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RAPD and ISSR Markers Related to Drought Tolerance of Regenerated Plants in Wheat Double Haploids and Varieties

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ABSTRACT

To verify the most drought-tolerant genotypes and to determine the appropriate concentration of polyethylene glycol (PEG) that could be used in callus induction and plant regeneration, 10 double haploid (DH) genotypes and five varieties of bread wheat were grown in the field under drought stress conditions and germinated in Petri dishes under 0, 10, 20 and 30% PEG. Five DH genotypes and two varieties (Giza 168 and Sakha 93) that were characterized by their high drought tolerant were selected to use as sources of mature embryos to produce calli. the growing calli were transferred to callus culture medium with 0, 10 and 20% PEG 6000 to induce drought stress for 30 days. Two of the five selected DH genotypes and the two wheat varieties induced enough calli and successfully regenerated under drought stress. RAPD analysis using three random primers showed that 28 of 39 total amplified fragments were polymorphic with 72.8% polymorphism under 10 and 20% PEG, whereas 91.7% polymorphism in primer B-09 was higher than that observed for the other two primers B-14 and C-15 (66.7 and 60%, respectively). The four wheat genotypes revealed 19 specific markers for drought tolerance with polymorphic mean percentage 50.6%. ISSR analysis using four different primers for drought tolerance revealed 35 polymorphic fragments with 57.3% mean polymorphism from a total of 61 amplified fragments under salinity stress. The four wheat genotypes had 25 specific markers for drought tolerance with 41% mean polymorphism, Sakha 93 and DH-1 had the highest total number of markers at 10% PEG and Giza 168 at 20% PEG, while DH-1 had the highest total number of markers at the two PEG concentrations. RAPD and ISSR are significant methods to detect specific markers for drought tolerance and could be used in marker assisted selection (MAS) of wheat breeding programs to predict the most tolerant genotypes.

Keywords: homozygous pure lines, PCR-markers, PEG-6000

INTRODUCTION

Wheat is one of the most abundant sources of energy and proteins for the world population. Ninety-five percent of wheat grown today is a self-pollinating hexaploid (*Triticum aestivum* L.) and the remaining 5% is durum (tetraploid) wheat (Patnaik and Khurana 2001).

Improving drought tolerance of wheat has long been a major objective of most breeding programs around the world because water deficits during some part of the growing period are common to most regions of the world. Wheat breeders are looking for genetic diversity in which drought tolerance and yield potential can be combined. Under such conditions, an improved cultivar is one of the key factors for increasing wheat productivity. Cultivar development and improvement of self-pollinating species, of which wheat is the prime example, can be achieved by selection from both natural populations and artificially segregating populations produced by hybridization. However, classical breeding for abiotic stress tolerance is quite difficult, time-, space- and labor-consuming (Brisibe *et al*. 2000).

It appears that *in vitro* selection for stress tolerance will continue to have its significant place in the strategy of establishing plant systems with optimal stress reaction and productivity (Dragiiska *et al*. 1996). The improvement of high yielding wheat varieties suitable for growing under different stress conditions by plant breeding usually requires genetically pure lines; either to be used as parents or to be distributed as new cultivars produced from breeding programs to be grown by farmers. The production of these pure lines by conventional breeding practices is a time-consuming process that needs 7-12 generations and could lead to a delay in new varieties production. One of the solutions for this problem is the production of double haploid plants via anther culture, which are developed by colchicine-induced chromosomal doubling and which leads to the direct production of completely homozygous lines from heterozygous plants in a single generation and could accelerate the production of new varieties with improved traits (Liu *et al*. 2002).

On the other hand, biotechnology is emerging as one of the latest tools of agricultural research and the development of *in vitro* technologies to complement conventional methods of wheat breeding in generating genetic variability are necessary for creating novel cultivars with desirable characters. The utilization of biotechnology is largely dependent on callus induction and subsequent plant regeneration from various explant sources (Zheng and Konzak 1999).

DNA molecular markers, such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSRs) have become excellent tools for plant breeders (Lima-Brito *et al*. 2006). RAPD technique gives fast results but also has limitations, such as low reproducibility (Fernández *et al*. 2002). In contrast, ISSR markers are more reproducible and the distribution of ISSRs in the eukaryotic genome makes them highly informative (Bornet *et al*. 2002) with no prior information of the sequence. In cereals, ISSR markers have been used to study genetic diversity and phylogenetic relationships (Matos *et al*. 2001), for gene mapping (Kojima *et al*. 1998) and for DNA fingerprinting (Carvalho *et al*. 2005). The aim of the present study was to develop RAPD and ISSR markers associated with drought tolerance in the regenerated plants derived from calli of bread wheat double haploids and varieties.

