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# Differential Expression for Genes in Response to Drought and Salinity in *Ruta graveolens* Plantlets

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**Abstract**

Abiotic stress-induced genes may lead to understand the response of plants and adaptability to salinity and drought stresses. Differential display reverse transcriptase – polymerase chain reaction (DDRT-PCR) was used to investigate the differences in gene expression between drought- and salinity-stressed plantlets of *Ruta graveolens.* Direct and stepwise exposures to drought- or salt-responsive genes were screened in *R. graveolens* plantlets using the DDRT technique. Gene expression was investigated both in the control and in the salt or drought-stressed plantlets and differential banding patterns with different molecular sizes were observed using the primers OPA-01 (646,770 and 983 pb), OPA-08 (593 and 988 pb), OPA-11 (674 and 831 pb), OPA-17 (638,765 and 1000 pb), and OPA- 15 (645 and 900 pb) indicating the expression of new genes amplified under stress conditions or of genes that already exist. Accordingly, DDRT-PCR seems to be a versatile and sensitive method, capable of detecting transcriptional changes at the mRNA level in plants**.**

**Keywords**: Gene expression, Drought and salinity stress, Callus culture, *Ruta graveolens*

## 1. Introduction

Differential display reverse transcriptase DDRT is one of the methods designed for analyzing differences in gene expression level. It has been successfully used to identify new genes in various tissues or cells. This technique is considered simple, quick, sensitive, and powerful for screening cDNA (Alves *et al.,* 1998; Rodriguez *et al.,* 2005). *Ruta graveolens* (Rutaceae), commonly known as the rue plant is an odorous medicinal and aromatic plant, grown in the Mediterranean region for outdoor decoration due to its beautiful yellow flowers. Rue contains many secondary metabolites such as flavonoids, furocoumarins, acridonealkaloids, furoquinolines, and coumarins which are used to treat many diseases such as anthelmintic, vitiligo, antispasmodic, multiple sclerosis, and emmenagogue in veterinary medicine (Ahmad *et al*., 2010; Zuraida *et al.,* 2014). Abiotic stresses negatively affect plant growth, and reduce crop productivity worldwide including salinity and drought. When plants are under salt stress, the result is an ionic imbalance and hyper osmotic stress (Munns and Tester, 2008). Imbalance in homeostasis usually occurs at the cell and whole plant levels (Rodriguez *et al.,* 2005). Tolerance depends on the ability of a plant to sustain growth even when conditions are unfavorable for basic plant developmental processes. This strategy involves certainly physiological and biochemical modification at the cellular and molecular levels (Xiong and Ishitani, 2006; Peleg *et al.*, 2011). Tolerance level differs from one plant to another and from one species to another species, but the mechanism starts with stress tolerance followed by gene products that are involved in cellular protection and may be a repair mechanism (Rao *et al.,* 2006). A gradual salt built up in the soil increases the osmosis in soil solution leading to accumulative decline in nutrients' absorption and thereafter a growth inhibition. The duration and severity of the stress (acute vs. chronic) influence the developmental stage of the plant, particularly when salinity is accompanied by drought stress (Cramer *et al.,* 2011; Peleg *et al.,* 2011; Leva *et al.,* 2012). This study is aimed at examining the response of *Ruta* callus cultures to varying levels of mannitol and saline water using two types of selection: (a) shock treatment, in which cultures are directly subjected to different concentrations for the stress agent, (b) stepwise long-term treatment, in which the cultures are exposed to stress with a gradual increase in the concentrations of the selected agent. It has been reported in this work that RNA, isolated from plantlets under stress conditions, then compared to the RNA isolated from the control plantlets and the DNA of intact plant, permits an increase in the gene expression level in the plantlets under stress.

## 2. Materials and Methods

*Ruta graveolens* plants were purchased from one of Baghdad nurseries. The plant stems were first washed under running tap water for thirty minutes, and were then subjected to surface sterilization using 1.5 % (v/v) NaOCL (Clorox) for twenty minutes with vigorous shaking. Stem segments (1.0 cm in length) were excised aseptically and transferred to Petri dishes (20 mL/dish) containing agar

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solidified MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L Kin. All cultures were incubated at 25 ± 2 ºC, 16/8 h (light/dark) photoperiod under a light intensity of 1000 lux. (Ahmed *et al.*, 2010).

