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## SPECIALTY SECTION

This article was submitted to  
Plant Abiotic Stress,  
a section of the journal  
Frontiers in Plant Science

RECEIVED 11 September 2022

ACCEPTED 28 December 2022

PUBLISHED 25 January 2023

## CITATION

Pakzad R, Fatehi F, Kalantar M and  
Maleki M (2023) Proteomics approach to  
investigating osmotic stress effects on  
pistachio.

*Front. Plant Sci.* 13:1041649.

doi: 10.3389/fpls.2022.1041649

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# Proteomics approach to investigating osmotic stress effects on pistachio

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Osmotic stress can occur due to some stresses such as salinity and drought, threatening plant survival. To investigate the mechanism governing the pistachio response to this stress, the biochemical alterations and protein profile of PEG-treated plants was monitored. Also, we selected two differentially abundant proteins to validate *via* Real-Time PCR. Biochemical results displayed that in treated plants, proline and phenolic content was elevated, photosynthetic pigments except carotenoid decreased and MDA concentration were not altered. Our findings identified a number of proteins using 2DE-MS, involved in mitigating osmotic stress in pistachio. A total of 180 protein spots were identified, of which 25 spots were altered in response to osmotic stress. Four spots that had photosynthetic activities were down-regulated, and the remaining spots were up-regulated. The biological functional analysis of protein spots exhibited that most of them are associated with the photosynthesis and metabolism (36%) followed by stress response (24%). Results of Real-Time PCR indicated that two of the representative genes illustrated a positive correlation among transcript level and protein expression and had a similar trend in regulation of gene and protein. Osmotic stress set changes in the proteins associated with photosynthesis and stress tolerance, proteins associated with the cell wall, changes in the expression of proteins involved in DNA and RNA processing occur. Findings of this research will introduce possible proteins and pathways that contribute to osmotic stress and can be considered for improving osmotic tolerance in pistachio.

## KEYWORDS

dehydration, osmotic stress, pistachio, proteomics, stress response

## Introduction

Since plants are sessile, they could not change their location and are continuously subjected to various stresses that threaten their survival. Osmotic stress, which results from abiotic stresses such as salinity, drought, and cold, and is one of the most common stresses in nature, is caused by a decrease in water potential in the environment around the roots (Xiong

and Zhu, 2002; Zang and Komatsu, 2007; Toorchi et al., 2009), which limits the plant's ability to absorb water and restricts water accessibility (Zang and Komatsu, 2007). Osmotic stress appears in various morphological, physiological, and biochemical dimensions in the plant. Tolerance to stress is a complicated phenomenon. To deal with this stress, plants trigger a variety of response mechanisms that require three steps of stress recognition, signal transduction, and the generation of related response components (Zang and Komatsu, 2007; Zhou et al., 2012). These responses enable plants to save water and reprogram cell metabolism for adaptation to stress (Ngara et al., 2018).

Plant survival against stress requires the rearrangement of many molecular processes and reregulation of many genes. Examination of mRNA expression is not sufficient to predict the events that occur in the plant during exposure to stress because there is a low correlation between the abundance of mRNAs and proteins (Wang et al., 2020). Moreover, proteins play important roles in all cellular processes such as gene regulation, transcription, translation, cell detoxification, protection of macromolecules, and osmotic adjustment (Pasaribu et al., 2021). Hence, studying the expression of proteins provides us with more information about plant behavior under stress. Numerous studies show that proteomics is a beneficial tool for analyzing osmotic stress induced changes. Zang and Komatsu (2007) showed that the accumulation of 15 proteins under stress was altered in rice, most of which were involved in lipid accumulation, proteasome regulatory pathway, and glyoxalase system. Applying osmotic stress, in addition to altering the expression of 37 proteins, including acetyl-CoA-O-methyltransferase and 20S proteasome alpha subunit A, led to reduced root and hypocotyl lengths in soybean (Toorchi et al., 2009). It has been reported that some main stress-responsive genes and proteins involved in ROS scavenging, phytohormone and protein metabolism, membrane stability, transport and signaling were active under osmotic stress (Zhang and Shi, 2018; Wang et al., 2020).

Iran, as an origin area of pistachio and its largest producer, is located in the arid regions, where environmental stresses which cause osmotic stress, constantly threaten agriculture (Esmailpour et al., 2015). According to the report of the Food and Agriculture Organization (FAO), countries such as Iran, America, Turkey, China and Syria respectively have the largest production of pistachios in the world (FAO, 2020). Pistachio is one of the most important strategic products of Iran, which has decreased in recent years due to the increase of osmotic stresses such as salinity stresses (Rahimi et al., 2021). According to the FAO report, the amount of production of this valuable product in Iran has decreased from 575 thousand tons in 2016 to 190 thousand tons in 2020 (FAO, 2020). Iran is the principal exporter of pistachio crop in the world, which recently its production due to over salinity water and soil has been reduced. According to this fact, in the present study, our aim is to identify important pathways related to osmotic stress through investigating changes of the proteome profiling of pistachio leaves using 2DE-MS under osmotic stress.

## Materials and methods

The seeds of *Pistacia vera* L. cv. Akbari were obtained from the Iranian Pistachio Research Institute (IPRI), Rafsanjan, Iran. The seeds

were soaked in water for 24h and germinated for a week in 9cm petri dishes with double layers of Whatman filter paper. The germinated seeds were sown in 5L pots containing perlite and irrigated by Hoagland solution for 10 weeks in a controlled greenhouse (25°C, 16h light/8h dark photoperiod with 30% relative humidity) (Esmailpour et al., 2015). Then, plants were divided into two groups, control group and osmotic treatment group, each treatment with three replicates (three plants per pots and two pots per replication). Pre-experiments was conducted to select the osmotic treatment. The osmotic treatment (-1.5 MPa) was applied by adding polyethylene glycol 6000 (PEG6000) to Hoagland solution as described by Khoyerdi et al. (Khoyerdi et al., 2016) and maintained for two weeks. The fully expanded leaves from the tip of each plant were frozen in liquid nitrogen prior to being stored at -70°C for physiological measurements and proteomics study.

## Biochemical assays

For three biological replicates of each treatment, proline was quantified following Carillo and Gibon (2011). Determining the concentration of phenolic compounds was performed according to Ainsworth and Gillespie (2007). Malondialdehyde (MDA) content were measured based on the study of Velikova et al. (Velikova et al., 2000). Photosynthetic pigment's assay (chlorophyll a, chlorophyll b, and carotenoids) was carried out according to the method of Lichtenthaler and Buschmann (2001).

## Protein extraction

According to modified Hurkman and Tanaka (1986) method, after homogenizing 500mg of fresh leaves in liquid nitrogen for three biological replicates of each treatment, 1 mL of cold extraction buffer comprising of 20mM Tris-HCl, (pH 7.5), 1mM EGTA, 1mM PMSF, and 1mM DTT was prepared and added. Then, the sample was incubated at 4°C for 90 min and centrifuged at 20,000×g for 45 min. Four volumes of cold acetone containing 0.08% β-mercaptoethanol and 12% TCA was added to the supernatant as it was incubated at -10°C for 15h. After that, the sample was centrifuged at 20,000×g for 45min. The pellet was washed by cold acetone including 0.08% β-mercaptoethanol seven times at -10°C for 4h and then lyophilized. Finally, the pellet was resolved in lysis buffer comprising of 7M urea, 2M thiourea, 4% CHAPS, 35mM TRIS-HCl, 1% w/v DTT, and 1% v/v Ampholyte, pH 3.5–10) and incubated at 25°C for 1h and then centrifuged at 12,000×g for 15min. The supernatant containing proteins was stored at -80°C. Proteins amounts were assayed by Bradford (1976) method.

