The gene expression fluctuations of glyceraldehyde-3-phosphate dehydrogenase in drought-stressed-basil cultivars

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as one of the key enzymes in glycolytic pathway in higher plants, was identified as an important objective protein in oxidative stresses condition, which can undergo thiolation reaction in the active site. However, several genes encoding of cytosolic isoenzymes of GAPDH have been identified and characterized in some plants, to investigate the role of GAPDH under drought stress, cloning of full-length cDNA, and expression profile of *Ocimum basilicum GAPDH* (*ObGAPDH*) cDNA were investigated. The full-length *ObGAPDH* was 1315 bp long, its deduced protein consists of 399 aminoacids with a predicted molecular mass of 42.54 kDa and a pI of 6.01. Phylogenetic tree study exhibited a ObGAPDH shared evolutionary relationship with the protein sequence of ObGAPDH reported in *Salvia splendens* and *Sesamum indicum*. *In silico* identified oxidative function of ObGAPDH in response to stimuli and docking illustrated inaction of protein with H_2O_2 as ligand. Expression profiling analysis exhibited different transcription ratios of *ObGAPDH* gene under water shortage stress. Overall, our findings revealed that the *ObGAPDH* regulation appears to be a cultivar- and drought stress-dependent mechanism.

1. Introduction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), participating in the alteration of glyceraldehyde 3-phosphate to 1,3- bisphosphoglycerate reaction in the existence of NAD^+ and inorganic phosphate as a major enzyme in the breakdown of glucose and production of energy in the glycolysis, thus involved in providing energy and various intermediates for the cell's metabolism (Sirover et al. 2011).

For decades, GAPDH was assumed as an ordinary "housekeeping gene" and has been utilized in transcription profiling and protein surveys solely as a reference, located primarily in the cytoplasm (Nicholls et al. 2012). This enzyme is found in all organisms in such a way that could be classified into three major subgroups in plant cells based on their various subcellular positions as follows: GAPA/B as the first group, encoded by *gapA* and *gapB* genes, which are a type of chloroplastic phosphorylating NADP-specific GAPDHs, GAPC, taking part in photosynthetic carbon assimilation; which is encoded by the cytosolic gene (*gapC*) as a phosphorylating GAPDH catalyzing the key step in glycolysis process; GAPCp, encoded by *gapCp*, playing a role in the production of glycolytic energy in nongreen plastids. GAPDH, a tetrameric protein with similar and specific subunits, can have a severe impact on both cellular redox and general metabolism (Tristan et al. 2011; Kim et al. 2005; Sirover et al. 2012).

In addition to its pivotal role in glycolysis, GAPDHs as moonlighting proteins, have been complicated in several functions in cells (Muñoz-Bertomeu et al. 2009). In animal cells, GAPDHs was shown to carry out additional nonmetabolic functions, influencing by the most common post-translational modifications on highly conserved residues, including signal transduction cascades, retention of DNA integrity, and apoptosis (Wang et al. 2013). Recent studies indicates that GAPDH plays important roles not only in glycolysis pathways, but also in several developmental functions, hormone signal transduction, and particularly on the exposure to abiotic stresses. For example, in *Populus tremula*, some increase in

GAPDH expression has been described under water shortage stress (Kapachari et al. 2015). Over expression of a cytosolic GAPC3 isoform has also been found in saline-tolerant line of rice. In addition, GAPDH forms immuno complex with protein kinases activated under osmotic stress in cells in salt-treated tobacco cells (Kosová et al. 2013). Recent studies have demonstrated that duplication of whole and segmental of genome might consider for the presence of various isoforms of *GAPDHs* in wheat, in which transcriptional analysis showed its key role in tolerance of plants under abiotic stress (Zeng et al. 2016(.

Drought stress, one of the adverse environmental conditions, has been considered as the most important factor in growth restriction development and many plants' yield (Bartels and Sunkar 2005; Farooq et al. 2012). Despite observation of reactive oxygen species (ROS) production as by-products of metabolic pathways under ordinary values, the objective has been extensively received that an excess of intracellular ROS, one of the impacts of general abiotic stresses, is too detrimental to the cells (Sharma et al. 2012). Hydrogen peroxide (H_2O_2) is the most abundant ROS and plays a signaling role involved in in stress responses in plants. The signification of ROS (chiefly H_2O_2) in cellular redox also common metabolism derives their sufficiency to induce post-translational modifications of objective proteins from the formation of oxidative stress conditions (Zaffagnini et al. 2013). H_2O_2 is regarded to alter target protein activities through S-thiolated, oxidizing of thiol groups of highly reactive catalytic Cysteine residues to sulfinic or sulfonic forms, over extended oxidative treatment. GAPDH, as a major target of oxidative stress, is hardly susceptible to the oxidization of active-site catalytic Cys residue (Cys^{152}) by H_2O_2 (Hwang et al. 2009). As an alternative, redox modification of active site cysteines by this irreversible reaction is likely to assist GAPDH associations with other protein cysteines or with glutathione thereby disulfide bonds, to regulate the intensity and duration of redox cellular signaling pathways in response to hydrogen peroxide stress (Bayek et al. 2008; Zaffagnini et al. 2012b). Previous studies indicated that, because of high catalytic reactivity of Cys residue, all phosphorylating cytosolic GAPDHs as a goal of various types of redox changes can endure numerous post translational modifications in response to ROS (Bedhomme et al. 2012). Recent studies that GAPDH remarkably can interact with phospholipase Dδ (PLDδ) and transducing the H_2O_2 signals in mediating water deficit and ABA-induced stomatal closure in *Arabidopsis thaliana* (Guo et al. 2012), providing a direct connection between many functions and control of plant growth in the plant response to ROS stress. Besides, the enhanced accumulation of cytosolic and plastidial GAPC1 in cadmium-stressed roots tip cells and sugar and amino acid balance were investigated in Arabidopsis (Vescovi et al. 2013; Muñoz-Bertomeu et al. 2009). The earlier works demonstrated that H_2O_2 also prevents the activity of two enzymatic recombinant GAPC1 and GAPA1 proteins (Zaffagnini et al. 2007).

Ocimum basilicum L. from the Lamiaceae family, as well-known flavorful and aromatic plant, which is broadly spread in Iran (Ekren et al. 2012). It has been widely used as a fever-reducing, anti-malarial, analgesic, anti-depressant agent and in combination with Anti-rheumatic drugs in the pharmaceutical industry. So far 140 compounds have been identified in the essential oil of this plant, three compounds with the medicinal value of phenylpropanoid (methyl chavicol and methyl eugenol), monoterpene

(linalool) and sesquiterpene (germacrene D, γ-cadinene and β-caryophyllene) constitute the major volume of its essential oil (Gang 2005). Recent studies indicate that the concentration of the major compounds of essential oil of basil e.g., linalool, methyl chavicol, 1,8-cineole and *trans-α*-bergamotene were significantly increased under drought stress, as a non-enzymatic defense systems to maintain cultivars of basil to oxidative stresses (Radcási et al. 2010; Khakdan et al. 2016). Since the chemical synthesis of these volatile compounds is difficult, complex, and not economically viable, relying on plants to extract these compounds as the only economic way to produce them is a very valuable way (Krasensky and Jonak 2012; Rejeb et al. 2014).

Because plants have limited machinery to avoid stress, they need flexibility tools to overcome the detrimental changes of the environment. Responses and adaptations to different types of stress in plants are highly variable and complex. In this regard, plants express a set of genes, structural changes of membranes, changes in metabolic processes, production of secondary metabolites, suppression of many active genes, expression of regulatory and stress- responsive genes for adaptation employs in adverse environmental conditions (Foyer et al. 2006). Therefore, detection of major genes implicated in the tolerance of stress in plants is essential for better comprehend of controlling of their tolerance based on genetic contents, in this way, the improvement of modified cultivars can be done under stress conditions.

Irrespective of numerous previously studies have been performed for basil in various fields (Renu et al. 2014; Khakdan et al. 2016, 2017, 2018, 2021), according to the best of our knowledge, there is no consideration precisely concentrating on the expression profiling of cytosolic *GAPDH* in the three cultivars under oxidative stress conditions imposed by the three levels of drought stress to understand protective machinery to overcome different types of stresses. To examine this, we report cDNA cloning, gene identification, investigate the bioinformatics properties and the basil *GAPDH* gene expression patterns at the transcriptional level.

2. Materials And Methods

2.1. Plant materials and treatment conditions

The mature basil (*Ocimum basilicum* L.) seeds belong to two different areas of Iran; i.e., Jahrom (Cultivar 1) and Amol (Cultivar 2 and 3) cities were sterilized by *Sodium Hypochlorite* solution *5* % w/v for 3 min and sown in single pots containing sandy-loam soil, with the beginning time of Mid-May, 2019. The total RNA extraction and various drought stress treatments were carried out from leaf of two-week-old seedlings of basil. Plants in the drought stress treatment group were subjected with three different stress levels nominated as *C*: 100% FC, *W1*: 75% FC, *W2*: 50% FC and *W3*: 25% FC (Khakdan et al. 2016). Sampling was performed after 30 days of water shortage stress treatment. All treated specimens of were collected and directly frozen in liquid nitrogen and stocked at -80 ° C, until further analysis.

2.2. Determination of the relative water content (RWC)

For RWC determination, the appendix process was used: the fresh middle leaves (three replications per plant) either from the water shortage treatment or control plants were gathered in flowering stage, directly weighted and nominated as "*fresh weight*" (FW). Afterwards, *saturated weight*" (SW) was obtained from the soaking and weighting of all leaf's samples of each treatment in an adequate quantity of distilled water near 4 °C for 24 h. The *dry weight* (DW) samples were derived from drying the treated samples for 72 h at 70 °C and weighting of each dried specimens. Finally, RWC was calculated by the appendix formula (Slayter 1967):

RWC (%) = [(FW-DW)/(SW-DW)]×100

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted using pBiozol reagent (Sigma) using the method followed the constructor's instruction. Using a Revert-AidTM First Strand cDNA Synthesis Kit (Fermentas, Germany), first-strand cDNA was synthesized according to the constructor's protocols.