*2.1. Treatments with Salinity and Drought Stress Agents*

Small pieces of calli weighing 100 mg each were re-cultured onto a callus maintenance medium containing 1.0 mg/L 2,4-D and 0.5 mg/L Kin, supplemented with different concentrations (0.0, 6, 12, 18, 24 or 30 %) of mannitol as osmoticum or (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 $dS/m^{-1}$) of saline water collected from drainage channels for direct screening and selection method. Callus cultures were recultured three times onto the same medium. For a gradual exposure to stress agents, callus pieces weighing 100 mg were re-cultured onto the maintenance medium containing 1.0 mg/L 2,4-D; 0.5 mg/L Kin, and were subjected to a gradual increase in mannitol concentrations (0.0, 6, 12, 18, 24 or 30 %) or (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 $dS/m^{-1}$) of saline water. All stressed callus pieces were transferred into MS regeneration medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA after screening cycles.

*2.2. Extraction of Genomic DNA and Total RNA*

The method of Ahmad *et al.*, (2010) was followed; briefly approximately 50 mg of dried leaves were ground with a mortar and pestle. The homogenized tissues were transferred to 600 µL of 2 % CTAB DNA extraction buffer mixed with 1.25 µL of β-mercaptoethanol in 1.5 mL Eppendorf tubes, and were incubated at 65ºC for thirty minutes in a water bath. Three microliters of RNAse were added and incubated at 37 ºC for one hour. Then, aliquot of 200 µL chloroform: isoamyl alcohol (24:1) was added to the solution, and was mixed well. The emulsified mixture was centrifuged at 13000 rpm for fifteen minutes, and then the aqueous phase was placed into a new sterilized Eppendorf tube. Aliquots of 600 µL isopropanol and 150 µL of sodium acetate were added, and then centrifuged at 13,000 rpm for ten minutes. The supernatant was discarded, the precipitated DNA was washed with 600 µL of 70 % ethanol, centrifuged at 13,000 rpm for five minutes, and then the supernatant was discarded. DNA was air-dried for two minutes and was dissolved in 150 µL of TE buffer and incubated at 65ºC for one hour in a water bath. DNA concentrations were then measured. Total RNA (in 100 mg of leaf samples) was isolated from plantlets exposed previously to abiotic stresses and from control regenerated plantlets using Geneaid total RNA Mini Kit (Vogelstein and Gillespie, 1979).

*2.3. Synthesis of cDNA*

The total RNA extracted from different calli samples (stressed and control) was used as a template to synthesize cDNA by AccuPower® RT Premix. The primer Oligo–dt (promega, USA) was prepared to obtain 100 pmol, which was mixed with DEPC 0.1 %. It was mixed well with 5 µL of template 0.5 – 1.0 µg RNA and Oligo $dt_{15}$ primer 100 pmol, and was incubated at 70˚C for five minutes, and was placed on ice. The incubated mixture was transferred into 5 µL AccuPower® RT Premix tube, then the volume was completed with 5 µL DEPC water. The cDNA synthesis was performed using a thermos cycler reaction at 42˚C, for sixty minutes (cDNA synthesis) then 95˚C, for five minutes (RTase inactivation).

*2.4. Differential cDNA Display*

AccuPower© PCR PreMix (Bioneer, Korea) ready to use, master mix (20 µL reaction) was used; it contained 250 µM of deoxyribonucleoside triphosphate (dNTPs), 30 mM of KCl, 10 mM of Tris- HCl (pH 9.0), 1.5 mM of $MgCl_2$, 1 Unit of Top DNA polymerase and a tracking dye. Random primers OPA-01, OPA-05, OPA-08, OPA-10, OPA-11, OPA-15, OPA-17, OPB-05, OPC-04, OPE-08 were provided (Operon model - Promega, USA). The sequences of the polymorphic primers are presented in Table 1. The primers were purchased in a lyophilized form, and were dissolved in sterile distilled water to give a final concentration of 100 ng/µL as recommended by the supplier. RAPD-PCR conditions were 94˚C for four minutes, 40 PCR cycles PCR products, and 100 bp DNA ladder (i.e., the cycles were performed at 94˚C for one minute, 36˚C for one minute and 72˚C for one minute) and were followed by a ten-minute extension step at 72˚C.