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Original Research Paper

MATERIALS AND METHODS

Plant material

Hexaploid bread wheat (*Triticum aestivum* L.) materials used in this study were comprised of 10 double haploid (DH) genotypes (DH-1 through DH-10) that were kindly provided by Dr. Tarek Hewezi from Laboratoire de Biotechnologie et d'Amélioration des Plantes (BAP), Castanet Tolosan, France and five varieties (Giza 157, Giza 168, Sakha 93, Sakha 8, Sids 1) provided by the Agricultural Research Center, Giza, Egypt.

Selection of drought-tolerant wheat genotypes

Wheat materials were grown under drought stress conditions at the Experimental Station of the National Research Center, El-kalyobia governorate and then germinated on filter paper in Petri dishes under different polyethylene glycol (PEG) 6000 concentrations (0, 10, 20 and 30%), in order to determine the appropriate concentrations of PEG and to verify the most drought-tolerant genotypes, which will be used in callus induction and plant regeneration. From previous initial field and Petri dish experiments five (DH-1 through DH-5) of the initial 10 DH genotypes and two (Giza 168, Sakha 93) out of five wheat varieties that were characterized by their high drought tolerance were selected to use as sources of mature embryos.

In vitro selection and plant regeneration of drought tolerant cell lines

Mature seeds were harvested from main spikes, surface-sterilized in 70% (v/v) ethanol for 10 min and in 5% sodium hypochlorite for 20 min and then washed several times in sterile distilled water. They were aseptically excised from the caryopsis and placed with the scutellum upwards on solid agar medium in sterile Petri dishes for 14 days at 26 ± 1°C in continuous darkness. The agar medium contained Murashige and Skoog (1962) (MS) mineral salts, 30 g/l sucrose, 2 mg/l 2,4-D and 0.7% (w/v) agar. The media was adjusted to pH 5.8 and autoclaved for 20 min at 121°C and 1.1 kg/cm^2 pressure. After callus induction, calli were subcultured at four-week intervals on fresh MS medium. One month-old calli were transferred to MS medium with PEG 6000 at 0, 10 and 20% for 30 days to induce drought stress (Bajji *et al*. 2000). Two of the five selected DH genotypes and two wheat varieties were successfully induced calli under drought stresses. The calli of the four genotypes (DH-1, DH-2, Giza 168, Sakha 93) were transferred to regeneration medium which contained MS basal salts without 2,4-D (Bregitzer 1992) and maintained for 5 weeks at 26 ± 1°C under 16-h cool-white fluorescent lighting (36 µmol m^{-2} s^{-1}, Philips) to induce shoots.

DNA extraction, RAPD and ISSR reaction conditions

DNA extraction using CTAB method was performed from the leaves of four regenerated wheat genotypes according to Doyle and Doyle (1987). RAPD analysis was performed using three 10-mer random primers (Metabion, Martinsried, Germany) and ISSR primers were procured from Integrated DNA Technologies Inc. (San Diego, CA, USA) based on core repeats anchored at the 5′ or 3′ end as shown in **Table 1**.

Amplification reactions for RAPD and ISSR analyses were used in a final volume of 25 µl containing 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 9.0), 2 mM dNTPs, 10 mM primer, 50 ng of template DNA and 0.5 U of *Taq* polymerase (promega, USA). Reactions were performed in a thermocycler (biometra, gmbh). RAPD-PCR was performed according to Williams *et al*. (1990) as one cycle of 94°C for 2.5 min (denaturation), 30 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min (annealing) and with a final extension of 10 min at 72°C. ISSR amplification was performed according to Zietkiewiez (1994) with an initial denaturation of 2 min at 94°C, followed by 40 cycles of 94°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 7 min. PCR products were analyzed using 1.2% agarose gel electrophoresis and visualized with 10 µg/µl ethidium bromide staining. The sizes of the fragments were estimated based on a DNA ladder of 100 to 2000 bp.

Table 1 Names and sequences of RAPD and ISSR primers used for PCR analyses.

