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Utilization of Differential Display Reverse Trancrptase- DDRT for detection of salt tolerant clones of Potato *Solanuim tuberosum* L.

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Differential Display Reverse Trancrptase- DDRT was applied to study the genetic markers related with salt tolerance for mutant clones [plants induced from salt tolerant calli (planted at salt levels 8, 10, 12 dS. m^{-1}) and plants induced from non salt tolerant calli (which planted at salt level 6 dS. m^{-1})] and their parental cultivars (Riviera and Burren) by using 10 randomized primers. RNA was extracted using AccuZolTM Total RNA Extraction Solution. The results were analyzed according to the molecular weight of the bands, percentage of polymorphism, percentage of discriminating ability and percentage of efficiency of each primers with the diagram of the Dendrogram. Results showed that The concentration of RNA in plant samples ranged 103.0 - 226.0 nano gram micromole^{-1} with purity ranged 1.86-2.01 respectively. Differential Display Reverse Trancrptase- DDRT with Primers OPA-10, OPA-11, OPC-4, OPC-8, OPC-15, OPF-8 and OPH7 succeeded to amplify DNA fragments involved in salt tolerance. Cluster analysis separated genotypes in groups which indicate the existence of a range of genetic variability among them.

Keywords: Potato; Plant tissue culture; Differential display; primers; salinity.

INTRODUCTION

Molecular markers such as other markers (phenotypes, enzymes, protein and cellular) are considered one of the effective indicators in the distinction between varieties, DNA identification, genetic compatibility and detection of genetic variability of plants susceptible to mutagenesis by comparing the results of the DNA analysis with the origin as well as the reliability of the primers used and considered as a function of Environmental stress (AL-Daoude et al.,2008) There are several types of primers that vary depending on the type of the markers. They may have universal sequences and can be used with all organisms, such as Randomly Amplified Polymorphic DNA (RAPD), or may be specifically designed to identify a site specialist distributed randomly within the genome, such as Simple Sequence Repeat (SSR) technique and Inter Simple Sequence Repeats-ISSR (AL-Hussaini, 2016 ,(Al-Judy and Majeed 2013) and (AL-Kazaz ,2001)

In recent years, as part of the development of genetic engineering technologies, the technique of differentiation and analysis of genetic expression at the level of mRNA has been added, known as differential display reverse transcriptase – DDRT, in which genetic expression patterns are studied in experiments involving exposure of plants to stress conditions and comparing with non exposed, which considered as one of the techniques currently used to analyze differences in gene expression (Alves et al.,1998), (De Almeida et al.,2012)and (Fernández et al.,2002)

,**(**Liang P,2002) (Liang and Pardee 1992).This

technique was developed by (Liu et al.,2013)and described the speed, simplicity, sensitivity and efficiency of detecting high expression and regulatory genes (De Almeida et al.,2012), (Liu and Baird , 2003) and (Lutts et al.,1998) This technique is based on three basic steps, starting with the extraction of mRNA, amplification of the polymerase chain reaction (PCR) and ending with electrophoresis of polymerase reaction products using polyacrylamide gel or agrose gel (De Almeida et al.,2012) and (Fernández et al.,2002)

