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Estimation of TPM Values for MAPK Genes, Some Amino Acids and Chlorophyll Content under Salt Stress of some Iraqi Wheat Cultivars

[1]Waleed Kh. AL-Saadi, [2]Majid Sh. Hamdalla, [3]Wisam A. Radhi

[1]Directorate of Seed Testing and Certification, Ministry of Agriculture
[2] Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad
[3]Department of Plant Protection, Faculty of Agriculture, University of Kufa

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Abstract: Most plants encounter stress conditions such as salinity that adversely affect growth, development and crop productivity. The expression of the stress related-genes extends throughout various protective mechanisms in plants allows them to adapt to unfavorable environmental conditions. As a result of salinity, these effects are considered crucial signs of plant damage. In this study, some Iraqi wheat cultivars were examined against two salinity levels, the chlorophyll concentration and amino acids were estimated during booting stage. Also, the expression of Mitogen Activated Protein Kinase (MAPK) genes was measured at the same stage. RNAseq and bioinformatics analyses were used to analyze salinity stress-related genes. The Studied cultivars showed high values of transcripts per million (TPM) with a related five related genes in Dijlah cultivar and three genes in Ibaa 99 cultivar. (MAPK) studied genes were very obvious in their significant expression in the Dijlah cultivar than the Ibaa 99 cultivar, and showed variable numbers of TPMs between both wheat cultivars. Most amino acids increased under salt stress for both cultivars, but the rate was higher in the tolerant cultivar,Dijlah, especially for serine, threonine and cysteine.

Keywords: Amino acids, Chlorophyll, Gene expression, MAPK, Salinity, TPM, Wheat.

Corresponding author: (E-mail: Waleedbarber1988@gmail.com, Majid.s@ige.uobaghdad.edu.iq.)

Introduction

Wheat (*Triticum aestivum* L.) is one of the main cereal crops that meet the global energy requirements of the human diet. The protein found in wheat grains contains vital gluten, which is necessary for the production of bread (1, 2). Abiotic stresses like salinity, extreme temperature are the major impediments restraining plant growth and resulting in significant reductions in crop productivity (3). The response and adaptation of plants to such conditions are very complex and highly variable. Gene expression changes are one of the main chemical reactions to stress that plants exhibit, relating to several pathways involving the sense of stress, signal transduction, regulators, and synthesis of several compounds (3, 4). To fully understand the molecular foundations of plant stress tolerance, it is common practice to identify and characterize genes activated by abiotic challenges. Recent years have seen a fast development in genomic technology, which has increased our understanding of how plants' global gene expression changes under stress (5). Screening and testing

local wheat cultivars under several levels of salinity could generate tolerant genotypes. The objective of this study was using bioinformatics technique to characterize the molecular behavior of some MAPK genes belonging to different functional categories using metatranscriptomic data, analyzed by transcripts per million (TPM) values, without using techniques of polymerase chain reaction (PCR) for two wheat cultivars during salinity stress.

Materials and methods

Field experiment

The present study was carried out at Institute of Genetic Engineering and Biotechnology for postgraduate Studies/ University of Baghdad during season 2021- 2022. Two cultivars of wheat were obtained from the Seed Testing and Certification Office / Ministry of Agriculture - Iraq. The current study was carried out in the plastic pots filled with 7 kg of sandy loam sieve soil. Physical and chemical properties of the soil samples were measured. Seeds from each cultivar were placed in plastic seedling plate suggested for nurseries then transparent to field pots (6). Grains of wheat cultivars were sown manually in their respective pots. Fertilizers used were urea (46% N) at 200 kg ha-1 and super phosphate (46% P2O5) at 100 kg ha-1. The crop was managed according to the recommended conventional agronomical practices. The field equipped with rain fall shelter to avoid rain. The configuration of the experiment was Randomized Block Design (RCBD) with three replications. Two treatments of NaCl, i.e. 3 and 15 dSm^{-1}were added to the water irrigation.

The data were recorded at booting stage for six empirical plants were chosen randomly from each replication. Analysis of variance was used to statistically analyses all data, and the least significant Difference (LSD) test at $P \leq 0.05$ was used to differentiate between treatments average.

Biochemical parameters

Chlorophyll determination

Estimation of total chlorophyll were calculated according to Arnon (7). Nine local cultivars (in south and middle of Iraq) are planted.

