



Gene Profile Analysis and Molecular-physiological Evaluation of Tomato Genotypes under Drought Stress

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Received October 9, 2021; Accepted December 18, 2021; Online Published September 10, 2022

Abstract

Introduction: It is believed that the identification of the differentially-expressed genes is extremely important for the clarification of the complex molecular mechanisms under drought conditions. This study aims to identify candidate genes in tomato genotypes under drought stress through transcriptomics analysis, investigate the expression of these genes, and also some physiological parameters.

Materials and Methods: To the analysis of transcriptome profiles of sensitive and tolerant tomato genotypes under drought stress, three up-regulated genes were selected, including Chlorophyll a-b binding protein3 (CAB3), S-adenosylmethionine decarboxylase proenzyme (SAMDC), and 1-aminocyclopropane-1-carboxylate synthase 9 (ACS9). After bioinformatics analysis, the tomato genotypes were subjected to drought stress and gene expression was determined using Real-Time PCR. Physiological parameters of genotypes were also measured by spectrophotometer-based methods.

Results: According to the results, these three genes play a key role in stress tolerance. Expression of the *CAB3* gene in both sensitive and tolerant genotypes was not significantly different compared to the control. This is while the *SAMDC* gene decreased in both genotypes, the *ACS9* gene decreased in the sensitive genotype and also increased in the tolerant genotype. The physiological analysis also showed that under stress conditions, the photosynthetic system of the plant was disrupted and the chlorophyll content was reduced. However, proline content and antioxidant enzymes activity increased, in which their quantity in the tolerant genotype was significantly higher than sensitive.

Conclusions: In accordance with the obtained findings, it can be stated that under drought stress, due to damage to the lipid membrane, malondialdehyde content also increased, in which the sensitive genotype was more affected.

Keywords: Bioinformatics Analysis, Drought Stress, Gene Expression, Physiological Parameters, Tomato, Transcriptome

Citation: Ghanbari Moheb Seraj R, Tohidfar M, Ahmadikhah A. Gene Profile Analysis and Molecular-physiological Evaluation of Tomato Genotypes under Drought Stress. J Appl Biotechnol Rep. 2022;9(3):747-62. doi:10.30491/JABR.2021.309641.1459

Introduction

Water deficit represents a major global abiotic stress that limits plant productivity by inhibiting plant growth and development.¹ Plants, as sessile organisms, have developed different systems to survive under undesirable and fluctuating stress conditions, such as drought avoidance, escape and tolerance.² The germplasm of most of the cultivated crops contains several unknown drought stress tolerant genes. Besides, many reports indicated the wild species of most modern cultivars possess abiotic stress tolerance genes.³ Therefore, characterizing the differentially expressed genes is important to the clarification of the complex molecular mechanisms under drought conditions in wild and agronomic plants.⁴ In addition to genes, physiological parameters are also affected by drought stress. Some physiological changes induced by drought include chlorophyll, proline, antioxidant enzymes,⁵ and high production of Reactive Oxygen Species (ROS) in cells, which causes cell damage and plant death.⁶ Tomato is an economically important crop worldwide, which is sensitive to a series of abiotic stresses, especially drought,⁷ and is a plant that requires a large amount of water

for its development.⁸ Adequate genetic variation for abiotic stress tolerance exists within the cultivated tomato (*S. lycopersicum*), as well as in its related wild species such as *S. pimpinellifolium*.⁹ Despite their wide natural variation, rather limited efforts have been devoted to identifying these variations in tomato to produce drought-tolerant cultivars.¹⁰ Therefore, in this study, we identified candidate genes in tomato genotypes under drought stress through transcriptome analysis. We then examined the expression of these genes under water stress using real-time PCR. Finally, to further confirm, some important physiological traits were also measured and compared with gene expression.

Materials and Methods

Identification of Candidate Genes

Transcriptome profiles of sensitive (*Solanum lycopersicum*) and tolerant (*Solanum pimpinellifolium*) tomato genotypes under control and water deficit conditions were obtained from GEO, accession number GSE39894. The data were analyzed using the GEO2R program and the expression ratio

of treated genes to control genes was calculated and determined by log FC. Then, the probes with log FC above +2 and below -2 were selected. Subsequently, the probe sequences were received from the PLEXdb site and BLAST was performed on them. The most similar sequence accession was obtained and by using the NCBI or UniProt database, its UniGene code was determined and recorded. At the same time, this sequence was also BLAST at the TAIR site and *Arabidopsis* UniGene was also received. This operation was conducted for all probes. Heat map analysis was used to compare the expression of the genes, that this analysis was performed using R software and the *pheatmap* package. The functional category was determined by ShinyGO v0.61 and comparing gene lists was performed by Venny 2.1.0 program. The program STRING was used to determine the interaction of proteins, in which the UniGene codes of *Arabidopsis* were introduced into the program. Then, the node1 and node2 data were saved and used to plot the gene network by Cytoscape software. The gene network was plotted using the CytoHubba plugin, based on three MNC, degree, and closeness algorithms. Finally, the networks obtained from the three algorithms were compared and the genes with the highest interaction were selected.

Bioinformatics Analysis of Genes

KEGG database was used to study the photosynthetic pathway and metabolism of selected genes. The location of protein activity and placement was determined using the online software WOLF PSORT and Target p, respectively. Gene expression changes in different plant tissues and developmental stages of tomato were investigated using the Genevestigator software. The stability and instability, hydrophobicity, and isoelectric point of the proteins encoded by these genes were distinguished using the ProtParam server software. The enzymatic reactions and enzyme products and reaction pathways were determined using the BRENDA database.

Molecular Experiments

Plant Material and Stress Treatment

The seeds of two tomato (sensitive and tolerant) genotypes were prepared from the company of Pakanbazar, Isfahan, Iran. Seeds were surface sterilized with an aqueous solution of 70% ethanol for 30 s and 30% Sodium hypochlorite for 5 min and finally washed with sterile distilled water 2-3 times. For *in vitro* germination, seeds were cultured in the Murashige and Skoog (MS) medium with vitamins, sucrose (3% w/v), and solidified with 0.6% Agar (Merck, Germany). Before autoclaving at 121 °C for 20 min, the pH of the medium was set to 5.8. All of the cultures were maintained at 25 °C with a photoperiod of 16 h using a Photosynthetic Photon Flux Density (PPFD) of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps for 30 days. After germination

and reaching the four-leaf stage, the plantlets transferred to pots containing perlite and covered with polyethylene bags and were irrigated every day for 8-10 days and were kept in the culture room with the above condition. The covers were gradually removed and plants were transferred to large pots comprising garden soil for 20 days.

Then 6-8 leaf seedlings were transferred to field conditions. The field site was located in Shahid Beheshti University, Tehran, Iran (51.23°N, 35.48°E, and 1769 m above mean sea level). Air temperature and relative humidity were recorded from the meteorological site (Table1). The soil composition consisted of 1/3 clay, 1/3 sand, and 1/3 leaf composts. The field area was 70 m². Furrows were created to implement this study. The space of plants on furrows was 1 m and between furrows was 1 m. In general, seven furrows were created and cultivation was performed as follows: treated plants of tolerant genotype, empty furrow, control plants of tolerant genotype, empty furrow, treated plants of sensitive genotype, empty furrow, and control plants of sensitive genotype. Ten plants were cultured on each furrow, so the total number of plants cultivated on the field was 40. After 20 days (before the flowering stage), drought stress was applied to the plants, in which the plants were subjected to drought stress (without irrigation) for five days, but control treatments were irrigated by the previous method.¹¹ Then, at 9 a.m., the medium-sized intact leaves were separated from the middle part of the plant stem and placed in sterile foil and transferred to a -80 freezer. The number of replicates for each treatment was three replicates. The collected samples were used for molecular and physiological measurements.