2.4. Cloning of the full-length cDNA of *GAPDH*

Based on the similarity of the amino acid sequences of plant GAPDH accessioned in GenBank, the two degenerate oligonucleotide primers (GADPS- F and GADPS- R; see Table 1) from the conserved regions was designated for the amplification of primary fragment of *ObGAPDH* cDNA. Therefore, PCR reaction was done using cDNA as a template under the following program applying: 94 °C (pre-denaturation) for 3 min, followed by 35 cycles of amplification (94 °C for 1 min, 59 °C for 45 s, 72 °C for 1 min) and the 72 °C elongation step for 5 min. An initial amplified fragment of 671-bp cDNA fragment was extracted from the gel by the gel extraction kit, ligated into cloning vector pTG19-T (Vivantis, Korea), and then transformed into *E. coli* cells (DH5α) (Novagen, USA). The extracted plasmid sequence was performed by Bioneer Biotechnology Co. Ltd (Korea)). For obtaining the partial *ObGAPDH*, three primers [(the two forward gene-specific primers; Ob-GAP- F1 and GAP- F2) and reverse primer (R (anchor)) (as the anchored RT primer)], according to the obtained conservative sequence, was used to amplify the 3' end of *ObGADPH* by 3' RACE-PCR through PCR protocol (2 min at 94 °C (1 cycle) followed by 30 cycles of amplification (30 s at 94 °C, 1 min at 59 °C, 1.30 min at 72 °C, and 5 min at 72 °C). The nested amplified fragments were purified, ligated, and sequenced as described. Then, full-length sequence cDNA was amplified with primers; GAD-2-F 5' and GAD-2-R 5' (Table 1). Touchdown-PCR program was: 1 cycle (94 °C for 1 min), 5 cycles (94 °C for 30 s); 1 min at 62 °C, 1 min at 72 °C in the initial cycle). The temperature of anneal step was declined 1 °C per cycle. Next, the temperature situations were modified to program: 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C for 24 cycles); 7 min at 72 °C. The GAPDH PCR product was cloned into pTG19-T (Vivantis, Korea) and the isolated plasmid utilized for sequencing as described.

Table 1. Primers sequences utilized in the current study

Primer name	Primer sequences
GADPS- F	5'- GGR AGG AAC TTC TTG AGG TG- 3'
GADPS- R	5'- GTCATGGTYCCCTTGATG- 3'
GAP- F1	5'-GCT CGY CAG AAG AGY GG- 3'
GAP- F2	5'-GGA CTW CTT TGG GAC ATG ATC AC-3'
(R) Anchor	R 5´- ACGCTACGTAAGGCATGACA-3´
GAD-2-F 5'	5'- CTTCGCCTCTACCACTCCCC- 3'
GAD-2-R 5'	5'- CTC CGG TGT CGT TAA TGG C- 3
GAP-F	5'-CGT GTT CGT GGA CAG AGA AGG -3'
GAP-R	5'-GGGGCTGTAGTCATCGGCG-3'
Actin-F	5'- TCTATAACGAGCTTCGTGTTG-3'
Actin-R	5'- GAGGTGCTTCAGTTAGGAGGAC-3'

2.5. *In silico* sequence analysis

For aligning the sequenced gene segments, the obtained sequences of 3’ and 5’ regions of the *ObGAPDH* gene were analyzed using EditSeq software, which were then investigated by the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Prediction of longest open reading frame (ORF) were carried out using the ORF Finder tool (http//:www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced protein sequence was predicted using online server (http://www.expasy.ch/tools/dna.html). Physiochemical properties such as the predicted pI and molecular mass of protein sequence of ObGAPDH were determined on the website of Expasy (http://web.expasy.org/protparam). Some important functional and structural properties were acquired in ObGAPDH protein sequence via PROSITE, InterProScan, ProDom, and TrEMBL. MotifScan tool was used for identifying any functional conserved motif and domains. The hydrophobicity plot was determined by ProtScale with Kyte and Doolittle method (Kyte and Doolittle 1982). (Gasteiger et al. 2005). Protein secondary structure based on homology modeling was specified by SOPMA tool (https://npsa-prabi.ibcp.fr/). The protein subcellular localization was predicted by the Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (Hall 2002) and Prosite (http://psort.hgc.jp). Analysis for determining of N-terminal pre-sequences: signal peptide (http://www.cbs.dtu.dk/services/TargetP/) was done by TargetP-2.0 server. For recognition of transmembrane helix TMHMM serve, v .2.0 was used (Krogh et al. 2001). The functional domains were knew using the hmmscan online server (https:// www.ebi.ac.uk/Tools/hmmer/search/hmmscan).

2.6. Phylogenic analysis, prediction properties of CDS, codon usage, and miRNA targeted- ObGAPDH

The multiple alignments of deduced ObGAPDH and other known GAPDH sequences from different plants from GenBank was performed by Clustal W. A phylogenetic tree of the GAPDH gene was draw using

neighbor-joining method with 2000 bootstrap trials using the MEGA 6.0.

The deduced ObGAPDH sequence were surveyed for GC content at the 2nd and 3rd codon position (GC2 and GC3s), codon bias index (CBI), effective codon number (ENC), and relative synonymous codon usage (RSCU). Scaled Chi-square (SChi2) methods were analyzed by DnaSP program (Rozas et al. 2003). The psRNATarget database (http://plantgrn.noble.org/psRNA Target/) by the subsequent parameters: max expectation 3 and target accessibility (UPE) 25 was used to investigate ObGAPDH targeted miRNAs in the CDS sequence. Also, cystoscope software was used for visualizing miRNAs (Draghici et al. 2007).

2.7. Predicted tertiary structure, molecular docking and gene ontology of ObGAPDH The tertiary structure of the ObGAPDH protein sequence was detected by I-TASSER server based on the homology modeling method by the Ramachandran diagram and online version of VADAR 1.8 (Volume, Area, and Dihedral Angle Reporter) server. Two backbone dihedral phi (j) and psi (y) angels were measured based on the Ramachandran diagram.

The ligand structure of H_2O_2 was gained from the PubChem database for analyzing molecular docking using Molegro Virtual Docker software, in which the best situation with decreasing energy was chosen. Then, for determining the amino acids and the protein-ligand interactions Molegro Virtual Viewer software was used. Also, MetaGO server was used to determine gene ontology in the three levels of molecular function, biological process, and compartment structure.

2.7. Quantitative real-time PCR analysis

Gene expression analyses were performed to relatively quantify the expression ratios of *GAPDH* gene expressed in three levels of water shortage stress through qRT-PCR. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design the sequence of primer (Table 1). qRT-PCR amplification was done in a 20 µL PCR reactions volume containing appropriate diluted cDNA (50 ng), 0.25 µM of each two specific primers, 8 µL EvaGreen Master Mix (containing EvaGreen Dye) (SolisBioDyn, Germany). The qRT-PCR was performed in the Rotor-Gene Q real-time PCR system (QIAGEN, Germany), and under the protocol contains (15 min at 95 °C (1 cycle), 15 s denaturation at 96 °C, 15 s specific annealing temperature at 63 °C, and 15 s at 72 °C, 45 cycles. The *Actin* gene was amplified as a reference gene (Renu et al. 2014) and the quantification of expression level of the *ObGAPDH* gene were standardized to the expression level of the *Actin*, as the reference value. All reactions were performed were performed in triplicate and repeated twice.

2.8. Statistical analysis

All obtained data of biological experiment were obtained from the average of three experiments were analyzed by analysis of variance (ANOVA) and Duncan's multiple range method ($p \leq 0.05$). The mean ± standard deviation (SD) was used to demonstrate the values.

3. Results

3.1. RWC

Generally, RWC is assumed as the most alternating measure of water shortage stress resistance in plants in terms of cellular dehydration. The abundance of RWC of all the three cultivars enhanced significantly upon varying in water contents (Fig. 1). The maximum value of RWC abundance (a mean of 96 %) were obtained under control condition (100 % field capacity). Unlike, under the three levels of water shortage stress, as expected, the lower amount of RWC were remarkably decreased (p<0.01), declining to its minimum level in severe drought stress (25 % FC). Regarding difference between control group and *W1* level, no significant ratio was detected, but for *W2* and *W3* levels a number of reductions (p< 0.01) were detected (Fig. 1), indicating that *W2* and *W2* could be noticed as notable water stress levels. The highest ratio of RWC in cultivar 1 under the last level of water shortage stress (*W3*) as compared to the two other cultivars, represent that this cultivar is much more resistant to water deficit status than cultivar 2 and 3.

3.1. Cloning of the full-length cDNA of ObGAPDH

A 671 bp *ObGAPDH* fragment, as consensus sequence amplified was obtained using degenerate oligonucleotide PCR primers of a *ObGAPDH* cDNA, which alignment analysis exhibited most identity to the *GAPDH* disclosed in different plant species (data not shown). The cDNA 3' end was obtained from the fragment of plant *GAPDH* genes with higher identity by RACE-PCR. The full length of *ObGAPDH* gene was amplified by PCR with different numbers of gene specific primers based on amplified conserved partial sequence and highly conserved sequence of 5' end *GAPDH* gene. The full-length *GAPDH* cDNA sequence was acquired from *O. basilicum* (GenBank accession no. KU375117.1). It had a 1315 bp complete CDS with a poly (A), contains a 1200 bp ORF encoding a 399-amino acid protein.

