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Original Article

# Study of Cannabinoids Biosynthesis-Related Genes in Hemp (*Cannabis sativa* L.) under Drought Stress by in Vitro and in Silico Tools

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**Abstract**

**Introduction:** Cannabinoids can be found as the specific secondary metabolites of hemp (*Cannabis sativa* L.), including Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabichromene (CBC). There are many enzymes, particularly cannabichromene synthase, cannabidiolate synthase and Δ9-tetrahydrocannabinolate synthase, contributing to the biosynthesis of the cannabinoids. Environmental stress, particularly drought, can induce secondary metabolites. In the present study, we have tried to investigate and understand the key factors such as drouth-induced Transcriptional Factors (TFs) involving in the pathway by employing *in vitro* and *in silico* tools.

**Materials and Methods:** After providing the genes' names and IDs from the National Center for Biotechnology Information (NCBI), Transcription Start Sites (TSS) and TATA-box were predicted by the TSS Plant website, as well as involved transcriptional factors. The expression of the genes was assayed under drought conditions by *in silico* and *in vitro* tools, R software and Real-time PCR, respectively.

**Results:** The findings identified all the genes contributing to biosynthesis cannabinoids in drought conditions. There were actually six TF sites and four TF sites for the gene of olivetolic acid cyclase and *AAE1*, respectively.

**Conclusions:** Drought stress can induce overexpression of the genes encoding B3 domain-containing proteins, MLP28, MYB binding site, transcriptional repressor OFP7, and WAK1 as TFs respond to biotic and abiotic stresses in *Cannabis sativa* plants.

**Keywords:** *Cannabis sativa* L., Cannabinoids, Δ9-tetrahydrocannabinol, Cannabidol, Drought, Gene Expression, R Program, Real-time PCR
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## Introduction

Hemp (*Cannabis sativa* L.), categorized as a member of the *Cannabacea* family, is a gramineous, dioecious and annual plant. The leaves of *C. sativa* are claw-shaped including 5-7 toothed leaflets. The plant usually grows up 1-3 meters and has several varieties with a pleasant and strong smell.[1] *C. sativa* is considered as one of the oldest and first plants that has been cultured since the Neolithic period, according to archeological evidence. In the holy book of Hinduism, Vedas, and other remained books from 700-600 BC, Persia was mentioned as one of the main habitats of *C. sativa.* Moreover, the plant was used as medication by Greek physicians about 200-130 BC, as well as Chinese about 100 BC. So, *C. sativa* is always considered as a pharmaceutical–industrial crop, originated from central Asia, which has been universally used for food, fuel, fiber, medication and drug for millennia.[2] There are currently many R&D departments in many business organizations which study and utilize this plant and its secondary metabolites to produce novel medications.[1]

More than 480 secondary metabolites have been identified in the extract of *C. sativa*, such as cannabinoids, alkaloids, flavonoids, stilbenoids, terpenoids, and lignans.[3] Cannabinoids are 22-carbon terpenophenolic compounds, only identified in *Cannabis sp*. The most important identified cannabinoids in *C. sativa* include cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC), cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC).[4] Histochemical and immunochemical studies have indicated that cannabinoids, particularly THC, are mainly biosynthesized in the trichomes of the female *C. sativa*, however, they can be found in stems, pollen, seeds and roots.[5] There are two defined pathways for cannabinoids biosynthesis; the main pathway is the polyketide pathway synthesizing olivetolate as the final production and the precursor of cannabinoids.[4] At first, hexanoyl-CoenzymeA (CoA) synthetase uses ATP to catalyze hexanoyl-CoA from hexanoate and CoA. Then, 3, 5-dioxodecanoyl-CoA is biosynthesized by 3, 5, 7-trioxododecanoyl-CoA (olivetol) synthase. After that, 3, 5-dioxodecanoyl-CoA is used to produce olivetol by the contribution of olivetol synthase. Next, olivetolic acid cyclase catalyzes the biosynthesis of olivetolate.[5] Cannabigerolate (CBGA), as the first cannabinoid, is biologically produced

by olivetolate geranyltransferase. CBGA is considered as the precursor of other cannabinoids including cannabichromete (CBCA), cannabidiole (CBDA), and Δ9-tetrahydrocannabinolate (THCA), biosynthesized in order to cannabichromete synthase, cannabidiole synthase and Δ9-tetrahydrocannabinolate synthase.[4] Finally, CBC, CBD, and THC are biologically synthesized by spontaneous decarboxylation and protonation of CBCA, CBDA, and THCA, respectively (Figure 1).[5]