**Table 1.** Random primers used for the amplification of cDNA and genomic DNA.

| Primer's name | Sequence 5'------ 3' |
|---|---|
| OPA-01 | CAGGCCCTTC |
| OPA-05 | AGGGGTCTTG |
| OPA-08 | GTGACGTAGG |
| OPA-10 | GTGATCGCAG |
| OPA-11 | CAATCGCCGT |
| OPA-15 | TTCCGAACCC |
| OPA-17 | GACCGCTTGT |
| OPB-05 | TGCGCCCTTC |
| OPC-04 | CCGCATCTAC |
| OPE-08 | TCACCACGGT |

The optimization of the PCR reaction was accomplished after several trials, then, the mixture listed below was adopted as PCR products and 100 pb DNA ladder was determined by electrophoresis. Aliquot of 10 µL of the product was loaded on 1.0 % agarose gel and run at 80 volt for one hour. Bands were visualized on UV trans-illuminator and were photographed. The molecular weight of the bands was determined using the photo Capt MW program.

| Component | Concentration | Volume (µL) |
|---|---|---|
| $ddH_2O$ | ---------- | 12.0 |
| *AccuPower*®PCR PreMix | 1X | 5.0 |
| Primer | 10 pmol | 1.0 |
| DNA sample | 100 ng/ µL | 2.0 |
| Final volume | | 20.0 |

## 3. Results

Polymorphisms at cDNAs levels in assuming drought and salinity tolerant *R. graveolens* regenerated plantlets were compared with cDNA control plantlets (regenerated plantlets which are not subjected to stress) and DNA isolated from intact plants. The cDNA was amplified with ten base arbitrary primers. The polymorphic fragments capable of differentiating the tolerant lines from the

stressed regenerated plantlets were generated by most primers. New bands were observed in salinity- and drought-stressed regenerated plantlets which were not detected in cDNA obtained from the control treatments. The primer OPA-01 generated a profile distinguished between tolerant lines in stressed regenerated plantlets and the control ones (Figure 1). The intensity of the bands increased in the regenerated stressed plantlets. The amplification by with this primer revealed the presence of a 646 bp fragments in the tolerant lines (lanes 3, 4, 5, and 6) compared with the control. On the other hand, fragments at 676 bp in the DNA extracted from intact plants (lane 1) and at 688 and 1014 bp in c DNA from control plant (lane 2) were not detected in the tolerant lines. A new band with a molecular size of about 770 bp was observed in plants produced from direct salt-screening method (lane 3) and plantlets produced from direct and stepwise drought-screening methods (lanes 5 and 6). A band at a size of 983 bp was detected in plantlets which resulted from the direct salt-screening method (lane 3) and plantlets regenerated from direct drought screening method (lane 5), while a band at 955 bp was detected in plantlets obtained from stepwise drought-screening method (lane 6). However these bands were not found in plantlets regenerated from control treatment (lanes 1 and 2). The marker OPA-08 amplified seven bands, of which five were polymorphic and two were monomorphic. The primer has an amplification product with molecular sizes about 988 and 593 bp fragments visualized in all tolerant plantlets (lanes 3, 4, 5 and 6) and in DNA from the intact plant (lane 1), but they were absent in the cDNA control plantlets (lane 2) as shown in Figure 2.

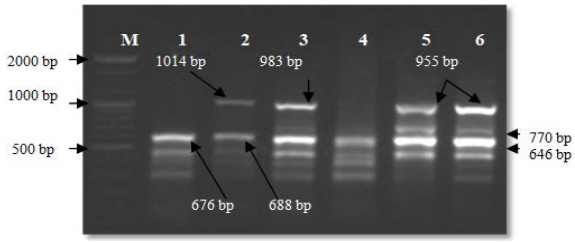


**Figure 1.** Amplification profile of differential display obtained using the primer OPA-01, lane (1): DNA from intact plant, lane (2): cDNA from regenerated-non stressed plantlet, lanes (3, 4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5, 6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: DNA ladder.

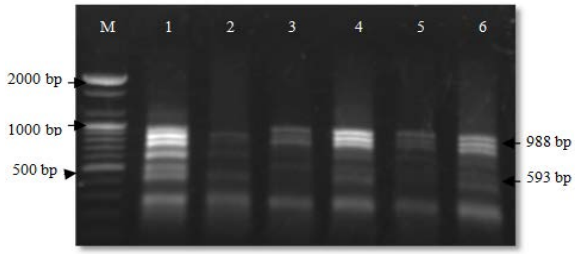


**Figure 2.** Amplification profile of differential display obtained using the primer OPA-08, lane (1): DNA from intact plant, lane (2): cDNA from regenerated non-stressed plantlet, lanes (3, 4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5, 6): cDNA from regenerated plantlets subjected to direct or gradual mannitol, M: DNA ladder.

