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Molecular analysis of somaclonal variations in chili pepper (*Capsicum annuum* L).

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Morphological and biochemical changes were recorded in one somaclone derived from calli induced from hypocotyls of Chili pepper (*Capsicum annuum* L.) grown on MS medium with salt stress of 9 EC supplemented with 2 mg/L of each IAA and Kinetin from our previous work. Thus RAPD and SSR primers were used to assess somaclonal variations in the unique somaclone at the DNA level to confirm the genetic changes. The results showed that 100% polymorphism was obtained with RAPD primer OPF18 while the primers OPA1 and OPF9 showed 84.6% and 91.7% polymorphism respectively. In addition SSR analysis showed that the primer CAM-864 did not amplify any fragments in the parental plant. However, the SSR primers GPMS 113 and GPMS 161 showed 75% and 66.67% polymorphism respectively. In conclusion, RAPD and SSR markers confirmed molecular somaclonal variations in a selected chili pepper somaclone with desired morphological and biochemical characteristics which represents the addition of a new germplasm that can be used to improve the production of the important medicinal chili pepper Iraqi cultivar. Moreover, it is recommended to use several types of markers to reveal more variations in the somaclones.

Keywords: Chili pepper; Hypocotyls; Somaclonal variations; Salt stress; RAPD and SSR.

INTRODUCTION

Chili pepper belongs to the Solanaceae family. Several studies were concentrated on the importance of this plant due to its edible fruits and their industrial and medicinal uses. The plant is rich with secondary metabolites (Kumar, 2006).

Tissue culture has been used to propagate medicinal plants and to enhance the production of their secondary metabolites. Variations in plants regenerated from tissue culture have been documented at morphological, chromosomal, biochemical and molecular levels (Hashim et al. 1990; Karp, 1995; Bairu et al. 2011). DNA Polymorphisms were found even among the phenotypically normal somaclones of many plant species such as Tomato (Soniya et al. 2001) date palm (Saker et al. 2000) and pepper (Bello-Bello et al. 2014).

DNA – based markers have been used to reveal somaclonal variations in regenerated plants and to verify the fidelity of the micropropagated plants of many species (Teixeira da Silva et al. 2007). Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR_S) were used to study somaclonal variations and proved to be very effective (Teixeira da Silva et al. 2007; Khatab and El-Banna, 2011). Although many researchers reported the negative aspect of somaclonal variations, others reported their importance in crop improvement and as a source for genetic diversity (Bairu et al. 2011; Grozeva and Todorova, 2015). Very limited previous studies

reported somaclonal variations in pepper (Hossain et al. 2003; Anu et al. 2004; Bello-Bello et al. 2014). In our previous work which aimed to improve capsaicin production in the local chili pepper, several somaclons showed morphological variations and higher amount of capsaicin than the prenatal plant (Al Ajeel et al. 2016). Therefore the objective of this study was to confirm the genetic changes in one unique chili pepper somaclon with desired characteristics regenerated from hypocotyls as compared with the parental plant using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR_S) techniques.

MATERIALS AND METHODS

The current research was conducted at The Ministry of Science and Technology/ Directorate of Agricultural Research, Genetic Engineering Department, during the year 2016. Chili pepper plants of Iraqi cultivar were used in this experiment.

Calli were induced from hypocotyls grown on MS (Murashige and Skoog, 1962) medium with salt stress of 9 EC supplemented with 2 mg/L of each IAA and Kinetin (El Kaaby et al. 2015). Plants were regenerated on MS hormone free medium (Al Hattab et al. 2015). Some of the regenerated plants showed morphological variations as compared with the parental plants. To confirm the genetic changes in these somaclones, two molecular markers were used namely RAPD and SSR. Genomic DNA was extracted from the seedlings of the parental plants and from the second generation of a selected unique regenerated plant at 3-4 true leaves stage by CTAB following Graham et al. (1994) method with slight modifications. DNA concentration was estimated by NANOSPEC CUBE and DNA purity was measured from the OD ratio 0f 280 and 260 Nanometer.