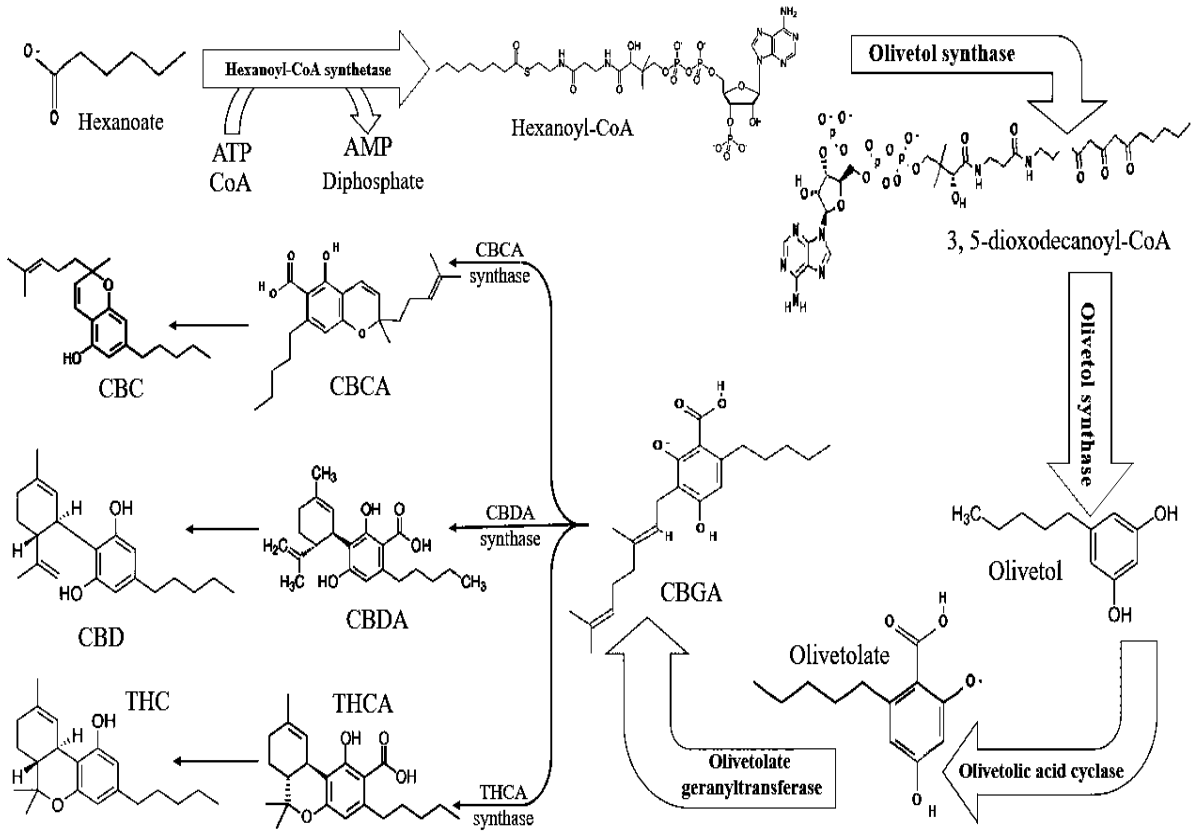


**Figure 1**. The Pathway of the Cannabinoids Biosynthesis.

All these metabolites can be effective for pain relief and declining the symptoms of diseases, as well as complications of medical treatments.[6] CBD, as one of the main secondary metabolites of *C. sativa*, can effectively relieve chronic and non-chronic pain and treat febrile convulsion, inflammation, anxiety, and nausea. Recent studies have demonstrated that the compound can collaborate in the remedy of psychological disorders such as schizophrenia neurological diseases particularly neurodegenerative diseases[7] including Parkinson's, Alzheimer's diseases and Multiple Sclerosis (MS) where the nerve cells are damaged.[8] Besides, antiviral activity has been reported for CBD. CBD can reduce the symptoms of Acquired Immune Deficiency Syndrome (AIDS)[9] especially anorexia nervosa and emaciation, as well as remedy a broad range of cancer including skin, breast, prostate, lung, pancreas, blood and brain tumors.[10] On the other hand, the most prominent cannabinoids is THC as a psychedelic substance binding Cannabinoid (CBs) receptors in many reigns of the brain including the hippocampus, cortex, cerebellum, the limbic system, thalamus, hypothalamus, and brainstem.[11] There are many studies to indicate apoptotic and toxic effects of THC to induce cell death in hippocampus by fragmentizing DNA, and producng free radicals such as reactive oxygen and nitrogen by activating cyclooxygenase (COX).[12] However, cannabinoids are generally beneficial for alleviating pain, treating local contractions, convulsion, and facilitating sleep.[7]

The development and growth of the plants are permanently influenced by environmental stresses. Generally, stresses include unfavorable environmental conditions, as abiotic stress, containing salinity, heavy metal contamination, nutrient deficiency, low and high temperatures, and biotic stress derived from the infection of the pathogens such as bacteria, virus, fungi, nematode, and parasitic plant.[13] The stress derived from water shortage is a constant risk for plant life. Actually, water stress is an environmental condition, caused by lack of precipitation that is known as "drought stress". A common characteristic between drought stress and other water stress such as salinity and osmotic stress is considered to be low water potential.[14] Many complex mechanisms have been evolved in plants resistance against stress for surviving in tough environmental conditions. The biosynthesis of the

