought, along with nutrient deficiency of the soil and other kinds of biotic and abiotic stresses. This challenge results, if not always but frequently, in a partial to drastic reduction of yield. It was reported that breeding for quantitative traits like salinity, drought and heat tolerance would be facilitated by the development of a procedure to be used in Marker-Assisted Selection (MAS) that is capable of identifying high performing genotypes in early generations (Schneider *et al*., 1997; Frova *et al*., 1999; Quarrie *et al*., 1999). In addition, Forster *et al*. (2000) reported that greater variation to abiotic stresses exists in primitive landraces in which molecular markers could be used in molecular breeding approaches to improve the drought tolerance of many crops. The need for rapid and accurate identification of the stress tolerant genotypes in wheat prompted the search for markers associated with this complex trait. Welsh and McClelland (1991) developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (Weining and Langridge, 1991). Schneider *et al*. (1997) identified five RAPD markers associated with drought resistance in common bean (*Phaseolus vulgaris* L.). Molecular markers were used in improving drought resistance in maize (Frova *et al*., 1999; Quarrie *et al*., 1999) and Inberseem clover (Fahmy *et al*., 1997). Protein, isozymes and RAPD markers were also used to study the genetic variation, polymorphism and interrelationship between different wheat cultivars and accessions (Joshi and Nguyen, 1993a, b; Farooq *et al*., 1994; Shah *et al*., 2000; Cao *et al*., 1999, 2000). The DNA sequences or RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide primers (Ovesna *et al*., 2002). The RAPD-PCR are currently used as genetic markers quite useful in breeding programs for assessment of genetic variability between genotypes (Hillel *et al*., 1992; Kahraman, 1999). Recent introduction of DNA technology has generated a large number of molecular markers. Molecular markers have significant value in breeding programs to characterize and evaluate genetic variability in germplasms and to identify varieties (Chao, 2006). The major goal of the present study was assessing genetic diversity in 20 Iranian bread wheat genotypes. Randomly Amplified Polymorphic DNA (RAPD markers) were used to determine the genetic differences between genotypes and to determine the molecular markers associated with drought tolerance.

MATERIALS AND METHODS

The present study was carried out at Department of Plant Breeding, Faculty Agriculture from Broujerd Azad University in time span between 2008-2009.

Table 1: Bread wheat genotypes and their drought tolerance status

Genotype No.	Genotype name	Collected center	Response to drought
1	C-D-5502	Arak	Tolerant
2	C-D-5505	Ardebil	Tolerant
3	C-D-5509	Eghlid	Tolerant
4	C-D-5517	Hamedan	Tolerant
5	Verbey	Karadj	Tolerant
6	N-78-11	Ahwaz	Semi-tolerant
7	S-80-18	Darab	Semi-tolerant
8	S-82-10	Dzful	Semi-tolerant
9	N-84-14	Gorgan	Sensitive
10	N-84-17	Sari	Sensitive
11	N-82-13	Gorgan	Sensitive
12	Nl785	Karadj	Tolerant
13	Baw898	Hamedan	Sensitive
14	N-80-6	Moghan	Sensitive
15	5806-6	Eghlid	Sensitive
16	N-78-14	Gorgan	Sensitive
17	7007-2	Sari	Sensitive
18	7107-6	Gonabad	Sensitive
19	S-83-3	Zabol	Semi-tolerant
20	S-83-4	Iranshahr	Semi-tolerant

DNA Extraction

A total of 20 bread wheat genotypes including tolerant, semi-tolerant and non-tolerant were used (Table 1).

Six of the genotypes were drought tolerant. DNA was extracted from 10 days old seedling CTAB method (Murry and Thompson, 1980). The material was subjected to molecular evaluations for determining their DNA based diversity. This was done by using RAPD primers and the protocol is described below:

In the growth room 5 to 7 cm long pieces of fresh leaf material were cut from the plants (3 week-old seedlings) and were placed in 1.5 mL eppendorf tubes. The tubes were subsequently dropped in the liquid nitrogen to rapidly freeze the leaf material. The plant material was then crushed to a fine powder with a knitting needle while still inside the tube. The 500 μL DNA extraction buffer (1% SDS, 100 mM NaCl, 100 mM tris base, 100 mM Na_2EDTA, PH: 8.5 by HCl) was added to each eppendorf tube containing the crushed leaf material and was mixed well with the help of a knitting needle. The 500 μL phenol: chloroform: isoamylalcohol (in the ratio of 25:24:1) was added and tubes were well shaken until a homogenous mixture is made. Samples were then centrifuged at 5000 rpm for 5 min. The aqueous phase (supernatant) was transferred to a fresh tube. To precipitate the DNA 50 μL 3 M sodium acetate (pH = 4.8) and 500 μL isopropanol was added to the tube and mixed gently. To make the DNA pellet, samples were centrifuged at 5000 rpm for 5 min. After pouring the supernatant, pellet was washed with 70% ethyl alcohol. Pellet was dried at room temperature for an hour and was resuspended in 40 μL TE buffer (10 mM Tris, 1 mM EDTA and PH: 8.0) (Weining and Langridge, 1991). To remove RNA, DNA was treated with 40 μg RNAase-A (0.20 μL of commercially supplied RNAase-A purchased from Gene Link, USA) at 37°C for 1 h. After RNAase treatment, DNA samples were run on 1.0% gel to check the quality of DNA and then was stored at 4°C. To use in Polymerase Chain Reaction (PCR) a 1:5 dilution of DNA was made in doubled distilled, deionized and autoclaved water.

Polymerase Chain Reaction

PCR reactions were carried out in 25 μL reaction containing 50-100 ng total genomic DNA templates, 0.25 μM of each primer, 200 μM of each dATP, dGTP, dCTP, dTTP,

50 mMKCl, 10 mM Tris, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase. The amplification conditions were as; an initial step of denaturation for 1 min at 94°C followed by 45 cycles each consisting of a denaturation step of 1 min at 94°C, an annealing step of 1 min at 34°C and an extension step of 2 min at 72°C. Seven minutes were given after the last cycle to the extension step at 72°C to ensure the completion of the primer extension reaction. GeneAmp PCR system 2700 was used for all amplification reactions.

Gel Electrophoresis

For electrophoresis of the amplification products, 1.5% agarose/TBE gel was used. Gels were visualized by Ethidium Bromide under the UV light chamber and observed using the computer program UVIPhotoMW.

DNA Marker

The GeneRuler^{tm} 1 kb DNA Ladder (Catalogue # SM0313, Lot: 00018968, Concentration: 0.1 μg μL^{-1}) by Ferments was used for sizing and approximate quantification of wide range double stranded DNA fragments on agarose gel. The ladder was premixed with 6X loading dye solution for direct loading on gel. Each amplification was performed using a single primer and gels scored for the presence and absence of products.

RESULTS

Results of this study indicated that, 27 primers produced scroable amplification products. Three primers (P14, P15 and P26) produced vague un-scroable bands. From 380 amplified band 155(40%) were polymorphic and primers P13, P1 and P26 produced more polymorphic bands. Band sizes were between 300 to 1950 bp. Primers P16 (TCGGCGGTTC) and P17 (CTGCATCGTG) that shown in Table 2 produced a 900 and 750 bp bands present in some of drought tolerant genotypes (C-D-5502, C-D-5505, C-D-5509, C-D-5517, Verbey and Nl785). These bands may be associated with drought tolerance in bread wheat (Fig. 1, 2) and they may be used in selection of tolerant genotypes in breeding plans. Hydroponic experiment performed with 20 genotypes of bread wheat under water deficit stress (-5 and -8 bars) exerted by PEG 6000, confirmed drought tolerance of the 6 tolerant genotypes shown in Table 1. Strategy for P16 and P17 products is to sequence the 900 and 750 bp bands and search against stress-related ESTs (expressed sequence tags) in public databases (Boguski *et al*., 1993) to identify the gene involved.

Table 2: Primer nucleotide sequence used to amplify DNA

Primer designation	Sequence 5'-3'	Primer designation	Sequence 5'-3'
1	CCATTCCCCA	16	TCGGCGGTTC
2	GGTGAACGCT	17	CTGCATCGTG
3	CTCCCTGAGC	18	TGAGCCTCAC
4	TTCCGGGTGA	19	TCGGCACGCA
5	GAGCTCGCGA	20	CTGCGCTGGA
6	CCTGGGCTTC	21	TACGTGCCCG
7	CCTGGGCTTG	22	GGCTAGGGGG
8	CCTGGGCCTA	23	TAGCCGTGGC
9	CCTGGGCCTA	24	CCGTGCAGTA
10	TGCCCCGAGC	25	ATCGGGTCCG
11	ACACAGAGGG	26	GGGGGTTAGG
12	CCTCTCGACA	27	GAGGTCCAGA
13	TCTCAGCTGG	28	AGGGGCGGGA
14	GTGTGCCCCA	29	GAGGGCGGGA
15	CCACGGGAAG	30	TTCCCCGACC

