Screening of some rice *Oryza sativa* L. genotypes for drought tolerance using SSR-PCR technique

غربلة بعض التراكيب الوراثية للرز *Oryza sativa* L. لصفة تحمل الجفاف بأستخدام تقانة المؤشرات الجزيئية للدنا SSR-PCR

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Abstract

Simple Sequence Repeat (SSR) markers were used to investigate the variations between drought sensitive and drought tolerant genotypes. Patterns obtained using RM328 and RM302 detected polymorphism between T_{14}, T_{15} and Amber33, Amber Baghdad genotypes and those obtained using RM316 and RM201 for the tested genotypes suggested that these primers may have the ability to produce drought tolerance markers. According to the patterns obtained using RM189, RM3825 and RM212 primers indicated that these primers cannot be relied on as markers for drought tolerance.

Key words: ***Oryza sativa,*** **drought tolerance, genotype**

المستخلص

وظفت تقانة المؤشرات الجزيئية للدنا (SSR) للكشف عن التغايرات الوراثية بين التراكيب الوراثية الحساسة و التراكيب الوراثية المتحملة للجفاف واثبتت التراكيب الناتجة من استخدام البادئات RM328 و RM302 وجود تباين وراثي بين التراكيب الوراثية الحساسة و التراكيب الوراثية المتحملة. كما اثبتت التراكيب الناتجة من استخدام البادئات RM201 و RM316 قابليتها على تكوين مؤشرات مرتبطة بصفة تحمل الجفاف في الرز . بينما اثبتت التراكيب الناتجة من استخدام البادئات RM212, RM189 و RM3825 عدم قابليتها على تكوين مؤشرات جزيئية ذات ارتباط بصفة تحمل الجفاف في الرز.

الكلمات المفتاحية : الرز ، تحمل الجفاف ، التراكيب الوراثية

Introduction

SSR or Microsatellites is a class of repetitive sequences which are widely distributed in all eukaryotic genomes. They consist of arrays of tandem repeated short nucleotide motifs of 1-4 bases, and are called mono, di-, tri- or tetranucleotide repeats respectively. It was known that such arrays of short DNA elements repeated in tandem tend to be imprecisely replicated during DNA synthesis and generate new alleles with different numbers of repeated units [1, 2].

SSR has been widely applied for rice genome mapping because of numerous quantity and highly polymorphism among rice cultivars [3]. SSR molecular markers for identification and purity test of super hybrid riceused by [4]. It has been reported that more than 3000 DNA SSR markers are now available, which makes the SSR system a convenient and efficient tool for rice molecular genetics and breeding studies [5]. The PCR primer pairs are designed on the bases of sequences flanking the repetitive tract. The primers are used to amplify DNA with the use of genomic DNA as a substrate. Change in number of repeats gives the length polymorphism which is revealed by designing primers for the sequences flanking the microsatellite repeat motif followed by PCR amplification and visualization in agarose or denaturing polyacrylamide gel. This type of the markers systems has the advantage over other molecular markers; (i) multiple SSR alleles may be detected at a single locus using a simple PCR based screen, (ii) SSRs are evenly distributed all over the genome, (iii) they are co-dominant, (iv) very small quantities of DNA are required for screening, and (v) analysis may be semiautomatic [2]. Huseynova and Rustamova [6] used SSR technique as a molecular marker in screening wheat genotypes for drought stress tolerance.

Materials and Methods

DNA isolation protocol [2]

Rice *Oryza sativa* L. seeds of T_{14} (code: IRg59007-191-1-b) and T_{15} (code: IR60080-AB-A) genotypes were kindly supplied by International Rice Research Institution (IRRI) in Manila/Philippine, while Amber 33 and Amber Baghdad genotypes were kindly supplied by Genetic Engineering Dept., Agricultural Research Directorate, Ministry of Science and Technology, Baghdad, Iraq. Approximately 50mg of dried