Primer Design

After selecting the genes, their nucleotide sequences were obtained from the NCBI site and then BLAST was performed. Next, high-similarity sequences were selected and aligned using T-Coffee software. From the conserved areas, near the end of poly adenine, the primer design was performed. The Polymerase Chain Reaction (PCR) product length was considered to be between 150 and 250 bp. Homodimer, heterodimer, stem-loop, GC percent, and TM temperature were measured using Vector NTI and Oligo7 software. The primers were then synthesized at Bioneer Company of South Korea. Primer sequences of the candidate genes, as well as the housekeeping gene, are demonstrated in Table 2.

Total RNA Isolation and cDNA Synthesis

Total RNA of each sample was isolated from approximately 0.2 g of fresh leaves using a total RNA kit (RB1001, RNA, Iran). After RNA extraction, gel electrophoresis (1% Agarose) was used to visually assess the quality of the extracted RNA. Before cDNA synthesis, RNA were treated with DNase I (RB125A, RNA, Iran) to remove any genomic DNA contamination.

Table 1. Different Growth Stages of Tomatoes Along with the Exact Date and Average Temperature and Humidity of the Growing Environment

Stages	Start Date	Average Temperature	Average Humidity
Seedling	2018.06.22	25 °C	50%
Vegetative	2018.07.23	25 °C	50%
Budding	2018.08.23	26.1 °C	25%
Initiation of drought stress	2018.09.06	26.1 °C	25%
Sample collection	2018.09.11	26.1 °C	25%

Table 2. Primer Sequence of Candidate and Housekeeping Genes

Primer Name	Primer Sequence	PCR Product Length(bp)	TM	PCR Amplification Efficiency
ACS9	F:ATGCGACATCTACTAAGTTC R:GATTCTGGTGATTAGTTGCGTTG	152	57 61	1.83
SAMD	F:TGAACCTGCCGATGCCAATAATGTG R:GCTCTGAAATCGTAACCCACTGCT	140	66 65	1.99
CAB3	F:GTAATGGTGTCAAGTTCGGTGAG R:GGTCTTCAGCAAGGCTAATG	157	63 61	2.05
18SrRNA	F:ATGATAACTCGACGGATCGC R:CTTGGATGTGGTAGCCGTTT	200	56.7 57.2	1.97

Subsequently, the first-strand cDNA was synthesized in a 20 µl reaction mixture (RB125A, RNA, Iran) following the manufacturer's instructions. After cDNA synthesis, PCR was performed using the primers, and products were visualized by agarose gel electrophoresis.

Gene Expression Analysis

Quantitative Real-Time PCR amplification was performed in 48-well plates with an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems, CA, USA) using SYBR® Green Real-Time PCR Master Mix (RB120, RNA, Iran). The PCR reactions were prepared in 20 µl volumes containing: 1 µl of synthesized cDNA, 10 µl 2 × SYBR, 1 µl of 1:20 diluted each primer, and 7 µl deionized water. The reactions consisted of an initial step of 95 °C for 5 min, followed by 40 denaturation cycles at 95 °C for 1 min and primer annealing at 62 °C for 1 min (for all genes). Next, the melting curves ranging from 60 °C to 95 °C were evaluated in each reaction to investigate the specificity of the amplicons. Biological triplicates of all samples were used for the qRT-PCR analysis, and three technical replicates were applied for each biological sample. The threshold cycle (Ct) was calculated using the $2^{-\Delta\Delta C_t}$ method.¹² Efficiency and cycle threshold values were computed using the LinRegPCR quantitative PCR data analysis program,¹³ and relative expression levels were computed using the REST method.¹⁴

Physiological Experiments

Chlorophyll a, b and a + b

Chlorophyll a and b content were determined according to Arnon¹⁵ and Mackinney¹⁶ methods, respectively. A total of 0.2 g of a plant sample was added to 2 ml of 80% acetone and grounded to a homogenate. Then, the solution volume with acetone 8% reached 25 ml and was centrifuged at 4,000 rpm for 10 min. The absorbance of the solutions was recorded at 646 and 663 nm and their amount was calculated using the following equations:

$$chla(mg/g\ FW) = (12.25(A663) - 2.55(A646)) \times V/W \times 100$$

$$chlb(mg/g\ FW) = (22.31(A646) - 4.91(A663)) \times V/W \times 100$$

$$\%v/w = \frac{\text{weight of solute (in g)}}{\text{volume of solution (in mL)}} \times 100$$

Proline Content

Proline content was extracted from a sample of 0.5 g fresh leaf material samples in 3% (w/v) aqueous sulphosalicylic acid and was estimated using the ninhydrin reagent according to the method of Bates et al.¹⁷ The absorbance of the soluble was measured at 520 nm using a spectrophotometer. A calibration curve was created with the proline as a standard. Finally, the proline content was measured by the following equation:

$$\text{Proline } (\mu\text{mole/gr of fresh weight}) = \frac{\text{proline}(\mu\text{g/ml}) \times \text{toluene (ml)}}{115.5 \mu\text{g} / \mu\text{mole}} \bigg/ \frac{\text{g sample}}{5}$$

Malondialdehyde (MDA)

Lipid peroxidation was specified by the Stewart and Bewley¹⁸ method. In this stage, 0.5 g of leaf sample was homogenized in 4 ml of 1% (w/v) trichloroacetic acid (TCA), and then centrifuged at 15,000 g for 10 min. Then, 4 ml of 0.5% (w/v) TBA in 20% (w/v) TCA was added to 2 ml of the supernatant. The mixtures were heated at 95 °C for 30 min and then quickly cooled in an ice bath. Finally, the mixture was centrifuged at 10,000 g for 10 min, then the absorption of samples was read at 532 nm and the quantity of nonspecific absorption was read at 600 nm and subtracted from ABS 532. The MDA content was measured by the extinction coefficient of 155 cm⁻¹mmol⁻¹.

Antioxidant Enzymes

Preparation of Leaf Enzyme Extract

Fresh leaves (0.2 g) were homogenized in 1200 µl of 0.2 M Na-phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Then, the homogenate was centrifuged at 15000 g for 20 min at 4 °C. The supernatant was used for APX and CAT activity

assay. To avoid enzyme inactivation, all procedures for enzyme extraction and activity determination were carried out on ice bath.

Ascorbate Peroxidase (APX)

The activity of the APX enzyme was measured by using Nakano and Asada's¹⁹ method. The reaction mixture contained 100 µl potassium phosphate buffer 1 M (pH 7.8), 50 µl hydrogen peroxide, 10 mM and 50 µl ascorbate 10 mM, and 10 µl enzyme extract, in a total volume of 1 mL. The reaction started by adding H₂O₂ and was performed at 25 °C. The oxidation rate of ascorbate was determined by the decrease in absorbance at 290 nm for 3 min. Reaction mixture without enzymatic extracts was used as a spectrophotometer blank. Finally, the activity of the enzyme was calculated using the following equation:

$$\text{Enzyme activity (Units/L)} = \frac{(\Delta \text{Abs} \times \text{Total assay volume})}{(\Delta t \times \epsilon \times l \times \text{Enzyme sample volume})}$$

Where Δt is the time of incubation (min), ΔAbs is the change in absorbance, ϵ is the extinction coefficient of substrates ($2.8 \text{ M}^{-1} \text{ cm}^{-1}$), and l is the cuvette diameter (1 cm). Enzyme activity (Unit) was defined as the amount of enzyme that oxidized 1 µmol of substrate/min.

