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Analysis of Differential Gene Expression under Salinity through Differential Display Reverse Transcription Polymerase Chain Reaction (DDRTPCR) Technique: A Review

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Review Article

Abstract

The current ongoing studies and elucidations of control mechanisms of stress tolerance at molecular level that might facilitate the usage of molecular tools for developing further tolerant floras, is mainly centered on the expression of specific genes related to stress. Differential Display Reverse Transcriptase polymerase chain reaction (DDRT-PCR), a competent, profound and reproducible technology, is more advantageous in numerous ways than other approaches of gene expression analysis. Until 1992, the only and single technique applied for isolation of differentially expressed genes was subtractive hybridization or differential hybridization. In 1992, a different and innovative PCR-based method called Differential Display (DDRT-PCR) was developed by Liang and Pardee. The technological simplicity and wider applicability made this technique very novel. It has successfully utilized in a number of organisms starting from yeast to mammals. This technique was introduced and developed to accelerate the identification of differentially expressed genes to overcome the shortcomings of earlier known methods which were sensitive to error, unresponsive and strenuous. The present review emphasized the multidimensional applications of DDRTPCR in studying the differentially expressed gene under biotic and abiotic stress especially the salinity stress.

Keywords: DDRTPCR; Salinity; Differential gene expression.

Abbreviation: DDRTPCR: Differential Display Reverse Transcriptase Polymerase Chain Reaction

1. Introduction

Plants undoubtedly get to face several environmental influences in the course of their advancement [1]. They are noticeably sensitive towards environmental conditions. As a result, they have developed the capacity to go through adaptive physiological and developmental variations as a response to the diverse environmental inducements [2]. Therefore, these various environmental stresses frequently activate almost same cellular responses and cell signaling pathways, namely, stress protein/enzyme production, up-regulation of different anti-oxidants along with accumulation of compatible solutes [3-9]. The composite response of plants to stress conditions comprises several genes and their biochemical-molecular pathways.

Since an organism's or a cell's physiological changes are accompanied by changes in gene expression pattern, gene expression analysis is essential in most of the biological researches. More over the functional characteristics of any compound produced in the cell under stress could possibly be the consequence of the effect on expression of gene. Thus gene expression analysis could be utilized to get comprehensive insights of the biochemical and physiological significance of the modifications in gene expression. This may also lead to the development of novel biomarkers for studying the stress tolerance in plants.

The current ongoing studies and elucidations of control mechanisms of stress tolerance at molecular level that might facilitate the usage of molecular tools for developing further tolerant floras, is mainly centered on the expression of specific genes related to stress. One of the central aims of molecular biology is the analysis of the gene which is expressed differentially in different cells or when subjected to diverse conditions. Interest in changes in the gene expression patterns has enhanced the quest for appropriate ways and means to make out and ascertain the authentic variations between the control and treatment conditions [10].

2. Approaches of Analysis of Gene Expression

Pioneering methods for analysis of gene expression

are classified into two systems-closed and open architecture systems. Whereas closed systems involve analysis of already known and characterized genes only whereas the open system facilitates the analysis of expression of unknown genes. Extent of earlier knowledge determines the merit for closed systems. In a closed system, a detailed transcriptome study depending on thoroughness of knowledge is carried out. On the other hand, theoretical information on transcriptome is not needed in open systems. Thus, open systems are able to recognize new transcripts when details for the varied expressions of said transcripts are absent [11].

2.1 Differential display reverse transcription polymerase chain reaction (DDRTPCR) technique

The only and single technique applied for isolation of differentially expressed genes was subtractive hybridization or differential hybridization until 1992; no other method to designate the aggregate figure of genes answerable to up/ down-regulation in certain situations was known While it is a consistent technique, it is tiresome, time taking and tough to execute, moreover, it involves mRNA in hefty amounts which in many conditions can be limited [10,12].

In 1992, a different and innovative PCR-based method called Differential Display (DDRT-PCR) was developed by Liang and Pardee [13,14]. The technique concentrated on detection of differentially expressed genes amongst about 15000 discrete mRNA sequences in mammalian cells. It was firstly defined for comparison among messages which vary between tumor and normal cells.

