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Use of RAPD and ISSR Assays for the Detection of Mutation Changes in Wheat (*Triticum aestivum* L.) DNA Induced by Ethyl-Methane Sulphonate (*EMS*)

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Abstract: This study was conducted at the National Research Center, Department of Genetics and Cytology. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) were used to determine the mutation changes in three bread wheat lines treated with 0.3 % of ethyl-methane sulphonate (EMS). Seven RAPD and four ISSR primers were used to detect the mutation changes of the lines. The RAPD primers produced total of 57 bands under control. 27 out of them were polymorphic. They produced 17 new bands after treatment with EMS. Used four ISSR primers give change ratio 0.08% and showed 4 different new alleles.
Key words: Bread Wheat, Mutagenesis, Molecular breeding, RAPD and ISSR– PCR markers.

Introduction

Wheat (*Triticum aestivum* L.) is the first important and strategic cereal crop for the majority of world's populations. It is the most important staple food of about two billion people (36% of the world population). Worldwide, wheat provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally[1].

Plant breeding requires genetic variation of useful traits for crop improvement. Often, however, desired variation is lacking. Mutagenic agents, such as radiation and certain chemicals, then can be used to induce mutations and generate genetic variations from which desired mutants may be selected. Mutation induction has become a proven way of creating variation within a crop variety[2,3].

RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeat) analyses are wide used for the genetic mapping, taxonomic and phylogenetic studies of many organisms. They can be also applicable for the detection of DNA alterations after influence of many genotoxical agents as well[4]. Ethyl-Methane Sulphonate produces point mutations. These different types of DNA damages must be detected by changes in RAPD and ISSR profiles.

The reverse genetic technique TILLING (Targeting Induced Local Lesions IN Genomes has been successfully applied in the detection of mutations in several plant species. These include *Arabidopsis*[5,6,7,8]**;** rice [9,10,11,12]; maize[13]; wheat[14,15]; sugar beet[16]; barley[17]; soybean[18]; pea[19]; beans[20,21]; tomato[22]; wheat[23] and in the vegetatively propagated banana[24].

Targeting Induced Local Legions IN Genomes (TILLING) is relying on genotyping specific loci and correlating discovered mutations to a phenotype [25]. Interesting in mutagenesis and TILLING has been growing in wheat with some early successes in new trait variations[25,26,27,28,29].

A modified RAPD method with ISJ primers was used for screening populations of mutant wheat[23]. RAPD assay was also used to detect the mutation changes in plant DNA induced by UV-B and γ-rays[40].

The aim of present study was to investigate the possibility mutation changes in plant DNA after the influence of ethyl methane sulphonate (EMS) with using RAPD and ISSR methods.

Materials and Methods

Plant material:

Three lines of bread wheat namely L1, L2 and L3 were kindly provided by[30] L1 and L3 are Egyptian lines, while L2 is exported from the U.S.A during 1994. The normal seeds of the three lines were treated with 0.3% of Ethyl-methane Sulphonate (EMS) mutagen.

Extraction of DNA:

The genomic DNA was extracted from fresh leaf samples collected from the three lines after and before treated with EMS according to the protocol of Biospin plant genomic DNA extraction Kit (Bio basic).

RAPD assay:

RAPD amplification was carried out using seven random primers (Metabion International AG.). The sequences of 10-mer primers are given in Table (1). The PCR reaction was performed in a total volume 25µL containing 5 µL of 5x buffer, 3.0 µL of dNTPs (2.5mM), 3 µL of Mgcl2 (25 mM), 3.0 µL primer, 0.3 µ L of Taq polymerase (5U/ µL), 2.0 µL of genomic DNA (50 ng/ µL) and 8.7 µL H2O. Amplification was performed in PTC-100 PCR version 9.0 from M J Research-USA. Amplification involved initial denaturation of DNA template at 94°C for 5 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 40°C and 2 min extension at 72°C. Amplification was finished by incubation at 72°C for 7 min[31]. PCR amplification products were analyzed in 1.5 % (w/v) agarose gel in 1xTAE buffer. PCR products (25 µl per sample) were mixed with 3–5 µl Gel Loading Dye Solution (Fermentas, Lithvuania) and 7 µl were loaded onto the agarose gel, containing ethidium bromide (0.5 mkg/ml)[32]. DNA ladder (1 Kb) was used for each agarose gel. Electrophoresis was carried out at 80 V for 45 min, and then the results were visualized under UV light and documented using documentation system, Biometra - Bio Doc. Analyze. Only clear, unambiguous and reproducible bands were considered for data analysis. Each band was considered a single locus. Data was scored as (1) for the presence band and (0) for the absence one. The similarity coefficients were generated by SPSS program version-10 to construct a dendrogram by the unweight pair group method with arithmetical average (UPGMA)[33].