Catalase Enzyme (CAT)

The activity of CAT was determined by the Aebi²⁰ method. The reaction mixture contained, 0.01 M H₂O₂ and 0.05 M potassium phosphate buffer pH 7.0 in a total volume of 1 ml. The reaction was initiated by the addition of 20 µl enzyme and the rate of utilization of H₂O₂ was measured for an initial 3 min by measuring a decrease in absorbance at 240 nm. Reaction mixture without enzyme extract was used as a control. The following formula was used to measure enzyme activity:

$$A = \frac{\Delta A_{240} \times df \times 1000 \times 2}{\epsilon t}$$

A = Specific activity of enzyme according to µM decomposed H₂O₂ in one minute and one ml of protein extract

$$\Delta A_{240} = A_{\text{Start}} - A_{\text{End}}$$

df = Dilution factor (df=50)

1000 = convert mM to µM

2 = volume of Cuvette (2 ml)

t = Reaction time

ϵ = Extinction coefficient of hydrogen peroxide ($40 \text{ Mm}^{-1} \text{ cm}^{-1}$)

Statistical Analysis

Statistical analysis for all molecular and physiological data was performed using R version 3.5.3²¹ and RStudio version 1.1.463.²² Data were analyzed by ANOVA for a completely

randomized design with treatments as fixed effects and replicate as random effect. Mean values were compared using Duncan test function provided in the *agricolae* package²³ at 5% significance level of probability.

Results

Identification of Candidate Genes

To compare the expression of identical genes (common genes) in the two genotypes, heat map analysis was used, in which red color indicated up-regulate and blue color indicated down-regulated genes (Figure 1). According to Figure 1, heatmap clustering refers to the task of grouping together a set of samples based on the similarity of their gene expression patterns, and as much as gene expression is different, they fall into more distant clusters (Figure 1).

Stress-responsive genes were identified using functional analysis. In sensitive genotype, up- and down-regulated genes had 13.88% and 25% of stress response genes, respectively, whereas in tolerant genotype, up- and down-regulated genes were 18.91% and 19.60%, respectively. It is clear that in the tolerant genotype, stress-related genes are more active. As shown in Figure 2, 27.7% of the down-regulated genes were common between sensitive and tolerant genotypes, whereas only 6.4% of the up-regulated genes were common between the two genotypes. Interestingly, one gene showed increased expression in the tolerant genotype and decreased expression in the sensitive genotype, whose unigene code was at .2572 (Figure 2).

According to the Figure 3, in drought stress conditions, *FBA2*, *CEL2*, *HSP21*, *CAB3*, *ASN3*, *GAD5*, *HSA32*, *SSP5*, etc. genes in sensitive genotype and *HSP21*, *HSA32*, *PSY*, *ACS9*, *SAMDC*, *GAD5*, *SGB1*, *ABCF1*, *IAA19*, *FBA2*, *SMC2*, etc. genes in tolerant genotype showed increased expression, while *DOX1*, *EXLB1*, *XTR6*, *OSM34*, *LAC7*, *LRX1*, *PPC3*, *ICL*, *RHS19*, *LAC7*, *APR1*, *XTH26*, *SKU5*, *ETR2*, *RTE1*, *ERF1*, *EFE*, *VND7*, etc. genes in sensitive cultivar and *HRGPI*, *RHS19*, *FLS5*, *LAC7*, *DOX1*, *FRU*, *ICL*, *PPC3*, *CHI*, *EFE*, *XTH7*, *PUP4*, *ARP4*, *CBSX3*, *XTH25*, *PME3*, *XPL1*, *SMO2*, etc. genes decreased in tolerant cultivar. STRING only shows the associations between proteins in a pathway or protein complex with a high, uniform score (0.900), and in order to find the hub genes with a high degree of connection, we need to draw a gene network for these proteins.

We plotted a gene network based on a CytoHubba plugin (Figure 4). CytoHubba was employed to explore PPI network hub genes. It actually provides a user-friendly interface to explore important nodes in biological networks and calculates using 11 methods. In the present study we used three algorithms of Closeness, Degree, and MNC.²⁴ The genes that were most common in the three algorithms have been selected and listed in Table 3. In this study, three genes that were overexpressed in two tomato species were selected

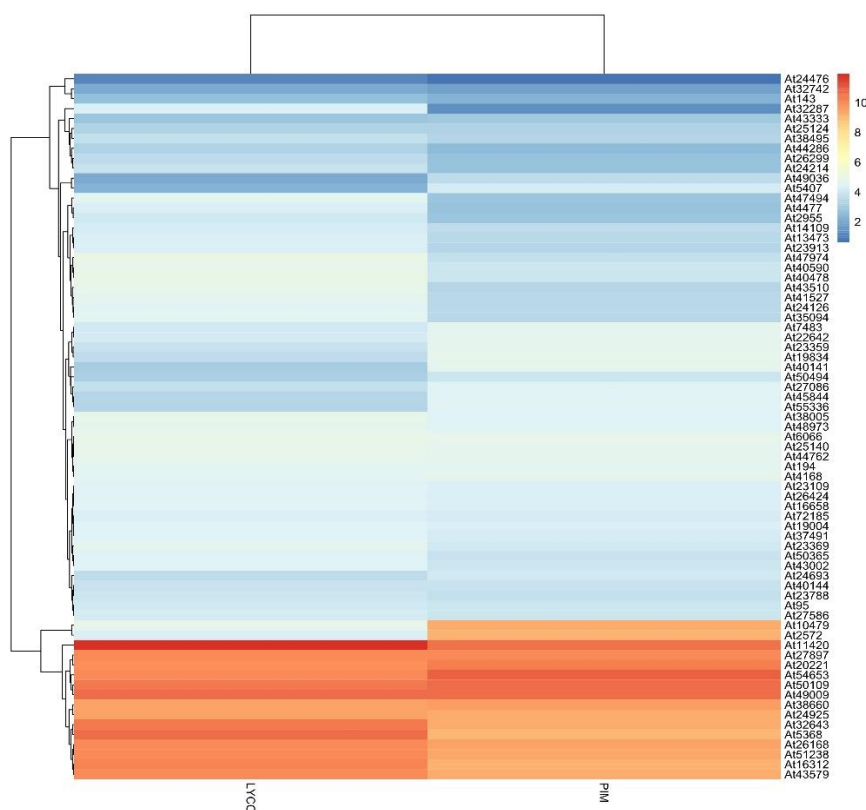


Figure 1. Heat Map for Identical Genes in Two Genotypes of Sensitive and Tolerant with Expression Values as LogFC+7 (To have positive values, seven were added to all values). Red color represents up-regulate genes and the blue color represents down-regulate genes.

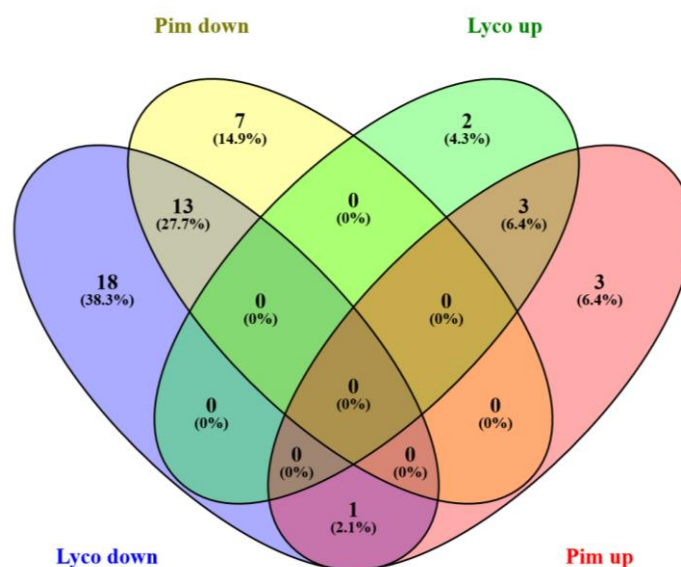


Figure 2. Comparing List of Up- and Down Regulate Genes in Sensitive and Tolerant Tomato Genotypes.