## Detection of proteins by 2-dimensional gel electrophoresis

120μg protein was added to 320μg rehydration buffer including 8M urea, 2% CHAPS, 0.018M DTT, 2% IPG buffer (pH 3–10), and 0.002% bromophenol blue. Rehydration buffer was loaded to 17 cm IPG linear gradient strips (Bio-Rad) with pH 4–7 in a rehydration tray

at 25°C for 12–16h. Isoelectric focusing (IEF) was carried out on a Multiphor II electrophoresis system (Amersham Pharmacia Biotech) at 20°C pursuant to the following conditions: 150Vh at 0–300 V, 300Vh at 300–500 V, followed by 2000Vh at 500–3500 V and finally 39,500Vh at 3500V. A maximum of 50µA per strip was used for the electric current. Equilibrium buffer comprising of 50mM Tris-HCl (pH 8.8), 6M urea, 30% glycerol (v/v), 2% SDS, 1% DTT, and bromophenol blue was used for balancing IEF strips for 15 min. Afterwards, strips were put onto 12.5% SDS-PAGE gels on Protein II Xi Cell (Bio- Rad) apparatus. Gels were stained using silver nitrate according to Blum et al. (Blum et al., 1987) protocol. After staining, the gels were scanned using Bio-Rad's GS800 densitometer and then converted into TIF format using PDQuest software. Melanie software (version 7) was used for quantitative and qualitative evaluation of protein spots in different treatments (Fatehi et al., 2012; Fatehi et al., 2013). Only spots with reproducible alternations (at least 1.5-fold change) in three biological replicates were used in further analyzes.

## In gel digestion and of protein Identification by MALDI/TOF/TOF MS

In-gel digestion and mass spectrometry were carried out according to Pakzad et al. (Pakzad et al., 2019). Briefly, spot were manually excised from the gels and destined for 1 h at 28 C by fresh wash solution (50% acetonitrile 50 mM ammonium bicarbonate (50:50 v/v)). Then washing solution were eliminated and spots dried for 30 min at 37°C. Protein reclamation and alkylation were carried out by 10 mM dithiotreitol (DTT) and 55 mM iodoacetamide (IAA), respectively, and then tryptic digestion were done in 50mM ammonium bicarbonate (pH 8) using MassPREP automated digester station (PerkinElmer). Peptides were extracted using a solution containing 2% acetonitrile and 1% formic acid and lyophilized. Using a solution including of 0.1% TFA (trifluoroacetic acid) and 10% acetonitrile, lyophilized peptides were solved. The peptide mixed in 5 mg/mL of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) (MALDI matrix), 50% acetonitrile, 6mM ammonium phosphate monobasic and 0.1% trifluoroacetic acid. The Mass Spectrometry information were obtained using an AB Sciex 5800 TOF/TOF System, MALDI/TOF/TOF (Framingham, MA, USA) with a 349 nm Nd : YLF OptiBeam On-Axis laser.

## Protein characterization and classification

Mass spectrometry data was analyzed by MASCOT software (Version 2.2, Matrix Science, London, UK) against Swiss-Prot. Protein properties were gained from UniProt database (<https://www.uniprot.org/>). More information about the function of proteins was obtained from the scientific literature.

## Bioinformatics analysis

The Gene Ontology (GO) analysis of identified proteins were investigated via Uniprot (<http://www.uniprot.org>) and string database (<https://string-db.org>). Protein-protein interaction was evaluated by the search tool for interactions of chemicals (STITCH) (<http://stitch.embl.de>).

## Validate identified proteins using quantitative real-time PCR

Based on the proteomics results, we validated three identified proteins via q-PCR. Total RNA was extracted from control and treated pistachio leaf by RNX – plus kit (Sinaclon, Iran). Synthesis of cDNA and qRT-PCR were done as illustrated by Sadeghi, Mirzaei (Sadeghi et al., 2022). Primers were designed using Primer 3 software (Table 1). Three biological and technical replicates were considered for each sample. The *EF1 $\alpha$*  gene was considered as an endogenous control (Moazzam Jazi et al., 2016) and normalization of the CT value of each gene was done by REST software (Relative Expression Software Tool). Change of transcription levels were quantified through the Pfaffl method (Pfaffl et al., 2002).

## Statistical analysis

To evaluate the significant differences between mean values of control and osmotic treatment t-test were performed using SAS software v9.1 (SAS Institute Inc., Cary, NC, USA). The measurements are presented as mean  $\pm$  standard deviation (SD) of 18 samples.

TABLE 1 The sequence of primers designed in this study.

Gene	Forward and Reverse primers sequence (5' - 3')	GC%	Annealing temperature (°C)	Amplicon size (bp)
Actin	F: GTCAGCCACACTGTCCCAT	60	62.13	91
	R: GGGCGTCAGTAAGGTCACGA	60	61.87	
Catalase isozyme 1	F: CAGGCGGACAAATCACTGGG	60	61.31	135
	R: ACAGCAGTCATCCTTCCCGT	55	61.19	
Abscisic acid receptor PYL9	F: CCAAACCCAACCCAAGGTGA	52.38	60.97	131
	R: CTCTGGGCTCGTGTCTGTGA	60	61.53	
Aspartokinase 2	F: AGTGAGTTGTGAGGGAGCGA	55	60.83	132
	R: CTCTCAGCAGAGGACACGGA	60	60.96	

## Results

### Biochemical parameters

MDA concentration was assayed as a lipid peroxidation product. Osmotic stress did not change its concentration, while it increased the level of phenolic compounds. Proline content was dramatically increased when osmotic stress was applied. Stress affected photosynthetic pigments except carotenoid, so that chlorophylls were degraded under stress (Figure 1).

### Identification of differentially regulated proteins

In present study, for finding the impact of osmotic stress, pistachio seedlings were treated with PEG6000 to apply osmotic stress for two weeks. Then, proteins of three biological replicates for treatment and control were extracted from leaves and separated by 2-DE (Figure 2). Silver nitrate and Melanie software were used for gel staining and analyzing, respectively. Only differentially accumulated protein spots that represented reproducible alterations were used for further analysis by MS.

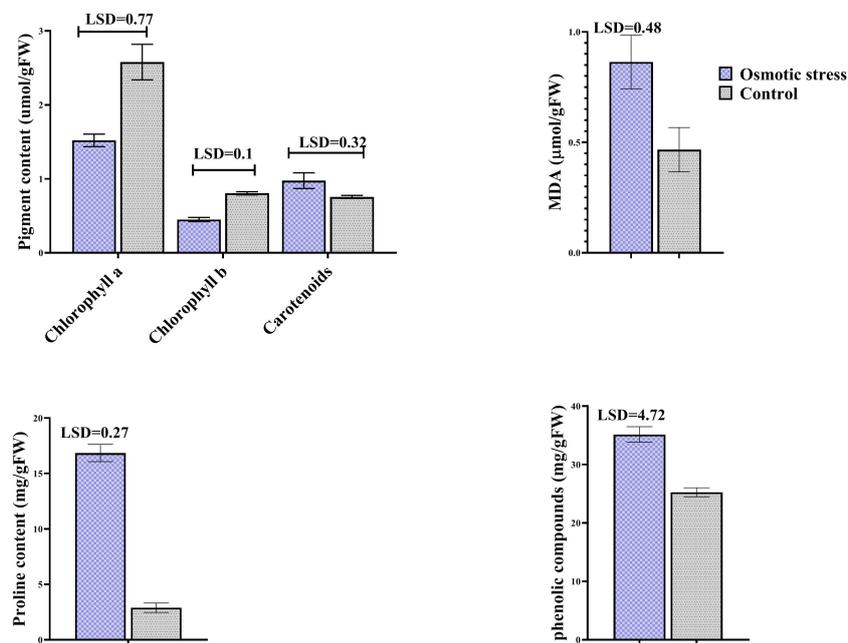


FIGURE 1 The effect of osmotic stress on photosynthetic pigments, MDA, proline and phenolic compounds in leaves of pistachio under osmotic stress compared with control. Bars indicated the SD (n = 3). Statistic were carried out at p = 0.05 according to the t test.