In this field Mahdi S (2015).tested this method with 12 random primers for dry stress treatment and 24 random primers for salt stress, as well as a comparison treatment of RNA isolated from the Triumph 545sunflower hybrid, the results of the electrophoresis on the polyacrylamide gel showed differences in the pattern of bands, this was attributed to differences in gene expression. Seventeen bands (sizes 125-378 base pair) were isolated and tested for the sequence of nitrogen bases and then registered at the National Center for Biotechnology-NCBI with registration numbers BG734514 to BG734530 to find out which of these genes are represented by these bands, these sequences were exposed to the gene bank and found that 6 of these sequences had a similarity with other plant genes. The similarities were between 37-71%, while he found that 13 isolates showed a genetic expression for salt or drought stress using Northern Gel blot analysis and the interaction of the quantitative real-time polymerase chain reaction. While (De Almeida et al.,2012) tested the differential display technique with 5 random primers of *Spartina anglica* plants (saline plant growing normally on the coasts of the sea) to salt stress (2% sodium chloride) as well as a comparison treatment (0 sodium chloride), The results showed that 4 bands were characterized by stress in comparison with those non exposed to salt stress. A sequence of nitrogen bases was then studied for a single sequence called KDI, which showed 63% similarity with the osmotin-like gene PA9 (NCBI M84468.1) in the *Atriplexnummularia,* he concluded that the differential is efficient in testing the gene expression of plants exposed to salt stress. As (Liang and Pardee 1992).exposed the callus of Ruta plants to different concentrations of NaCl and PEG. After regenrating plants, the DDRT was tested with short random intervals and observed differences in gene expression of exposed and non exposed using the OPA-08, OPA-01 , OPA-11, and OPA-17 primers when relaying the reaction products of these populations on the agrose gel, in a study by (McGregor et al.,2000)

to identify the genes responsible for salinity tolerance in potato plants, they pointed out in their study that although the techniques of multiple randomized replication polymorphisms (RAPD) and simple ISSR sequences succeeded in detecting the degree of genetic diversity and relationship Between mutant clones of potatoes but failed to detect genes for salinity tolerance in those mutant.

In Iraq, potato was considered a widely consumer crop and due to spread of the salinity in large areas and the urgent need to salt tolerant genotypes, as well as the lack of studies in the field of the development genetic variation of salt tolerance in the potato by using tissue culture and there is not enough available information on salt tolerant genes. Therefore, the aim was to study genetic indicators associated with tolerance by identifying and analyzing variation in gene expression among salt tolerant and non tolerant mutant by applying differential display reverse transcriptase – DDRT technique.

MATERIALS AND METHODS

This study was carried out in the laboratories of the Department of Genetic Engineering of the Directorate of Agricultural Research, Ministry of Science and Technology, Baghdad, Iraq, 2015-2016.

Explant production

Potato plantlet of Bureen and Riviera cultivars were established on semi-solid medium MS Murashige and Skoog (1962) .Plantlets were exposed to Gamma rays (source Co 60) at dose 12 and 18 Gy. Respectively, the internod cuttings (stem segments approximately 1- 1.5 cm length, without node) from *in vitro* irradiated plantlets were used for callus induction, which cultured on petri dish containing MS with 3% sucrose, 7% agar and 0.1, 100, 0.5, 0.5,0.5, 2, 2 mg L^{-1} of Thiamine – HCL, Inositol, Glycine, Nicotinic Acid, Pyridoxine-HCL, Benzyl adenine, 2,4-D, respectively. after month Calli were transferred to regeneration media containing MS salt supplemented with mg L^{-1} of 0.4 Thiamine HCL, 100 Inositol, 0.5 Glycine, 0.5 Nicotinic Acid, 0.5 Pyridoxine-HCL, 3 BA, 0.5 GA3, 0.03 NAA and 30 gm L^{-1} sucrose, with different levels of NaCl to generate EC at 8, 10, 12 dSm^{-1}, the EC of the control treatment (MS basal medium, without adding NaCl) was 6 dSm^{-1}.

Table 1. Mutant clones were formed in growth room chamber after 60 days

Number	Samples
	Non salt tolerant clone
1	Plants of non Irradiated Riviera which induced from non salt tolerant Calli (at 6 dS m-1 NaCl)
4	Plants of non Irradiated Bureen which induced from non salt tolerant Calli (at 6 dS m-1 NaCl)
	Salt tolerant clone
2	Plants of non Irradiated Riviera which induced from salt tolerant Calli (at 12 dS m-1 NaCl)
3	Plants of Irradiated Riviera at18 Gy which induced from salt tolerant Calli (at 10 dS m-1 NaCl)
5	Plants of Irradiated Bureen at 12 Gy which induced from salt tolerant Calli (at 8 dS m-1 NaCl)
6	Plants of Irradiated Bureen at 12 Gy which induced from salt tolerant Calli (at 12 dS m-1 NaCl)
	Parental cultivars
7	in vitro non irradiated Riviera plantlets which planted at 6 dS m-1 NaCl
8	in vitro non irradiated Bureen plantlets which planted at 6 dS m-1 NaCl

All cultures were incubated in the growth room chamber at 25°C±2 under a 16 h light and 8 h dark. Plants were formed after 60 days as a mutant clone, (Table. 1) (AL-Daoude et al., 2008).