secondary metabolites is included as one of the effective mechanisms responding against abiotic and biotic stresses.[15]

The aim of the present study was to identify the effective factors, derived from drought stress, contributing to the pathway to produce cannabinoids, especially THC and CBD, as a natural pharmaceutical compound.

## Materials and Methods

### *Primary Bioinformatic Analysis*

Gene names and IDs of the enzymes involved in cannabinoids biosynthesis were extracted from the NCBI (https://www.ncbi.nlm.nih.gov/). For this purpose, 2000 nt of upstream sequences from the start codon of each gene were used for analysis. TATA box and the TSS were identified for recognizing promoter sites by the TSS Plant website (http://www.softberry.com), as well as involved TFs and regulatory sequences.

### *Genes Expression Assay by in silico Tool*

The gene expression was analyzed by R software (DFvis) (version 3.6.3) as the *in silico* tool, with SSR markers related to drought stress.

### *Plant Culture*

The *C. sativa* seeds were cultured in some pots under greenhouse conditions (Humidity: 50%, Temperature: 25 °C). After germination and growing up to 15 cm, the plants were treated by drought. Irrigation was decreased to 30%. Then, the plants were utilized for *in vitro* assay of the gene expression.

### *Genes Expression Assay by in vitro Tool*

RNA was extracted by NORGEN kit (Cat.51800) for RT-PCR, along with random hexamer sequences in the reaction solution of a kit (K1622, Thermo). The reaction solution was incubated at 42 °C for 60 min then at 70 °C for 5 min. Real-time PCR, as the *in vitro* approach, was employed to analyze gene expression by using a kit (Intron, 25344) containing SYBER Green I dye in a thermocycler (Applied BioSystems StepOne, USA). Also, 40 cycles were completed at 64 °C as the annealing temperature, with three repetitions for each treatment. The results were statistically analyzed by using the SPSS software.