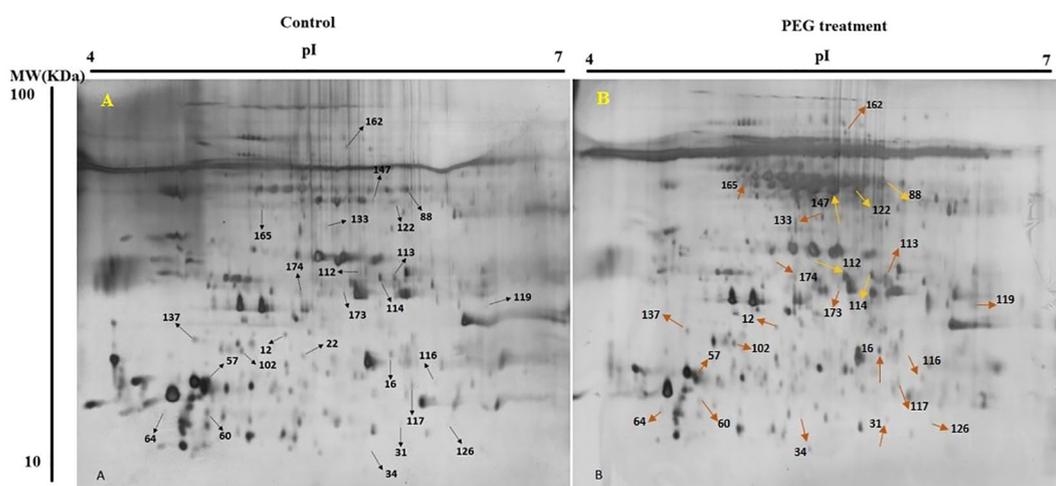


FIGURE 2 2-DE gel images of protein profiles from pistachio leaves. Control (A) and PEG treatment (B). 17cm IPG strip (pH 7-14) was used for loading proteins and SDS-PAGE was done with a 12% gel. Gel was stained using CBB G-250. Proteins with differential regulation levels are marked by arrows.

Out of 280 detected spots, 25 protein spots were significantly altered in response to osmotic stress, accounting for about 8.9% of the detected spots. Among them, only four proteins (spots 57, 64, 102, and 137) were down-regulated and the others were up-regulated (Table 2).

MALDI-TOF/TOF MS was applied to distinguish possible identities of differentially expressed spots. Mascot search engine

searched the Swiss-Prot database, while a higher score as well as higher sequence coverage was our criteria for selection.

The calculated pIs of nearly half of the identified proteins were in the acidic pH range and those of the other half were in the neutral and alkaline pH range. 64% of them were distributed in the range of 10,000 – 100,000 Da. whereas, monoisotopic mass of protein 31 was below 10,000 Da and those of proteins 60, 88, and 162 were above 100,000 Da.

TABLE 2 Protein properties of differentially expressed proteins of pistachio leaves under osmotic stress.

Annotations	Spot No.	Accession No.	Score	Coverage%	MW (Da)	pI	Accumulation Status
<b>Photosynthesis and metabolism</b>							
Ribulose biphosphate carboxylase large chain	57	P28458	58	12	52115	5.91	–*
Ribulose biphosphate carboxylase small subunit	64	P07180	58	28	20446	6.73	–
Oxygen-evolving enhancer protein 1	102	P85194	57	29	34487	5.4	–
Photosystem I assembly protein Ycf4	137	Q8WI09	60	24	21398	9.83	–
Aspartokinase 2, chloroplastic	133	O23653	62	22	60137	6.31	+*
Glyceraldehyde-3-phosphate dehydrogenase A	113	P19866	52	28	43338	7.62	+
Putative cytochrome c oxidase subunit II PS17	31	P84733	41	100	1707	9.62	+
Thiosulfate/3-mercaptopyruvate sulfurtransferase 1	112	O64530	48	16	42152	5.95	+
Cytosolic sulfotransferase 4	12	Q8RUC1	54	35	32368	8.68	+
<b>Stress response</b>							
Abscisic acid receptor PYL9	116	Q84MC7	41	31	21173	5.89	+
Phospholipase D alpha 3	122	P58766	41	7	93931	6.36	+
18.1 kDa class I heat shock	117	P19037	40	25	18123	6.77	+
Catalase-2	119	P25819	63	24	57237	6.63	+
Catalase isozyme 2	173	Q9XHH3	43	19	57141	7.71	+
Probable serine/threonine-protein kinase CST	34	P27450	51	19	46482	9.58	+
<b>DNA and RNA processing</b>							
DEAD-box ATP-dependent RNA helicase 7	16	Q39189	62	24	73187	9.29	+
DNA repair protein RAD50	162	Q9SL02	68	22	153632	5.98	+
Replication protein A 70 kDa DNA-binding subunit D	174	Q9FME0	58	33	70676	6.1	+
Protein argonaute MEL1	60	Q851R2	66	8	117987	9.34	+
<b>Cell wall biosynthesis</b>							
Prolyl 4-hydroxylase 5	147	Q24JN5	42	11	32842	7.75	+
Probable galacturonosyltransferase 3	165	Q0WQD2	58	15	78178	7.27	+
<b>Transporting and movement</b>							
Putative aluminum-activated malate transporter 11	22	Q3E9Z9	38	24	17149	9.55	+
Kinesin-like protein KIN-7H	88	F4JZ68	57	27	122375	5.53	+
<b>Signal transduction</b>							
Guanine nucleotide-binding protein alpha-1 subunit	126	P18064	59	32	44860	5.96	+
<b>Other</b>							
F-box/kelch-repeat protein At3g17530	114	Q9LUP5	48	26	44807	7.43	+

\*+: Up-regulated expression, -: Down-regulated expression, ND, no data.

## Functional classification of differentially regulated proteins

As shown in Figure 3, the study of biological functions of differentially accumulated proteins led to their classification into seven diverse groups. Most of them contributed to photosynthesis and metabolism accounting for 36% then followed by stress response (24%), DNA and RNA processing (16%), and cell wall biosynthesis (8%), transporting (8%). Furthermore, the remaining proteins were associated with signal transduction (4%) and other (4%).

## Protein-chemical interaction

In this study, we evaluated the network of protein-protein/chemicals interactions involving in osmotic stress in pistachio leave using STITCH database against *Arabidopsis thaliana*. All of 25 identified proteins were detected with the STITCH database. The PCI network indicated a strong interaction network between identified proteins and several chemical compounds in different pathways (Figure 4). Identified chemical compounds related to plant response under osmotic stress were included proline, guanosine triphosphate, arginine, nicotinamide, H<sub>2</sub>O<sub>2</sub>, pectin, glucan, glutamate, phosphoglycerate kinase 1, phosphate, glucose, chitin, topoisomerase II, estradiol, malondialdehyde, cytochrome p450 72c1, cytochrome oxidase 2, cytochrome c oxidase subunit 3,1,4-beta-D-xylan synthase, allene oxide cyclase 2, putative nucleolar GTP-binding protein 1, ATP-dependent RNA helicase DHX8/PRP22, silencing defective, large subunit ribosomal protein L24e, cell wall-associated kinase, alpha-ketoglutarate-dependent dioxygenase alkB, ethylparaben, replication factor A1, G protein alpha subunit 1, magnesium chloride, hypersensitive to ABA1, pescadillo-related protein, putative xyloglucan glycosyltransferase 8, phosphoglycerate kinase 1. Also STITCH database were predicated that various pathways controlled by hormones and their crosstalk, consisting of brassinolide, gibberellin, ethylene, salicylic acid, ABA, auxin, and jasmonate.