Primers	Name	Sequences
RAPD	B-09	5′ GGG TAA CGC C ′3
	B-14	5′ GTT TCG CTC C ′3
	C-15	5′ TTT GGG GGG A ′3
ISSR	17898-A	$(CA)_6$ AC
	844-B	$(CT)_8$ GC
	HB-10	$(GA)_6$ CC
	HB-12	$(CAC)_3$ GC

RESULTS AND DISCUSSION

The results of callus induction and *in vitro* selection of drought-tolerant genotypes were previously reported by Abdelsamad *et al*. (2007).

RAPD markers of regenerated drought-tolerant wheat genotypes

A total of 39 amplified DNA fragments ranging in size from 50 to 2000 bp were observed using the three random amplified polymorphic DNA (RAPD) primers; B-09, B-14 and C-15, whereas 28 fragments were polymorphic and the other amplified fragments were commonly detected among the four wheat genotypes under 10 and 20% PEG (**Table 2**). The three primers; B-09, B-14 and C-15 showed a mean polymorphism of 72.8%, whereas the percentage polymorphism of primer B-09 was higher (91.7%) than the other two primers B-14 and C-15 (66.7 and 60%, respectively). primer B-09 resulted in 12 fragments whose sizes ranged from 100 to 1380 bp (**Fig. 1, Table 2**), whereas 11 fragments were polymorphic and one fragment with molecular size 400 bp was common among the four wheat genotypes under different PEG concentrations. Wheat genotypes treated with 10 and 20% PEG revealed some induced amplified bands at each or at both of the two PEG concentrations, which did not exist in the controls. For instance, a 1380 bp band in Giza 168 and another 210 bp band in Sakha 93 and DH-1 were displayed at 10% PEG while four bands of 1200, 750, 210 and 100 bp in size in Giza 168 and one band of 670 bp were displayed in DH-1 at 20% PEG. In addition, Sakha 93 and DH-2 showed some induced amplified bands at both 10 and 20% PEG, whereas 1100 and 100 bp bands were observed in Sakha 93 and bands 1100, 800 and 550 bp in size in DH-2.

Primer B-14 revealed 12 fragments with sizes ranging from 50 to 1550 bp (**Fig. 1, Table 2**), whereas eight fragments were polymorphic and the other four fragments with molecular sizes 550, 450, 290 and 50 bp were not affected by the two PEG concentrations. Some distinctive amplified bands were uniquely displayed at 10% PEG, such as 1550 and 1200 bp in DH-1 and Sakha 93, respectively while three induced bands with 1200, 850 and 800 bp were observed at 20% PEG in Giza 168. Five amplified bands with different molecular sizes were induced at 10 and 20% PEG, whereas two of them (800 and 180 bp) were detected in Sakha 93 and the other three bands (1550, 1300 and 850 bp) in DH-2. Primer C-15 revealed 15 fragments with sizes ranging from 2000 to 100 bp (**Fig. 1, Table 2**), whereas nine fragments were polymorphic and the other six fragments were not affected by PEG treatments. Two amplified bands (790 and 590 bp) were induced at 10% PEG in Giza 168 and DH-2, respectively and one band (650 bp) was induced at 20% PEG in DH-1 while two bands (590 and 200 bp) were induced at both 10 and 20% PEG in Sakha 93. Such induced bands which resulted from RAPD-PCR amplified products using the three primers could be considered as RAPD markers for drought tolerance in wheat.

Table 2 RAPD analysis of variable (polymorphic) bands of regenerated plants for four wheat genotypes at 0, 10 and 20% PEG using three random primers; B-09, B-14 and C-15.

Primer name	P%	Bs	Giza 168			Sakha 93			DH-1			DH-2		
		(bp)	0	10	20	0	10	20	0	10	20	0	10	20
B-09	91.7	1380		+		+								
		1200			+	+	+		+					
		1100					+	+	+				+	+
		900	+	+		+								
		800	+	+	+	+	+	+	+				+	+
		750			+				+	+	+			
		670	+	+		+	+	+			+	+	+	+
		550	+	+	+	+	+	+	+	+			+	+
		510							+	+	+	+		
		210			+		+			+				
		100			+		+	+						
	Variable bands = 11		4	5	6	6	7	5	6	4	3	2	4	4
	Total = 12		5	6	7	7	8	6	7	5	4	3	5	5
B-14	66.7	1550								+			+	+
		1300											+	+
		1200			+		+		+	+		+	+	+
		850			+								+	+
		800			+		+	+	+	+		+	+	+
		520	+	+	+				+	+		+		
		200	+	+	+	+								
		180					+	+	+	+	+	+	+	+
	Variable bands = 8		2	2	5	1	3	2	4	5	1	4	6	6
	Total = 12		6	6	9	5	7	6	8	9	5	8	10	10
C-15	60	2000	+	+		+	+	+	+	+		+	+	
		1750				+	+							
		1600	+	+	+	+	+	+	+	+	+	+	+	
		1200							+	+	+	+	+	
		790		+										
		650	+	+	+	+	+	+			+	+	+	+
		590					+	+	+	+	+		+	
		200	+	+	+		+	+	+	+	+	+	+	+
		100	+	+	+	+	+	+	+	+	+	+	+	
	Variable bands = 9		5	6	4	5	7	6	6	6	6	6	7	2
	Total = 15		11	12	10	11	13	12	12	11	12	12	13	8
Total variable bands = 28 Overall total bands = 39								Polymorphism (%) = 72.8*						