The protocol of Tilahun et al. (2013) was adopted for RAPD using seven primers which were obtained from Bioneer. The names and the sequences of the primers are shown in (table 1).

Table 1. RAPD primers codes and sequences

(RAPD) Primer code	Sequence (5´-3´)
OPA 1	CAGGCCCTTC
OPF 9	CCAAGCTTCC
OPF 18	TTCCCGGGTT

Reaction mixture PCR – RAPD was consisted from 5 µL Master Mix (Bioneer), 3µL RAPD primers, 4 µL DNA samples, 8 µL D.D H_2O. The Master Mix consisted from 30 mM KCL buffer, 1.5 mM $MgCl_2$, 250 µM DNTPs, 1U Taq DNA Polymeriase, 10 mMTris-HCl (pH9.0). The Thirmocycler was programmed as the following: Initial Denaturation at 94 C ^{0} for 4 min, 45 cycles of 1 min Denaturation at 94 C ^{0}, 1 min Annealing at 36 C ^{0} and 1 min Extension at 72 C ^{0}. The last step was one cycle of Final Extension for 10 min at 72 C ^{0}. The amplicons were separated on 1 % Agarose gel along with 100bp Ladder at 70 Volt for 120 min. Gels were examined on Gel Documentation at 254 Nanometer.

Tilahun et al. (2013) method was used to reveal the variations in the DNA of some Simple Sequence Repeats (SSR). Three pairs of SSR primers were used which were obtained from Bioneer and their codes and sequences as shown in Table 2. The reaction mixture for PCR-SSR was consisted from 5 µL Master mix (Bioneer), 1.5 µL of each F (forward primer) and R (reverse primer), 4.0 µL DNA samples and 8.0 µL D.D H_2O. Thirmocycler was programmed as the following: Initial Denaturation at 94 C ^{0} for 3 min, 35 cycles of 1 min Denaturation at 94 C ^{0}, 1 min Annealing at 55 C ^{0} , 2 min Extension at 72 C ^{0} and one cycle of Final Extension for 10 min at 72 C ^{0}. PCR-SSR products were separated on 10% polyacryleamide gel of 29:1 Acrylamide: Bis Acrylamide in TBE buffer (0.32 M Boric acid, 0.5 M Tris-Base, mM EDTA-Na_2). The gel was polymerized with Tetra Methyl Ethylene Diamine (TEMED) and 10% Ammonium persulfate. Samples were loaded on the gels along with DNA Ladder. The gels were stained with Ethidum bromide and visualized with Gel Documentation. Clear reproducible bands from both methods were scored and identified using Photocapt program.

Table 2. The codes and the sequence of SSR primers

Primer code (SSR)	Sequence (5´-3´)
CAMS-864	F: CTGTTGTGGAAGAAGAGGACA R: GCTTCTTTTTCAACCTCCTCCT
GPMS 113	F: GCACAAGTCAATCCAAACGA R:CAAAAAGATGATGATGGATGAGA
GPMS 161	F:CGAAATCCAATAAACGAGTGAAG R: CCTGTGTGAACAAGTTTTCAGG

RESULTSAND DISCUSSION

The somaclone with the most morphological differences in comparison with the parental plant was selected for this study. It has larger leaves, longer stem and larger fruits as well as higher amount of capsaicin as previously reported (Al-Ajeel et al. 2016). Those variations were stable in the second generation therefore the somaclone was subjected to molecular analyses to confirm the genetic changes. The results of the quality and the quantity analyses of genomic DNA extracted from the parental and the regenerated plants are shown in (Table 3). The OD 260/280 were 1.85 and 1.84 for parental and the regenerated plants respectively which indicated the DNA purity of both samples. High purity DNA has an OD 260/280 of 1.8 (Holme and Peck, 1998). DNA extraction by CTAB method was very effective even without using liquid nitrogen.