leaves of rice genotypes seedling [7] were ground with a mortar and pestle. The homogenized tissues were transferred to 600µl of 2% CTAB DNA extraction buffer with 1.25µl of β-mercaptoethanol in 1.5ml Eppendorf tubes. Incubation at 65ºC for 30min in a water bath. Three Microliters of RNAse was added and incubated at 37ºC for 1hr. Then, a liquot of 200µl chloroform: isoamyl alcohol (24:1) was added to the solution, and mixed well. The emulsified mixture was centrifuged at 13000Xg for 15min, and then the aqueous phase was placed into new sterilized Eppendorf tube. Aliquot of 600µl isopropanol and 150µl of sodium acetate were added, and then centrifuged at 13,000rpm for 10min. The supernatant was discarded. The precipitated DNA was washed with 600µl of 70% ethanol, centrifuged at 13,000rpm for 5min, and then the supernatant was discarded. DNA was air dried for 2min and dissolved in 150µl of TE buffer. Incubation for 1hr at 65ºC in a water bath. DNA concentrations were measured.

Analysis of genomic DNA

Specific primers

The microsatellite markers RM189, RM201, RM212, RM302, RM316, RM328 and RM3825 were selected according to [8,9]. A pair of specific primers was provided (Alpha DNA-Canada) for each marker, the sequences of the polymorphic primer pairs and their chromosomal locations are presented in table (1). Primers were provided in a lyophilized form and were dissolved in sterile distilled water to give a final concentration of 100ng/µl as recommended by the supplier.

Taq Master Mix (2X) for SSR-PCR

Taq Master Mix ready to use mixture was used which containing *Taq*DNA polymerase, 2X Vibuffer A, $MgCl_2$ and pure deoxynucleotiedes (dNTPs). *Taq* Master Mix was provided by (Vivantis, Germany). Amplification was performed on ice under aseptic conditions inside a laminar air flow using 0.2ml tight cap Eppendorf tubes. In order to achieve homogeneity of reagents and reduce the risk of contamination, a master mix for all samples was prepared containing all the components of the reaction except the genomic DNA (template DNA), mixed gently with sterile distilled water to achieve the appropriate volume.

Table (1): Specific primers, their sequences and chromosomal location used in this work.

Primer		Forward (F) and Reverse (R) Primers Sequence	chromosomal location
RM189	F	CGTCTTCCCCAACGCTAAAA	
	R	CGCGGGGCTTCGCTTC	Ch 1
RM201	F	CTCGTTTATTACCTACAGTACC	
	R	CTACCTCCTTTCTAGACCGATA	Ch 9
RM212	F	CCACTTTCAGCTACTACCAG	
	R	CACCCATTTGTCTCTCATTATG	Ch1
RM302	F	TCATGTCATCTACCATCACAC	
	R	ATGGAGAAGATGGAATACTTGC	Ch 1
RM316	F	CTAGTTGGGCATACGATGGC	
	R	ACGCTTATATGTTACGTCAAC	Ch 9
RM328	F	CATAGTGGAGTATGCAGCTGC	
	R	CCTTCTCCCAGTCGTATCTG	Ch 9
RM3825	F	AAAGCCCCCAAAAGCAGTAC	
	R	GTGAAACTCTGGGGTGTTCG	Ch 1

Simple sequence repeat-PCR compounds

Optimization of SSR-PCR reactions was accomplished after several trials; thus the following mixture was adopted.

Component	Concentration	Amount (µl)
D.W.	_____	7.5
TaqMaster Mix	2X	12.5
$MgCl_2$	50mM	2
Primer A (forward)	100ng/µl	1
Primer B (reverse)	100ng/µl	1
DNA sample	100ng/µl	1
Total volume		25µl

SSR-PCR program

To detect the variation between the two drought sensitive rice genotypes and the other two drought tolerant genotypes, the following program was adopted.

Step	Temperature (°C)	Time (min)	No. of cycles
Initial denaturation	**94**	**4**	**35**
Denaturation	**94**	**1**	**35**
Annealing	**55**	**1**	**35**
Extension	**72**	**1**	**35**
Final extension	**72**	**7**	**35**

Analysis of PCR products [10]

PCR products and 100bp DNA ladder were determined by electrophoresis. Aliquot of 3µl of loading dye plus 7µl of the product were mixed and loaded on 2.0% agarose gel and run at 70 volt for 1hr. Bands were visualized on UV transsiluminator and then photographed.