(+) = Present of amplified bands, Bs = Molecular size by base pair, P% = Polymorphism percentage, Total = Total number of amplified bands.
* (91.7 + 66.7 + 60 = 218.4), then Average = 218.4/3 = 72.8%

Among a total of 28 polymorphic fragments, 19 were specific markers for drought tolerance either at 10 or at 20% PEG with a 50.6% percentage polymorphism. It is interesting to note that the four wheat genotypes varied considerably in their drought tolerance markers, but at 10% PEG Sakha 93 revealed the highest number (8 marker bands) followed by DH-2 with 7 markers, while the other two genotypes showed only two marker bands. At 20% PEG, Giza 168 revealed the highest number (7 marker bands) followed by Sakha 93 and DH-2 with 6 markers (**Table 4**). These results confirmed the importance of RAPD-PCR analysis to detect molecular markers for drought tolerance, which are in agreement with the findings of Wenzel (1992), who emphasized the potential for DNA marker-based diagnosis of abiotic stress tolerance in plants.

Progress toward genetic improvement for drought tolerance using traditional breeding techniques has been slow because of the complexity of character expression and its quantitative inheritance. Identification of markers which are associated with drought tolerance may facilitate future breeding efforts aimed at improving this trait through marker assisted selection (MAS). Thus, RAPD analysis was extensively used to detect and generate molecular markers for drought tolerance in different plant species. For example, Abdel-Tawab *et al*. (2003) used RAPD analysis to detect markers for drought tolerance in wheat, Tuinstra *et al*. (1992) used RAPD markers to identify quantitative trait loci (QTLs) associated with drought tolerance within recombinant inbred sorghum lines, Schneider *et al*. (1997) identified four RAPD markers in one population and five in another associated with drought resistance in common bean (*Phaseolus vulgaris* L.). They reported that breeding for a quantitative trait like drought resistance would be facilitated by the development of MAS that is capable of identifying high performing genotypes in early generations. Malik *et al*. (2000) detected 87 reproducible polymorphisms using 160 RAPD primers between two wheat genotypes, PK81 (drought resistant) and ABA18 (drought susceptible) and their study revealed that RAPD has great potential to find DNA-based polymorphisms between genotypes of the same species. Boopathi *et al*. (2001) found a RAPD-positive marker that could be used to identify drought-tolerant genotypes in segregating rice populations. Toorchi *et al*. (2002) identified two RAPD markers which explained the considerable amount of variation in maximum root length of rice under water stress. One of the markers identified under low moisture stress conditions was also able to explain variability in maximum root length in the mean environment. RAPD analysis indicated the appropriateness of this PCR-technique for determination of polymorphism among 21 lines of chickpea under rainfall conditions (Farshadfar and Farshadfar 2007).

ISSR markers of regenerated drought tolerant wheat genotypes

A total of 61 amplified fragments ranging in size from 70 to 2000 bp were observed using the four ISSR primers; 17898-A, 844-B, HB-10 and HB-12, whereas 35 fragments were polymorphic and the other amplified fragments were com-