RAPD analysis showed that out of the seven selected RAPD primers only three amplified clear reproducible bands on the agarose gel which were OPA1,OPF9 and OPF18 (Figure 1). OPA1 amplified 10 DNA fragments in the selected somaclone with sizes ranged from 236 bp to1699 bp while 5 DNA fragments in the parental plant with molecular sizes ranged from 175 bp to 458 bp (Table 4). The parental and the somaclone sheared only two fragments of 236 bp and 392 bp. The primer OPF18 amplified 6 fragments as a total number from both samples (Figure 1 and Table 4). Four fragments in the somaclone and 2 frgments in the parental plant. All the fragments were polymorphic and their moleculare weights ranged from 551 bp in the somaclone sample to 159 bp in the parental sample. In summary, the results showed that the primer OPF18 gave 100% polymorphism while the primers OPA1 and OPF9 gave 84.6 and 91.7 polymorphic percentage respectively.

Table (3): DNA concentration and purity extracted from the young leaves of chili pepper parental and the regenerated plants

Samples	OD280(abs)	OD260(abs)	OD260/280(abs)	Concentration of DNA (µg. µl^{-1})
Parental plant	2.32	4.31	1.85	215.8
Tissue culture plant	1.62	3.0	1.84	150.1

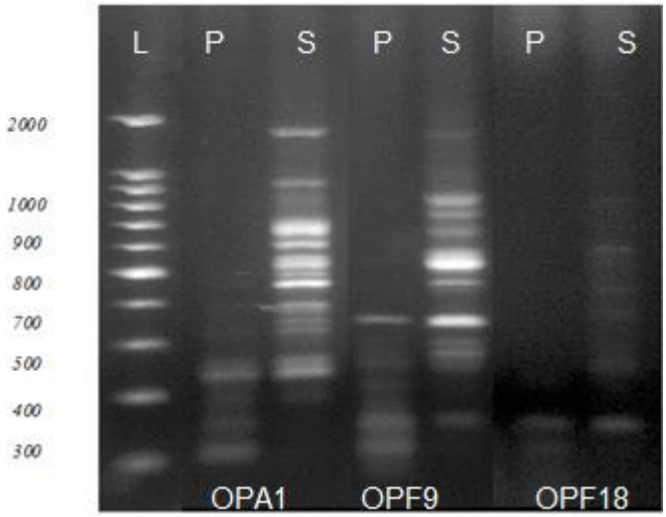

Figure 1. The RAPD-PCR amplification patterns produsedusing OPA1, OPF9 and OPF18 primers separeted on 1% Agarose gel (L: DNA Ladder, P: Parental, S Somaclone)

Table (4): The amplified DNA fragments and their sizes produced by OPA1, OPF9 and OPF18 RAPD primers

Primer	Fragments (bp)	parental	somaclone	Polymorphism %
OPA1	175	+	-	84.6%
	180	+	-	
	236	+	+	
	351	-	+	
	392	+	+	
	458	+	-	
	463	-	+	
	522	-	+	
	600	-	+	
	685	-	+	
	835	-	+	
	929	-	+	
	1699	-	+	
OPF9	175	+	-	91.7%
	180	+	+	
	254	+	-	
	273	-	+	
	293	-	+	
	351	-	+	
	358	+	-	
	458	-	+	
	528	+	-	
	534	-	+	
	817	-	+	
	1571	-	+	
OPF18	159	+	-	100%
	177	-	+	
	178	+	-	
	212	-	+	
	242	-	+	
	551	-	+	