3.2. Analysis of the predicted protein sequence and phylogenic analysis

Blast P online as homology tool (http://www.ncbi.nlm.nih.gov/BLAST) and Clustal W result showed considerable homology to other plant GAPDH. It was found that the deduced ObGAPDH amino acid sequence presented 95.49 %, 94.24 %, 93.73 %, 91.48 %, 90.07 %, and 89.78 % identity to *Salvia splendens*, *Sesamum indicum*, *Paulownia fortune*, *Buddleja alternifolia*, *Nicotiana benthamiana*, and *Erythranthe guttata*, respectively (Fig. 2).

A Neighbor-joining phylogenic tree was created for additional recognized the relationship between the protein sequence of ObGAPDH and known GAPDH amino acid sequences of other plants by MEGA 6.0 (Fig. 3). The phylogenetic tree was divided into two groups and was found ObGAPDH associated with *Salvia splendens* and then to *Sesamum indicum* with identities 95.42 %, and 94.24 %, respectively (Fig. 3).

For analyzing physicochemical properties such as pI, molecular weight, etc. of the ObGAPDH amino acid sequence, ScanSite and ProtParam tools (http://www.Expasy.ch/tools/protparam.html) was used. The ObGAPDH protein molecular formula, molecular mass, and molecular weight was predicted to be $C_{1877}H_{3012}N_{522}O_{581}S_{11}$, 42.54 kDa, and 6.01 computed by the pI/MW online tool. Instability index of the

protein has been calculated 24.35, indicating ObGAPDH is a stable protein (instability parameter <40 considered for stable proteins). Because of the presence of methionine at the N-terminal end of ObGAPDH, the protein half-life in *in vitro* condition was computed 30 h. Also, aliphatic index, as a main agent in distinguishing the protein stable to heat was obtained 94.01. The hydrophobicity pattern was illustrated by ProtScale with Kyteand Doolittle method (Kyte and Doolittle 1982).The highest score was 2.144 for valine in position 280 and the lowest score was -2.433 for glycine in position 382 that are associated with the strongest and weakest condition of hydrophilicity. The hydrophilic and hydrophobic domains were showed below and above the zero line, respectively (Fig. 4). Results of SOPMA tool for prediction of the protein secondary structure revealed that predicted ObGAPDH protein mainly consisted of 129 α-helix (32.33 %), 99 extended strand (24.81 %), 27 β-turn (6.77 %), and 144 random coil (36.09 %) (Fig. 5). The results of Plant-mPLoc server, Prosite, and Target P to predict of subcellular localization revealed at least one chloroplastic signal peptide in ObGAPDH protein. TMHMM tool showed no transmembrane helix at the ObGAPDH protein. The results of HMM scan showed ObGAPDH protein has functional domain contain glyceraldehyde 3-phosphate dehydrogenase with two accession numbers PF02800.20 and PF00044, which are C-terminal and NAD binding domains, respectively.

Moreover, the predicted amino acid sequence had conserved consensus active site, NADB_Rossmann domain (GXGXXG) to GAPDH activities conserved in different plant species. Also, the multiple alignment results showed that Gp_dh_C domain as the C-terminal area of ObGAPDH protein sequence revealed the maximum identity in length and composition of protein sequence. The results of MotifScan server showed that the protein sequence of ObGAPDH had 10 functional domains includes of AMIDATION (*Amidation site*), ASN_GLYCOSYLATION *(N-glycosylation site)*, CAMP_PHOSPHO_SITE *(cAMP- and cGMP-dependent protein kinase phosphorylation site*), PKC_PHOSPHO_SITE *(Protein kinase C phosphorylation site), E4PD_MF_01640 (D-erythrose-4-phosphate dehydrogenase), DapB_N (Dihydrodipicolinate reductase, N-terminus), GFO_IDH_MocA (Oxidoreductase family, NAD-binding Rossmann fold), Gp_dh_C (Glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain), Gp_dh_N (Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain), and FARP (FMRFamide related peptide family).*

3.3. Analysis of codon usage and miRNA-targeted ObGAPDH

The codon usage of cDNA as a marker in molecular characterization of ObGAPDH was performed. The results showed the ENC was 42.11 in ObGAPDH. However, in overall, the ENC range is between 21 to 61, which the lower the value, indicates the use of only one codon decoded the amino acid, and *vice versa* (De Mandal et al. 2020). The value of codon bias index (CBI) is reported to be between zero and one and for ObGAPDH was 0.49. The RSCU value was utilized to recognition overrepresented and underrepresented codons for each amino acid (Fig 6). The higher RSCU value exhibits a greater frequency. Schi2 is the discrepancy between the quantity of observed codons and the anticipated codon of the same amino acids. This value for ObGAPDH was 0.52 that the mean of value. The percentages of GC and GC in third position of codon were 0.57 and 70, respectively.

19 miRNA-targeted ObGAPDH that 18 of them regulated gene expression in cleavage site was found, but results showed only mtr-miR2089-5p can be a regulated agent of gene expression at translating level. So, these two actions can alter amounts of gene levels after that transcription. Also, the miRNA interaction to ObGAPDH was illustrated in Fig 7.

3.4. Predicted tertiary structure, molecular docking and gene ontology of ObGAPDH

Tertiary structure of the deduced ObGAPDH protein sequence was surveyed using I-TASSER online server using homology procedure. Results revealed that among models, the best model with estimated RMSD (8.6±4.5A●), S-core (-0.76), and estimated TM-Score (0.61±0.14) was selected (Fig. 8a). This model had 0.85 % coverage and 0.66 % identity with template (6ghrB), in which it contains 117 (29 %) strands, 193 (49 %) coils, 56 (14 %) turn, and 88 (21 %) helices. The selected model was measured according to the Ramachandran plot, in which 67.9 %, 23.1 %, and 4.3 % of residue were in the core, allowed, and generous areas, respectively and also 4.6 % of these were placed in the external area.

Molecular docking results showed the most appropriate protein interaction and pose is belonged to the first ligand binding pose with the lowest energy -31.6258 in docking procedure. Afterwards, this pose submitted to survey the detailed interaction between ligand and protein (Fig. 8b), which contains four amino acids with distinct positions Try 245, Glu 330, Pro 301 and Asn 302, with hydrogen inaction and ester bond.

Gene ontology results revealed that *ObGAPDH* had the phosphorylation activity (NADP-dependent glyceraldehyde-3-phosphate dehydrogenase), an important role, as high statically level of molecular function verified this feature (Fig. 9). This result confirms the consequences of experiments which derived from *in silico* and *in vivo* conditions. As previous studies showed, the protein with 0.5 statically level is sufficient to mediate glucose metabolic process, reductive pentose phosphate cycle, response to temperature stimulation, response to chemical material, responses to stress, response to abiotic condition and, oxidation–reduction process, as exactly mention in different resources.

3.5. Transcription profile of *ObGAPDH*

In order to discover a preliminary insight into the potential function of *GAPDH* in basil under drought stress, the expression ratio of *ObGAPDH* were examined at various levels of drought stress using qRT-PCR. The transcript level of actin gene as the reference gene was put for normalization the transcription level under water shortage stress. As Fig. 10 is demonstrated, the transcript ratio of *ObGAPDH* boosted at the level of water shortage stress (*W1*; 4.14-fold), and slightly enhanced 5.32- and 5.34 times in *W2* level and increased sharply ~6.28- and 6.34-fold change upon severe shortage water treatment level in both Cultivar. 1 and 3, respectively. Regarding Cultivar 2, the transcription level of *ObGAPDH* was slowly increased during the first, second, and third levels of drought treatment (*W1, W2, and W3*; up to 5.52, 5.38, and 5.68-fold change).

4. Discussion

In nature, plants are inevitably attempting to universal warning and consequent water shortage stress, directing frequently to the production of an immense availability of volatile compounds to reduce the adverse effects into least proportion (Sandalio et al. 2009, Kosová et al. 2011). The excessive ROS production, the outcomes of biotic and abiotic stresses, moreover its fluctuation via plant cellular mechanism is too harmful agent for the diverse normal activity in living cells e.g., physiological and biochemical mechanisms (Ahanger et al. 2020). It has long been identified that functioning cytosolic and chloroplastidic metabolic enzymes reversibly inactivated as the generation of H_2O_2 has induced by various environmental factors. Therefore, the identification and expression profiling of *GAPDH* as an important metabolic enzyme that was a target for H_2O_2 in plants to modulate the overproduction of ROS which are certainly harmful in greater values and may end in cell apoptosis. The full length of *ObGAPDH* cDNA sequence was cloned for the first time and the transcription level analyzed under water shortage stress.

The *in silico* survey exhibited that the *ObGAPDH* protein sequence was with highly similarity to GAPDH sequence from different plant species, e.g., 95.49% identity to SsGAPDH, 94.24% sequence identity to SiGAPDH, 93.73% sequence identity to PfGAPDH, 91.48% sequence identity to BaGAPDH, 90.07% NbGAPDH, and 89.78% sequence identity to EgGAPDH (Fig. 2). All plant cells, clearly from eukaryotes, have two enzymes with glyceraldehyde 3-phosphate dehydrogenase activity in the cytosol. There are tree group of group glyceraldehyde-3-phosphate dehydrogenase (Bostos et al. 2008). All of the proteins been studied owing to their ability to have NAD-dependent glyceraldehyde 3-phosphate dehydrogenase, a particular enzyme participated in glycolysis pathway and production of NADPH, encoded by the *gapC* gene based on gene ontology prognostication glycolysis process (Bustos et al. 2008). *ObGAPDH* gene is deposited in GenBank and analyzed by bioinformatic tools, with affirmation on the structural characterization.