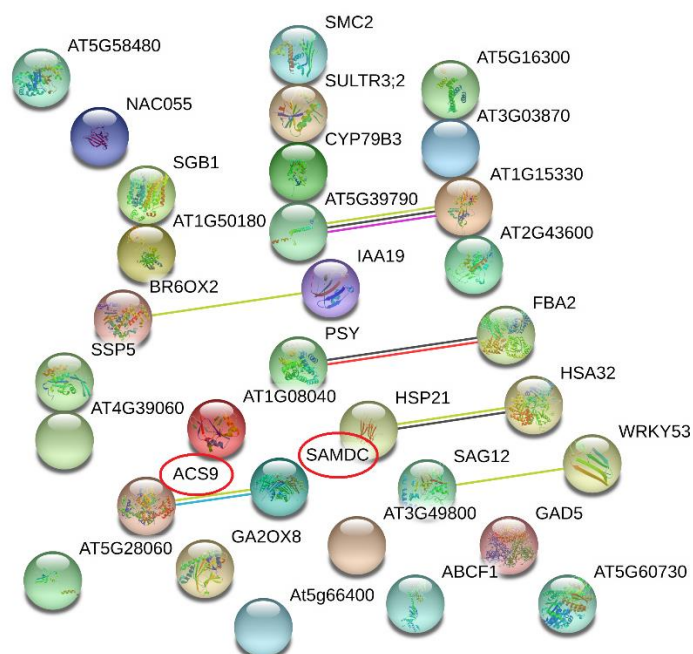
and analyzed. The selected genes in *Solanum lycopersicum* include *CAB3*, and in *Solanum pimpinellifolium* include *SAMDC* and *ACS9*.

Bioinformatics Analysis of Selected Genes

The *CAB3* protein acts on the photosynthetic pathway in chloroplasts and plays a role in receiving light and transmitting

energy to the plant. *SAMDC* protein is involved in two metabolic pathways including, cysteine-methionine and arginine-proline. In both pathways, this enzyme (S-adenosylmethionine decarboxylase) catalyzes S-adenosyl-L-methionine to the S-adenosylmethionine amine. The *ACS9* protein is also involved in the cysteine-methionine metabolism pathway. This enzyme catalyzes the conversion of S-adenosyl-L-methionine to

(c)



(d)

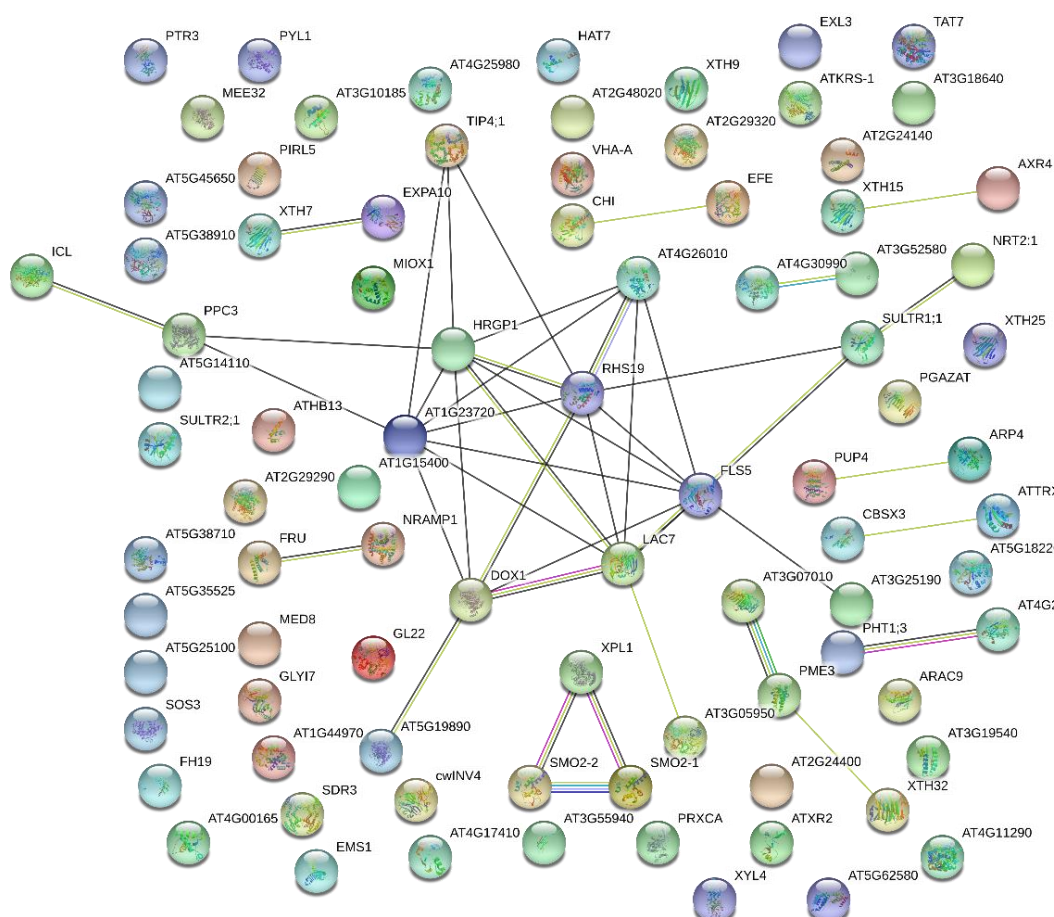


Figure 3. Protein Interaction of Two Sensitive and Tolerant Tomato Genotypes with Increased and Decreased Expression. (a) Up-regulate genes in *Solanum lycopersicum*. (b) Down-regulate genes in *Solanum lycopersicum*. (c) Up-regulate genes in *Solanum pimpinellifolium*. (d) Down-regulate genes in *Solanum pimpinellifolium*. In these interactions, each node indicates a protein and the connecting lines of the nodes demonstrate the interaction between those proteins. The color of the connecting lines (strings) in figure 2 represents: Known interactions from curated databases and experimentally determined interactions are denoted by blue and purple strings, interactions based on gene neighborhood, gene fusions and gene co-occurrence are indicated by green, red and dark blue strings while interactions on the text mining, co-expression and protein homology shown by yellow, black and dark purple strings, respectively.