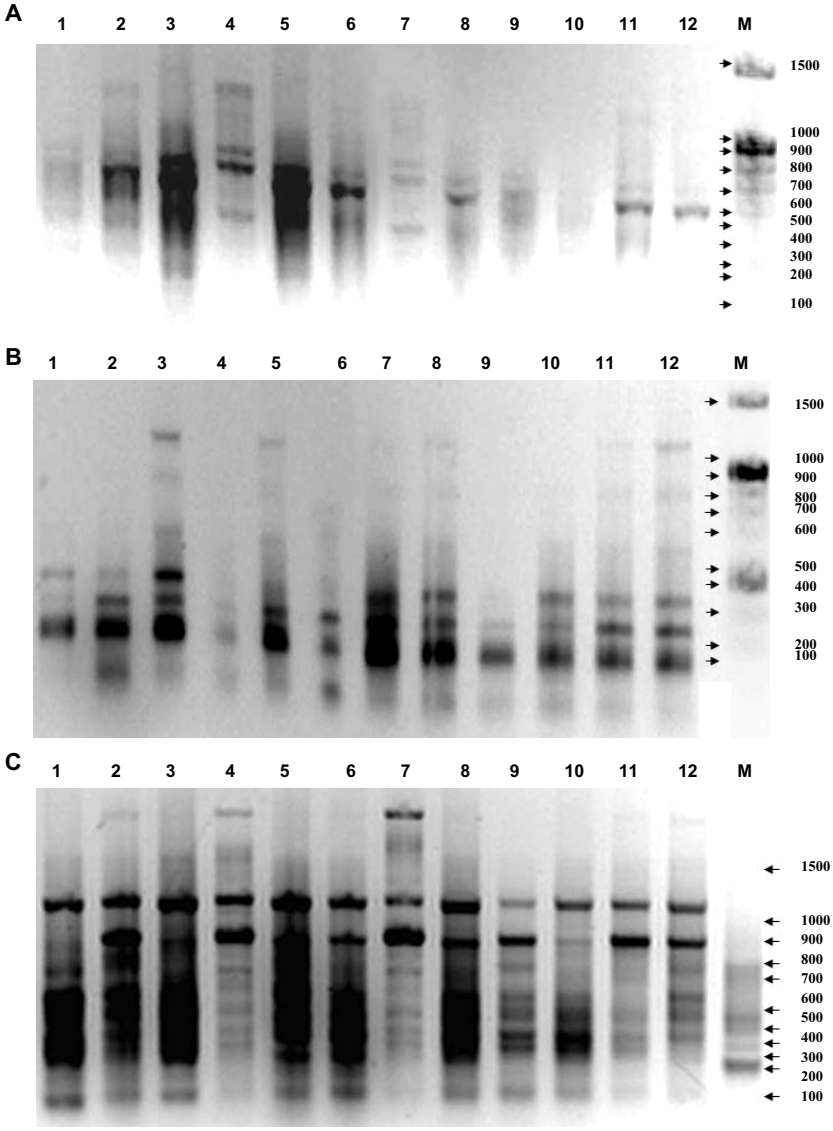

Fig. 1 RAPD amplified products of regenerated plants for four wheat genotypes. Giza 168, Sakha 93, DH-1 and DH-2 at 0, 10 and 20% PEG (lanes 1-4, 5-8 and 9-12, respectively) using three random primers: B-09 (**A**), B-14 (**B**) and C-15 (**C**).

monly detected among the four selected wheat genotypes under 0, 10 and 20% PEG (**Table 3**). The four ISSR primers exhibited a mean polymorphism of 57.3%, whereas this percentage in primer 844-B was higher (80%) than the other three primers HB-12, HB-10 and 17898-A (52.9, 53.3 and 42.9%, respectively).

Primer 17898-A pattern revealed 14 fragments with molecular sizes ranging from 80 to 1550 bp (**Fig. 2, Table 3**), whereas six fragments were polymorphic and the other eight fragments were common among the four wheat genotypes under different PEG concentrations. Three of the four wheat genotypes treated with 10 and 20% PEG revealed some induced amplified bands at each concentration or at both PEG concentrations, which did not exist in the controls. For example, Giza 168 exhibited a band with 1550 bp at 10% PEG and another with 1250 bp at 10 and 20% PEG; Sakha 93 showed two bands at 10% PEG and two other bands with 890 and 90 bp at 10 and 20% PEG while, DH-1 revealed two bands at 20% PEG and four bands with 1000, 890, 550 and 90 bp at 10% PEG. However, DH-2 did not produce any induced amplified bands.

The results of ISSR analysis using primer 844-B showed 15 amplified fragments with molecular sizes ranging from 70 to 1500 bp (**Fig. 2, Table 3**), whereas 12 fragments were polymorphic and the residual three fragments ordinarily appeared in the four wheat genotypes under 0, 10 and 20% PEG. Induced amplified bands that did not exist in the untreated (control) displayed remarkable variability among the four genotypes. For instance, a 490 bp band appeared in Giza 168 at 20% PEG and at the same time appeared in Sakha 93 at 10% PEG and in DH-1 at 10 and 20% PEG, while it disappeared in DH-2 at 20% PEG. Therefore, the percentage polymorphism was 2-fold higher than expected than the predicted drought-tolerant markers (80% vs. 40%), respectively as shown in **Table 3**.