Amplification products using the primer OPA-11 (Figure 3a) which showed the presence of new bands with molecular sizes of 831and 674 bp in the lanes 3, 4, 5, 6, and in lane 1 of the control, but they were missing in lane 2 in the control. The primer amplified a band with a molecular size of 719 bp which showed high intensity as visualized in lanes 3, 4, 5, 6, and 1, but it exhibited very low intensity in lane 2. Two bands with the molecular sizes of 541 and 300 bp were visualized in all tolerant lines, but not in control plantlets (lanes 1 and 2). Amplification with the primer OPA-17 revealed the presence of 1000, 765, and 638 bp fragments in the tolerant plantlets with a molecular size of 900 bp as visualized in the plantlets which resulted from the direct salt-screening method (lane 3) and those produced from the direct and stepwise drought-screening method (lanes 5 and 6) in the control plant (lane 1), but this band was absent in the plantlets regenerated from stepwise salt-screening method (lane 4), and in the cDNA control plantlets (lane 2). Lanes 3, 4, 5, and 6 compared with the control in lane 1, but those were absent in the cDNA of the control regenerated plants in lane 2 (Figure 3b).

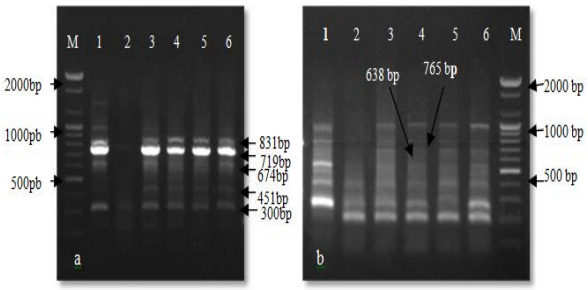


**Figure 3.** Amplification profile of differential display obtained using the primer OPA-11 (a) and OPA-17 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non-stressed plantlet, lanes (3, 4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5, 6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200bp.

The primer OPA-15 amplified three monomorphic bands detected in all of the samples (Figure 4a).

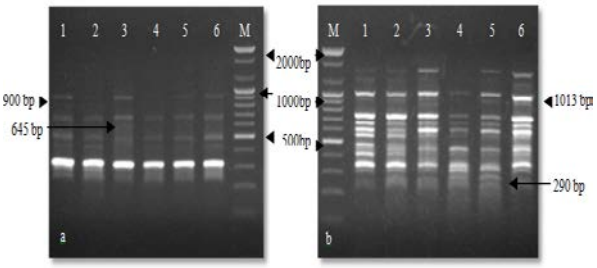


**Figure 4.** Amplification profile of differential display obtained using the primer OPA-15 (a) and OPB-05 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non-stressed plantlet, lanes (3, 4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5, 6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: DNA ladder.

Another band was detected in lane 3 only with a molecular size 645 bp. amplification with the primer OPB-05 (Figure 4b) confirmed the presence of a band at size 290 bp only in plantlets regenerated from stepwise salt and direct drought-screening methods (lanes 4 and 5). Meanwhile, a band with a molecular size of 1013 bp was visualized only in the plantlets produced from stepwise

drought screening method (lane 6). Many bands amplified by the primer OPA-05 with molecular sizes 100-300 bp appeared in all tolerant plantlets, but were missing in the control ones (Figure 5a). The intensity in the band at 870 bp appeared clearly in all tolerant plantlets, while a lower intensity band was exhibited in the control plantlets (lanes 1 and 2). The primer OPA-10 generated a profile differentiating the tolerant plantlets from the control ones (Figure 5b). Amplification products with this primer illustrated the presence of 1000 and 500 bp bands visualized in salt tolerant plantlets (lanes 3 and 4) and in those regenerated from stepwise drought-screening method (lane 6), while 255 bp band was clear in lanes 4 and 6 only. These bands were absent in the control ones. The two primers OPC-04 and OPE-08 showed many amplification products in all of the tested samples; however, no new bands appeared in the regenerated tolerant plantlets (Figure 6a and b).