## Gene ontology analysis

According to the analyses of GO enrichment, investigated proteins were in various ranges of biological processes (Figure 5), included of metabolic process (16.78%), response to stimulus (16%), cellular component organization or biogenesis (9.48%), oxidation-reduction process (8%), DNA metabolic process (5.1%), meiotic cell cycle (4.38%), DNA repair (4.38%), reproduction (4.38%), carbohydrate biosynthetic process (4.38%), DNA recombination (3.65%), photosynthesis (3.65%), reductive pentose-phosphate cycle (2.2%), carbon fixation (2.2%), double-strand break repair (2.2%), meiotic nuclear division (2.2%), telomere maintenance (2.19%), reciprocal meiotic recombination (2.19%), meiosis I (2.19%) and mitotic recombination (1.46%).

Findings of the GO enrichment analyses displayed that identified proteins, under osmotic stress, mainly located in intracellular part (22%), intracellular membrane-bounded organelle (19.64%), cytoplasm (17.26%), macromolecular complex (6.54%), organelle envelope (5.35%), intracellular organelle lumen (4.16%), nuclear part (4.16%), thylakoid (3.57%), plastid envelope (3.75%), nuclear lumen (3.75%), chloroplast stroma (3.57%), stromule (2.97%), Mre11 complex (1.19%), heterotrimeric G-protein complex (1.19%) and cytoplasmic side of plasma membrane (1.19%) (Figure 5).

The enrichment of molecular functions illustrated the most processes related to catalytic activity (42.25%), ion binding (35.21%), oxidoreductase activity (15.49%), cytochrome-c oxidase activity (4.22%) and ribulose-bisphosphate carboxylase activity (2.81%) (Figure 5).

Evaluation of KEGG pathways demonstrated that differentially accumulated proteins enriched in metabolic pathways (35.29%), microbial metabolism in diverse environments (14.70%), Carbon fixation in photosynthetic organisms (11.76%), homologous recombination (11.76%), carbon metabolism (11.76%), glyoxylate and dicarboxylate metabolism (8.82%) and non-homologous end-joining (5.88%) (Figure 5).

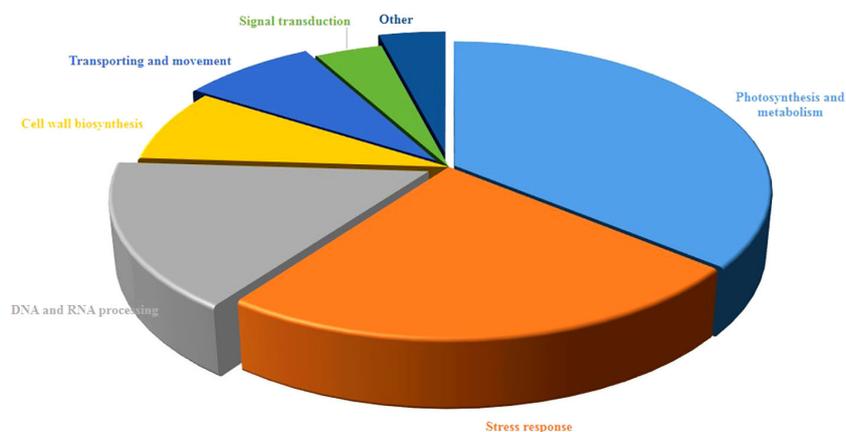
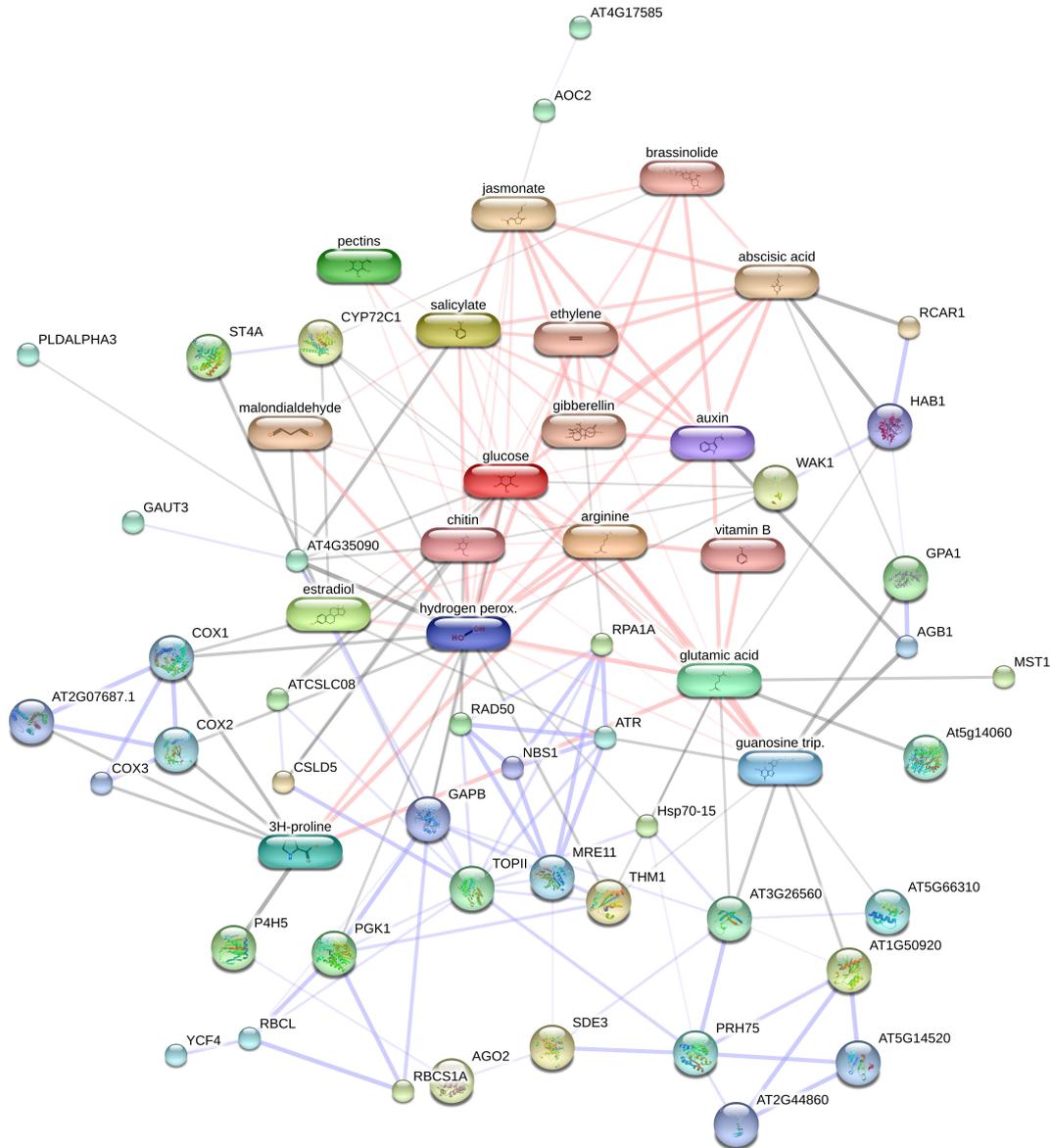


FIGURE 3

Functional classification of differentially regulated proteins of pistachio leaves under osmotic stress condition. The percentage of annotated proteins related to each pathway illustrated in pie charts.