According to the ProDom and TrEMBL, conserved domain database, results exhibited ObGAPDH protein include the main domain; Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) and Gp_dh_C have been specified in all of ObGAPDH protein (Lu et al. 2020). These functional domains, 6 amino acids allocated at the C-terminal of the polypeptide, may be for NAD binding catalysis, catalytic active site, chemical binding site. The amino acid composition of the active site in the functional domains are also conserved in the glyceraldehyde 3-phosphate dehydrogenase family polypeptide, in which diverse group of proteins assign an functional domain; Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain. This domain is participated in multiple types of dehydrogenases reaction of several metabolic pathways many redox enzymes involved in glycolysis (Lanteri et al. 2011). The Rossmann fold is a super-secondary and functional structure that is specified by the multiple noncovalent bonds such as hydrogen bonding and van der Waals interactions, mainly a hydrogen bond of amino acids chains between the first strand and the next helix in a turn structure. This turn structurally demonstrated a consensus sequence like GXGXXG, indicating 2 glycines of the first codons take part in NAD(P)-binding, and the other glycine in the third position simplify adjacent forming the packet contains the α-helix and the β-strand. Also, the proteins from this large family include a main second domain plus to the NADB domain, which take part in pivotal

functions e.g., substrate binding and enzymatic reaction catalyzing (Guo et al. 2012). The previous studies showed these catalytic active sites is known to be an important motif with regulation and functional roles of the cytosolic plant GAPC *in vivo* (Hajirezaei et al. 2006, Holtgrefe et al. 2008, Rinus et al. 2008, Munoz-Bertommeu et al. 2009, Zeng et al. 2016). Such outcomes from searching conserved domains in ObGAPDH are consistent with previous results, which similar conserved peptide domains were apperceive for GAPDH from *Sesamum indicum*, *Paulownia fortunei*, and *Buddleja alternifolia* (Wang et al. 2014, Ma et al. 2021; Cao et al. 2021), although some differences in amino acid sequences of these domains observed. The alignment results of GAPDH protein sequence from various plant species showed ObGAPDH protein sequence is constructed of the conserved N-terminal domain which is a Rossmann NAD(P) binding fold, which is a distinctive signature of glyceraldehyde-3-phosphate dehydrogenase protein family (Huang et al. 1989).

The various functions of GAPDH mentioned, studies confirm the effect of this molecule in the process of cell death under conditions of oxidative stress. For the first time in rat cells, researchers have reported that catalytic cysteine nitrosylation occurs in GAPDH under stress conditions, reacting with the ubiquinone kinase in the cell and pushing GAPDH into the nucleus to form a complex. It can thus extend the transmission activities of redox signals under stress conditions (Hyslop et al. 2022). The present study, based on gene ontology and molecular docking analysis of protein further confirms the oxidation–reduction activity, multi-action, and interaction of ObGAPDH with H_2O_2 in oxidation–reduction, and it suggests that ROS is a common element involved in oxidation signaling in plants (Schneider et al. 2018; Kappachery et al. 2021).

To our knowledge, thorough considerations of the role of cytosolic GAPDH, a part of the glycolytic pathway, in mediating H_2O_2 effects as a cellular antioxidative tool in transcriptional responses to drought stress are not recognized for *O. basilicum*. So, to obtain a primary understating and consideration of the GAPDH role in the regulation of drought stress responses, its transcriptional profiling and H_2O_2 accumulation (data from our previous study) was discussed in the three Iranian cultivars of basil under the three levels of water shortage stress (Khakdan et al. 2016).

Accordingly, on the subject of cultivar 1 and 3, as compared to the control, the lowermost values of *ObGAPDH* transcript were found for *W1* (-*W1*; 4.14-fold), and slowly incremented for *W2* (5.32- and 5.34-fold, respectively), and eventually the lowermost transcript value of the identical gene was apperceived, as *W3* specimen were considered (~ 6.28 and 6.34, respectively). As for Cultivar. 2, expression ratio of *ObGAPDH* was not significantly different under drought stress (Fig. 9).

High levels of ROS under oxidative stress in several environmental conditions, however, provide a destructive condition in damaging of lipid, carbohydrates, DNA, and proteins (Baek et al. 2008). Thus, production of some antioxidant's compounds beside numbers of ROS scavenging enzymes may lead to diminish and remove ROS accumulation (Loretoa et al. 2014). Previously, we have also been reported that the activity of antioxidant enzymes e.g., SOD, CAT, APX, GPX, and PPO reduces the accumulation of H_2O_2 in these three cultivars of basil under described stress (Khakdan et al. 2016). Now, in this report, we found

that ObGAPDH is involved in cellular redox regulation, although recently it has pointed significant changes in H_2O_2 contents to induce the activity types of antioxidant systems. We interpreted an enhancement in the generation proportion of volatile terpenoids is important to prevent over production of H_2O_2. This increase is correlated with up-regulation of multiple genes participating and even biosynthesis of terpenoids and phenylpropanoids determined by qRT-PCR and GC-MS experiments (Khakdan et al. 2017, Khakdan et al. 2021). Previous studies showed that when photosynthesis is inhibited, the stored carbon source can even be mobilized to maintain volatile production under stress conditions, proposes a logistic connection among the transcriptional level of *ObGAPDH* as the key factor, the decrease of carbon assimilation and redux regulation during drought stress.

In our previous results, we reported decreasing of H_2O_2 reposition in cultivar 1 with increment of the drought stress levels. In this study, it seems that GAPDH has further new function that is participated in the regulation of cellular redox. This result is according to our findings that (1) accumulation of H_2O_2, (2) an affirmative relationship between the reposition of H_2O_2 and *ObGAPDH* transcripts and enhanced tolerance to drought stress by enhancing the levels of shortage of water. The previous study reported the excited effect of dehydration and ABA treatment on the cytosolic GAPDH from *Craterostigma plantagineum* (Hancock et al. 2005), so that these conditions are recognized to enhance the H_2O_2 content in plant species.

Previous studies demonstrated that H_2O_2, as a mediator agent in signaling to downstream effectors in plant cell exposure to oxidative stresses rigorously prevented the CO_2 assimilation in extracted spinach chloroplast, and this inhibitory activity was contracted by the excess of antioxidating components. Such this previous research has been suggested that carbohydrate metabolism is changed by H_2O_2, thereby modifying cysteine amino acids at the active site with the catalytic role in GAPDH protein sequence. This change terminated in both inactivation and structural modification of GAPDH enzyme, which permit GAPDH to take part in protein-protein interaction, operating as a signaling intermediating between H_2O_2 and phospholipase D (Guo et al. 2012).

Also, *Arabidopsis* cell extracts was shown to inhibited GAPDH activity in the presence of H_2O_2, indicating GAPDH as a straightforward objective of H_2O_2, in which labeling experiments results showed H_2O_2 was ready to modify GAPDH directly in the cytoplasm. Furthermore, Kapachari et al. (2015) exhibited that the GAPDH transcripts abundance enhanced in *Populus tremula*, in response to water shortage stress (Kapachari et al. 2015). It has lately been demonstrated that saline-tolerant saline GAPDH gene was an important aim of phosphorylation and up regulated as time and concentration-dependent manners (Kosová et al. 2013). In another previous research, the behavior of GAPC1 and GAPC2, two cytosolic GAPDH isoforms, under the condition with production of ROS induced by cadmium-contaminated soil in Arabidopsis was investigated. For this purpose, the expression of these two isoforms in the mentioned conditions was examined. The results showed that oxidative stress conditions increased the activity of GAPC1 promoter and GAPC2 protein accumulation. It has also been shown that in the root of

Arabidopsis, oxidative stress induced by cadmium causes massive accumulation of *GAPC1* gene copies in the nucleus (Vescovi et al. 2013).

In conclusion, isolation, cloning and sequencing of the full-length *ObGAPDH* cDNA sequence and transcriptional profiling of *GAPDH* were determined in the three Iranian cultivars of basil (*Ocimum basilicum)* exposure the three levels of water shortage stress. Our finding affirmed that the transcriptional profiles of *ObGAPDH* gene are fluctuated under water shortage stress. The isolation of drought-induced *GAPDH* gene from basil will help in our perception of the role of this gene in mediating of redox regulation, and in the improvement of new tools thereby by the advancement of modified cultivars which can carry out better in stressful environments in future.

Abbreviations

ANOVA Analysis of variance

CBI Codon Bias Index

ENC Effective Codon Number

FC Field capacity

GC3 GC content at 3rd site position

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

RACE Rapid amplification cDNA ends

ROS Reactive Oxygen Species

RSCU Relative Synonymous Codon Usage

RWC Relative water contents

Quantitative real-time PCRqRT-PCR

SChi2 Scaled Chi-square

Declarations

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The authors have no relevant financial or non-financial interests to disclose

Author Contributions

F.Kh. and M.R. conceived, designed and performed the experiments; F.Kh. supervised experiments; M.R. and Z.Sh. analyzed the results; F.Kh. and Z.Sh. wrote the manuscript.