Pardee, et al. first showed that combinations of random primers with attached cDNA primers can be used effectively to produce a set of fragments from cDNA resulting from the total mRNA of a cell (Figure 1) [13-15]. Under the proper conditions, the form of fragments resulting from one type of cells is reproducible and a comparison with that of another cell type can be done [13,15,16]. The hypothetical designs as well as experimental outcomes confirmed that the method can create patterns of bands which might denote almost all expressed genes and can also reproducibly identify differences in gene expression among two or more cell types or conditions [16]. Several researchers already have applied this method named "differential display" or DDRT-PCR effectively, using limited number of primers [13,17].

A powerful tool, the differential-display reverse transcription-PCR (DDRT-PCR) technique, improved lately, It has extensively been used to isolate genes in plants which expressed differentially in several stress conditions [18-21].The technique is apt, cost effective for identifying differentially expressed genes [13,16,22]. It also enables to compare plant responses for stress intensities and durations [23-25].

Unlike differential hybridization, DDRTPCR could give a comprehensive idea regarding the display

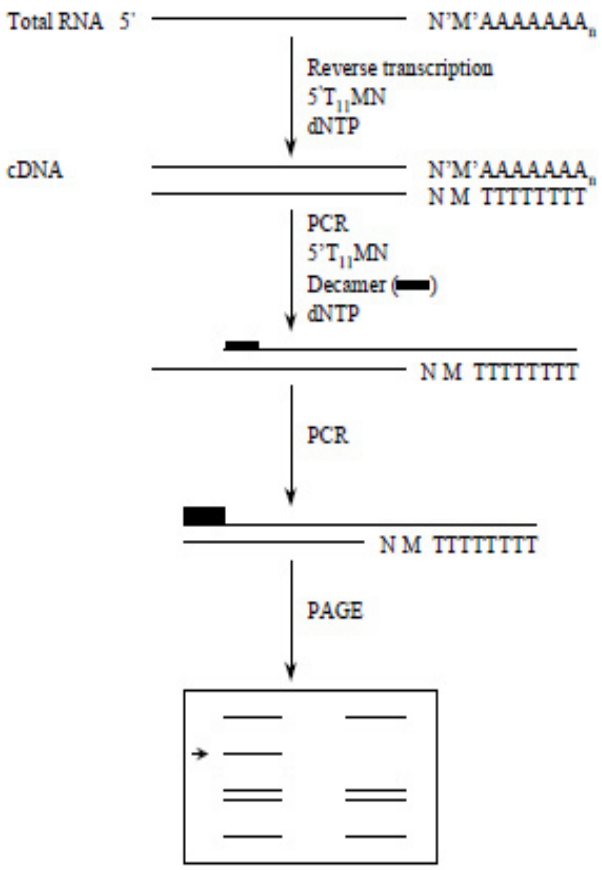

Figure 1. Schematic representation of DDRT-PCR. $T_{11}MN$, degenerate oligo (dT) primer; M indicates A, C or G (degenerate); N can be A, C, G or T. (adapted from Liang & Pardee, 1995b). Arrowhead indicates a differentially expressed fragment.

pattern of differentially expressed genes along with their regulation in different stress conditions in a shortest possible time.

2.2 Multidimensional application of DDRT-PCR

The technological simplicity and wider applicability made this technique very novel. It has successfully been utilized in a number of organisms starting from yeast to mammals [26]. It has to been applied in studying the differentially expressed genes in frogs and also in plants [27-30]. Among plants, this technique has been used in *Aneurolipidium chinense* for PIP kinase, in other plants at different stages like fruit ripening stage; flower senescence; seed development or by treatment with ozone or hydrogen peroxide; induction through touch regulated genes in common bean for the expression of CA7 and NCED genes [31-38]. The differentially expressed genes or cDNAs during heat stress, senescence and development have also been reported. About 90% of cultivable lands are estimated to face different environmental stresses [39-42]. More than 50% of the crop loss is caused by abiotic stress worldwide [43,44]. A wide and extensive research using the DDRT-PCR technique has been carried out in the field of biotic and abiotic plant stress.

2.3 Application of DDRT-PCR in studying salinity responsive genes

Agricultural productivity globally is affected with growing environmental constraints, especially with salinity owing to its high impact and widespread distribution [45,46]. Salt stress, arising due to excessive salt accumulation by plants, limits the productivity of agricultural crops; moreover, it is in evitable result of high concentration of ions [47]. The growth and development as well as the survival of the plant are reduced due to multiple effects of high salinity viz., water and oxidative stress, ion toxicity, nutritional imbalance, reduced or changed cellular and metabolic processes, etc. [4,43,48-50]. However; the intensity of adverse and injurious effects of salinity stress depends upon the nature of plant species, concentration and duration of salt stress, plant developmental stage and mode of salt application to the crop [51,52].The severity of the effect of salinity is depending on the growth stage at which the plant is exposed to salinity. The lack of reliable parameters for screening salt tolerance makes it difficult to develop salt tolerance in target crops. As salinity stress acts as the chief limiting factor of growth of plants, it has always been an area of greater interest to the researchers.