Table (1): Sequences of the 10-mer primers (5'-3') used in experiments.

No.	Primer code	Sequence (5`→3`)
1	OPA-02	CAGGCCCTTC
2	OPA-07	AATCGGGCTG
3	OPB-10	GAAACGGGTG
4	OPO-10	GGTGACGCAG
5	OPO-13	CTGCTGGGAC
6	OPO-14	TCAGAGCGCC
7	OPO-19	GTCAGAGTCC

ISSR assay:

ISSR amplification was carried out Using four inter simple sequence repeat (ISSR) primers. The sequences and names of the ISSR primers are shown in Table (2). The PCR amplification was carried out using a Master Cycler Gradient PCR (Germany). 25 µl reaction mixture containing 50 ng genomic DNA, 1x PCR buffer, 0.2 mM of each dNTPs, 2.5 mM MgCl2, 0.5 unit Taq DNA polymerase (Promega, USA) and 10 pmol of ISSR primers. The thermal cycling condition involved initial denaturation of DNA template at 94oC for 2 min followed by followed by 38 cycles of 30 seconds at 94 ºC, 30 seconds at annealing temperature and 1 minute at 72 ºC. Amplification was finished by incubation at 72 ºC for 10 min. The amplification products were separated on a 1.5% (w/v) agarose gel in 1xTAE buffer. 1kb DNA ladder (Promega, USA) was ran simultaneously. The agarose gel was documented using the UV-gel image acquisition camera (Geliance 200, Perkin Elmer).

Table (2): Sequences, annealing temperature and GC content of ISSR primers used in experiments.

	No.	Primer Code	Sequence (5`to 3`)	AT* (°C)	GC Content%
ISSR	1	UBC-811	5` (GA) 8 C 3`	63	52.94
	2	UBC-817	5` (CA) 8 A 3`	61	47.06
	3	UBC 814-32	5` (CT) 7CCTA 3`	64	50.00
	4	UBC 876-32	5` (GATA) 2 (GACA) 2 3`	54	37.50

*****Annealing temperature

ISSR data analysis:

The DNA bands produced at different loci were determined and named for each DNA sample. Banding profiles generated were converted into a binary data matrices on the basis of present (1) or absent (0) of bands. Data scoring is based on several criteria: (1) locus is assumed as independent or non-allelic, (2) there is no bias in scoring monomorphic fragments versus polymorphic fragments, (3) amplified loci are expected to be in the range of 140bp to 1700bp, and (4) the similarity of fragment size is assumed to be the indicator of homology. The binary matrices were used to estimate genetic diversity of the lines. Genetic data analysis was performed using POPGENE version 1.32 software by assuming Hardy-Weinberg.

Results and discussion

RAPD Markers:

The main changes observed in the RAPD profiles have been resulted both in an appearance or disappearance of different bands (Figure 1 A and B) with variation of their intensity as well. These effects might be connected with structural rearrangements in DNA caused by different types of DNA damages.

Seven RAPD primers were used to evaluate the mutation changes of three wheat lines (Figures 1 A and B). They produced total of 57 bands under control. 27 out of them were polymorphic. After treatment with EMS, some new bands were appeared (Tables 5 and 6). These results agree with[34]. They used the same technique to detect the genetic variability in barley. ISSRs were more useful for the analysis of diversity in terms of quality and quantity of data output as compared to RFLP and RAPD[35]. Significantly, the efficiency of the technique was evident in characterization even at the varietal level of a species. Whenever, the molecular nature of the polymorphisms can be known only if the fragments extracted from the gel are sequenced. But ISSRs have also been used along with AFLP and RAPD markers in the mapping by[36].