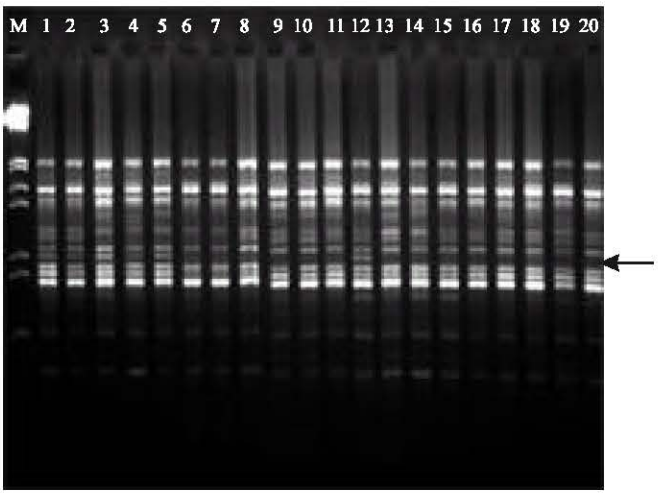

Fig. 1: Electrophoretic pattern generated by RAPD primer P17 (CTGCATCGTG). Arrow shows a 750 bp band present in drought tolerant (absent in others) genotypes

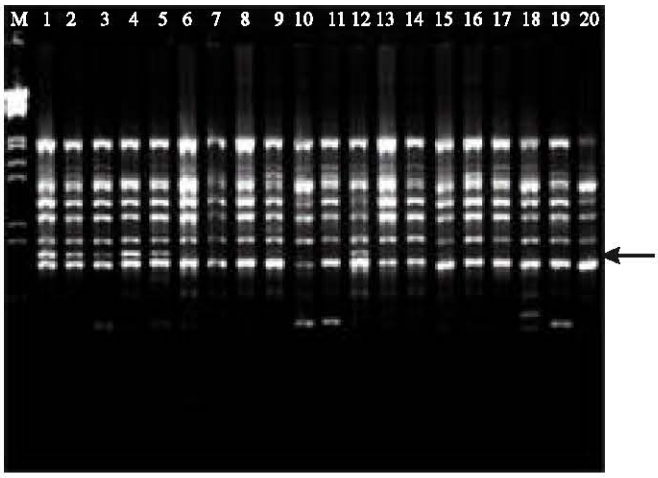

Fig. 2: Electrophoretic pattern generated by RAPD primer P16 (TCGGCGGTTC). Arrow shows a 900 bp band present in drought tolerant (absent in others) genotypes

DISCUSSION

Over the past century, the development and successful application of plant breeding methods has produced high yielding crop varieties upon which modern agriculture is based. New varieties are usually bred by crossing a set of genetically related modern varieties, followed by an intensive selection in succeeding generations (Ceccarelli *et al*., 1987). Introduction of valuable genes from exotic donors via wide crosses has been proposed to broaden the genetic base of many crop plants with known and closely related wild relatives (Zohary *et al*., 1969). Forms in the primary gene pool have genetic proximity to the genomes A, B and D of bread wheat thus all conventional wheat breeding utilizes its cultivars that