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Figures

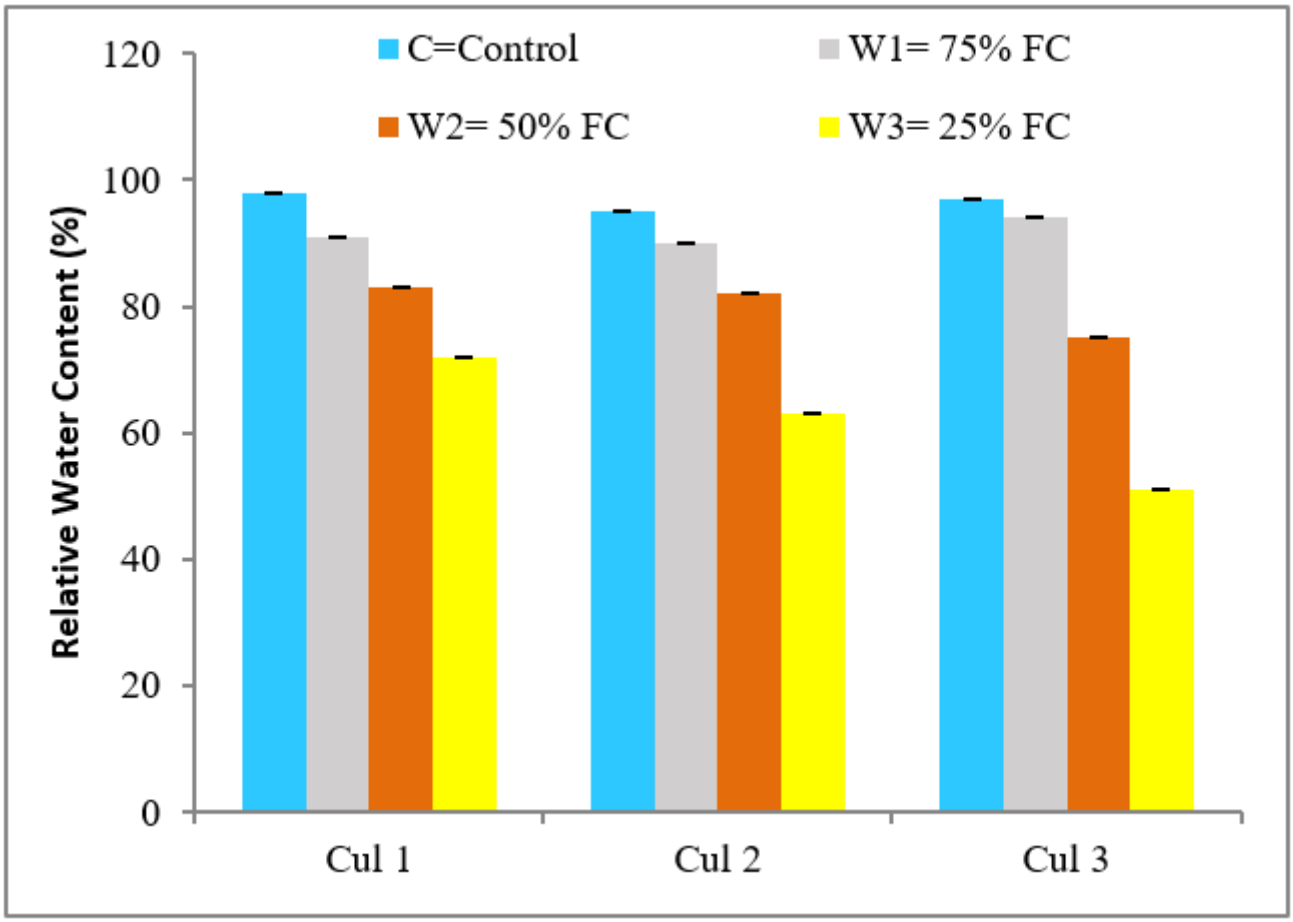

Figure 1

The Relative Water Content of leaves in the three Iranian cultivars of basil under water shortage stress. The values are mean ± standard error (SE) of three replications. *C*=control (100 % FC); *W1*=75 % FC; *W2*= 50 % FC, and *W3*= 25 % FC.

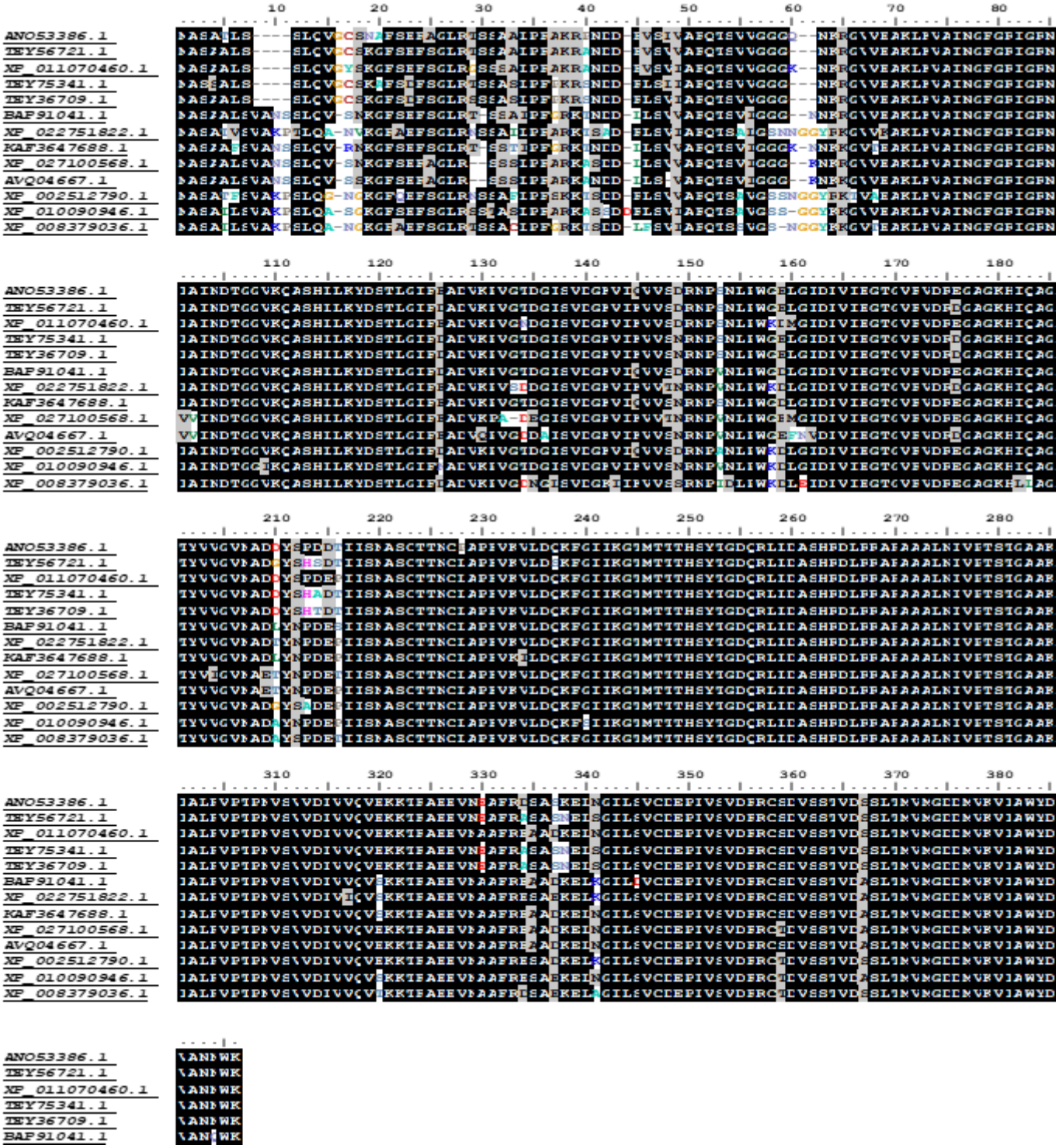

Figure 2

Sequence multi-alignment of the predicted ObGAPDH protein sequence with other GAPDHs. The completely identical amino acids are indicated with black foreground and white background. ANO53386.1 (*Ocimum basilicum*), TEY56721.1 (*Salvia splendens*), XP_011070460.1 (*Sesamum indicum*), TEY75341.1 (*Salvia splendens*), TEY36709.1 (*Salvia splendens*), BAP91041.1 (*Nicotiana benthamiana*), AEO45783.1 (*Scoparia dulcis*), XP_022751822.1 (*Durio zibethinus*), KAF3647688.1 (*Capsicum annuum*), XP_027100568.1 (*Coffea Arabica*), AVQ04667.1 (*Neolamarckia cadamba*),

XP_002512790.1 (*Ricinus communis*), XP_010090946.1 (*Morus notabilis*), XP_008379036.1 (*Malus domestica*).