## Results

Four genes, involving in the cannabinoids biosynthesis in *C. sativa*, were identified and used in the study. Notably, the information of the *AAE1* gene was used because there is no data for the gene from *C. sativa* (Table 1). For the recognition promoter region and related elements, 2000 nt of upstream sequences of the genes were assayed by the TSS Plant website. The results of the primary bioinformatics assay represented that there is no significant difference among TSS score of the genes, however, the lowest TATA-box score (4.64) belongs to *AAE1*. This is while the genes encoding olivetolic acid cyclase and CBDA synthase showed the highest score (5.7) for TATA-box (Table 2). About 500 nt of the TSS upstream containing TATA-box was considered as the promoter for identifying TFs. Most TFs, including GCBP-2, AREB2/ABF4, NtWRKY12, UPAAvrBs3Drep16, PacC, and IbNAC1, were predicted for the promoter of the CBDA synthase and lowest TFs (BPC1, FLC, RAVL1, and ANAC092) were observed for the promoter of the gene which encodes THCA synthase. Also, there were six TF sites (Alfin1, BPC1, MADS-box proteins, STF1/HY5, NSP1, and TBF1) and four TF sites (BPC1, FLC, RAVL1, and ANAC092) for the gene of olivetolic acid cyclase and *AAE1*, respectively (Table 3).

### *R Analysis*

According to the bioinformatic analysis of gene expression by the R software, the amount for expressing each locus was predicted, as well as increasing or decreasing gene expression. In Figure 2, each spot represents a locus and also positive and negative numbers on Y-axis respectively show an increase and decrease in gene expression.

Moreover, the heat map plot of Differentially Expressed Genes (DEGs), containing 38 loci, was drawn. The map displays the loci in rows and SSR markers, playing roles in drought stress, in columns. Also, the color and the boxes intensity represent changes in gene expression. About half of the loci were decreasingly expressed by all SSR markers. It is interestingly notable that the loci expression which was increased by SRR1258317 and SRR1258318 were decreased by SRR1258319 and SRR1258320, vice versa. The expression of gene-LOC115698845 was only enhanced in the presence of SRR1258319. The highest expression was observed by SRR1258317 for gene-LOC115712191, gene-LOC115714524, and gene-LOC115700995, as well as gene-LOC115712191 by SRR1258318 and gene-LOC115721097 by SRR1258319 and SRR1258320 (Figure 3).

**Table 1**. Genes and Enzymes Involved in Cannabinoids Biosynthesis

| No. | Enzyme Name | Species Name | Gene Name | Gene ID |
|---|---|---|---|---|
| 1 | hexanoyl-CoA synthetase | *Arabidopsis thaliana* | AAE1 | 838644 |
| 2 | olivetolic acid cyclase | *Cannabis sativa* | LOC115723438 | 115723438 |
| 3 | CBDA synthase | *Cannabis sativa* | LOC115697762 | 115697762 |
| 4 | THCA synthase | *Cannabis sativa* | LOC115697880 | 115697880 |

**Table 2**. Information of TSS and TATA-box for Genes

| Gene Name | TSS Position | TSS Score | TATA-box Position | TATA-box Score |
|---|---|---|---|---|
| AAE1 | 1021 | 1.97 | 988 | 4.64 |
| LOC115723438 | 1344 | 1.97 | 1311 | 5.7 |
| LOC115697762 | 624 | 1.98 | 592 | 5.7 |
| LOC115697880 | 248 | 1.95 | 216 | 4.72 |

**Table 3.** Predicted TFs in Genes

| Gene Name | TFs |
|---|---|
| AAE1 | BPC1, FLC, RAVL1, ANAC092 |
| LOC115723438 | Alfin1, BPC1, MADS box proteins, STF1/HY5, NSP1, TBF1 |
| LOC115697762 | GCBP-2, AREB2/ABF4, NtWRKY12, UPAAvrBs3Drep16, PacC, IbNAC1 |
| LOC115697880 | RNFG1, tomato HsfA2, B3 AFL TFs |