Differential Display Reverse Trancrptase-DDRT technique

Molecular differences between the parental cultivars (Riviera and Burren) and salt tolerance mutant clones [plants induced from salt tolerant calli (planted at salt levels 8, 10, 12 dS. m^{-1}) and plants induced from non salt tolerant calli (which planted at salt level 6 dS. m^{-1})] were studied which implemented with steps, from extraction and purification of total RNA and ending with electrophoresis of the polymerase chain reaction.

Extraction and purification of total RNA

Total RNA was extracted from potato shoots using the **AccuZolTM Total RNA Extraction Solution**, then DNA was removed from RNA extracted using RQ1 RNase -Free DNasePromega Kit.

Characterization of RNA and visualization

RNA was characterized by measuring the concentration of RNA (ngμl^{-1}) and its purity by using the nano-Drop spectrophotometer. RNA were visualized using gel electrophoresis by Preparing 1% agarose gel (fig. 1).

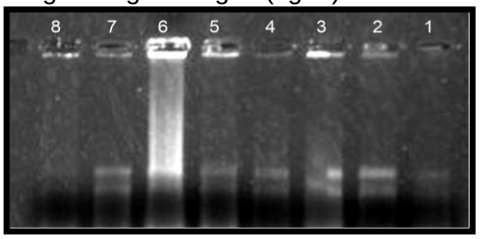


Figure 1.RNA electrophoresis from salt tolerance mutants of potato on 1% agrose gel.

Reverse transcriptase

In order to synthesize cDNA, the Rocketscript TM Reverse Transcriptase Kit (Korean origin) was used. First-strand cDNAs were synthesized in two steps: **a)Preparation of the reaction mixture** in a total volume of 20 µl containing of 9 µl total RNA, 1µl primer Oligo (dt)18 (100 n g μl^{-1}), 4µl RocketScript Reaction Buffer(5x), 2µl DTT(100mM), 2µl dNTP(10mM), 1µl RNase Inhibitor (200U μl^{-1}).

b) Reverse transcriptase reaction. Mixture was placed in Polymerase chain reaction (PCR) containing two phases: **1) Cyclic Reverse Transcription (CRT)** which designed as 12 cycles of 30 sec at 37 °C for Primer annealing, and 4 min at 50 °C for cDNA synthesis, and 30 sec at 55 °C for Melting secondary structure &cDNA synthesis, and 1 cycle of 5 min at 95 °C for Heat inactivation. **2) Fixed Temperature Reverse Transcription (FTRT).**which began in Primer annealing for 1 min at 37 °C, and cDNA synthesis for 60 min at 50 °C, and Heat inactivation for 5 min at 95 °C.

Polymerase chain reaction -PCR

To detect the difference in gene expression, cDNA was compared to plants produced from salt tolerant and non-tolerant mutant plants. The reaction was performed using the **AccuPower® PCR PreMix kit polymer** from Pioneer Korea, which had 96 tubes, each tube contained mM MgCl2 (1.5 mM), KCl (30 mM) ,dNTP) 250 µl , Tris – HCL (10 mM) and Taq DNA polymerase (1 U) .

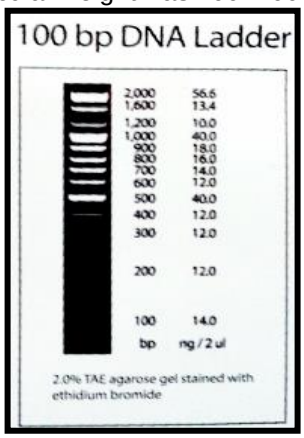
For PCR amplification randomly selected oligonucleotide primers (Operon Technologies, Pioneer, Koren) were used (Table 2). A PCR experiment was set up by adding to each tube (AccuPower® PCR PreMix kit polymer) 200 ng genomic cDNA, 100 ng primer, and 12 µL water.