SSR analysis showed that there are diffrences between the parental and the selected somaclon in the number of the amplified DNA fragments by the selected SSR primers (Figure 2 and Table 5). The primer CAM-864 did not amplify any fragments in the parental plant while there were two fragments in the somaclon with moleculare weights of approximately 35 bp and 579 bp (Table 5). The primer GPMS113 amplify one monomorphic fragment with a molecular weight of approximately 300 bp and 3 polymorphic fragments; 2 in the parental plant with molecular weights of approximately 110 bp and 55 bp and one in the somaclone with a molecular weight of 224 bp approximately. While the primer GPMS161 amplify one monomorphic DNA fragment with a molecular weight of approximately 10 bp and two polymorphic fragments; one of them in the parental plant of approximately 20 bp and one in the somaclone of approximately 35 bp. The results showed that there were a wide range of variations between the somaclone and the parental plant in the fragments patterns. The selected RAPD and the SSR primers showed clear polymorphism between the plants under investigation. The changes in the studied DAN fragments confirmed the inheritance of the variations in the morphology and the yield parameters of the selected somaclone compared with the parental plant as previously explained (Al-Ajeel et al, 2016). The polymorphic DNA fragments indicated the genetic changes in the somaclone. The variations in the regenerated plants are known as somaclonal variations (Larkin and Scowcroft, 1981). Such variations resulted

from the changes in the chromosome numbers and structure, deletion, inversion, addition, point mutation and DNA amplification. The changes in the nucleotides arrangements of the genomic DNA are responsible for the presence or the absence of the amplified fragments of the PCR markers. Nucleotides rearrangements might occur due to the deletion, insertion or inversion as well as point mutation and DNA methylation (Zhong et al. 2009; Demirkiran et al. 2013).

Moreover, unequal crossover within the microsatellite is responsible for the polymorphism increase in the regenerated plants (Oliveira et al. 2006). There are many factors responsible for the DNA changes in the regenerated plants. The growth regulators, number of subcultures and medium components have great effect on the genetic stability of the regenerated plants (Brar and Jain, 1998; Leva et al. 2012; Cristea et al. 2010; Tiwari and Rana, 2015).

Although many researchers reported no variations among the regenerated plants using molecular markers which confirmed the genetic fidelity of the somaclones as compared with the donor plant (Cristea et al. 2010; Mallaya and Ravishankar, 2013), other researchers used molecular markers to detect the DNA changes in the regenerated plants of many plant species.