**FIGURE 4**  
 Analysis of the network of protein – chemical of identified proteins using STITCH 5.0. AT2G44860, large subunit ribosomal protein L24e; AT3G17530, F-box and associated interaction domain-containing protein; WAK1, Serine/threonine-protein kinase; At5g14060, aspartokinase 2; SDE3, SILENCINGDEFECTIVE; AT1G11780, alpha-ketoglutarate-dependent dioxygenase alkB; ST4A, sulfotransferase 4A; AGO2, argonaute 2; P4H5, prolyl 4-hydroxylase 5; AOC2, allene oxide cyclase 2; THM1, thioredoxin M1; RPA1A, replication factor A1; YCF4, unfolded protein binding; GPA1, G protein alpha subunit 1; NBS1, nijmegen breakage syndrome 1; PLDAPHA3, phospholipase D; RBCS1A, ribulose biphosphate carboxylase small chain 1A, AT4G35090, catalase 2; TOPII, topoisomerase II; AT4G17585, aluminum activated malate transporter family protein; HAB1, HYPERSENSITIVE TO ABA1; CSLD5, 1,4-beta-D-xylan synthase; AT5G14520, pescadillo-related protein; MST1, thiosulfate sulfurtransferase; COX1, cytochrome oxidase; AT3G26560, ATP-dependent RNA helicase DHX8/PRP22; RCAR1, abscisic acid receptor PYL9; RAD50, DNA repair protein RAD50; RBCL, ribulose-biphosphate carboxylases; ATCSLC08, putative xyloglucan glycosyltransferase 8; GAUT3, galacturonosyltransferase 3; PGK1, phosphoglycerate kinase 1; PRH75, DEAD-box ATP-dependent RNA helicase 7; AT5G66310, ATP binding microtubule motor family protein; ATR, serine/threonine-protein kinase ATR; AGB1, GTP binding protein beta 1; CYP72C1, cytochrome p450 72c1; AT2G07687.1, cytochrome c oxidase subunit 3; COX3, cytochrome c oxidase subunit 3; MRE11, MEIOTIC RECOMBINATION 11; COX2, cytochrome oxidase 2; Hsp70-15, Heat shock protein 70.

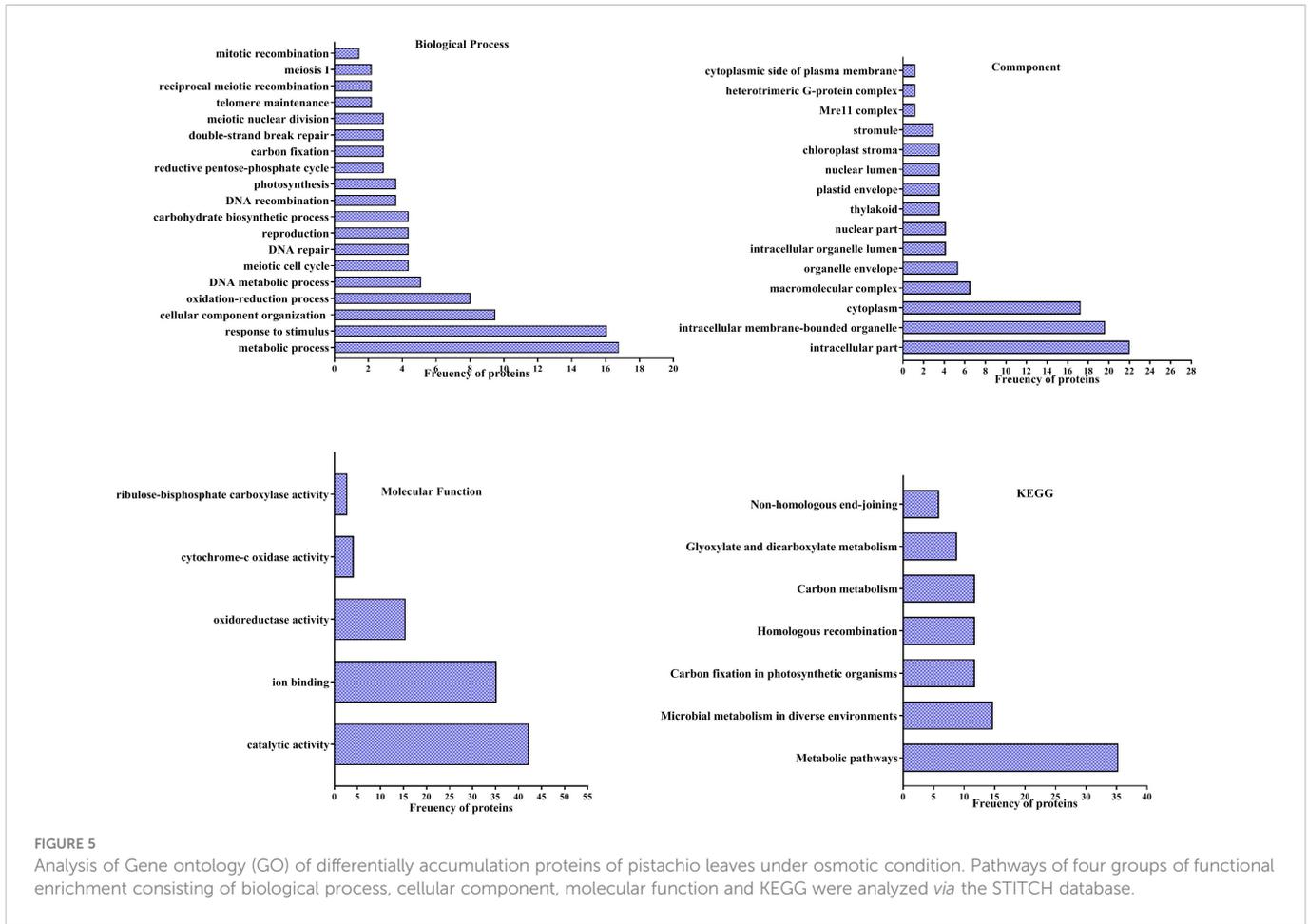
### Analysis of transcriptional expression change by qRT-PCR

Changes in the transcription level of three selected genes of differentially abundant proteins were investigated by qRT-PCR (Figure 6). Results of qRT-PCR analysis indicated that transcription level of genes related to spots 116 and 133 increased in response to

osmotic stress. The change of the expression level of representative genes was the same as their protein expression.