The pattern of primer HB-10 revealed 15 fragments with molecular sizes ranging from 70 to 1000 bp (**Fig. 2**). Eight fragments were polymorphic with 53.3% polymorphism, while the other seven fragments were commonly detected among the four wheat genotypes under different PEG concentrations (**Table 3**). Most of the wheat genotypes were characterized by several unique amplified bands. For instant DH-2 was characterized by three inhibited bands, two of them with molecular sizes 70 and 100 bp, which disappeared at 20% PEG, while they existed among the three genotypes at all PEG treatments. A band with 1000 bp disappeared in DH-2 only at 10 and 20% PEG. DH-1 was uniquely characterized by four induced amplified bands; two of them (590 and 390 bp) were induced at 20% PEG, band number 1 induced at 10% only and the 300 bp band was induced at 10 and 20% PEG. Giza 168 was characterized by an induced 850 bp band at 20% PEG that appeared in DH-2 at all PEG treatments and appeared at 10 and 20% PEG in Sakha 93 and DH-1. Such induced bands could be considered as positive markers for drought tolerance. The results of ISSR analysis using primer HB-12 showed 17 amplified fragments with molecular sizes ranging from 80 to 2000 bp (**Fig. 2, Table 3**). Nine fragments were polymorphic with 52.9% polymorphism and the residual eight fragments appeared ordinarily among the four wheat geno-

Table 3 ISSR analysis of regenerated plants for the four wheat genotypes at 0, 10 and 20% PEG using four ISSR primers; 17898-A, 844-B, HB-10 and HB-12.

Primer name	P%	Bs	Giza 168			Sakha 93			DH-1			DH-2		
		(bp)	0	10	20	0	10	20	0	10	20	0	10	20
17898-A	42.9	1550		+			+				+			
		1250		+	+		+				+			
		1000	+	+	+					+		+	+	+
		890	+	+	+		+	+		+		+	+	+
		550	+	+	+					+				
		90	+	+	+		+	+		+		+	+	+
	Variable bands = 6		4	6	5	0	4	2	0	4	2	3	3	3
	Total = 14		12	14	13	8	12	10	8	12	10	11	11	11
844-B	80	1500				+	+		+					
		1000			+	+	+	+	+	+		+	+	+
		890			+	+	+	+	+	+		+	+	+
		840				+			+					
		800				+	+	+	+			+	+	+
		600			+	+	+	+		+		+	+	+
		490			+		+			+	+	+	+	
		420			+	+	+	+	+					
		310	+	+	+	+	+	+	+	+		+	+	+
		290	+	+	+	+	+	+		+	+	+	+	+
		200	+			+	+	+	+			+		
		70	+			+	+	+	+	+	+	+		
	Variable bands = 12		4	2	7	11	11	9	9	7	3	9	7	6
	Total = 15		6	5	11	14	14	12	12	10	6	12	10	9
HB-10	53.3	1000								+		+		
		880		+	+		+	+		+	+	+	+	+
		850			+		+	+		+	+	+	+	+
		590									+	+	+	+
		390									+	+	+	+
		300	+	+	+	+	+	+		+	+	+	+	+
		100	+	+	+	+	+	+	+	+	+	+	+	
		70	+	+	+	+	+	+	+	+	+	+	+	
	Variable bands = 8		3	4	5	3	5	5	2	6	7	8	7	5
	Total = 15		10	11	12	10	12	12	9	13	14	15	14	12
HB-12	52.9	2000							+			+	+	+
		1200							+					
		960		+	+	+	+	+	+	+		+	+	
		930		+					+					+
		890							+				+	+
		830		+		+	+	+	+					
		790			+	+	+		+	+	+			
		700	+				+	+						
		180	+	+	+	+	+			+				
	Variable bands = 9		2	4	3	4	5	3	7	3	1	2	3	3
	Total = 17		10	12	11	12	13	11	15	11	9	10	11	11
Total variable bands =35														
Overall total bands = 61									Polymorphic (%) = 57.3**					

(+) = Present of amplified bands, Bs = Molecular size by base pair, P% = Polymorphism percentage, Total = Total number of amplified bands.
** (42.9 + 80 + 53.3 + 52.9 =2291), then Average = 229.1/4 = 57.3%

types under 0, 10 and 20% PEG. The four wheat genotypes reflected inconsistent responses in seven amplified bands (960, 930, 890, 830, 790, 700 and 180 bp) under drought stress. Each of the four genotypes was affected in a different manner for all seven bands. For example, each amplified band that existed in one genotype disappeared in another genotype, as shown in **Table 3**. Meanwhile, DH-1 was uniquely characterized by two bands that existed in the control and were absent at the two PEG concentrations, namely band numbers 1 and 2 while DH-2 was characterized by band number 1 that existed in all PEG treatments.