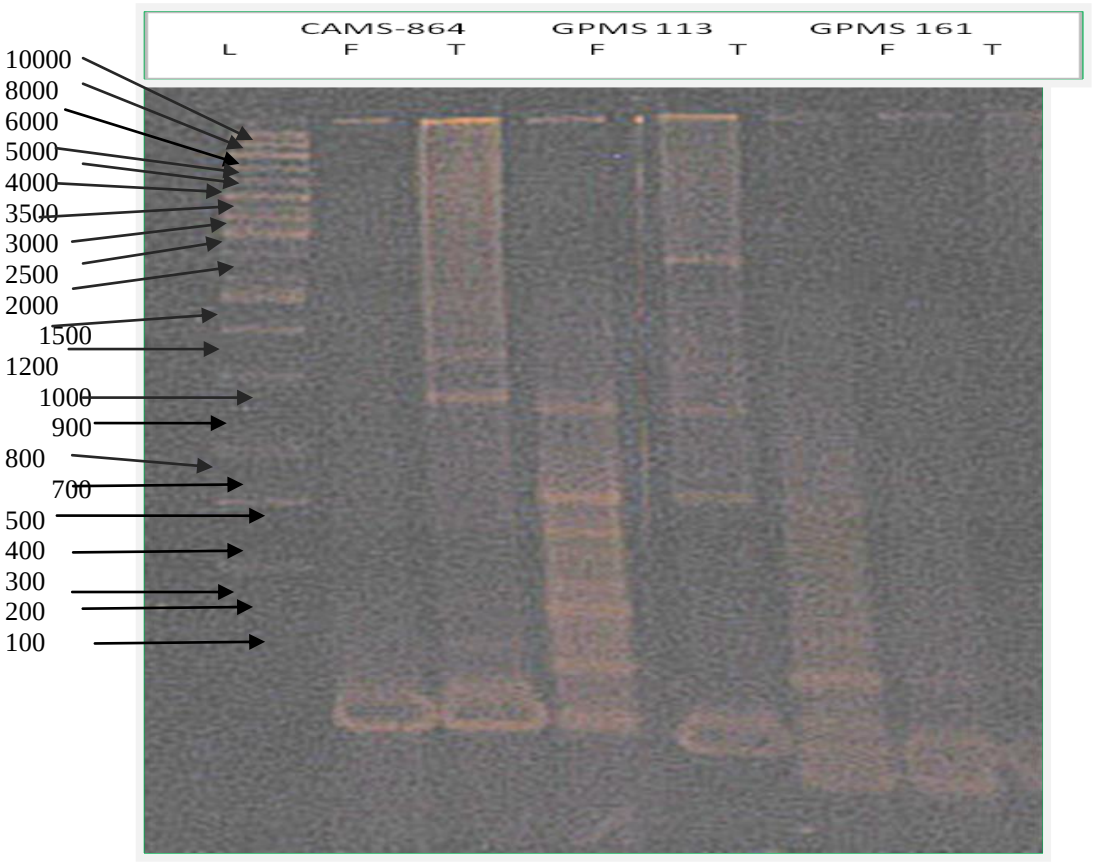

Figure 2: fragments' sizes (bp) which amplified by CAMS-864, GPMS 113 and GPMS 161 SSR primers in parental (F) and the somaclone (T) of chili pepper and DNA Ladder

Table (5): The SSR- PCR fragments and their sizes (bp) which amplified by CAMS-864, GPMS 113 and GPMS 161 SSR primers

Primer	Molecular weight (bp)	Parental	Somaclone	Number of Alleles	% of polymorphism
CAMS-864	35	-	+	2	100
	579	-	+		
GPMS 113	55	+	-	4	75%
	110	+	-		
	224	-	+		
	300	+	+		
GPMS 161	10	+	+	3	66.67%
	20	+	-		
	35	-	+		

The RAPD analysis is the most used technique to study the genetic changes in the somaclones of different plant species such as date palm (Saker et al. 2005) Tomato (Soniya et al. 2001) Banana (Khan et al. 2011) Olive (Leva et al. 2012) and Potato (Khan et al. 2014). In all those studies RAPD markers were used to confirm the differences between the somaclones and donor plant. RAPD-PCR and SSR were also used for the detection of genetic variations in micropropagated banana plants (Khan et al. 2011). Moreover, SSR marker technique showed 12% of the regenerated chili pepper plants were different compared with the parental plants (Ryu et al. 2007). The mutation in the microsatellite regions is different from the point mutation. All the SSR unit is lost or duplicated during the process of the DNA synthesis and may be the change occurred in more than one microsatellite unit. This explains the drastic changes in the patterns of the amplified fragments in the current study (Forster et al. 2015). Palombi and Damiano (2002) compared RAPD with SSR for the detection of molecular variations in kiwifruit (*Actinidiadeliciosa* A. Chev) and reported that DNA were amplified by both markers, however only SSR marker revealed variations among the plants under investigations.

CONCLUSION

The results of the present study indicated that somaclonal variations in chili pepper somaclones were in the genomic DNA as they were detected by RAPD and SSR markers. Desired and stable variations were identified in a unique chili pepper somaclone which represents the addition of new germplasm that can be used to improve the important medicinal Iraqi chili pepper plant. Moreover, it is recommended to use several types of markers to reveal more variations in the somaclones

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

The present research is part of Ph.D work of E. A .El- Kaaby. Z. N. Al Hattab is the co-supervisor suggested the problem and wrote the manuscript. S. A. Al Ajeel is the supervisor reviewed the manuscript. All authors read and approved the final manuscript.

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