### Discussion

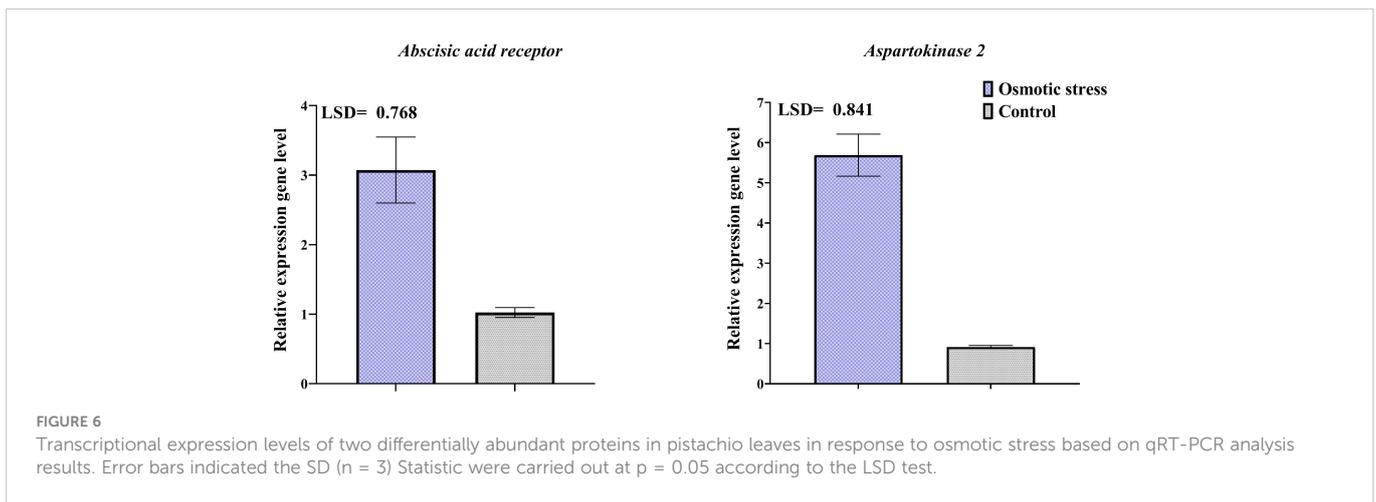
Plants have established numerous defense mechanisms against osmotic stress such as osmotic adjustment by ion transport



reregulation and osmoprotectants synthesis, preservation of membrane stability, activation of antioxidant defense, reregulation of cell cycle, and metabolic changes (Xiong and Zhu, 2002; Zhang and Shi, 2018). Polyethylene glycol (PEG), which has no toxic effect on the plant, induces osmotic stress by withdrawing water from the protoplasm and apoplast (Toorchi et al., 2009). In this study, the altered contents of some biochemical compounds and several differences in protein expression patterns due to dehydration resulted from PEG were detected in pistachio.

### Biochemical Parameters

Tolerant plants to osmotic stress have potency to sustain homeostasis of metabolic using increase of different solutes (Blum, 2017). In this study, accumulation of several organic solutes like proline and phenolics were increased, indicating a positive role of these compounds in the pistachio plant under osmotic stress. Proline acts not only as a compatible osmolyte but also as a ROS scavenger, a buffer for cellular redox potential, and a nutritional source under



stress (Hayat et al., 2012; Chun et al., 2018). An increase of about six-fold in proline content was observed here. An increase in proline accumulation as an osmolyte in response to dehydration has been observed in a wide range of plants (Skirycz et al., 2010; Benhassaini et al., 2012; Kim et al., 2016; Jungklang et al., 2017; Zegaoui et al., 2017; Koenigshofer and Loeppert, 2019; Mattioli et al., 2020). Pál et al. (Pál et al., 2018) had also confirmed the enhancement in the amount of proline under osmotic stress. Owing to ability to forgive hydrogen, decrease and extinguish radical oxygen, phenolics have oxidation virtues and have a main role as sweepers of ROS in plant under various stresses (Naikoo et al., 2019; Mechri et al., 2020). A potent relation exists among osmotic tolerance and increased accumulation of phenolic compounds (Dey and Bhattacharjee, 2020; Naikoo et al., 2019). Piwowarczyk et al. (Piwowarczyk et al., 2017) reported that concentration of phenols elevated in grass pea plant under PEG-induced osmotic stress, similar to our results. Several studies illustrated that concentration of proline and phenols increased under salinity and drought stress in pistachio plants (Khoyerdí et al., 2016; Akbari et al., 2018; Jamshidi Goharrizi et al., 2020). Therefore, considering the presented results and literature data, the increased accumulation of phenolics and proline may be propounded as a main elements related to the tolerance of pistachio to osmotic stress.

Lipid peroxidation of cell leading to produce malondialdehyde indicating severity of injury to the cell membrane (Morales and Munné-Bosch, 2019). Our data showed that malondialdehyde content did not changed that maybe due to the physiological adaptation and or elevated activity of antioxidant systems that diminished ROS levels and membrane injury. On the other hand, our result contradicts results of Khoyerdí et al. (Khoyerdí et al., 2016) and Goharrizi et al. (Goharrizi et al., 2020). The principal reason for this conflict is probably differences in type of reaction pistachio varieties to osmotic stress as well as differences in the way of implementing stress treatment.

The photosynthesis process and its severity rely on content of pigments such as Chl a, Chl b, and carotenoids and effect the biological productivity. Also, photosynthetic pigments harvest the light for photosynthesis process (Rahneshan et al., 2018; Lan et al., 2020). Osmotic condition can injury the chlorophyll content and prevent synthesis of chlorophyll pigments, therefore chlorophyll degradation is one of the subsequences of osmotic stress (Akbari et al., 2018; García-Morales et al., 2018). In this study, decline in chlorophyll content were observed under osmotic stress. In general, this reduction can be imputed to various factors, such as the sluggish synthesis or rapid degradation of the pigments in cells, decrease in synthesis of chlorophylls, derangement in the complex of pigment-protein and thought deficits in ions that are necessary for chlorophyll biosynthesis (Akbari et al., 2018; Behzadi Rad et al., 2021; Zhu et al., 2021). Lan et al. (Lan et al., 2020) reported that chlorophyll content remarkably reduced in wheat under PEG-induced osmotic stress, similar to our results. Also, our findings are in compliance with (Behzadi Rad et al., 2021) who reported that the chlorophyll content of pistachio leaves reduce under salinity condition. Carotenoids function as photoprotection by absorbing extreme light and protect chloroplasts from harmful ROS level therefore protect chlorophyll from major damage (Rahneshan et al., 2018). In this study, no change in carotenoids concentration was observed (Figure 1).

## Photosynthesis and metabolism related proteins

The most important enzyme in photosynthesis is ribulose biphosphate carboxylase (Rubisco), which consists of two types of large and small subunits, plays main role in the fixation of CO<sub>2</sub>.

The alteration in photosynthesis of plants highly related to the Rubisco activity. It has been reported that drought stress had a harmful impact on the function of Rubisco in various plants, led to decrease of biosynthesis and degradation of subunits and finally diminution of photosynthesis (Zhang et al., 2016; Shayan et al., 2020). In this study, subunits of Rubisco down-regulated which was agreement with our pervious study (Pakzad et al., 2019). Also, Jamshidi Goharrizi et al. (Jamshidi Goharrizi et al., 2020) using analyzing the leaf proteome profiling of pistachio demonstrated that ribulose biphosphate carboxylase/oxygenase large chains were down-regulated under salinity stress condition.