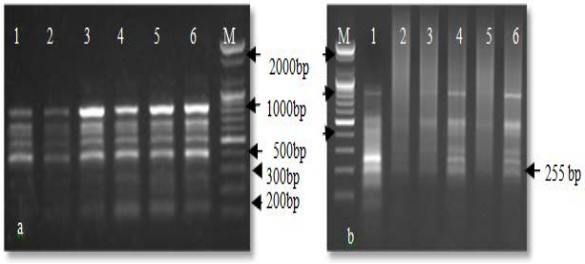


**Figure 5.** Amplification profile of differential display obtained using the primer OPA-05 (a) and OPA-10 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non-stressed plantlet, lanes (3, 4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5, 6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: DNA ladder.

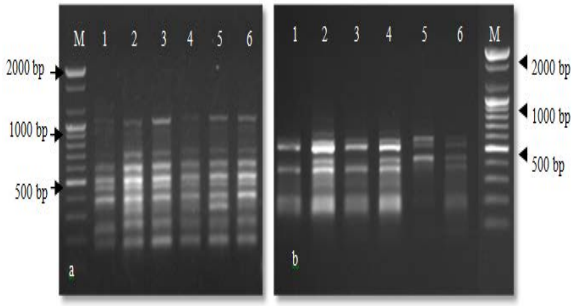


**Figure 6.** Amplification profile of differential display obtained using the primer OPC-04 (a) and OPE-08 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non-stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: DNA ladder.

## 4. Discussion

DDRT-PCR and RAPD techniques confirmed that the tolerant regenerated plantlets differed genotypically from the control ones conferring genetic polymorphism among the salt-selected and drought-tolerant lines. At annealing PCR conditions, even a slight base change at the primer-annealing site was clear in the presence or absence of bands produced by RAPD. It can be, therefore, concluded that under tissue culture conditions and the presence of stress selective agents, somaclonal variation may be generating genetic changes expressed among the selected plantlets as has been hypothesized by Larkin and Scowcroft (1981). This in turn plays an important role in varietal improvement. It is proven previously that some tissue culture variants can be superior to the donor clones in terms of abiotic tolerance (Shomeli *et al.,* 2011; Balkrishna and Shankarrao, 2013; Hadi *et al.,* 2014; Rastogi *et al.*, 2015). *In vitro*, cultures may exhibit somaclonal variation, gene inactivation, or reactivation of silent genes, and gene over expression (Muller *et al.,* 1990; Kaeppler *et al.,* 2000; Leva *et al.,* 2012; Mohammad. and Ibrahim, 2017). Activation of complex signaling pathway(s) may cause changes in the cellular gene expression which is a perquisite for plants to adapt to extreme conditions (Tong *et al.,* 2007; Tamirisa *et al.,* 2014). DDRT-PCR has been widely used to identify the expression patterns of uncharacterized genes in many different plant species. A large number of differentially expressed genes can be identified, particularly those expressed in plants under stress (Alves *et al.,* 1998). In the present study, the total amplification products generated by these primers ranged from 200 bp in the primer OPC-04 (Figure 6a) to approximately 1400 bp in the primer OPB-05 (Figure 4b). Results confirmed that cDNAs are differentially expressed in response to both drought and salinity stresses. Drought and salinity may activate certain sets of common genes ruta plant cells as has been proposed by Said *et al.,* (2015). Genes that are overexpressed under stress conditions are classified into two groups; the first group includes classes of proteins such as enzymes required for biosynthesis of various osmoprotectants, LEA proteins, chaperones, and detoxification enzymes, which protect plant cells. The second group includes signaling molecules, transcription factors, and protein kinases (Rai *et al.,* 2011; Lokhande and Suprasanna, 2012). Mahajan and Tuteja (2005) reported that each stress is controlled by many gens, therefore, the exposure of plant cells to stress agents may result in the alteration of a large number of genes as well as their products. This may explain the differences which occurred in the number and intensity of cDNA bands at different concentrations of salinity and drought compared with those bands visualized in the control plantlets. The current work confirms the findings of Tamirisa *et al.,* (2014) who proposed an evidence that the enhanced abiotic stress tolerance in Arabidopsis transgenic plants is due to a significant increase in the expression gene levels which agrees with the results of Kamal *et al.,* (2010) who reported on the function of proteins expressed by genes in stress- tolerant plants.

## 5. Conclusion

The findings of the present study indicate that the DDRT-PCR technique is suitable for detecting the expression of salt and drought genes in the tolerant regenerated plantlets of *R. graveolens*. This may apply to other members of Rutacea family.

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