reside in this gene pool (Coghlan, 2006). Several types of diversity can be measured in the context of breeding programs (Sorrells and Wilson, 1997). Apparent and latent genetic diversity are directly related to the performance of crops. Measures of apparent diversity are manifested in phenotypic differences of populations or cultivars in the field (Rajaram and van Ginkel, 1996). Latent diversity refers to parentage analysis and molecular measurements that are not necessarily expressed in crop performance or phenotypes. Consequently, the genetic variation of crop plants is continued to be reduced by plant breeding (Tanksley and McCouch, 1997). It is the plant breeding process itself that threatens the genetic base on which breeding depends (Russell *et al*., 1997). In the light of these developments, the main objective of this study was to examine the genetic diversity in modern wheat breeding materials and genetic resources provided by and stored at CIMMYT. There has been a large transformation in the productivity of wheat due to the application of Green Revolution technology (Byerlee and Moya, 1993). The development of DNA markers in wheat is somewhat problematic due to three features. First, the size of the wheat genome (16×109 bp, compared to barley or maize with 5×109 bp), which makes the application of several marker techniques difficult. Second, the hexaploid nature of wheat adds complexity to many marker assays (Chao *et al*., 1989). Three sets of bands usually appear (often in the same size range), which are difficult to manage and interpret. Third, there is a generally low level of polymorphism in wheat relative to other cereal crops. This implies that a larger number of markers must be screened than in the case of rice, barley or maize (Chao *et al*., 1989; Liu *et al*., 1999). Furthermore, the level of polymorphism is not consistent across genomes and crosses. Commonly, the D genome tends to have the poorest marker coverage (Chalmers *et al*., 2001). Lack of genetic polymorphism in crops such as wheat and soybeans and the consequent problems to identify molecular markers, has been a major limitation to the impact of Marker Assisted Selection (MAS) in wheat breeding (Sorrells and Wilson, 1997). Molecular markers detect variation of the DNA sequences among cultivars and therefore directly bypass problems connected with environmental effects (Cox *et al*., 1985; Maric *et al*., 1998).

The RAPD technique is quick (Colombo *et al*., 1998) cost effective (Fugang *et al*., 2003) and the ability to perform analysis without the need for prior sequencing of the genome (Huff *et al*., 1993). However, problems with reproducibility in amplification of RAPD markers and with data scoring have been reported (Jones *et al*., 1997). Although, major bands from RAPD reactions are highly reproducible, minor bands can pose difficulty to repeat due to random priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers (Tessier *et al*., 1999). It is hoped that the discovery of markers associated with drought tolerance whit the aid of the genes involved.Sequence data can also be used to develop more robust PCR primers as diagnostics for drought tolerance. Another strategy is to map the two products to confirm or otherwise their genetic co-location.

In this study, RAPD markers were able to discriminate some of drought tolerant genotypes (C-D-5502, C-D-5505, C-D-5509, C-D-5517, Verbey and Nl785). This high level of discrimination is also reported by Belaj *et al*. (2003) and Rajora and Rahman (2003).

In one study in Iranian wheat cultivars 58% polymorphism was reported using 8 RAPD primers (Abdolahie *et al*., 2003). A study carried out by Liu *et al*. (1999) used 54 RAPD markers generated by six primers to study their potential value in distinguishing between parents with different characteristics and predicting the yield performance of hybrids produced from these parents. Replication slippage is thought to occur more frequently than single nucleotide mutations and insertion\deletion events, which generated the

polymorphisms detected by RAPD analysis (Powell *et al*., 1996). Reliable RAPD data can be generated following a standard protocol, replication of amplification reactions and a conservative criterion of band selection (Belaj *et al*., 2003). The RAPD technique is quick (Colombo *et al*., 1998) cost effective (Fugang *et al*., 2003) and the ability to perform analysis without the need for prior sequencing of the genome (Huff *et al*., 1993). However, problems with reproducibility in amplification of RAPD markers and with data scoring have been reported by Jones *et al*. (1997). Although, major bands from RAPD reactions are highly reproducible, minor bands can pose difficulty to repeat due to random priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers (Tessier *et al*., 1999). In recent years, several molecular assays have been applied to assess genetic diversity among wheat cultivars (Chen *et al*., 1994). These molecular methods are different in principle, application, type, amount of polymorphism detected and in task and time requirements. Assays based on the Polymerase Chain Reaction (PCR) are considered to meet both the technical and genetic requirements for the characterization of plant and animal genetic resources (Powell *et al*., 1996). Present results suggest that molecular approaches along with quality studies may be used to evaluate genetic diversity and assess the genetic relationships between bread wheat genotypes with high accuracy. These results are in agreement with those of Khan *et al*. (2005). Similar results were found by Amein (2007), Salem *et al*. (2008) and Parakash and Joshi (2003). Therefore, the classification obtained for these Iranian wheat genotypes, based on quality traits and molecular markers will be a useful tool to Iranian breeders to plan crosses for positive agronomic characters by choosing genotypes with appropriate diversity.

CONCLUSION

In this study, RAPD markers were able to discriminate some of drought tolerant genotypes(C-D-5502, C-D-5505, C-D-5509, C-D-5517, Verbey and Nl785). The results indicated that in this research, two out of 27 primers detected associations to drought tolerance in tolerant genotypes which they may be related to involved QTLs.

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