Results and Discussion

SSRs markers have been used to distinguish between drought sensitive and drought tolerant rice genotypes. Accordingly, the results shown in figure 1 confirmed that Amber33, Amber Baghdad, T_{14} and T_{15} genotypes produce a PCR product with a molecular size ~140bp using RM189 primers pair. Amber33 and Amber Baghdad genotypes produce a PCR product with a molecular size ~200bp compared with T_{14} and T_{15} (0) using RM302 primers pair. Additionally, using RM201 primer pair, PCR products with a molecular size ~0, 190, 165 and 180bp appeared in Amber33, Amber Baghdad, T14 and T_{15} genotypes respectively.

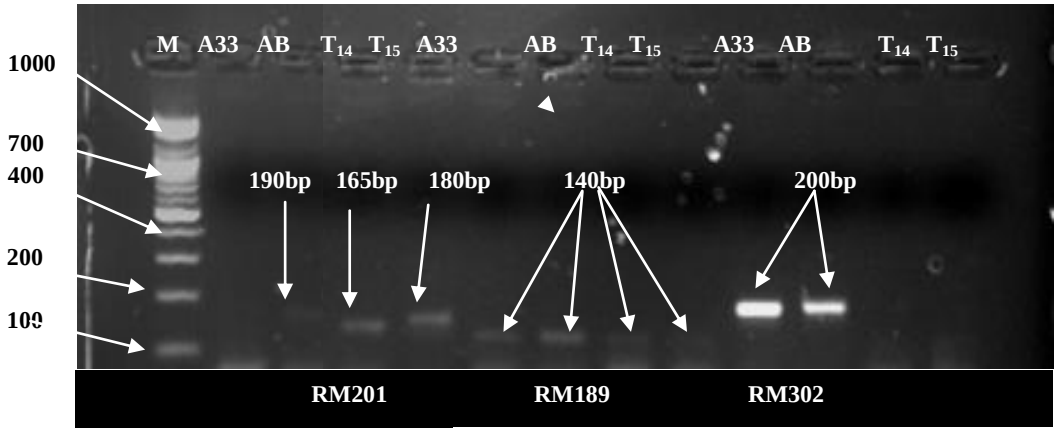


Fig. (1):Agarose gel electrophoresis of PCR reaction using specific primersRM201 and RM189 and RM302. Bands were separated by electrophoresis using 2.0% agarose gel (1hr, 70V, 1XTBE buffer) and visualized under U.V. light after staining with ethidium bromide. M: Bench top ladder, A33 (Amber33), AB (Amber Baghdad), T_{14} and T_{15} genotypes, 100ng/µl.

Figure (2) reveals that Amber33, Amber Baghdad, T_{14} and T_{15} genotypes produce a PCR product with a molecular size ~180bp using RM3825 and ~145bp using RM212 primers pair. Results also indicated that T_{14} genotype possesses a PCR product with a molecular size ~210bp compared with other genotypes that possess a PCR product with a molecular size ~200bp using RM316 primers pair.