Given its higher efficiency, The DDRT-PCR technique can be employed to identify a large number of differentially expressed genes in less time and acquiring knowledge about those genes that express in the controlled as well as stressed conditions. A study on sunflower employing DD technique revealed that the gene HaABRC5 (with 812 nucleotides, 423 ORF encoding 141 amino acids) having three ABA responsive elements playing a role in plant stress response, got up-regulated by exposure to high salinity [53]. Another DD study on plants of sunflower, *Helianthus annuus* L. for gene expression under control and drought or salt stress was conducted by [53]. They cloned and sequenced five drought-regulated and 12 salinity-regulated cDNAs of which certain genes were reported to respond to both the stressed conditions.

Dang et al. shows that 18 salt stress induced *Reaumuria trigyna* transcripts were identified which showed strong up-regulation due to salts pointing out the important roles of gene in survival of *R. trigyna* under salt stress. The differential expression of the vacuolar ATPase Subunit B in barley has been reported by Wei, et al. [54]. According to another study, 58 Differential Display products in barley were found to over express or disappear in the salt stressed samples indicating a qualitative and quantitative difference in the gene expression. Moreover a gene that encoded Na+/H+ antiporter that could sequester sodium into the vacuole in the salt tolerant barley was identified and it could be used as marker in the selection programme for salt tolerant germplasms. According to another work by Kadri et al., demonstrated that due to treatment of barley cultivars with 200 mM NaCl for two weeks, an altogether 2000 bands (300 to 900 bp) were generated out of which 134 were up regulated and 89 were found to be down regulated. *Bnuc1* that encodes a nuclease I was isolated by differential display method from salt-stressed barley leaves (*Hordeum vulgare* L. cv. Harunanijyo). The transcript of *Bnuc1* gene through Northern Blot Analysis was found to increase vividly in barley leaves under stress. Its level was much higher in older leaves than in younger [55].In the study by Kadri et al. distinctly different expression was observed for salt resistant and susceptible variety in *Hordeum vulgare*. Use of mere 10 primer combinations, DDRT-PCR identified numerous salt induced transcripts, revealing the strength and detection power of the method. Differences in gene expression of wheat under salinity stressed and normal conditions were studied where wheat asparagine synthetase (AS) gene, TaASN1 and TaASN2, were isolated (comparison with other organisms revealed several homologies). Liu et al. in their study in wheat cultivar Baofeng 7228 reported that 27 cDNA fragments were obtained through DD technique under salt stress out of which SR07 (with 561 bp ORF) fragment got noticeably induced by salt stress [56]. They suggested, expression of the said fragment may have a crucial role in regulation of water in plant. An mRNA for dehydrin protein family (Group2 LEA protein) were accumulated at higher level in salt-tolerant line (R1) in comparison to salt-sensitive line (S1) of wheat when exposed to 200 mM NaCl [57].Twenty five genes screened from leaves of cotton through DD technique, were found to be up regulated in the salt stressed condition in comparison to the control sequence. A study of DD technique in tomato root revealed the induction, promotion or repression of several gene expressions by salt treatment and cloned salt-induced mRNAs [58]. Angaji et al. conducted a study on the collective effects of gibberellic acid and salt stress on gene expression [59]. The differential display technique in two Iranian chickpea cultivars showed up regulation of twenty five gene fragments responding to salt stressed condition. Basyuni et al. suggested the function of the terpenoids in roots under salt stress [60]. They further, through their study, reported that terpenoids instead of phytosterols had a major role in coping with the salinity stress in mangrove root. The study provided additional evidences that terpenoids have a protective role also. Nine out of twelve transcripts were up-regulated under saline condition as reported by Banzai et al. reported four differentially up-regulated and one down regulated transcripts in salt stressed root tissue in Roselle [61,62]. A study on salt tolerance responsive genes in Pak-choi (*Brassica campestris* L. ssp. *chinensis* (L.) Makino var. *communis* Tsen et Lee) under salt stress using mRNA differential display showed the screening of transcript derived fragments (TDFs) associated to salt tolerance in tolerant and moderately tolerant Pak choi germplasm. Seven out of 78 cDNA sequences obtained were greatly homologous to certain identified expression genes linked to the