Among the 61 amplified bands, 35 were 57.3% polymorphic (**Table 3**) and among them 25 bands were specific markers for drought tolerance either at 10 or at 20% PEG with 41% (**Table 4**). It is interesting to note that the four wheat genotypes varied considerably in their drought tolerance markers: at 10% PEG Sakha 93 and DH-1 revealed the highest number (10 marker bands) followed by Giza 168 with 6 drought markers, while DH-2 revealed only one marker. In addition, at 20% PEG Giza 168 revealed the highest number (10 marker bands), followed by DH-1 with 8 markers and then Sakha 93 with 6 markers, while DH-2 displayed only two markers (**Table 4**).

In cereals, ISSR markers associated with both qualitative and quantitative traits, for instant salt tolerance in wheat (Lang *et al*. 2001) and drought tolerance in rice (Fahmy *et al*. 2007), are used to study genetic diversity and phylogenetic relationships (Qian *et al*. 2001), and gene tagging in MAS (Kaushik *et al*. 2003). However, genetic differences between 17 Polish rye varieties and five new breeding lines with different tolerance to drought stress applied on *in vitro* embryo cultures were determined using ISSR markers. The results showed that the majority of varieties were characterized by the lack of tolerance to drought and osmotic stress (Rzepka-Plevneš *et al*. 2008). As a general conclusion from the aforementioned results, RAPD and ISSR are considered good molecular markers for salt tolerance. The results confirmed that ISSR-PCR was the most useful method to detect molecular markers for salinity tolerance and could be used as MAS in wheat breeding programs to predict the most tolerant genotypes. ISSR primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no