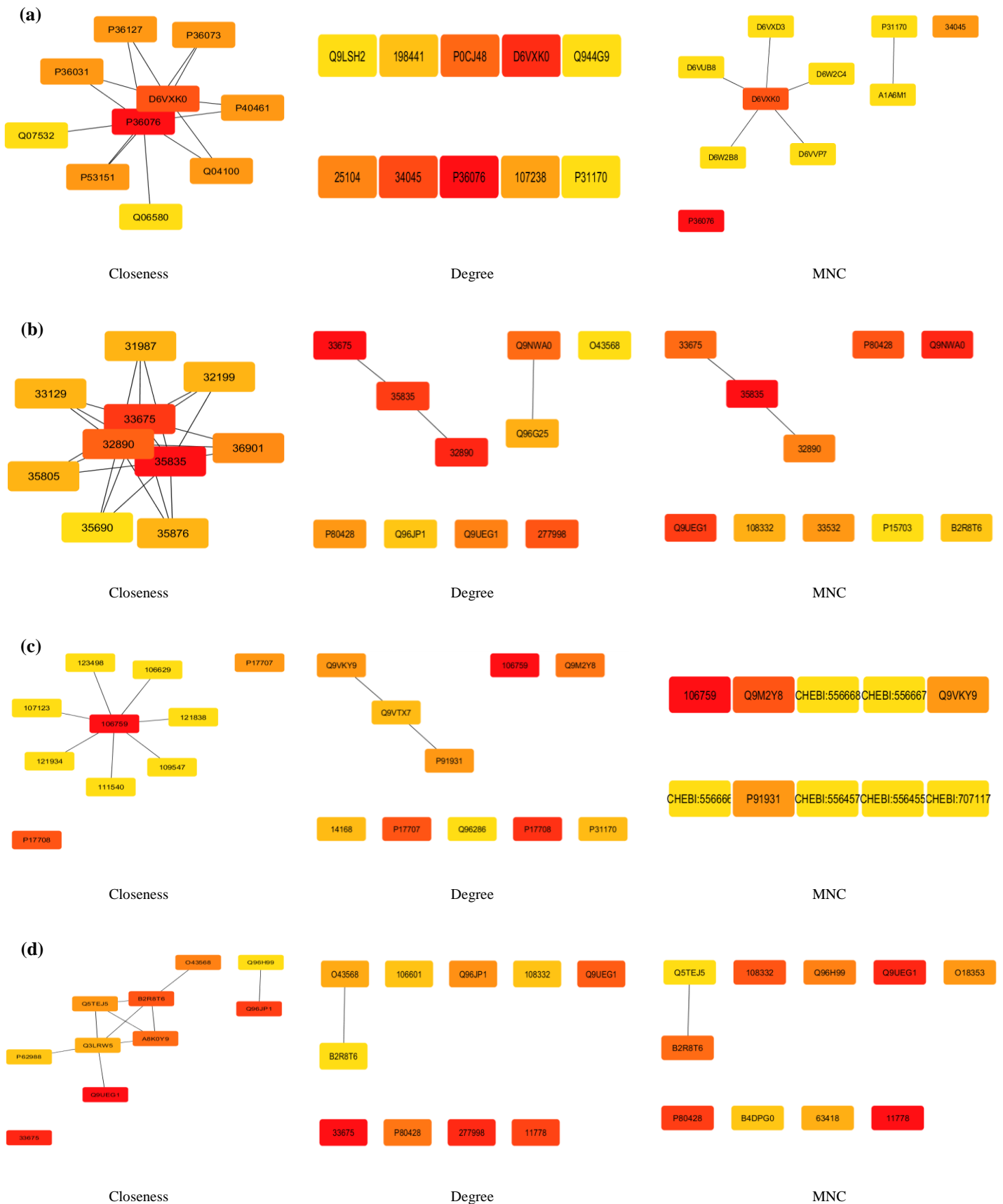


Figure 4. Gene Networks of Two Tomato Genotypes Based on Three Algorithms of CytoHubba Plugins. **(a)** *Solanum lycopersicum* & Up-regulate genes. **(b)** *Solanum lycopersicum* & Down-regulate genes. **(c)** *Solanum pimpinellifolium* & Up-regulate genes. **(d)** *Solanum pimpinellifolium* & Down-regulate genes.

Table 3. Most Common Genes in Three Algorithms of CytoHubba Plugin

Uniprot Accession	Gene Name	Gene Function
<i>Solanum lycopersicum</i> & Up-regulate		
P36076	<i>CAB3</i>	Coenzyme A biosynthesis protein 3
D6VXK0	<i>CAB3</i>	Chlorophyll a-b binding protein 2, chloroplastic
34045	<i>CAB3</i>	Chlorophyll a-b binding protein 2, chloroplastic
P0CJ48	<i>LHCB1.1, CAB3</i>	Chlorophyll a-b binding protein 2, chloroplastic
<i>Solanum lycopersicum</i> & Down-regulate		
33675	<i>ARP4</i>	Nuclear actin-related protein involved in chromatin remodeling; component of chromatin-remodeling enzyme complexes
32890	<i>MED8</i>	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation
35835	<i>CSE2</i>	Mediator of RNA polymerase II transcription subunit 9
<i>Solanum pimpinellifolium</i> & Up-regulate		
106759	<i>AMD1</i>	adenosylmethionine decarboxylase 1
Q96286	<i>SAMDC1</i>	S-adenosylmethionine decarboxylase proenzyme 1
Q9M2Y8	<i>ACS9</i>	1-aminocyclopropane-1-carboxylate synthase 9
<i>Solanum pimpinellifolium</i> & Down-regulate		
33675	<i>ARP4</i>	Nuclear actin-related protein involved in chromatin remodeling; component of chromatin-remodeling enzyme complexes
Q9UEG1	<i>UBC</i>	Ubiquitin C
Q96JP1	<i>SLX4</i>	Structure-specific endonuclease subunit SLX4
B2R8T6	<i>RAD51A</i>	DNA repair protein RAD51 homolog 1
277998	<i>arp42</i>	SWI/SNF and RSC complexes subunit arp42 (Actin-related protein 42) (Chromatin structure-remodeling complex subunit arp42)
11778	<i>GRF1</i>	general regulatory factor 1

1-aminocyclopropane-1-carboxylate (ACC). The location of protein activity and placement in CAB3, SAMDC, and ACS9 were predicted in chlorophyll, cytoplasm, and nucleus, respectively. According to the Genevestigator analysis (Figure 5), the expression levels of the CAB3 and SAMDC genes varied at different stages of development, but at ACS9, it was almost constant. Gene expression of SAMDC increased at vegetative and flowering stages but decreased at the fruiting stage, while, CAB3 was highest at the vegetative stage and decreased at the flowering stage. This is while it increased again at the fruiting stage and decreased at the fruit ripening stage (Figure 5a). The highest expression levels of CAB3, SAMDC, and ACS9 genes were observed in shoots, roots, and callus, respectively (Figure 5b). The instability index, hydrophobicity percentage, and isoelectric point of each gene have been given in Table 4. SAMDC plays a role in the polyamines (PAs) pathway and the biosynthesis of spermidine I, II, III, while, ACS9 is involved in ethylene biosynthesis and L-methionine salvages cycle.

Gene Expression Analysis

Results of gene expression analysis showed that the CAB3 gene in both sensitive and tolerant tomato genotypes did not have a significant difference compared to the control, but the SAMDC gene decreased in both genotypes and the ACS9 gene decreased in sensitive genotype and increased in the tolerant genotype. A comparison of gene expression in sensitive and tolerant genotypes showed that expression levels of all three genes in tolerant genotype were more than sensitive. The expression of CAB3 and SAMDC genes in tolerant genotypes was 2.26 and 2.42 times more than that of sensitive genotype, whereas the ACS9 gene expression in tolerant genotype was 413.23 times more than sensitive

genotype. The amount of ACS9 gene expression was significant to the sensitive genotype since this gene controls ethylene production, therefore appears to play a key role in drought condition tolerance (Figure 6).

Physiological Experiments

The ANOVA analysis of different physiological parameters in tomato genotypes that grew under drought stress conditions indicated that the single effects of genotype and treatment and also their interactions were highly significant ($p < 0.01$) in Chlorophyll a and b, Proline, MDA, CAT, and APX (Table 5).

Chlorophyll a and b

Under drought conditions, chlorophyll a content in the tolerant genotype decreased by 5% compared to the control, while in the sensitive genotype it was 15%. Chlorophyll a in the tolerant genotype was more than the sensitive genotype. The difference in chlorophyll a content in tolerant to sensitive plants was 0.007 in control samples and 0.015 mg/g-1 FW in treated samples, respectively (Figure 7a). Similarly, chlorophyll b decreased by 6% and 11% in tolerant and sensitive genotypes, respectively. In addition, chlorophyll b content in the tolerant genotype was 0.0014 (in control) and 0.0021 mg/g-1 FW (in treated samples), which was higher than the sensitive genotype. According to the above mentioned results, the tolerant genotype had a higher ability to retain chlorophyll a and b than the sensitive genotype (Figure 7b).