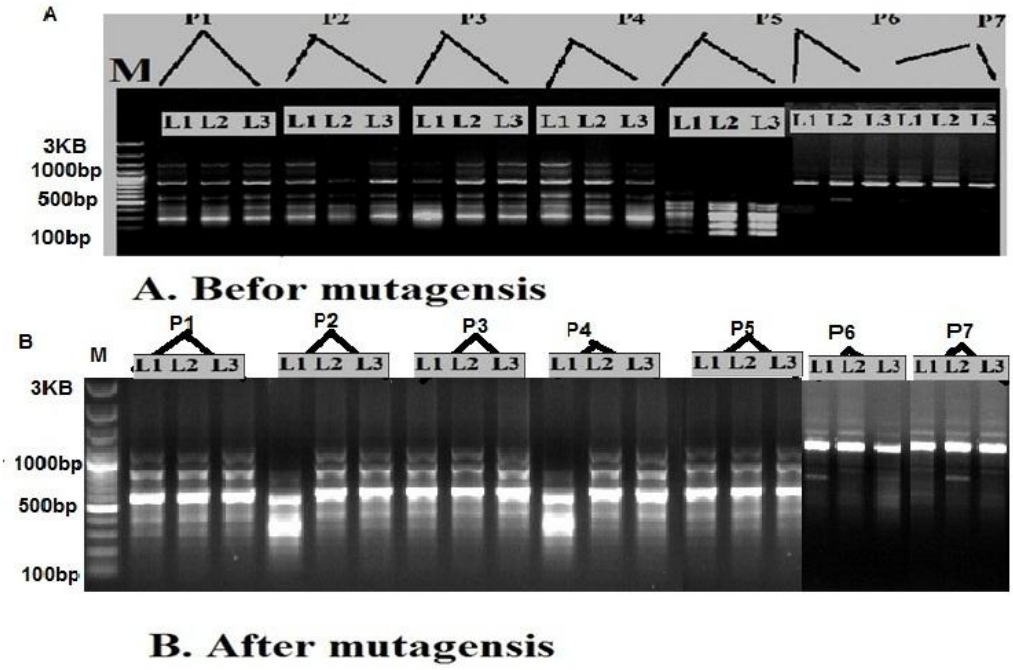

Fig. 1A and B: Amplicons of the three lines under control (A) and treated with EMS (B) using seven RAPD primers.

Table (4): The total number of bands, Monomorphic, polymorphic bands and % polymorphism of the three lines using seven RAPD primers under control.

Primer name	Monomorphic bands	Polymorphic bands	Total number of bands	%Polymorphism
OPA-02	3	2	5	40 %
OPA-07	6	2	8	25 %
OPB-10	4	4	8	50 %
OPO-10	5	4	9	44.44 %
OPO-13	5	3	8	37.5 %
OPO-14	5	6	11	54.54%
OPO-19	2	6	8	75%
Total	30	27	57	47.368%
Average	4.286	3.857	8.143	

Table (5): Number of bands in control and appearance of new bands after treated with EMS in the three lines using seven RAPD primers.

Primer	No. of bands in control	Appearance of new bands after treated with EMS	% Mutation
OPA-02	5	1	16.67%
OPB-07	8	3	27.27%
OPB-10	8	2	20%
OPO-10	9	3	25%
OPO-13	8	2	20%
OPO-14	11	4	26.67%
OPO-19	8	2	20%
Total	57	17	29.82%

Table (6) Summary of new allelic fragments using RAPD–PCR technique.

Primer RAPD	No. of new bands	Lines	Molecular size bp
OPA-02	5	L1	450
OPA-07	7	L2	230
OPB-10	4	L2	430
OPO-10	3	L1	650
OPO-13	6	L3	370
OPO-14	3	L1	780
OPO-19	2	L2	710

ISSR Markers:

The other technique was ISSR-PCR for detected mutations assay on the three lines under this study. To evaluations the new bands under EMS effect were in Tables (7 and 8).