Table 2. Primers with Sequence

Number of .Primer	Name of Primer	5 → 3 Sequence
1	OPA-1	CAGGCCCTTC
2	OPA-10	GTGATCGCAG
3	OPA-11	CAATCGCCGT
4	OPC-4	CCGCATCTAC
5	OPC-8	TGGACCGGTG
6	OPC-15	GACGGATCAG
7	OPD-2	GGACCCAACC
8	OPF-8	GGGATATCGG
9	OPH-7	CTGCATCGTG
10	OPZ-11	CTCAGTCGCA

The PCR was designed as 40 cycles of 4 min at 94 °C, 1 min at 94 °C, 1 min at 72 °C and 10 min at 72 °C. PCR products were then run on a 1% (w/v) agarose gel in TBE buffer at 90 V. After separation, bands were examined and documented under UV**.**

The samples were carried out with 4 µl of the DNA Ladder 100 pb (figure 2) of Pioneer Korea, whose molecular weight was 100- 2000 base pairs.



Analysis of results

Molecular Weight

Photo capt. was used to calculate molecular weight, a computer program that is accurate in calculating the bands sizes resulting from polymerase chain reactions by comparing the DNA Ladder 100pb with the samples.

The percentage of polymorphism.

Method of (Rodriguez et al., 2005) was followed, according to the following equations:

Percentage of polymorphism = (The number of different bands in the primer / total number of primer bands) x 100

Percentage of discriminatory capacity= (The number of different bands for the primer /The number of different bands for each primers) × 100

percentage of the efficiency of each primer = (total number of primer band / total number of bands of all primers) × 100

Dendrogram

In order to determine the dendrogram between parental plants and salt tolerant and non tolerant mutant plants of Bureen and Riviera potato, Reproducible and clear bands were scored as binary characters (presence (1) or absence (0). The SPSS version 20 was used to analyze data to draw the Dendrogram by using Dendrogram Linkage Method, which is one of the most important hierarchical Agglomerative Methods to simplify data by clusters in accordance with the similarity of the response pattern based on the Sokal and Sneath2 equation.

RESULTS

RNA concentration and purity

Table 3 showed that RNA concentration for mutant clones [plants induced from salt tolerant calli (planted at salt levels 8, 10, 12 dS. m^{-1}) and plants induced from non salt tolerant calli (which planted at salt level 6 dS. m^{-1})] and their parental cultivars (Riviera and Burren) ranged from 103 and 226.0 ng μl^{-1} while, purity ranged 1.86 and 2.01. The isolated RNA gave a visual perception of the efficiency of the method and the materials used in extraction (AccuZolTM Total RNA Extraction) and the purification and removal of DNA from the total RNA using RQ1 RN1 -Free DNase Promega, as the quantities and purity were appropriate and suitable for subsequent steps.

Differential Display Reverse Transcriptase-DDRT

Among the 10 RAPD primers examined in this

study, 7 (OPA-10, OPA-11, OPC-4, OPC-8, OPC-15, OPF-8 and OPH7) were succeeded to amplify DNA fragments involved in salt tolerance. The OPA-10 primer was identified three unique bunds at molecular weight 800, 449 and 385 bp in Plants of Irradiated Riviera at 18 Gy which induced from salt tolerant Calli (at 10 dS m^{-1}NaCl) (3), which were disappearing from all samples (figure 3).

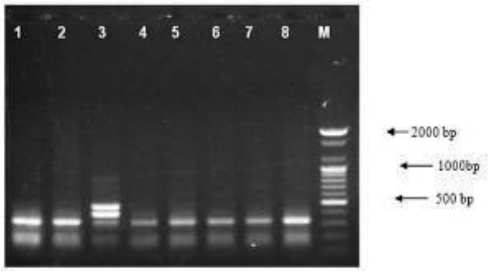


Figure 3; DDRT profiles obtained from the OPA-10 primer. M: Ladder, 1,4; non salt tolerant ,2,3,5,6; salt tolerant ,7,8 ; parental.