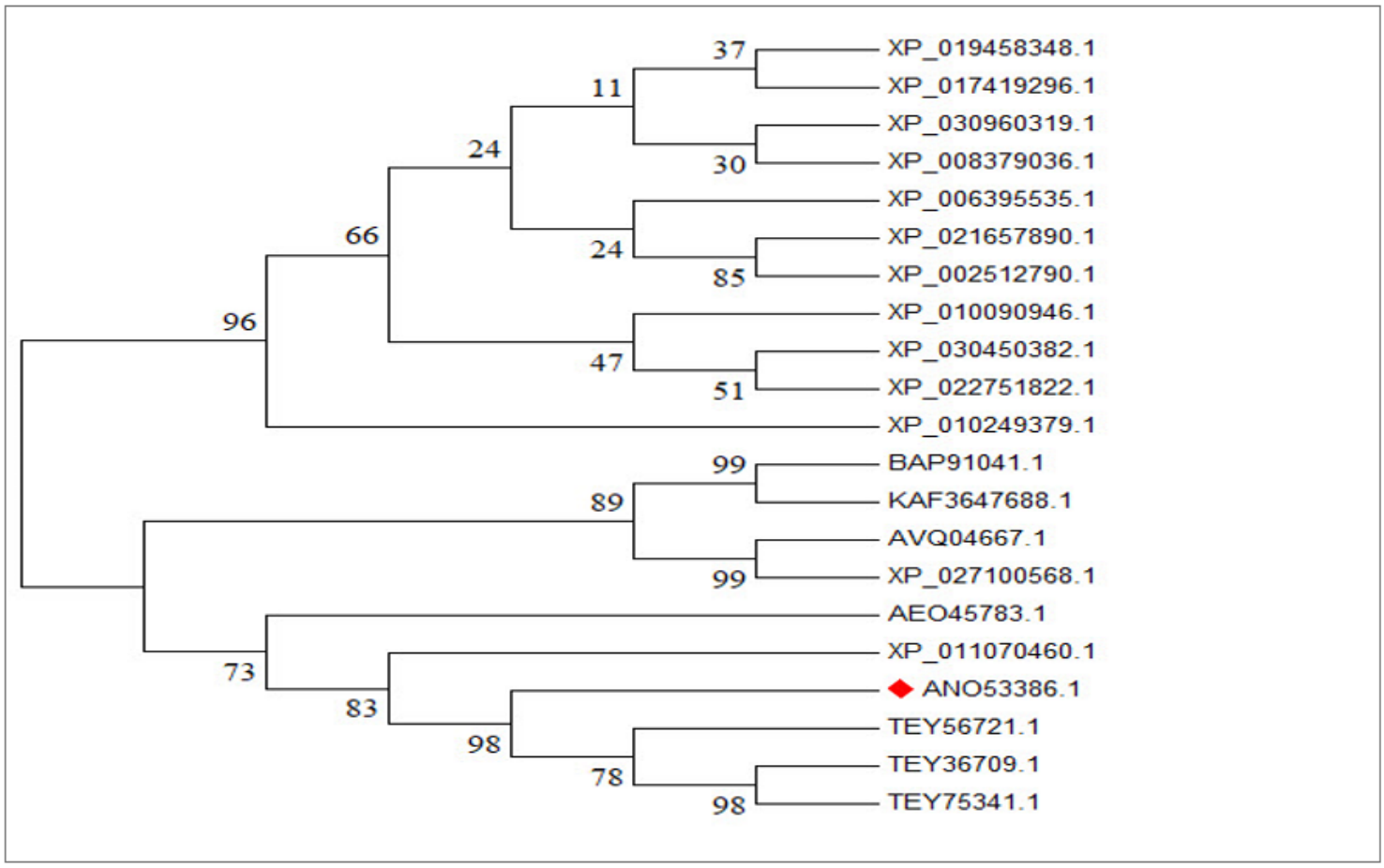

Figure 3

Phylogenic tree of ObGAPDH protein sequence (ANO53386.1) and the corresponding sequence of other various plants. ANO53386.1 (*Ocimum basilicum*), TEY56721.1 (*Salvia splendens*), XP_011070460.1 (*Sesamum indicum*), TEY75341.1 (*Salvia splendens*), TEY36709.1 (*Salvia splendens*), BAP91041.1 (*Nicotiana benthamiana*), AEO45783.1 (*Scoparia dulcis*), XP_022751822.1 (*Durio zibethinus*), KAF3647688.1 (*Capsicum annuum*), XP_027100568.1 (*Coffea Arabica*), AVQ04667.1 (*Neolamarckia cadamba*), XP_002512790.1 (*Ricinus communis*), XP_010090946.1 (*Morus notabilis*), XP_008379036.1 (*Malus domestica*), XP_021657890.1 (*Hevea brasiliensis*), XP_019458348.1 (*Lupinus angustifolius*), XP_006395535.1 (*Eutrema salsugineum*), XP_030450382.1 (*Syzygium oleosum*), XP_010249379.1 (*Nelumbo nucifer*a), XP_030960319.1 (*Quercus lobate*), XP_017419296.1 (*Vigna angularis*). Tree constructed using the neighbor-joining (NJ) method and MEGA 6.0 software and bootstrap values are indicated 1000 replicates.

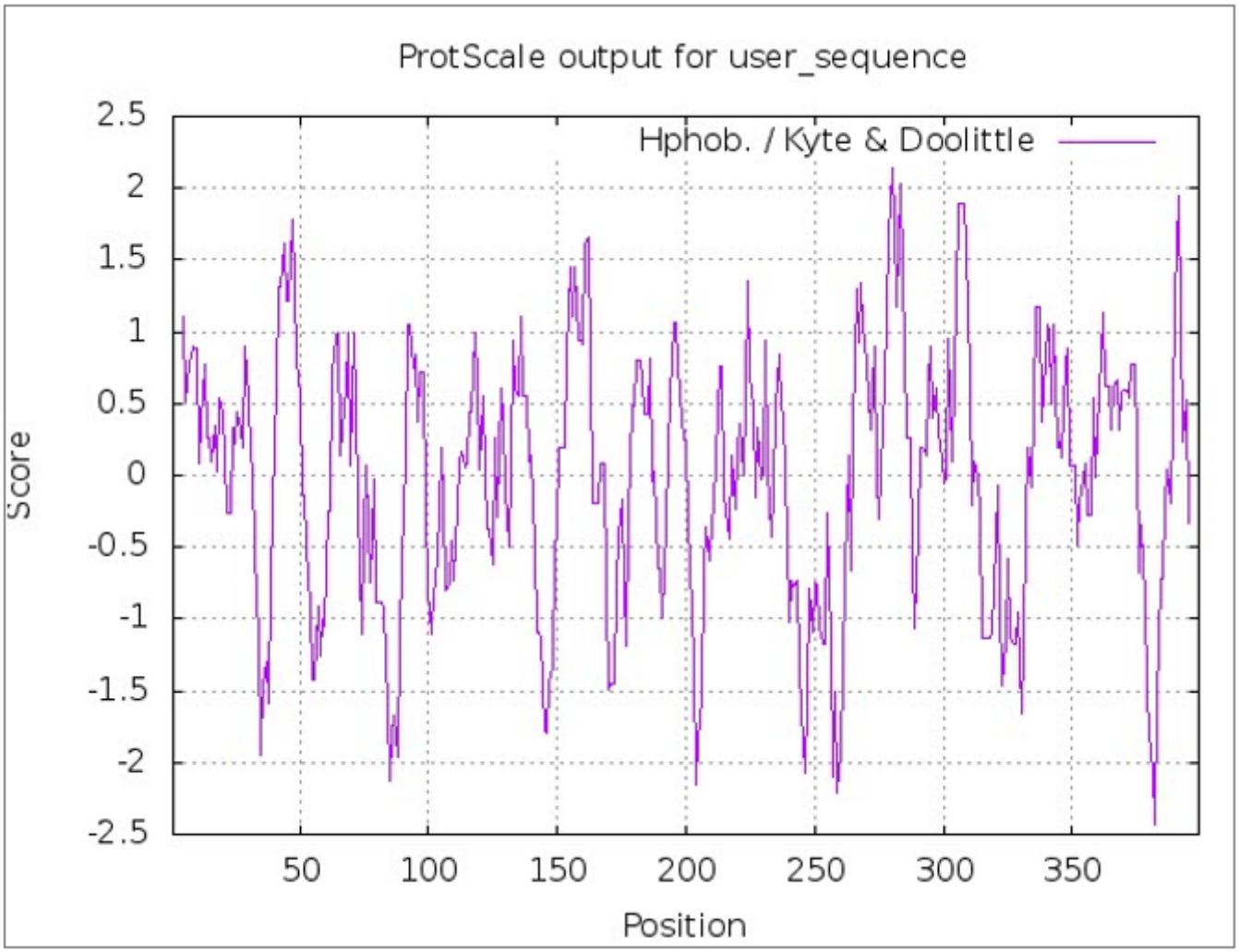

Figure 4

Hydrophobic-hydrophilic pattern of ObGAPDH protein. Hydrophobic domain is located above the zero line and hydrophilic domain located below the zero line