Amino acid determination

The method of digestion and extraction was as follows: weight of 0.2g of fresh leaves add to acid HCl 12 ml (6 molar), then put the sample in the oven on at (110˚C) for 24 hours, then wash the sample with distilled water and filter paper, then dry the sample by rotary device, and the washing is repeated by adding distilled water (10 mg). Finally, the sample is injected into the amino acid device after adding the (OPA) reagent: OPA is a very efficient amino acid derivatization agent. Because it rapidly interacts with amino groups to form highly fluorescent products when an excess of thiols are present (8).

RNA extraction

Two leaf samples of cultivars Dijlah, and Ibaa99. Flag leaves were collected for RNA extractions. To extract the RNA, the leaf samples sterilized by washing with 70% ethanol (v/v) for one minute then sodium hypochlorite 3% (v/v) for five minutes(9). After that, it was placed in an Eppendorf tube and immersed in 5x volume RNALater solution, and shipped to DNA-link Company, Republic of Korea. The company instruction extracted the total RNA using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) (Table 1).

Table (1): Concentration and purity of RNA extracted for both cultivars.

Sample	Nanodrop Conc. (ng/µℓ)	260/280	vol. (µℓ)	RIN	rRNA ratio
Dijlah RNA	529.38	1.98	35	5.6	0.4
Ibaa99 RNA	710.13	1.98	35	5.4	0.6

RNA sequencing of cultivars (Ibaa99 and Dijlah)

The RNA sample's quality was assessed using an Agilent 2100 Expert Bioanalyzer, to obtain the Entire RNA sequence, the best sample was sequenced using NovaSeq6 000, 2×101PE (Table2).

Table (2): RNA QC report and kits used in this study.

	Specification	Standard	Reagents and Equipment used for QC
RNA QC	Total RNA amout(µg)	1µg	ND-2000 spectrophotometer, Thermo Scientific 2100 Expert Bioanalyzer, Agilent RNA 6000 Nano Kit, Agilent
	RNA minimum conc.	20ng/µℓ	
	2100 Expert Bioanalyzer	RIN : 7	
		rRNA ratio : 1.5	
		No Degradation	

The compressed files

The compressed files RNA1 and RNA2 (raw reads) were obtained from the company, then the data were approached to the bioinformatics analyses.

Bioinformatics and computational analysis

In the current study Geneious prime software was used, as the one of the popular bioinformatics software created by Kearse *et al*. (10).

Map to reference

The forward and reversed reads of RNA Sequence data were paired and then,used in the map to reference runs of reference gene sequences using

Geneious RNA (Medium, low sensitiviy) using Geneious prime version 11 (10). Group of stress related-genes sequences Mitogen Activated Protein Kinase (MAPK) were examined during salt stress against the whole transcript reads, and the higher assembled sequences were chosen. After that, the consensus sequence was determined out, while the other lower mapped sequences were discarded.

The analysis of the RNA reads count per kilobase of sequence (RPK) divided by a million was used to get the transcripts per million (TPM)

value of the reference sequence. (11).

Normalizing expression measures is necessary for removing biases that can be introduced during sequencing, such as sequencing length and depth of the RNA transcript. Geneious determines three expression level measurements on individual samples, which are modified to allow comparison of genes expression in the same sample can be compared:

RPKM: Reads per kilobase per million normalizes the raw count by transcript length and sequencing depth.

FPKMP: Similar to RPKM, however only one of the mates is counted if the data is paired, i.e. Fragments are counted rather than reads.

TPM: Transcripts per million (as Wagner *et al.*, suggested (12) is a modification of RPKM developed to maintain consistency between samples.

It becomes typical by total transcript count instead of read count in addition to average read length.

TPM = (mean read length * CDS read count * 10^6) / (CDS length * total transcript count).

Pairing of forward and reverse RAW reads

The total paired end reads of RNA were 46,611,090 for Dijlah and 55,926,220 for Ibaa99 with GC% ratio 54.1% and 54.7% for Dijlah and Ibaa99 respectively (Table 3). Using BBDuk to produce clean reads, the trimmed reads of RNA were 46,257,366 and 55,372,464 with GC% ratio 53.9% and 54.5% for Dijlah and Ibaa99 respectively (Figure 1).