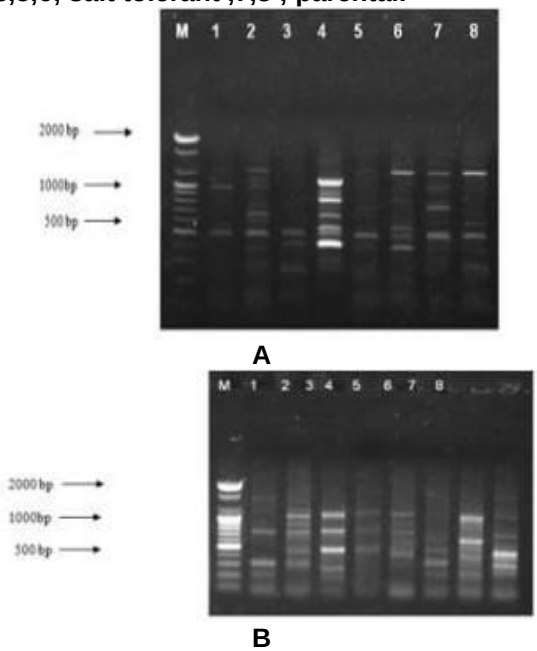


Figure 4. DDRT profiles obtained from the OPC-4 , OPC-15 primers. M: Ladder, 1,4; non salt tolerant ,2,3, 5,6; salt tolerant ,7,8 ; parental.

Due to absent these bands in the parental plants Burren and Riviera) and non Irradiated (Riviera, Bureen) plants which induced from non salt tolerant Calli .So it could be considered to be related to salt tolerance. Also the primers OPC-4 and OPC-15 identified only a single band possibly associated with salt tolerance, which appearance of salt tolerant plants. OPC-4 was identified band 400 bp in Plants of non Irradiated Riviera, which induced from salt tolerant Calli (at 12 dS m^{-1}NaCl) (2) and Plants of Irradiated Bureen at 12 Gy, which induced from salt tolerant Calli (at 12 dS m^{-1}NaCl) (6) (figure 4, A). OPC-15 was found the band at size 960 bp in Plants of the non Irradiated Riviera which induced from salt tolerant Calli (at 12 dS m^{-1}NaCl) (2) and Plants of Irradiated Bureen at 12 Gy, which induced from salt tolerant Calli (at 8 dS m^{-1}NaCl) (5) (figure 4, B).

Either the primer OPA-11 has been observed number of bands at different sites may be associated with salt tolerance, where the band at size 700 bp appeared in plants which induced from salt tolerant Calli (3 and 5) and an band of 279 bp in all tolerant saline plants for both genotypes (2, 3, 5 and 6) also recognized band at site 152 bp in the Plants of Irradiated Bureen at 12 Gy which induced from salt tolerant Calli (at 12 dS m^{-1}NaCl) (6). (Figure 5). OPH-7 primer was able to identify salinity-tolerant bands that appeared in salt tolerant plants and disappeared in both parental and non salt tolerance plants. Band at 2000 bp, 1115 and 1110 bp was appeared in the Plants (5), (2) and (3) respectively.(Fig. 6).

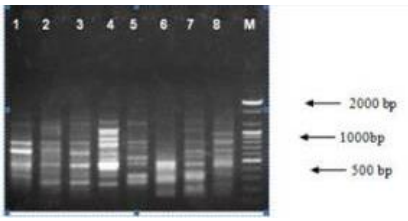


Figure 5; DDRT profiles obtained from the OPA-11 primer. M: Ladder, 1,4; non salt tolerant ,2,3,5,6; salt tolerant ,7,8 ; parental.