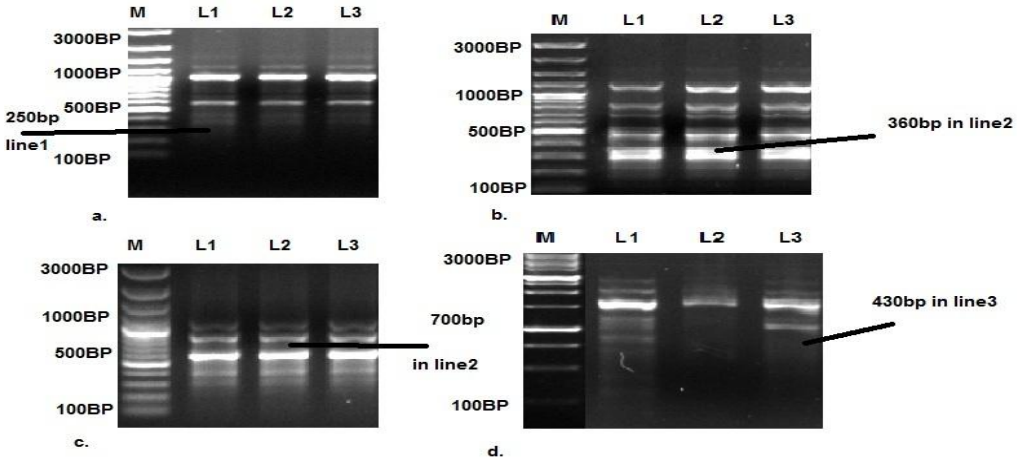

Fig. 2): ISSR Amplicons of the three lines under treatment with EMS using (a) UBC-811, (b) UBC-814, (c) UBC 814-32 and (d) UBC 876-32, M: 100bp ladder.

Table 7: Estimates of standard genetic diversity parameters of the three lines under treat with 0.3% (*EMS)* using four ISSR primers.

ISSR primers	Control Before treat with *EMS*		After treat with (*EMS*)0.3%	
	Monomorphic bands	No. of polymorphic bands	Polymorphic Bands	% Change ratio
UBC-811	3	10	11	7.14%
UBC-814	2	8	9	9.09%
UBC 814-32	4	5	6	10%
UBC 876-32	2	8	9	9.09%
Average	2.75 alleles/primer	7.75 alleles/ primer	8.75 alleles/primer	8.83%
Total	11(26.19%)	31(73.81%)	35(76.09%)	35.32%
Change ratio/primer				0.08%

Table (8) Summary of new allelic fragments using ISSR–PCR technique.

Primer ISSR	No. of new bands	Lines	Molecular size bp
UBC-811	5	L1	250
UBC-814	6	L2	360
UBC 814-32	3	L2	700
UBC 876-32	4	L3	430

Four ISSR primers were used to evaluate the mutation changes of three wheat lines (Figure2). ISSR markers have the advantage of relatively low cost, high polymorphism and good reproducibility.

The primer (UBC-811) gave 13 bands in control, while it showed 14 bands after the treatment with EMS. The primers (UBC-814) and (UBC 876-32) reveled 10 bands in control, while they gave 11 bands after treatment. On the other hand, the primer (UBC 814-32) gave 9 bands in the control, while it gave 10 bands after treatment. The percentage of mutational change was ranged from 7.14 to 10 % (Table 7 and Table 8).The genetic variations among the *EMS* treated lines are shown in (Table 7). These variations, the disappearance of some bands and the appearance of new bands, are due to the effect of *EMS*. The results in this study agree with results recorded by[38], where plants treated with different concentrations of *EMS*. The high-dose treatment that caused growth inhibition has been ascribed to the cell cycle arrest during somatic cell division and/or various damages in the entire genome[39]. In this study, the standard-dose 0.3% of *EMS* revealed high diversity among the lines.

Conclusion

Three bread wheat lines were used in this study to detect the mutation changes in DNA induced by 0.3% EMS. Seven primers of RAPD proved the appearance of new bands in mutant lines as markers and the ratio reached to 29.82 %. Four ISSR primers gave high diversity after using EMS. The variation in band intensity and disappearance of some bands may correlate with level of photoproducts in DNA template after genotoxical treatment, which can reduce the number of binding sites for *Taq* polymerase. Appearance of new bands can be explained as the result of different DNA structural changes (breaks, transpositions, deletions etc). We can estimate the existence of mutation and structural alterations in plant DNA after impact of different stressful factors on the bases of DNA patterns obtained after RAPD and ISSR with the set of primers. Line 2 was the high line in change ratio, while line 3 was the little in rate of mutations.

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