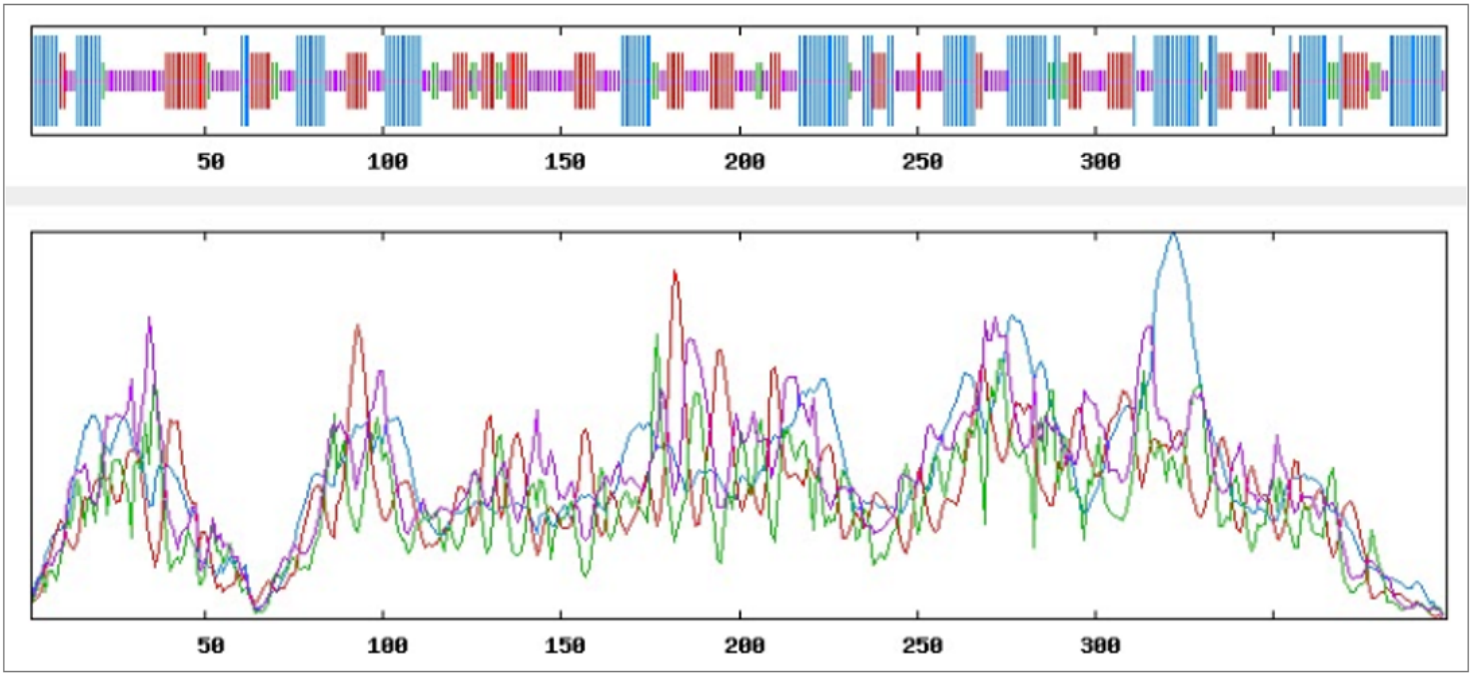

Figure 5

The diagram of secondary structure of ObGAPDH. The α-helix (blue lines), extended strand (red lines), β-turn (green line) and random coil (purple lines).

UUU-F	1 (0.14)	UCU-S	5 (0.94)	UAU-Y	2 (0.67)	UGU-C	0 (0.00)
UUC-F	13 (1.86)	UCC-S	11 (2.06)	UAC-Y	4 (1.33)	UGC-C	6 (2.00)
UUA-L	0 (0.00)	UCA-S	6 (1.13)	UAA-*	0 (0.00)	UGA-*	0 (0.00)
UUG-L	4 (0.77)	UCG-S	4 (0.75)	UAG-*	0 (0.00)	UGG-W	5 (1.00)
CUU-L	8 (1.55)	CCU-P	3 (0.75)	CAU-H	1 (0.40)	CGU-R	4 (1.33)
CUC-L	15 (2.90)	CCC-P	12 (3.00)	CAC-H	4 (1.60)	CGC-R	2 (0.67)
CUA-L	1 (0.19)	CCA-P	0 (0.00)	CAA-Q	3 (0.55)	CGA-R	0 (0.00)
CUG-L	3 (0.58)	CCG-P	1 (0.25)	CAG-Q	8 (1.45)	CGG-R	1 (0.33)
AUU-I	6 (0.86)	ACU-T	2 (0.35)	AAU-N	6 (0.63)	AGU-S	1 (0.19)
AUC-I	14 (2.00)	ACC-T	17 (2.96)	AAC-N	13 (1.37)	AGC-S	5 (0.94)
AUA-I	1 (0.14)	ACA-T	3 (0.52)	AAA-K	5 (0.42)	AGA-R	4 (1.33)
AUG-M	5 (1.00)	ACG-T	1 (0.17)	AAG-K	19 (1.58)	AGG-R	7 (2.33)
GUU-V	9 (0.77)	GCU-A	7 (0.78)	GAU-D	11 (0.69)	GGU-G	10 (1.14)
GUC-V	14 (1.19)	GCC-A	21 (2.33)	GAC-D	21 (1.31)	GGC-G	10 (1.14)
GUA-V	0 (0.00)	GCA-A	4 (0.44)	GAA-E	3 (0.46)	GGA-G	9 (1.03)
GUG-V	24 (2.04)	GCG-A	4 (0.44)	GAG-E	10 (1.54)	GGG-G	6 (0.69)

Figure 6

The color boxes indicate RSCU values. The RSCU is frequency of specific codon usage in each coding position.

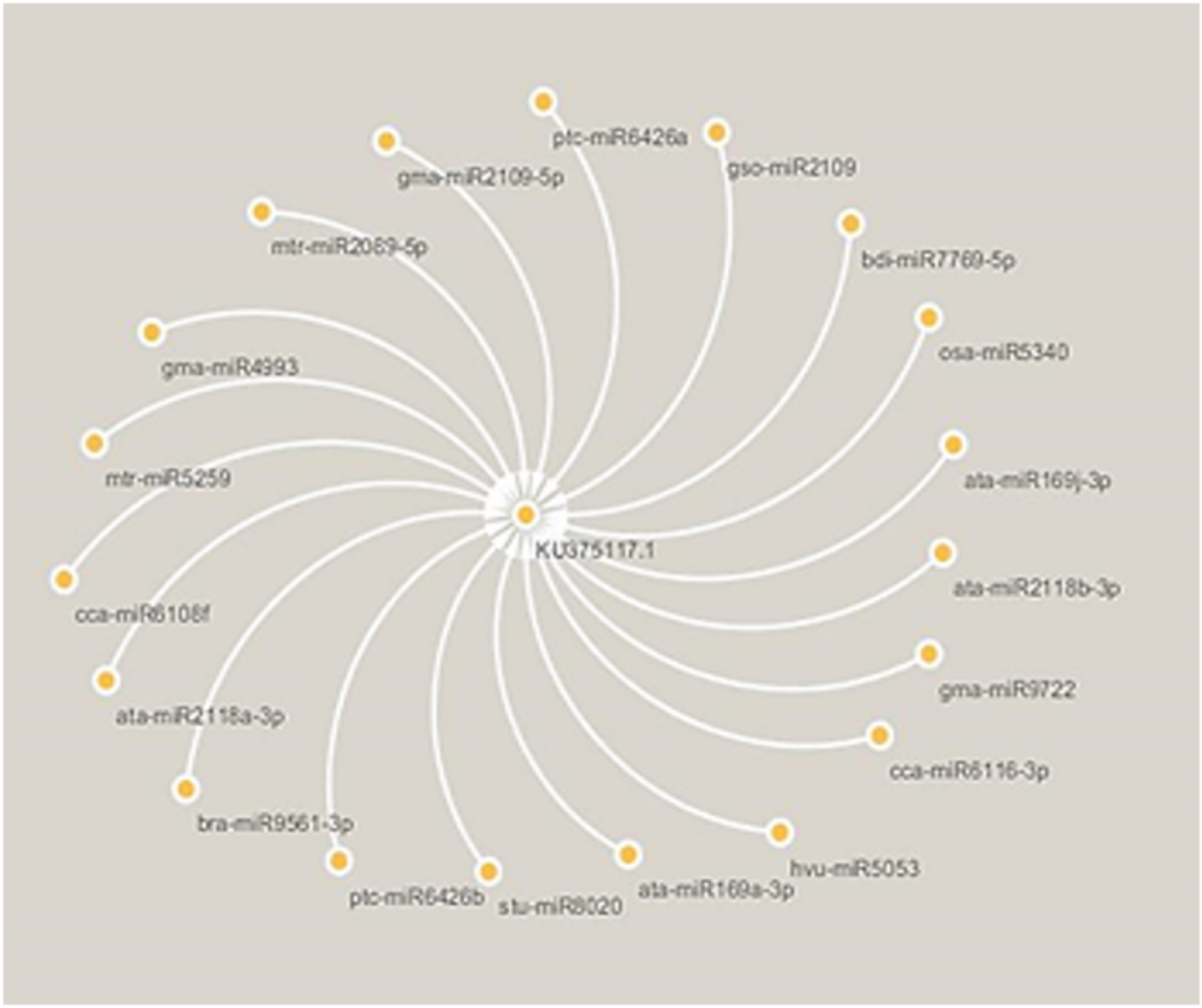

Figure 7

Interaction network of ObGAPDH-targeted miRNAs.