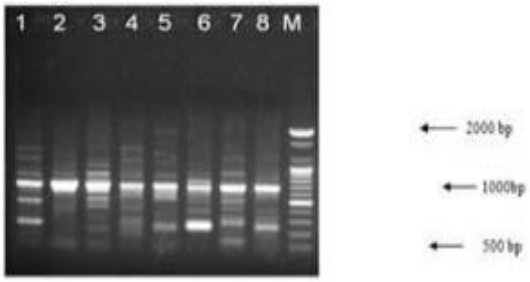


Figure 6: DDRT profiles obtained from the OPH-7 primer. M: Ladder, 1,4; non salt tolerant ,2,3,5,6; salt tolerant ,7,8 ; parental.

The primer OPC-8 showed bands related to salt tolerant, that identified band at 1350 bp that appeared in Plants (2), (6) and bands at sites 264, 800 and 1600 bp Which appeared in Plants (6) and band at 960 bp, in the Plants (5) (Figure 7). The OPF-8 primer also identified a number of bands, (2000, 1502, 1226, 736 and 490 bp) were showing in the Plants (2) (3), and (6) (figure 8).

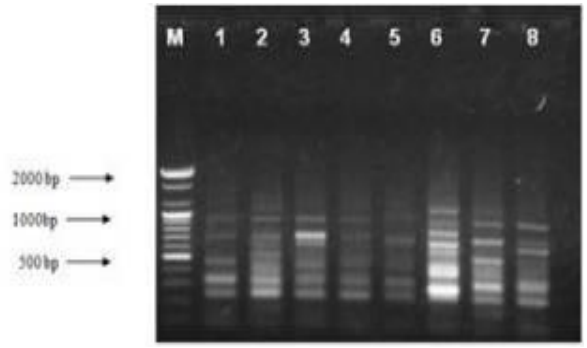


Figure7; DDRT profiles obtained from the OPC-8 primer. M: Ladder, 1,4; non salt tolerant ,2,3,5,6; salt tolerant ,7,8 ; parental.

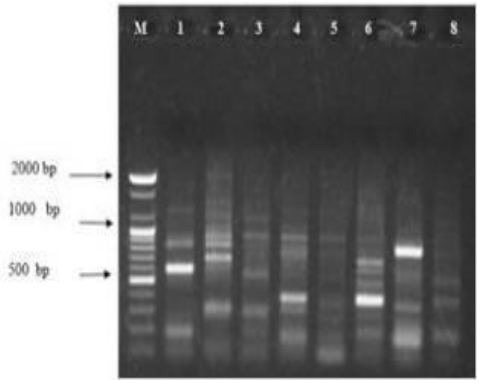


Figure 8; DDRT profiles obtained from the OPF-8 primer. M: Ladder, 1,4; non salt tolerant ,2,3,5,6; salt tolerant ,7,8 ; parental.

The ability of these primers to determine the bands which associated with salt tolerant between parental, salt and non salt tolerant mutant clones may be explained to the direct effect of radiation or to the occurrence of somaclonal variations (Saleem et al.,2005), Yamazaki and Saito (2002), Yaycili and Alikamanoglu (2012) which causing a change in the nucleotides of the DNA and its reflection on the sequence of messenger RNA nucleotides, and thus a change in the genetic code and gene expression.

Polymorphism, Discriminatory capacity and Efficiency of primers Percentage

All the primers proved to be effective in giving a polymorphism between the measured sites. From total150 bands, 142 bands gave polymorphism(Table 4). Primers differed in the number of bands and the lowest number of bands was appearing in an OPA-10 primer while, the highest number was shown in OPH-7 (23 sites). The percentage of polymorphic reached 100% in the OPD-2, OPF-8 and OPZ-11 primers. While the lowest percentage was 60% in the OPA-10 primer due to its number of bands varied approximately half of its total. The highest efficiency (15.33%) and the highest discriminating capacity (15.49%) were recorded in OPH-7.