Table (3): The difference in the number and qualities of reads before and after trimming in both cultivars

Cultivars	No. of reads before trimming	No. of reads after trimming	QTrimmed	KTrimmed	Total Removed	quality-trimming both ends
Dijlah (RNA)	46,611,090	46,257,366	8465 reads (0.02%)	537009 reads (1.15%)	353724 reads (0.76%)	Q6.0
Ibaa99 (RNA)	55,926,220	55,372,464	553756 reads (0.99%)	821312 reads (1.47%)	553756 reads (0.99%)	Q6.0

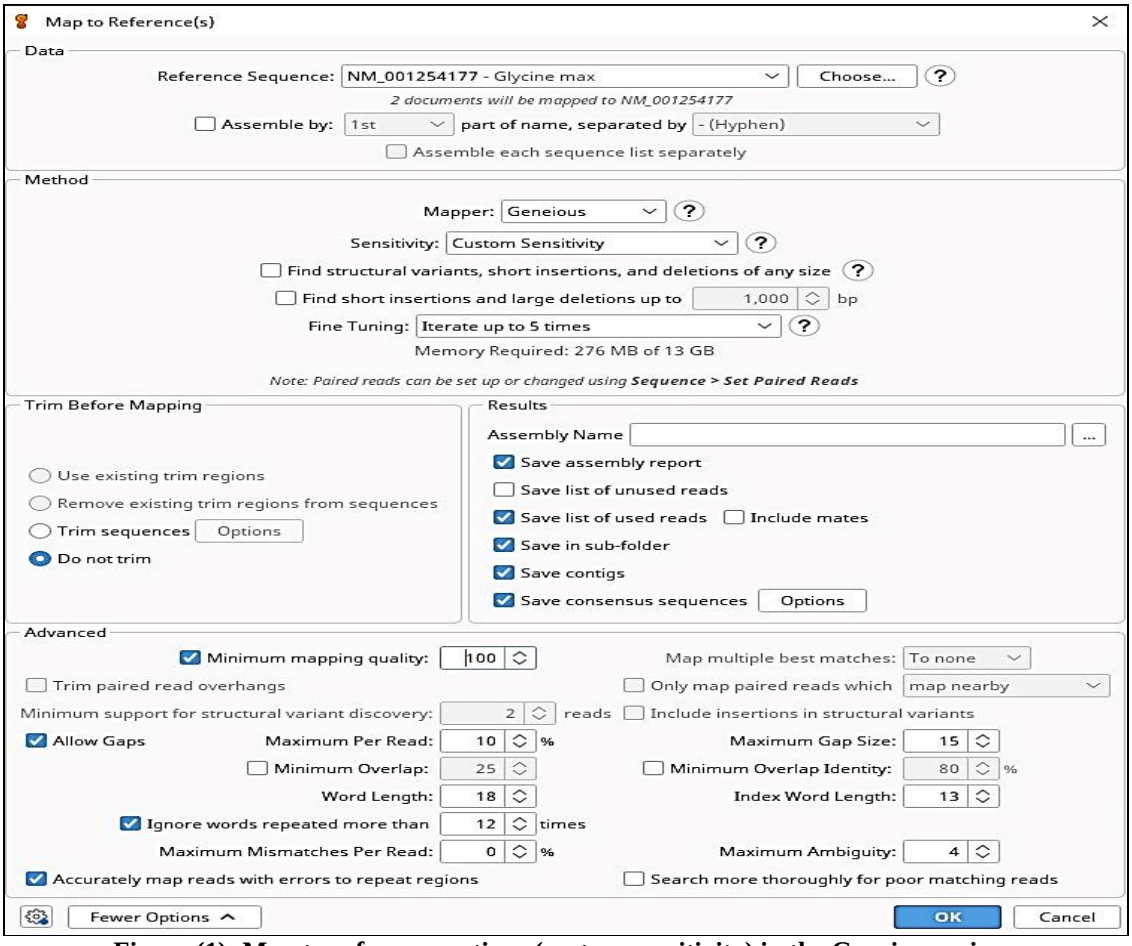

Figure (1): Map to reference options (custom sensitivity) in the Geneious prime.