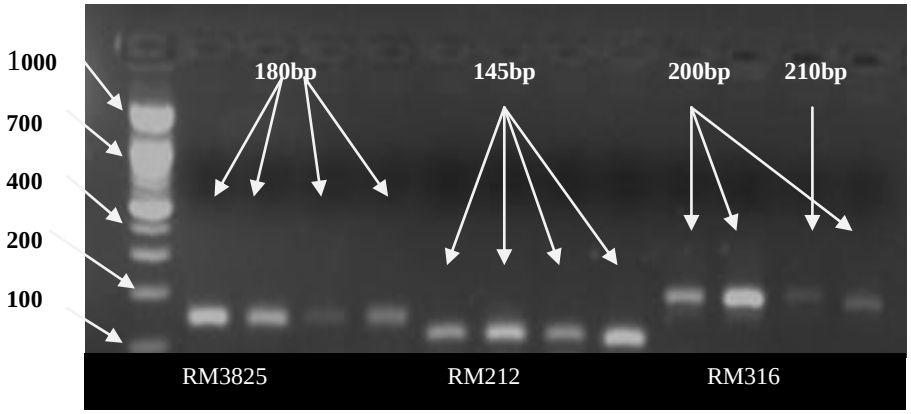
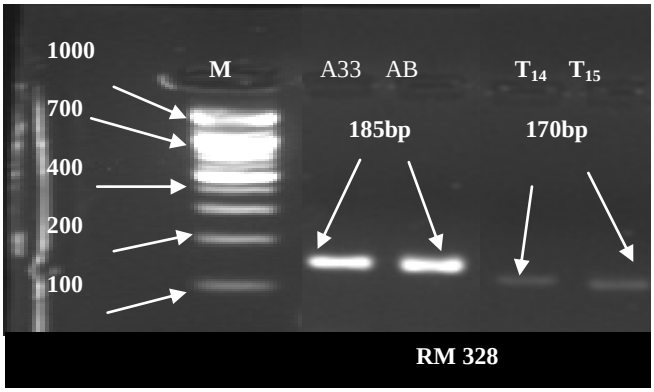


Fig (2): Agarose gel electrophoresis of PCR reaction using specificprimersRM3825, RM212 and RM316. Bands were separated by electrophoresis using 2.0% agarose gel (1hr, 70V, 1XTBE buffer) and visualized under U.V. light after staining with ethidium bromide.M: Bench top ladder, A33 (Amber33), AB (Amber Baghdad), T_{14} and T_{15} genotypes, 100ng/µl.

According to the results displayed in figure 3, The genotypes T_{14} and T_{15} possesses a PCR product with a molecular size ~170bp compared with Amber33 and Amber Baghdad genotypes, that posses a PCR product with a molecular size ~185 using RM328 primers pair.



Fig(3): Agarose gel electrophoresis of PCR reaction using specific primer RM328. Bands were fractionated by electrophoresis using 2.0% agarose gel (1hr, 70V, 1XTBE buffer) and visualized under U.V. light after staining with ethidium bromide. M: Bench top ladder, A33 (Amber33), AB (Amber Baghdad), T_{14} and T_{15} genotypes, 100ng/µl.

In this study, the analysis of PCR amplified DNA fragments relies on the differences in molecular weight and intensity of amplified bands, since DNA concentration was controlled at 100ng/µl evenly for each sample, which means that the number of DNA template copies was the same in each sample, that may not affect the intensity of the resulted bands [10].

SSRs markers on chromosome 9, including RM328 figure (3) detected polymorphism between T_{14}, T_{15} genotypes and Amber33, Amber Baghdad genotypes, this suggested that a number of repeated units of SSR markers linked to drought tolerance in these genotypes are different from the local rice genotypes. Patterns obtained from RM201and RM316 figures (1, 2) for the tested genotypes suggested that these primers may have the ability to produce drought tolerance markers, since they detected polymorphism between the tested genotypes. According to the pattern obtained with RM189, this primer cannot be relied on as marker for drought tolerance. These results in agreement with those obtained by [8] who expected that SSR markers including RM328, RM316 and RM201 located on chromosome 9 associated with drought tolerance in rice.

SSR markers on chromosome 1, including RM302 figure (1) detected polymorphisms between T_{14}, T_{15} genotypes and the other rice genotypes grown in Iraq, while the patterns obtained from RM3825 and RM212 figure (2) indicated that these primers cannot be relied on as a markers for drought tolerance and these results are disagreement with those obtained by [9] who reported that, three primers alone showed

polymorphism between the bulks of these three primers, viz. RM212, RM302 and RM3825 and these three primers were located on chromosome 1 of the rice between 135.8 and 143.7 cM., this region has been found to be linked with several drought tolerance traits such as plant height, biomass, deep root mass, basal root thickness, tiller number and deep root to shoot ratio.

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