OEE1 is one of member of the PSII related to photoreactions, and plays a role in stabilizing the cluster of Mn in the PSII that is initial locate of water splitting. A loss of this protein leads to a full inability for evolve oxygen in PSII (Chaves et al., 2009; Dubey, 2018; Heide et al., 2004; Mayfield, 1999). The decreased levels of this protein were occurred under osmotic stress. In contrast to our results Buendig et al. (Buendig et al., 2016), reported a decrease in the abundance of OEE1 in potato genotypes under osmotic stress. A diminish in the accumulation of OEE1 maybe therefore due to harm to PSII and represents a considerable decrease of efficiency photosynthesis under osmotic stress (Chaves et al., 2009; Dubey, 2018). PSI assembly protein Ycf4 (YCF4) plays a significant role in the assembly of PSI and its firm hold to the thylakoid membrane (Nellaepalli et al., 2018; Hui-Hui et al., 2019). In this study, we found that the accumulation of YCF4 were reduced under osmotic stress, illustrating that osmotic stress reduce quantity and integrity of PSI protein. Cytochrome c oxidase subunit II PS17 is the final enzyme related to respiratory chain, oxidizing cytochrome c and make molecular water using transfer electrons to molecular oxygen. It has been reported that alterations in expression of the cytochrome c oxidase level were attended with alterations in the accumulation of proteins related to photosynthesis and carbohydrate metabolism under stress condition (Çulha Erdal et al., 2021). Increased accumulation of this protein has been reported by Çevik et al. (Çevik et al., 2019) and Çulha Erdal et al. (Çulha Erdal et al., 2021) under drought stress. Abundance of this protein was elevated by osmotic stress in pistachio, indicating that maybe help to generation of energy *via* respiratory chain, leading to improve photosynthesis and carbohydrate metabolism levels. Totally, the decrease in the expression of OEE1, Ycf4, and ribulose biphosphate carboxylase, implicitly indicated destructive effect of osmotic stress on the photosynthesis process.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a vital role in the physiological plant function and energy production *via* glycolytic pathway and protect of photosystem II from ROS under stress condition (Fermani, 2007; Bertomeu et al., 2010). the study conducted by Kappachery et al. (Kappachery et al., 2021) indicated that Overexpression of gene encoding GAPDH in *Arabidopsis thaliana* transgenic elevate antioxidant enzymes, photosynthetic pigments and improve photosynthesis *via* increasing general PSII efficiency under salt stress. In our study, GAPDH accumulation elevated in pistachio leaves

treated with PEG6000, revealing that this enzyme may provide the way for obtaining extra energy for regulation of cellular homeostasis and also it maintain photosynthetic efficiency using protect photosystem II from adverse effect of osmotic stress.

Aspartate kinase (AK) is the primary and the most vital enzyme in phosphorylating L-aspartate, leading to biosynthesizing four necessary amino acids: methionine, threonine, lysine, and isoleucine (Jander and Joshi, 2009; Han et al., 2021). There are several evidences demonstrated AK involved in osmotic stress (Chefdor et al., 2006; Héricourt et al., 2013; Héricourt et al., 2016) and drought and nutritional stress (Curtis et al., 2018). In this study the increased accumulation of AK was determined, suggesting biosynthesis of numerous amino acid leading to improve plant adaption abilities under osmotic stress.

The function of the sulfotransferase superfamily is to transfer a sulfonyl group from the general donor, PAPS, to a hydroxyl group from a wide range of substrates, including glucosinolates, phenolic acids, flavonoids, brassinosteroids, coumarins, jasmonates, and terpenoids. They are involved in very diverse physiological functions such as a response to pathogen or detoxification (Hernández-Sebastiá et al., 2008; Hirschmann et al., 2014). In this study two proteins belong to sulfotransferase superfamily; Thiosulfate/3-mercaptopyruvate sulfurtransferase 1 and sulfotransferase 4 was found to be increased under osmotic stress. It has been reported that sulfurtransferases play a main role in ROS, cyanide, and heavy metals detoxification and contribute to metabolism of sulfur and cysteine (Papenbrock and Schmidt, 2000; Nakamura et al., 2000; Most and Papenbrock, 2015; Yamasaki and Cohen, 2016). Proteomic profiling on lettuce was conducted by Leitão et al. (Leitão et al., 2021) indicated that accumulation of sulfurtransferase was increased under stress induced by pharmaceutical contamination. Also, several studied illustrated that sulfurtransferases play a main role in plant response to abiotic stress such as osmotic, salt and hormone stress (Jin et al., 2019). Overall, increased expression of cytosolic sulfotransferase 4 and thiosulfate/3-mercaptopyruvate sulfurtransferase 1 indicated a wide range of changes associated with osmotic stress.

## Stress response related proteins

Phospholipase D is a most important enzyme involved in hydrolyzing membrane phospholipids, leading to generate phosphatidic acid which act as a signaling molecule so that promotes stomatal closure under osmotic stress (Saucedo-García et al., 2015; Rodas-Junco et al., 2021). Many evidences illustrate that PLD has an important role in plant tolerance under stress (Ji et al., 2018; Alferéz et al., 2019; Gnanaraj et al., 2021; Wei et al., 2022) and adjust plant defense response to osmotic stress (Liu et al., 2021; Liu et al., 2022; Hong et al., 2008). Our findings indicated that abundance of Phospholipase D was increased under osmotic stress, similar to results of (Urban et al., 2021).

Osmotic stress induce the ABA level in various plants, which is a well-known reality (Haider et al., 2018; Kai et al., 2019; Xing et al., 2016). In this study, the up-regulation of abscisic acid receptor PYL9 was observed, indicating activation of pathway related to ABA signaling in pistachio plant toward response to osmotic stress.

Transcriptomic analysis of grapevine leaves indicated that PYL9 induced under salt stress (Guan et al., 2018) Also, it Miao et al. (Miao et al., 2018) reported that PYL9 overexpression improves drought resistance.

Up-regulation HSPs, as chaperones, involved in facilitating protein conformation or refolding under stress conditions, because, denaturation of proteins occurs as a result of the reduction of water content in osmotic stress (Zang and Komatsu, 2007). The expressions of HSP genes are prompted by denatured or damaged proteins (Xiong and Zhu, 2002). Thus, their expressions are up-regulated in the stress or some stages of growth and development (Park and Seo, 2015). The elevated expression of a heat shock protein under osmotic stress was observed in the present study. This result is agreement to studies of Rahman et al. (Rahman et al., 2015) who reported increased accumulation of 18.1 kDa class I heat shock protein and other HSPs in transgenic sugarcane under drought stress induced with PEG.

Following many stresses, oxidative stress also occurs due to augmented ROS. Although ROS plays a key role in signaling and regulation of many genes (Xiong and Zhu, 2002), enhancing its concentration damages cellular structures seriously, so some mechanisms have been established in the plant to prevent the overproduction or to remove ROS (Toorchi et al., 2009), including catalase activation or biosynthesis, which sweeps away H<sub>2</sub>O<sub>2</sub> by its activity. Increasing antioxidants as a common result of most abiotic stresses, improves plant stress tolerance. Abiotic stress induces genes for various catalase isoforms (Xiong and Zhu, 2002). Catalase in interaction with plant natriuretic peptide (PNP) triggers the regulation of ROS levels and cell redox homeostasis during the salinity or drought stress (Turek et al., 2020). In this study two spots (119 and 173) identified as catalase enzyme that their expression were increased under osmotic stress. Augmented activity of the antioxidant system due to increased expression of relevant proteins during osmotic stress has been observed in other studies, as well (Toorchi et al., 2009; Zhang and Shi, 2018).

Serine/threonine-protein kinase CST is a receptor-like cytoplasmic kinase that acts as an inhibitor in such a way that limits the extent of cell separation signaling, and causes cells to be separated only in designated areas in abscission zone (Burr et al., 2011). Several researches have been proven positive role of Serine/threonine-protein kinases under stress condition in different plants (Mao et al., 2010; Sun et al., 2013; Rampino et al., 2017; Mao et al., 2010). In this study, activation of Serine/threonine-protein kinase CST was increased. The role of this protein in response plant to biotic stress was proved (Ghorbani et al., 2019).