Results and discussion

The total chlorophyll content drastically dropped as NaCl concentration rose in all genotypes. 3 ds m^{-1} treatment produced greatest total of chlorophyll by (20.88) µg/ml, while 15 dSm^{-1}produced the lowest average by (7.61) µg/ml. The chlorophyll content of the studied cultivars varied significantly and “Furat” and "Dijlah" owned highest rate while Ibaa 99 had the lowest rate. The two cultivars “Furat” and "Dijlah" gave the highest total chlorophyll under both with no significant difference between them, whereas "Ibaa 99" and “Uruk " had the lowest total chlorophyll under 3 ds m^{-1} and "Ibaa 99" and “Baraka " under 15 ds m^{-1} by (6.82) µg/ml and (6.85) µg/ml and respectively. (Table 4). However, the depression percentages, as a result of irrigation with 3 ds m^{-1} and 15 ds m^{-1} were not contrasting among the studied genotypes. According to (13, 14), a significant decrease in total chlorophyll content and a subsequent decrease in photosynthetic efficiency may be caused by the chloroplastids' cell membrane degeneration under salt stress. Chlorophyll has also been mentioned as a potential critical characteristic for determining whether crop plants are sensitive or tolerant of salt.

Table (4): Effect of NaCl on chlorophyll content (µg/ml).

Cultivars	Salinity levels		Mean	Depression%
	(control) 3 ds m^{-1}	15 ds m^{-1}		
Ibaa 99	16.19	6.82	11.50	58
Furat	24.69	8.30	16.49	66
Baghdad	21.53	8.37	14.95	61
Bohooth 22	20.05	7.24	13.65	64
Abo Ghareeb	21.11	6.94	14.02	67
Uruk	18.01	7.66	12.83	57
Adana	18.30	7.31	12.90	60
Baraka	18.14	6.85	12.50	62
Dijlah	24.59	8.18	16.38	67
Mean	20.88	7.61	14.25	46
LSD (cultivars)= 0.9 LSD (Salinity)= 0.4 LSD (cultivars x salinity)=1.2				

Most amino acids have increased under high salt concentration except of Isoleucine, which decreased by 50% in both cultivars and Glycine by 20% in Dijlah cultivar (Figure 2 and 3). The concentration of Isoleucine decreased remarkably in both cultivars, but it decreased more extremely in Ibaa 99 cultivar and to a lesser extent in the Dijlah. The lowest concentration was 0.2 % for Arginine amino acid in Ibaa 99 cultivar under 3EC and highest concentration was for 21.8 % for Cysteine amino acid in Dijlah cultivar under 15EC.

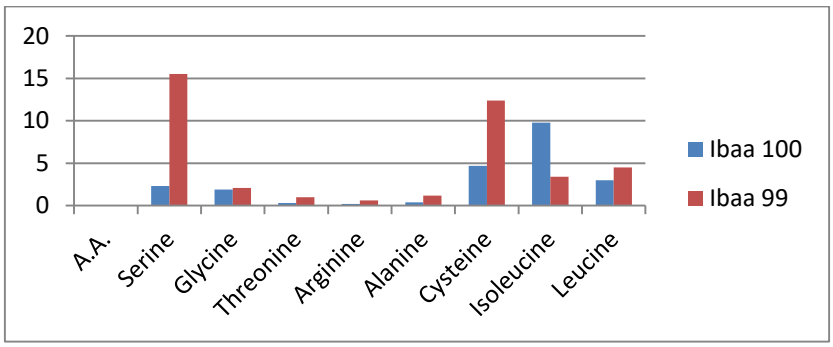

Figure (2): Amino acid concentration of Ibaa99 for two electrical conductivity (3 and 15 EC).

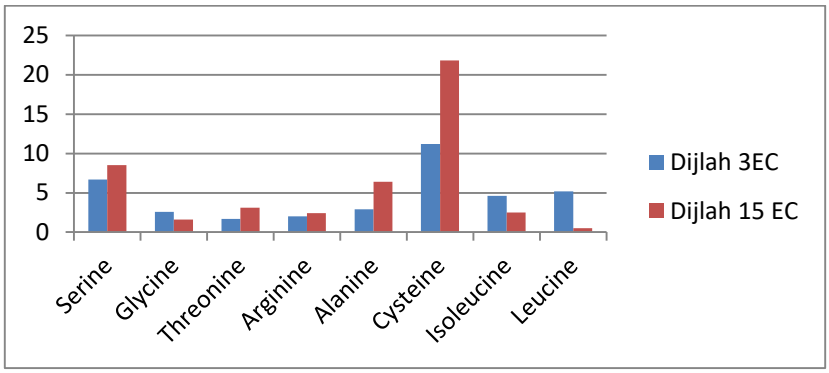

Figure (3): Amino acid concentration of Dijlah for two electrical conductivity (3 and 15 EC).