Proline

Under dehydration conditions, for osmotic regulation, the proline content of plants increases to prevent cell plasmolysis. In our experiment, under drought stress, proline content increased in both tolerant and sensitive genotypes. Proline

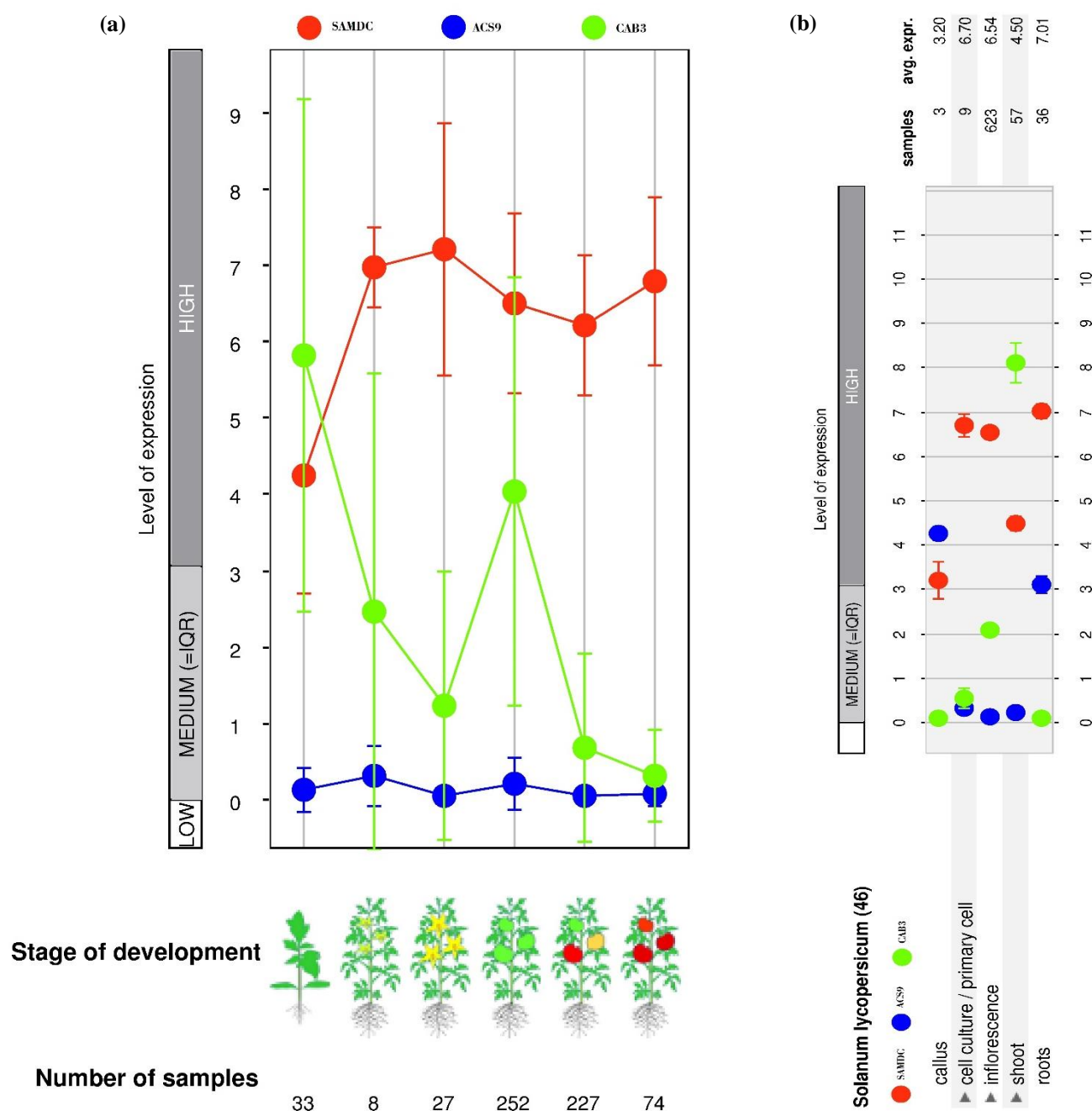


Figure 5. Expression Level of Candidate Genes in Different Tissues and Developmental Stages Using Genevestigator Software.

Table 4. Physicochemical Properties of Candidate Proteins using ProtParam Server

Protein	Instability Index (II)	Grand Average of Hydropathicity (GRAVY)	Theoretical pI	% G+C
CAB3	43.67 (unstable)	0.654	5.06	47.17
SAMD1	43.45 (unstable)	0.691	4.96	41.76
ACS9	45.21 (unstable)	0.820	5.01	40.84

content in tolerant to sensitive genotypes increased by 4% and 5% in control and treated samples, respectively (Figure 7c).

Malondialdehyde (MDA)

Water stress induces lipid peroxidation and MDA accumulation in plants. In both genotypes, MDA increased significantly

under stress conditions. Lipid peroxidation in sensitive and tolerant genotypes was 5.35 and 3.42 times higher than the control, respectively (Figure 7d).

Catalase Enzyme (CAT)

CAT is essential for catalyzing H_2O_2 into water and oxygen. Therefore, increasing this enzyme under stress conditions

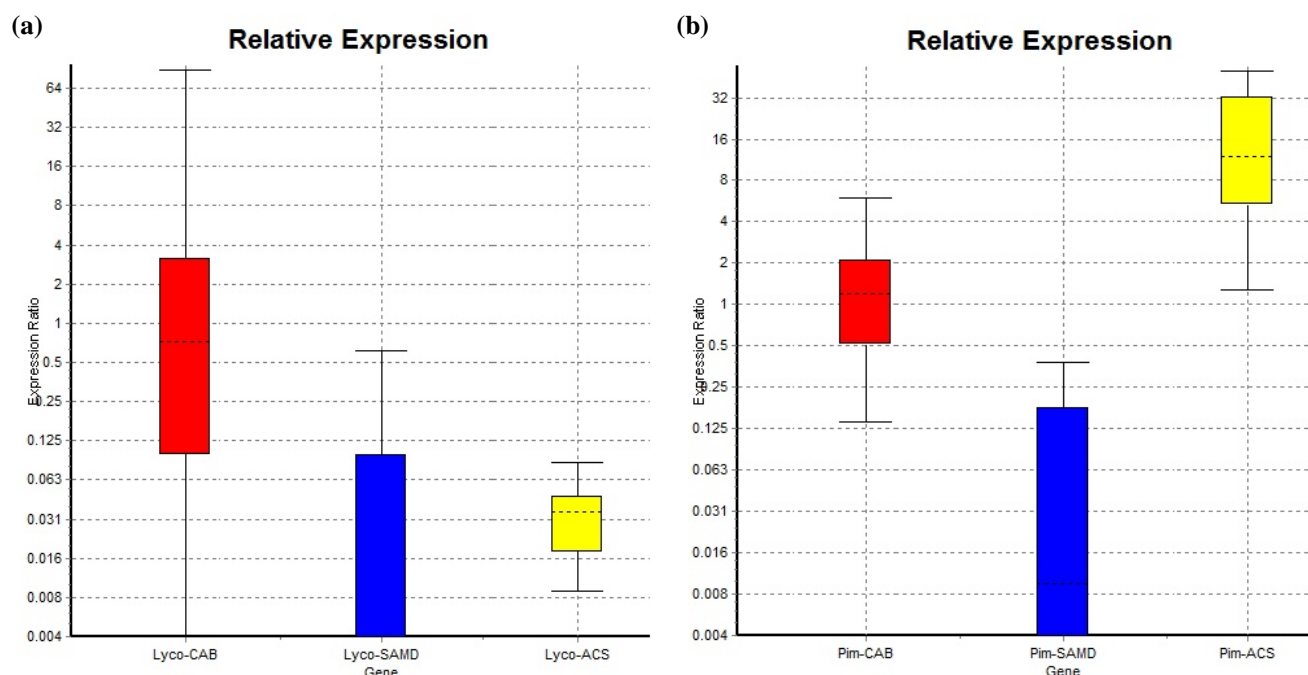


Figure 6. Expression Ratio of Three Genes in Tolerant and Sensitive Genotypes of Tomato. (a) Relative expression of *Solanum lycopersicum*. (b) Relative expression of *Solanum pimpinellifolium*.