## DNA and RNA processing related proteins

Plants to dominate the stable challenge from a swiftly altering environment have several particular adaptation mechanisms, among which DNA and RNA processing are main strategies (Wong et al., 2017; Song et al., 2021). In this study several proteins related to DNA and RNA processing were identified included of DEAD-box ATP-dependent RNA helicase 7, DNA repair protein RAD50, replication protein A 70 kDa DNA-binding subunit D, and argonaute MEL1 are proteins that participate in the processes assigned to DNA and RNA

(Nonomura et al., 2007; Takashi et al., 2009; Gherbi et al., 2001; Gallego et al., 2001; Aubourg et al., 1999). This display that plant to adapt under osmotic condition could increase several transcriptional and translation processes and seriously elevated the stability and variety of proteins (Aubourgt et al., 1999; Nonomura et al., 2007). The role of DNA and RNA processing has been indicated in studies related to various environmental stress (Gao et al., 2019; Li et al., 2020; Marondedze et al., 2020)

## Cell wall biosynthesis related proteins

Like other results (Ngara et al., 2018; Zhang and Shi, 2018; Wang et al., 2020), we also recognized some proteins associated with cell wall construction, including prolyl 4-hydroxylase 5 and probable galacturonosyltransferase 3, since the cell wall is the protective barrier and the first front of defense against stress. Cell division, which requires the formation of a new cell wall, is also inhibited under osmotic stress (Xiong and Zhu, 2002). Hence, cell metabolism changes to guide plant status from optimal growth to stress-adapted growth, which requires alterations in the expression and the activity of many proteins assigned to the intercellular space and cell wall (Ngara et al., 2018). The synthesis of pectin, which is a component of the cell wall, requires the activity of galacturonosyltransferase (Boustani et al., 2017). 4-hydroxyproline, which is an important component of cell wall glycoproteins, is produced post-translationally by the activity of Prolyl 4-hydroxylase 5 on proline-rich sequences in glycoproteins (Velasquez et al., 2011). In this study proteins related to cell wall stabilization was increased, indicating that these proteins might help to the more consolidation of cell wall in pistachio and leads to more osmotic tolerance and plant could regulate the osmotic potential using changes to the cell wall.

## Transporting and movement related proteins

Kinesin-like proteins are motors that perform microtubule-based movement, such as the transport of vesicles and organs, chromosome segregation, and signal transduction, thus play a key role in developmental and environmental processes (Ni et al., 2005). Changes in the expression of microtubule-related proteins may alter the morphology of stressed tissue. In this research, kinesin-like protein KIN-7H revealed higher abundance in the PEG treatment.

Aluminum-activated malate transporter, which belongs to the anion channels, is located in the membranes of different tissues and has a various and fundamental range of physiological functions such as aluminum resistance, signaling, anion homeostasis, osmotic adjustment, stomata regulation, and abiotic stress tolerance *via* transporting malate or inorganic anions (Palmer et al., 2016; Ramesh et al., 2018; Hejri et al., 2021). Maintaining sufficient amounts of water is essential for plant growth and development (Pasaribu et al., 2021). Therefore, the plant controls the ionic balance in the cell to regulate the osmotic pressure during the osmotic stress. Thus, aluminum-activated malate transporter 11 can play a significant role in this process by transporting malate and inorganic anions. Scientific reports suggest that abiotic stresses such as salinity

regulate transporters either at the protein or mRNA levels (Xiong and Zhu, 2002).

## Signal transduction related protein

Under osmotic stress, plants sense the stress through signal transmission networks and as a result start to react. They adapt to stress conditions through different signaling pathways that affect a wide range of protein expression (Zandalinas et al., 2020). Alterations were reported in levels of various proteins related to signal transduction under stress condition (Shan et al., 2018; Meena et al., 2019; Zhang et al., 2019; Wang et al., 2022). A main protein to take a notice is guanine nucleotide-binding proteins, named G proteins or GTPases. These proteins *via* activity of moderator or transducers in different signaling systems located in transmembrane adjust many cellular processes such as secretion, transport etc. (Patel et al., 2020). G-proteins consisting of the  $G\alpha$ ,  $G\beta$ , and  $G\gamma$  subunits, Based on our findings, The  $G\alpha$  subunit up-regulated under osmotic stress.  $G\alpha$  subunit is the vital the member of G-protein signal transduction so that activation of G-protein and downstream signal depended on  $G\alpha$  (Liu et al., 2021; Liu et al., 2018; Pandey and Assmann, 2004). Similar to our findings, several studies illustrated that increased expression of the  $G\alpha$  subunit plays a significant role in plant resistance to various abiotic stress such as salt (Misra et al., 2007), drought (Ferrero-Serrano and Assmann, 2016), heat and cold (Chakraborty et al., 2015; Chakraborty et al., 2015; Ma et al., 2015; Guo et al., 2020).

## Other protein

F-box kelch-repeat proteins can adjust biosynthesis of phenylpropanoid *via* regulating the turnover of phenylalanine ammonia-lyase and also play a main role in a main role in providing homeostasis *via* eliminating misfolded or injured proteins which could destroy cellular activations (Zhang et al., 2013; Kamireddy et al., 2021). In this study, the expression level of the F-box/Kelch-repeat protein (At3g17530) was elevated under stress. To date, the function of this protein is unclear but several studies indicated that proteins belong to F-box/Kelch-repeat protein family play an important role in improve of tolerance plant under stress condition (Curtis et al., 2013; Wang et al., 2017; Venkatesh et al., 2020).

## STITCH and GO analysis

Protein-protein/chemical interactions can notably modulate different cellular activities, such as replication, transcriptional regulation, defense responses, growth and development, processes of signaling, and consonance of numerous metabolic pathways (Fukao, 2012; Braun et al., 2013). In this study, the interaction networks proteins-proteins/chemicals in pistachio leaves treated by PEG were analyzed using STITCH. The STITCH network predicted 33 proteins and small molecules interacted with identified proteins using proteomic technic. Also STITCH analysis indicated that all of proteins and small molecules regulation by a set of hormones and their crosstalk.

According to KEGG analysis, the most proteins involved in response to osmotic in pistachio leaves enriched in metabolic

pathways that was similar to proteomic findings, indicating the osmotic stress mostly impacts the metabolic pathways.

## Conclusion

To better our knowledge about plant tolerance under osmotic stress and molecular mechanisms behind related responses, proteomics of pistachio leaves was performed. Osmotic stress imposed a change in the expression of 25 proteins. 21 proteins were highly expressed while four proteins were less expressed. These identified proteins function in several biological processes such as stress response, photosynthesis and metabolism, DNA and RNA processing, and cell wall biosynthesis which point out their roles in adaptation of pistachio under osmotic stress. Based on KEGG analysis, proteins related to metabolic pathways have the most vital role in pistachio response to osmotic stress. The decline in the expression of Rubisco, OEE1, and photosystem I assembly protein Ycf4 suggested the destructive effect of membrane dehydration resulted from osmotic stress on the photosynthesis process. Altered expression of some proteins associated with the cell wall was expected because the wall is the first defense barrier against stress, and cell division, which requires the formation of a new wall, is inhibited under stress as well. Some proteins involved in DNA and RNA processing were also overexpressed because osmotic stress activates signaling pathways such as the ABA-related pathway, which ultimately leads to altered gene expression and delayed cell division, and stress-induced ROS may also damage DNA.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

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## Author contributions

RP performed the experiments, analyzed the data and wrote the original draft. FF conceived and designed the study, supervised all steps, review and edit the manuscript, approved the final version to be published. MK investigate and MM analyzed the data, review and edit the manuscript.

## Funding

Research funding provided by FF and RP.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.1041649/full#supplementary-material>

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