signaling pathways under different abiotic stress [63]. Chen, et al. employed the differential display reverse transcriptase (DDRT)-PCR for screening genes in *Eucalyptus microcorys* that showed down-regulation during adaptation to salt stress [64]. Their major finding was that a cDNA which was greatly related to α-tubulin was readily repressed under salinity. In a study by Zhang, et al. DDRT-PCR technique was employed for identifying differentially expressed genes between wild type rice variety 77-170 (*Oryza sativa* var. *japonica*) and its salt tolerant mutant (M-20) under salinity [65]. A total of 13 cDNA (200-600 bp; designated as SIGR1-SIGR13) fragments that were salt inducible were identified and cloned. The genes were found to be highly homologous with an ABA-inducible gene of rice, Rab16. A comprehensive list of studying differential gene expression under salinity is shown in Table 1.

All the above studies collectively demonstrate

Table 1. List of studies included the differential gene expression under salinity with DDRT-PCR.

S. No.	Crop	Findings	References
1	Rice (*Oryza sativa)*	Expression of *SAMDC1* gene positively correlates with salt tolerance in rice.	Chen et al. [64].
2	Rice (*Oryza sativa)*	A Total of 13 cDNA (200-600 bp; designated as SIGR1-SIGR13) fragments that were salt inducible were identified and cloned. The genes were found to be highly homologous with an ABA-inducible gene of rice, Rab16.	Zhang et al. [65].
3	Rice (*Oryza sativa)*	Reported that random primers may be used as the molecular markers for identifying the response of rice under salt stress, providing an outstanding approach for incessant monitoring of growth and development of the plants in their natural habitat and rice improvement programs in future as well.	Varshikar et al. [68].
4	Rice (*Oryza sativa)*	Under Salinity the up regulation of OsIM1 genes was observed in rice salt-tolerant mutant M-20 by differential display.	Kong et al. [55].
5	Wheat *(Triticum aestivum)*	The full-length cDNA or Root Hair Defective- 3 gene (RHD3) from the salt tolerant wheat variety Shanrong No. 3 (Za3) was cloned and up-regulation of the gene under salinity was reported.	Shan et al. [69].
6	Wheat *(Triticum aestivum)*	Abundance of*TaASN1*mRNA in young spikes and anthers was higher than that in shoots and roots under normal growth conditions. *TaASN1* was induced by salinity in seedlings. *TaASN2*transcripts were very low in all detected tissues and conditions.	Wang et al. [70]
7	Wheat *(Triticum aestivum)*	27 cDNA fragments obtained through DD technique under salt stress out of which SR07 (with 561 bp ORF) fragment got noticeably induced by salt stress suggesting expression of the fragment may have a crucial role in regulation of water in plant	Liu et al. [5]
8	Wheat (*Triticum durum*)	An mRNA for dehydrin protein family (Group2 LEA protein) were accumulated at higher level in salt-tolerant line (R1) in comparison to salt-sensitive line (S1) when exposed to 200 mM NaCl	Masmoudi et al. [65].
9	Barley (*Hordeum vulgare*)	Identified clones encoding stress responsive proteins and wound-induced. Further some encoded genes linked to resistance to anion flow-related proteins.	Kadri et al. [49,50].
10	Barley (*Hordeum vulgare*)	BSVAP got induced under prolonged salt stress in the salt susceptible cultivar Maythorpe but less in the fairly salt tolerant Golden Promise; expressed more highly in Maythorpe under control	Wei et al. [71].
11	Barley (*Hordeum vulgare*)	58 Differential Display products were found to over express or disappear in the salt stress. Gene encoding Na+/H+ antiporter that could sequester sodium into the vacuole in the tolerant variety was identified and it could be used as marker in the selection programme for salt tolerant germplasms	Kadri et al. [49,50].
12	Barley (*Hordeum vulgare*)	Better expression of salt-inducible nuclease activity possibly analogous to the Bnuc1 gene identified	Muramoto *et al*. [72].
13	Barley (*Hordeum vulgare*)	An altogether 2000 bands (300 to 900 bp) were generated out of which 134 were upregulated and 89 were found to be down regulated	Kadri et al. [49,50].
14	Chickpea (*Cicer arietinum* L.)	The differential display technique in two Iranian chickpea cultivars showed upregulation of twenty five gene fragments responding to salt stressed condition	Angaji et al. [36]