Table 5. ANOVA Analysis of Some Physiological Parameters in Tomato Genotypes Under Drought Stress

Sources of Variation	df	Chla	Chlb	Proline	MDA	CAT	APX
Genotype (G)	1	0.001*	0.0008*	2.76**	1.32**	2084**	191**
Treatment (T)	1	0.002**	0.0023**	6.69**	12.8**	6800**	1176**
G×T	1	0.002**	0.0064**	0.13**	1.21**	1694**	963**
Error	6	0.001	0.002	0.003	0.035	34	5

*significance at $p < 0.05$, **significance at $p < 0.01$, ns no significance

protects the plants. In our experiment, the CAT activity had a significant increase in tolerant genotype under stress conditions. As shown in Figure 7e, the activity ratio of this enzyme in tolerant genotype was 2.41 times higher than the sensitive genotype (Figure 7e).

Ascorbate Peroxidase (APX)

The APX activity was increased by water deficit. According to the results, the highest APX activity was recorded in tolerant genotype under drought conditions (215.84 U/ml), and the lowest was obtained in the control treatment in sensitive genotype (127.95 U/ml) (Figure 7f).

Discussion

Water shortage represents a major global abiotic stress that limits plant production by inhibition of plant growth and development.²⁵ Drought induces ROS overproduction and leads to the disruption of membrane integrity and osmotic balance in plant cells. The result of these changes is a decrease in crop quality and quantity, which causes crop yield losses.²⁶ Therefore, it is important to identify the Differentially Expressed Genes (DEGs) under drought stress condition and their expression evaluation, as well as to examine the physiological parameters affected by stress,

including the photosynthetic system, osmolytes (proline), MDA, and antioxidant enzymes (CAT and APX). In this study, three genes *CAB3* (*LHCB*), *SAMDC*, and *ACS9* that up-regulated under drought stress conditions were selected and examined.

Regulation of *LHCB* expression is raised as one of the important plant mechanisms to modulate chloroplast functions.²⁷ Previous reports showed that the members of the *LHCB* family play an important role in plant adaptation to environmental stresses,¹¹ as well as their expression which is regulated by ABA.²⁸ The plants most affected by drought stress (sensitive plants) produce higher ABA levels, thereby increasing the expression of the *LHCB* gene. Liu et al., (2018) reported that *LHCB* genes expression were increased in *Saccharum narenga* in response to water deficit.²⁹ In cotton leaves, chlorophyll a/b-binding genes, which are involved in the light-harvesting complex of photosystem II (PSII) were significantly down-regulated by waterlogging stress.³⁰ Xu et al.,³¹ reported that down-regulation or disruption of any member of the *LHCB* family reduces the responsiveness of stomatal movement to ABA, and therefore results in a decrease in plant tolerance to drought stress in *Arabidopsis thaliana*. In contrast, our results showed that the expression of *LHCB* in both sensitive and tolerant cultivars of tomato

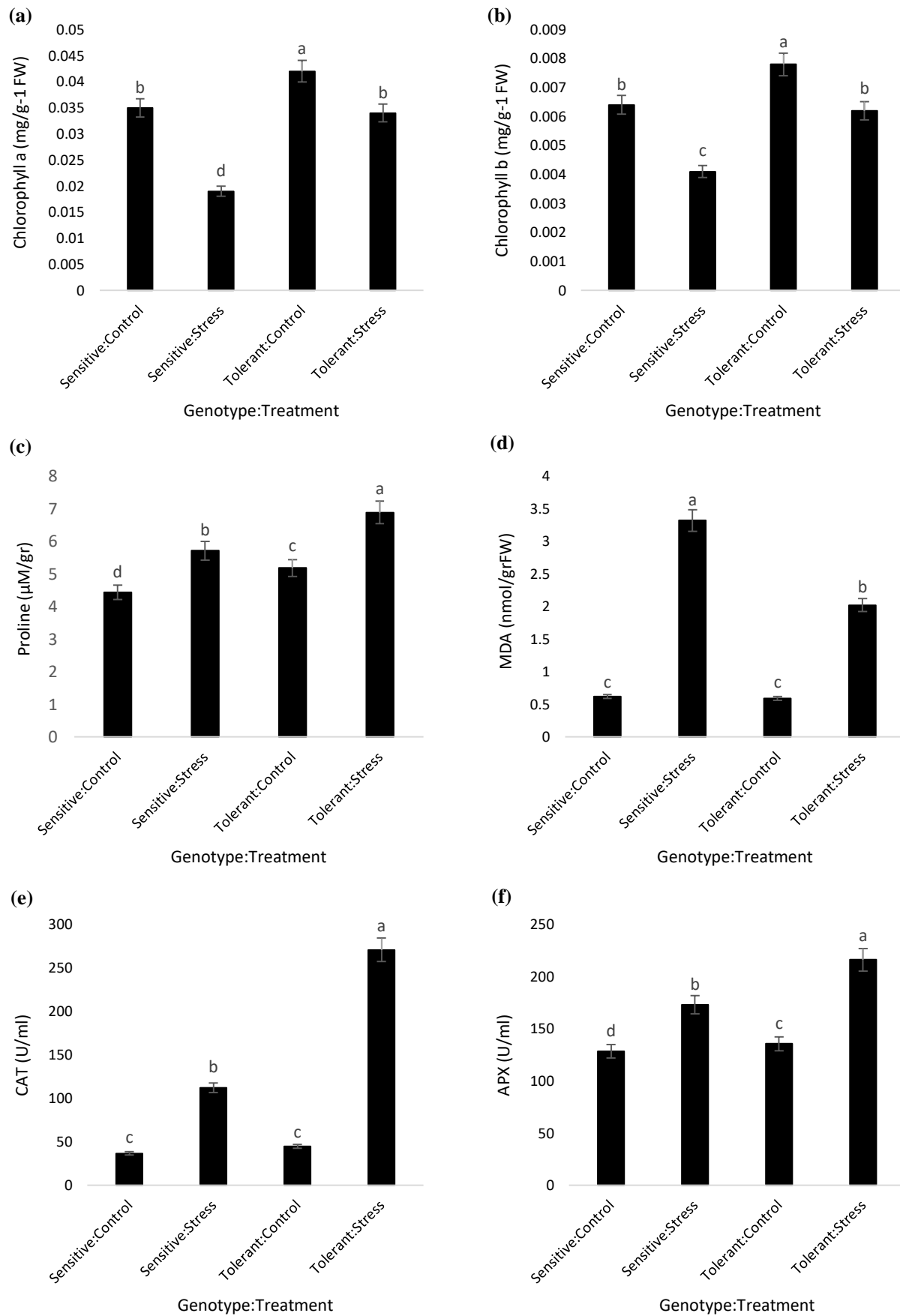


Figure 7. Interaction of Genotypes and Drought Stress Levels on Some Traits of Tomato.

was not significantly different compared to the control group, which could be due to different conditions such as stress duration, plant type, environment temperature, culture medium, etc.