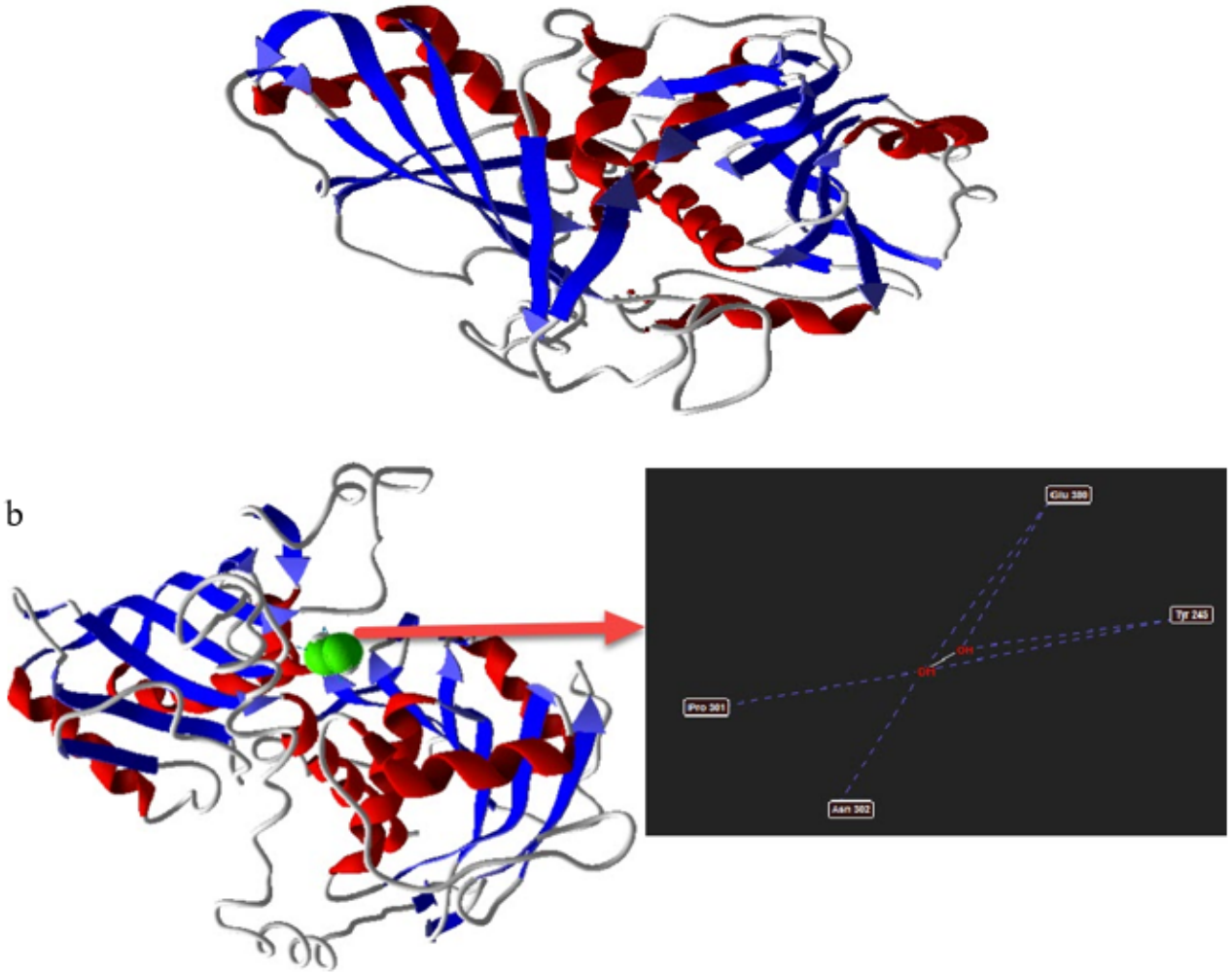

Figure 8

Protein structure and molecular docking of ObGAPDH (**a**) Homology modeling of the ObGAPDH protein structure using the I-TASSER software. **(b)** Protein and ligand docking and the interaction of the ligand-protein in this pose was through amino acid and ligand atoms.

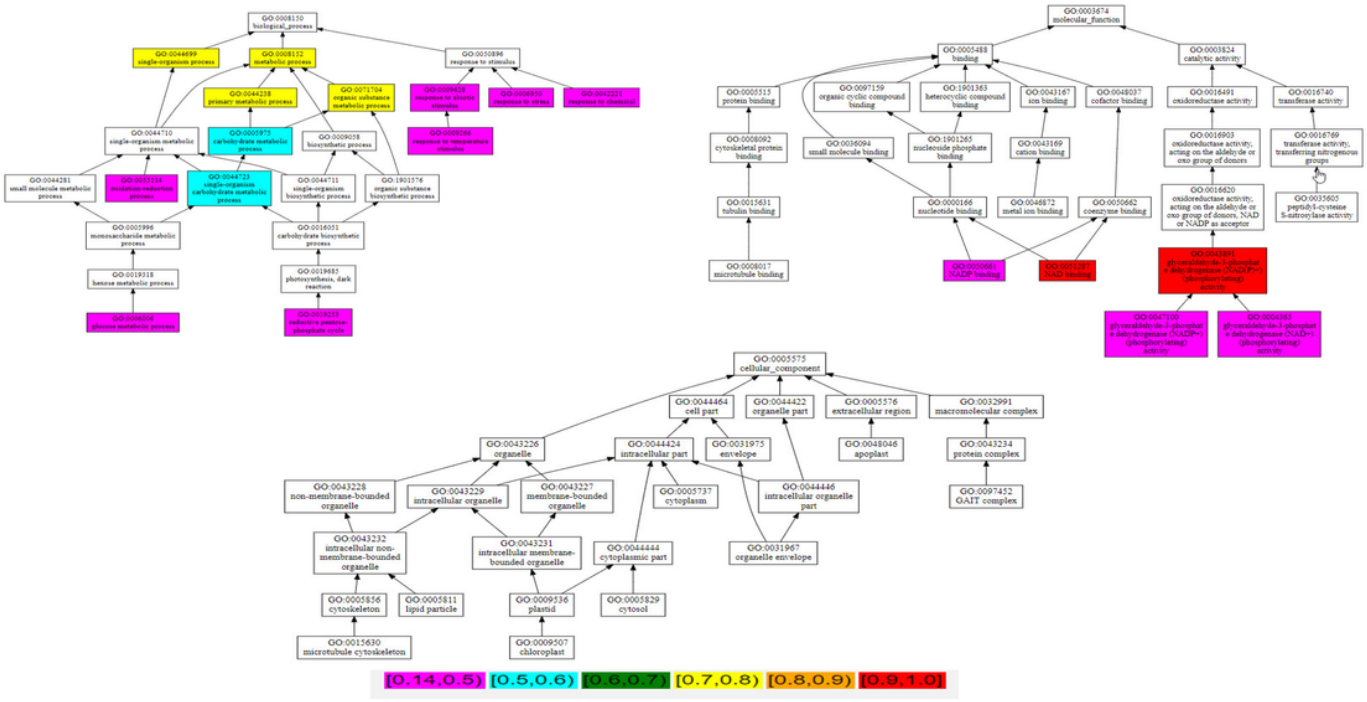

Figure 9

Gene ontology was exhibited in terms of molecular function, biological process, and cellular comportment of ObGAPDH protein using the MetaGO tool.

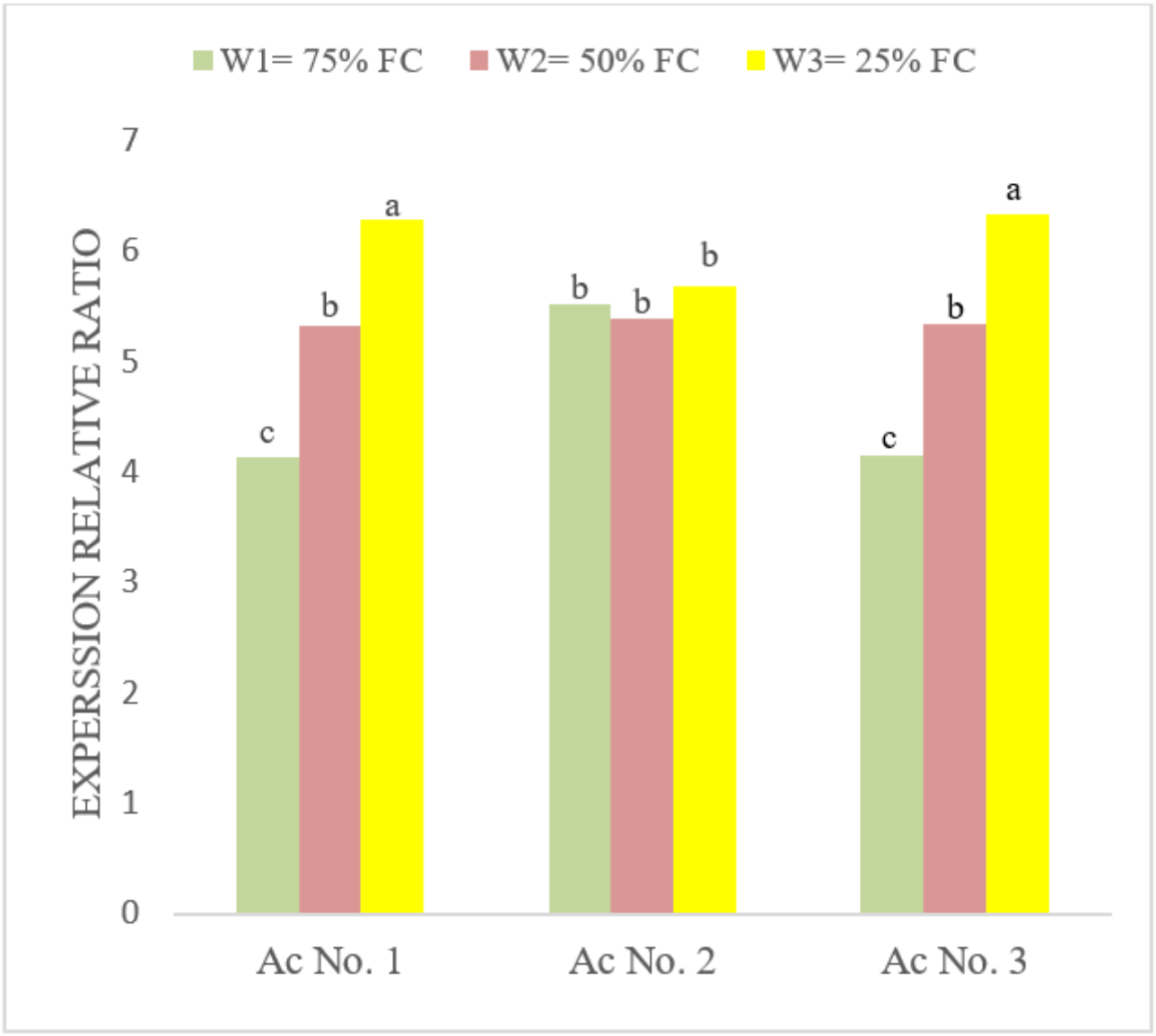

Figure 10

Gene expression levels of the *ObGAPDH* in the three cultivars of *Ocimum basilicum* under different treatments of water shortage stress (*W1*, *W2*, *W3* are 75, 50 and 25 % FC, respectively). Different letters (a, b, c) indicated above the bar represent statistically significant difference at $p \leq 0.05$ (Duncan's multiple range test).