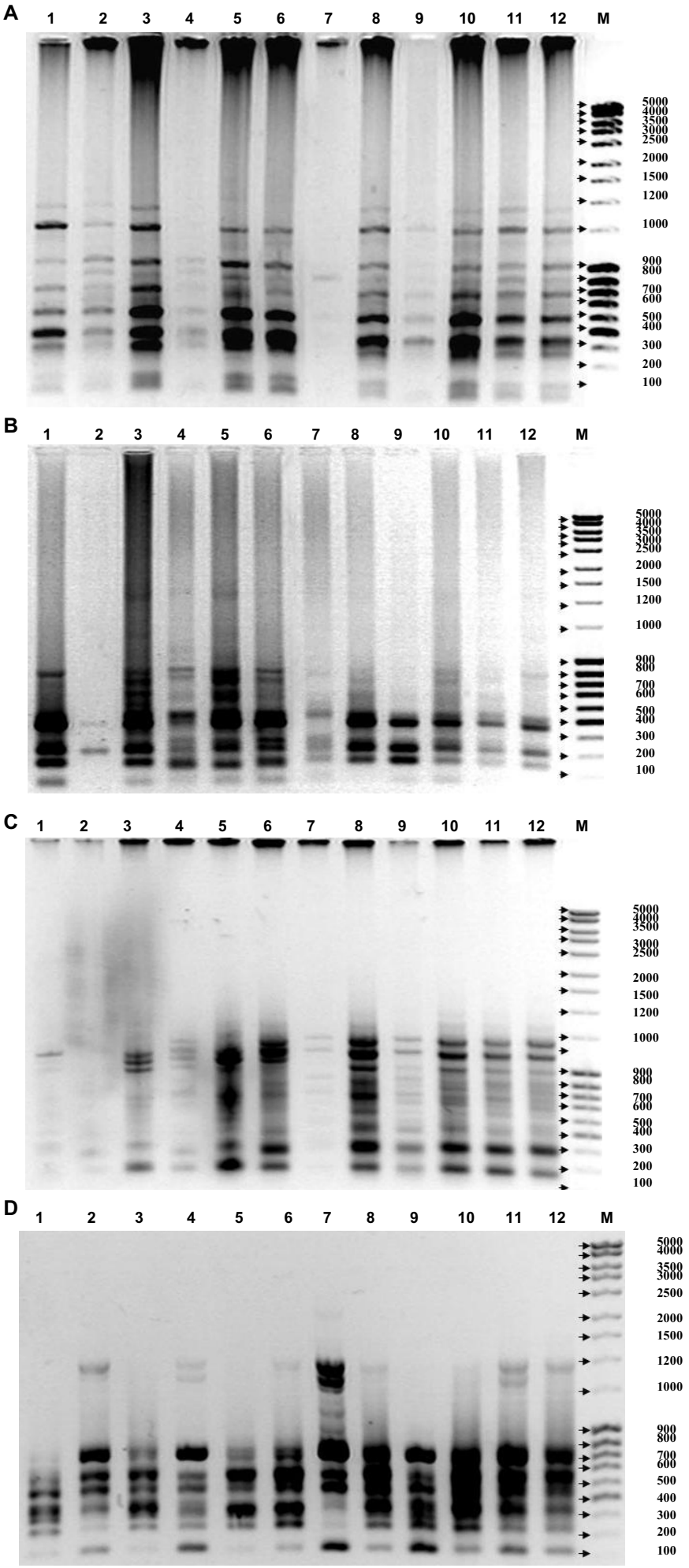

Fig. 2 ISSR amplified products of regenerated plants for four wheat genotypes. Giza 168, Sakha 93, DH-1 and DH-2 at 0, 10 and 20% PEG (lanes 1-4, 5-8 and 9-12, respectively) using four primers: 17898-A (**A**), 844-B (**B**), HB-10 (**C**) and HB-12 (**D**).

Table 4 RAPD and ISSR markers for drought tolerance in regenerated plants of four wheat genotypes at 10 and 20% PEG.

Marker type	Primer name	P%	№ and size (bp) of markers	Giza 168		Sakha 93		DH-1		DH-2	
				10	20	10	20	10	20	10	20
RAPD	B-09	75	1380	+							
			1200, 750		^{2}+						
			1100			+	+			+	+
			800, 550							^{2}+	^{2}+
			670						+		
			210		+	+		+			
			100		+	+	+				
			Total = 9	1	4	3	2	1	1	3	3
	B-14	50	1550					+		+	+
			1300							+	+
			1200		+	+					
			850		+					+	+
			800		+	+	+				
			180			+	+				
			Total = 6	0	3	3	2	1	0	3	3
	C-15	26.7	790	+							
			650						+		
			590			+	+			+	
			200			+	+				
			Total = 4	1	0	2	2	0	1	1	0
Total marker bands = 19											
Polymorphic mean (%) = 50.6*				2	7	8	6	2	2	7	6
ISSR	17898-A	42.9	1550	+		+			+		
			1250	+	+	+			+		
			890, 90			^{2}+	^{2}+	^{2}+			
			1000, 550					^{2}+			
			Total = 6	2	1	4	2	4	2	0	0
	844-B	40	490		+	+		+	+		
			1000, 890, 420		^{3}+						
			600		+			+			
			290			+	+				
			Total = 6	0	5	2	1	2	1	0	0
	HB-10	40	880	+	+	+	+	+	+		
			850		+	+	+	+	+		
			1000			+					
			590, 390						^{2}+		
			300					+	+		
			Total = 6	1	2	3	2	3	5	0	0
	HB-12	41.2	960	+	+						
			930	+							+
			890							+	+
			830	+							
			790		+						
			700			+	+				
			180					+			
			Total = 7	3	2	1	1	1	0	1	2
Total marker bands = 25											
Polymorphic mean (%) = 41**				6	10	10	6	10	8	1	2

P% = Polymorphic percentage of marker bands, Total = Total number of markers
+ = Presence of marker bands, ^{2}+ and ^{3}+ = Presence of two and three markers.
* (75 + 50 + 26.7) = 151.7), then Average = 151.7/3 = 50.6%.
** (42.9 + 40 + 40 + 41.2 = 164.1), then Average = 164.1/4 = 41%

prior knowledge of the target sequence is required for ISSR (Godwin *et al*. 1997). Also, the sequences targeted by ISSR primers are abundant throughout the eukaryotic genome and evolve rapidly. Consequently ISSR may reveal a much higher number of polymorphic fragments per primer than RAPD (Esselman *et al*. 1999). In addition, studies have indicated that ISSR produce more reliable and reproducible bands than RAPD because of the higher annealing temperature and longer primer sequence (Nagaoka and Ogihara 1997).

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