Another up-regulate gene, *SAMDC*, is the key enzyme for the biosynthesis of polyamines (PAs). Prior studies reported that PAs were involved in plant reactions to abiotic stresses such as cold, drought, and salinity.³² *SAMDC* protein is involved in two metabolic pathways: in the cysteine-methionine metabolism pathway, in the absence of this enzyme, S-adenosyl-L-methionine is converted to ethylene and homocysteine.³³ This enzyme inhibits ethylene production and aging in the plant,³⁴ therefore, the plants are less affected by adverse environmental conditions. In the arginine-proline metabolism pathway, this enzyme results in the production of both spermine and spermidine. These compounds in plants embrace a wide range of biological processes, such as cell division, growth, differentiation, aging, somatic embryogenesis, root and shoot formation, pollen development, flowering, fruit maturation, and response to environmental stress.³⁵ Marco et al.,³⁶ indicated that overexpression of the *SAMDC1* gene in *Arabidopsis* produces Spm accumulation and leads to plants with improved tolerance to salt stress. Results of Pál et al.'s study³⁷ showed that there is a positive feedback loop between ABA and PAs. Overexpression of the genes of PA synthesis enzymes, such as *SAMDC*, resulted in increased ABA biosynthesis. This is while ABA plays a central role in the improvement of plant drought resistance.³⁸ In our experiment, the expression of this gene decreased in both sensitive and tolerant cultivars. Perhaps one of the reasons that tomato is sensitive to drought conditions is to decrease the expression of this gene.

Finally, the last gene to be studied is *ACS9*. This enzyme eventually results in the production of ethylene and 2-exobutanate.³⁹ Developmental steps, stress conditions, and the presence of phytohormones (such as auxin and ethylene itself) are the principal inducers of transcriptional variations in *ACS* genes.⁴⁰ RT-PCR analysis revealed that abiotic stressors induce the expression of the wild-type *ACS* gene which is involved in ACC production. Under stressful conditions, plants can produce high levels of ACC that subsequently increase ethylene concentrations and lead to an inhibition of plant growth and development.^{41,42} Ethylene regulates a defense response, mostly in full-grown leaves, and growth response in young leaves.⁴³ Nascimento et al.,³⁹ report that ACC itself, independently of ethylene, may play a role as a signaling molecule that controls plant growth and development. It is interesting that ACC conjugates with other phytohormones such as jasmonic acid, which is closely linked to plant defense.⁴⁴ This suggests a role for ACC in phytohormone crosstalk and a possible effect in mediating some plant defense responses. Similar to the results of Eun et al.,⁴² in our results, the expression of *ACS* was increased

in wild cultivar (tolerant) but decreased in the sensitive cultivar.

Our three proteins have a positive index, so they are stable at high temperatures. The instability index could be used as an indirect degree of the *in vivo* half-life of a protein.⁴⁵ Proteins with a stability index of more than 40, have an *in vivo* half-life of fewer than 5 h, whilst, the proteins with a stability index of less than 40, have an *in vivo* half-life of more than 16 h.⁴⁶ According to Table 4, the stability index of our proteins is greater than 40, and therefore, they are unstable and their half-life is less than 5 h. The GRAVY index displays the solubility of the proteins, in which positive GRAVY is hydrophobic and negative GRAVY is hydrophilic proteins. The GRAVY index of our three proteins is positive, so they are among the hydrophobic proteins that are a current property in the membranous proteins.⁴⁷ The hydrophobic character of these genes is correlated with genes related to different stresses, such as the LEA,⁴⁸ heat shock protein chaperones, which do binds with non-native proteins and thus inhibit their aggregation during abiotic stress conditions.⁴⁹

Dehydration leads to the induction of signal transduction cascade and ultimately activation of transcription factors, which each activates a set of target genes, including those necessary for the synthesis of protective molecules and amplification of the plant defense system.⁵⁰ Photosynthetic pigments allow plants to attract energy from light, therefore foliar Chlorophyll content is a key factor affecting the performance of plant photosynthesis.⁵¹ In our experiment, dehydration led to a significant reduction in Chlorophyll levels in tomatoes, especially in sensitive genotypes. Several types of research have also explained that water stress, significantly reduces the Chlorophyll a and b of various crops.^{52,53} The decrease in Chlorophyll content under drought stress might be due to reduction in the synthesis of the principal Chlorophyll pigment complexes encoded by the *CAB* gene family, or to the demolition of the pigment-protein complexes that protect the photosynthetic system, or to oxidative damage of chloroplast lipids and proteins.⁵⁴

Proline protects plants from stress by contributing to cellular osmotic adjustment, ROS detoxification, protection of membrane integrity, and enzymes/protein stabilization.⁵⁵ Therefore, the accumulation of proline increases the tolerance of plants under stress conditions. Our results also indicated the accumulation of proline under stress conditions. The tolerant genotype had a higher proline content than sensitive, indicating that it is more tolerant. MDA is a product of peroxidation of unsaturated fatty acids in phospholipids, and the lipid peroxidation level is used as an indicator of free radical damage to cell membranes under stress.⁵⁶ Under stress conditions, MDA content increased in both tomato genotypes, which is significant in the sensitive genotype. Many antioxidants play a pivotal role in hindering the oxidative damage caused by unfavorable conditions, two of

which are CAT and APX. CAT activity in response to drought stress suggests a prominent role for this enzyme in the protection of leaf tissue against oxidative damage. APX also acts as the key enzyme to catalyze the reaction of H_2O_2 into H_2O .⁵⁷ The expression of APX may be activated by various factors, including water deficit.⁵⁸ In our experiment, both antioxidant enzymes increased under stress conditions. It can also be mentioned that the ratio of enzyme activity in tolerant to sensitive genotypes was higher in CAT enzyme than APX.

Conclusion

Tomato is a sensitive plant to drought conditions and so far, many methods have been developed to prevent crop reduction. Since genes in each organism play a decisive role in any situation, in this study we aimed to look for a way to identify stress-tolerant genes. In this study, after the transcriptome analysis, three key genes were found including *CAB3*, *SAMDC*, and *ACS9*. The results of our analysis showed that these three genes play an important role in stress tolerance. The *CAB3* gene lead to the synthesis of chlorophyll pigment complexes, so the decrease in the expression of this gene leads to a decrease in chlorophyll content. *SAMDC* gene is involved in the biosynthesis of polyamines. Polyamines have anti-senescence and anti-stress effects, antioxidant properties as well as membrane and cell wall stability. Therefore, the decrease in its expression led to a decrease in antioxidant enzymes and an increase in lipid peroxidation in sensitive to tolerant genotype. The *ACS9* gene is involved in the ACC production and subsequently in the production of ethylene. Under stress, *ACS9* inhibits plant growth and ACC itself, independent of ethylene, acts as a signaling molecule and controls plant growth. The *ACS9* gene significantly increased in tolerant genotype compared to sensitive cultivar. According to this result, the tolerant plant avoids drought and through ethylene production, it led to the closure of stomatal pores, reduced water loss, and reduced membrane damage. This method is one of the most basic defense systems in tolerant plants.

Authors' Contributions

RGMS performed material preparation, data collection and analysis. MT as the corresponding author read and approved the final manuscript. AA contributed to the study conception and design.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

We thank the Department of Biotechnology of Shahid Beheshti University for providing us with laboratory equipment.

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