Dendrogram

Upon evaluation of the dendrogram demonstrating genetic distances, The Dendrogram was showed the separation of the genotypes into major groups. The first major group included two subgroups, the first sub group included Riviera cultivar (non irradiated, irradiated at 18 Gy which induced from salt tolerant Calli (at 10 and 12 dS m^{-1}NaCl) and Riviera, Bureen cultivar (non irradiated, irradiated at 18 Gy which induced from salt tolerant Calli (at 6 and 8 dS m^{-1}NaCl). As for the second subgroup included Bureen cultivar (non irradiated, irradiated at 12 Gy, which induced from salt tolerant Calli (at 6 and 12 dS m^{-1}NaCl) and Bureen parental. While the second major group (Riviera parental) were genetically distant from all the groups (fig 9). The number of bands determines the proximity or genetic dimension between the studied genotypes, As the number of the bands increases, the genetic dimension decreases. That is, the common bands indicate a similarity in the genetic material in that area of the genome, which may represent similarities in some phenotypic traits and then the appearance of the genotypes within a specific group, The separation of genotypes into distinct groups indicates that there are genetic variations between the genotypes of these groups (McGregor et al.,2000).

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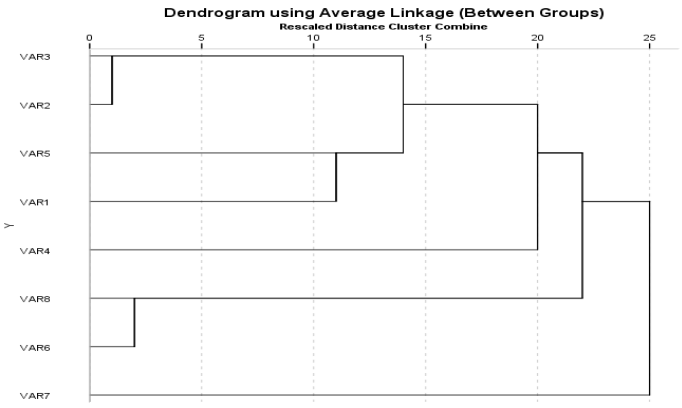

Figure9. Dendrogram of the mutant clones induced from salt tolerant and non tolerant compared with parental cultivars using Average linkage

Table 3. The concentration and purity of RNA, which extracted from leafs of potato

Samples	Concentration ng μl^{-1}	Purity
1	113.1	1.97
2	109.3	1.93
3	103.0	1.90
4	137.8	1.91
5	226.0	1.94
6	105.2	2.01
7	169.4	2.01
8	193.8	1.86

Table 4.Percentage of polymorphism, efficiency and discriminatory capacity of primers.

Primer name	Total number of bands	Number of bands polymorphism	% Bnds Polymorphism	% Primer efficiency	% Primer discriminary
OPA-1	**13**	**12**	92.31	8.67	8.45
OPA-10	**5**	**3**	60.00	3.33	2.11
OPA-11	**18**	**17**	94.44	12.00	11.97
OPC-4	**12**	**11**	91.67	8.00	7.75
OPC-8	**15**	**14**	93.33	10.00	9.86
OPC-15	**12**	**11**	91.67	8.00	7.75
OPD-2	**19**	**19**	100.00	12.67	13.38
OPF-8	**17**	**17**	100.00	11.33	11.97
OPH-7	**23**	**22**	95.65	15.33	15.49
OPZ-11	**16**	**16**	100.00	10.67	11.27
Total	**150**	**142**			

CONCLUSION

In conclusion, the Differential Display Reverse Trancrptase- DDRT technique with sufficient polymorphism can be successfully used for obtaining salt tolerant mutant clones and identify some of the primers as a function for salt tolerance, Which encouraged the continuation of its application and the detection of the stability under field conditions for several generations to be planted in high saline land, which is a model of land that suffers from salt stress.

CONFLICT OF INTEREST

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

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AUTHOR CONTRIBUTIONS

ZA performleed the experiments, data analysis and also wrote the manuscript, SY performleed the experiments and data analysis, SA reviewed the manuscript. All authors read and approved the final version.

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