The TPM of some MAPK-related genes showed high values in Ibaa99 than Dijlah especially in LOC123061583, LOC123136122, and LOC123169996, while tolerant cultivar "Dijlah" showed that five genes obtained higher values of TPM than Ibaa99 cultivar. In general, the MAPK was variable between the two cultivars with more effect in the tolerant cultivar (Table 5).

Table (5): The transcription per million of MAPK genes.

	Gene ID	Sequence length (bp)	TPM 'Dijlah'	TPM 'Ibaa 99'
MAPK	LOC100125895	6343	241090.945	206709.0535
	LOC123061583	13836	23154.95205	60812.19269
	LOC123068569	4602	58015.28349	48242.29854
	LOC543038	5339	275494.1868	213213.3003
	LOC123136122	18141	14931.2345	30876.41776
	LOC123146283	5527	51695.56278	39132.71557
	LOC123169996	2857	40741.11984	88426.20642
	LOC123171044	5456	276964.8606	223084.1406

The primary reason for the assoc iated decrease in chlorophyll content with increasing salt stress conditions is the excess energy absorption in the photosynthetic apparatus; this implies a lower capacity of leaf tissues for light harvesting and the production of reactive oxygen species; this might be avoided by degrading the absorbents pigments (15). According to studies by Mahlooji *et al*. (16), possible causes of the decrease in chlorophyll content in some genotypes under stress include (1) the loss of chloroplast membranes, (2)

distortion of the lamellae vesiculation, (3) excessive swelling, (4) pigment photo-oxidation, (5) damage to chloroplasts by ROS, and (6) their slower synthesis, faster breakdown, or dissociation.

Most amino acids increased under salt stress for both cultivars, but the content was higher in Dijlah cultivar. The most amino acids that showed higher raising under salt stress were serine (570%), threonine (230%) and cysteine (160%) in Dijlah cultivar, and alanine (120%), cysteine (94%) and threonine (80%) in Ibaa 99 cultivar. Interestingly, isoleucine has reduced under salinity condition in both cultivars. Patel *et al*., (17) discovered that under NaCl stress levels of total essential and non-essential amino acids significantly increased. Arg. is a major form of transport for organic nitrogen in plants in addition to its role as an amino acid for protein synthesis, and an essential metabolite for many cellular and developmental processes. Whereas the amino acid Cysteine (Cys.) has shown benefits for relieving abiotic stress in various kinds of plant crops. Under salinity stress, (Cys.) has a vital role in synthesis the carotenoids and Chlorophyll a, b, (18). The chloroplastic cysteine functions as a signaling molecule that both protects and regulates photosystems resulting in minimizes the negative impact of salt stress on plants. One of the two likely pathways might be the cause of these results. The first; is L-cysteine's impact on antioxidant ability to reduce the negative effects of free radicals produced by salt stress (19). The pyruvate that is produced from L-cysteine is the second pathway. (20), Pyruvate is then converted to acetyl CoA, which is considered to be the basic component for a number of, biological, compounds, including proteins, fatty acids , carotenoids, chlorophyll, and phytohormones. through a study of Hussein *et al*., (21), it has been found that exogenous cysteine can successfully overcome the negative impact of NaCl stress on the growth and development of plant. Salinity and drought stress triggers a complex molecular response in plants, involving the expression of various stress-related genes such as NAC, GSK, MAPK and DREB gene (22). *TaMAPK*s were induced by salt stress. This was consistent with previous research reporting that MAPK was strongly induced under high salt stress environment (23). MAPKs are signaling proteins involved in transducing stress signals from the cell membrane to the nucleus, leading to the activation of stress-responsive genes. In response to salinity stress, MAPKs can regulate various cellular processes, including gene expression, ion transport, and cell death. The MAPKs genes that showed high gene expression values (TPM) under salt stress in Dijlah could be an indicator selection for tolerant genotypes.

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