15	Tomato (*Lycopersicon esculentum)*	The study revealed the induction, promotion or repression of several gene expression by salt treatment and cloned salt-induced mRNAs	Wei et al. [71].
16	*Brassica campestris* L. ssp. *chinensis* (L.) Makino	Seven out of 78 cDNA sequences obtained were greatly homologous to certain identified expression genes linked to the signaling pathways under different abiotic stress	Qiu et al. [73].
17	*Sunflower (Helianthus annuus)*	cDNA (designated *Ha-RPS28*) encoding a protein component. *Ha-RPS28* expression was down-regulated in both seedling roots and shoots in response to high salinity.	Liu et al. [5].
18	Sunflower (*Helianthus annuus*)	Gene HaABRC5 having three ABA responsive elements played a role in plant stress response, got up-regulated by exposure to high salinity	Liu et al. [62].
19	Sunflower (*Helianthus annuus*)	Cloned and sequenced 12 salinity-regulated cDNAs of which certain genes were reported to respond to both the salinity and drought stressed conditions.	Liu et al. [61].
20	*Gossypium arboreum* L.	Twenty five genes screened were found to be up regulated in the salt stressed condition in comparison to the control sequence	Shahid et al. [74].
21	Jojoba (*Simmondsia chinesis*)	Salinity inhibits the processing of 16s rRNA; confirmed through Northern Blot.	• Ela Mizrahi Aviv et al. [67].
22	Jojoba (*Simmondsia chinesis*)	ScRab expression reduced in shoots grown in the presence of salt compared to shoots from non-stressed cultures	Mizrahi Aviv *et al*. [67].
23	Mangrove (*Bruguiera gymnorrhiza*)	Terpenoids instead of phytosterols had a major role in coping with the salinity stress in mangrove root. The study provided additional evidences that terpenoids have a protective role also.	Basyuni et al. [38].
24	Mangrove (*Bruguiera gymnorrhiza*)	Nine out of twelve transcripts in mangrove were up-regulated under saline condition	Banzai et al. [37].
25	Roselle (*Hibiscus sabdariffa* L.)	Four differentially up-regulated and one down regulated transcripts in salt stressed root tissue in Roselle	Mohamed et al. [74].
26	*Eucalyptus microcorys*	Down-regulation in genes noticed during adaptation to salt stress. A cDNA which was greatly related to α-tubulin readily got repressed under salinity.	Chen et al. [41].
27	*Reaumuria trigyna*	18 salt stress induced transcripts showed strong up-regulation due to salts pointing out the important roles of gene in survival under salt stress	Dang et al. [42].

and establish the versatility and the strength of the DD technique. Abundant researches have been made to understand the varied aspects of developmentally controlled genes which differentially express under stress conditions in plants [66]. The DDRT-PCR technique finds its great potential use in plant molecular biology research. Although a fairly great deal of focus has been made towards gene identification, molecular biology application to medicine or agriculture necessitates knowledge of gene function and this remains a main focus area of molecular biology nowadays.

3. Conclusion

Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR), a competent, profound and reproducible technology, is more advantageous in numerous ways than other approaches of gene expression analysis [6,67-74]. This simple method has extensively been used to ascertain and clone the genes which are differentially expressed at several developmental phases in a plant, prior genomic information of which is known. The enormous number of fruitful applications of this method establishes its strength. This technique was introduced and developed to accelerate the identification of differentially expressed genes and to overcome the shortcomings of earlier known methods which were sensitive to error, unresponsive and strenuous. For achieving this, the technique had to be easy, profound, methodical, and dependable. For a matter of fact, in molecular biology, the revolution mostly has been strengthened by the simple advances viz., DNA sequencing, recombinant DNA technology and PCR. False-positive results, sometimes, may produce an enormous amount of fake sequences which do not characterize the differentially expressed genes. Several methodological reforms were led to diminish the difficulty of false positives and to upsurge the reproducibility. With suitably designed and planned primers, DDRT-PCR could yield outcomes which actually reproduce gene

expression patterns of diverse tissues. The Medline database, in 1996, enumerated several hundreds of publications representing genes known using DDRT-PCR. Considerable improvements in salt tolerance of important crop species like barley, rice, pearl millet, maize, sorghum, alfalfa, and many grass species have been achieved in the past two decades.

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