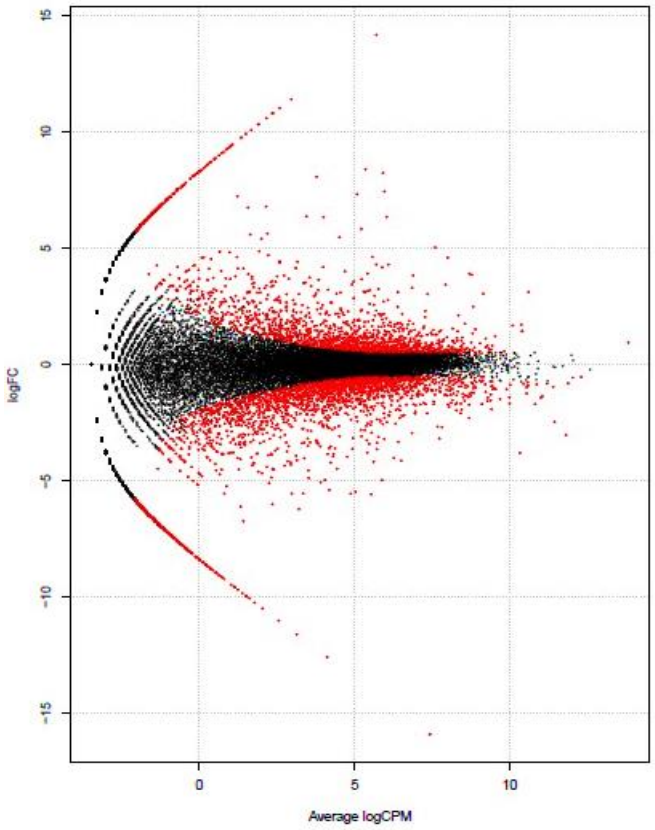


**Figure 2.** The Expression of the Genes Involved in Cannabinoids Biosynthesis.

### *Real-time PCR*

Among 38 loci, seven cases were selected for in vitro analysis of gene expression in drought conditions. The expression of all seven genes was significantly more than the control genes. The highest and the lowest expression were respectively observed in LOC115695737 and LOC115723934. On the other hand, it seems that there were no significant changes between the expression of LOC115695737 and LOC115713678, LOC115723934 and LOC115720447, also LOC115714427 and LOC115697922. Generally, the results show that drought stress can positively influence the expression of these genes which are involved in cannabinoids biosynthesis (Figure 4).

## Discussion

Secondary metabolites are categorized as the large and varied group of organic compounds produced in very low amounts (less than 1% dry weight). So, they are considered to be valuable herbal compounds. They are biologically produced as the byproducts of the biosynthesis of the primary metabolite. The most prominent secondary metabolites include alkaloids, phenols, steroids, lignins, tannins, flavonoids, and terpenoids.[15] These compounds play key ecological and physiological roles in the plants but are not involved in metabolic pathways. They are biosynthesized to make resistance in plants against abiotic and biotic stress including drought, salinity, heavy metal contamination, nutrient deficiency, low and high temperatures, and the infection of the pathogens such as bacteria, viruses, fungi, and nematode.[13] In addition, drought, as abiotic stress, is considered as one of the most effective environmental factors restricting plant productions. Global climate has been intensively changed to cause global warming and consequently the chance of drought stress occurrence. So, drought stress can be considered as one of the vital issues for cultivating the medicinal plant and producing secondary metabolites.[14] In this study, the expression of the genes involved in the production of the cannabinoids as the most important secondary metabolites in *C. sativa* influenced by drought stress, have been investigated through bioinformatics and experimental techniques.

Firstly, the data were analyzed by the R program with SSR markers related to drought stress, including SRR1258317, SRR1258318, SRR1258319, and SRR1258320. According to the results of the bioinformatic analysis, gene-LOC115712191, gene-LOC115714524, and gene-LOC115700995 were highly expressed by SRR1258317. The gene-LOC115712191 expression can also be enhanced by SRR1258318. Gene-LOC115712191 involves the expression of the B3 domain-containing proteins, found in abscisic acid insensitive3 (ABI3) TF, high-level expression of the sugar-inducible gene, RAV related to ABI3 TF, auxin response factor, and reproductive meristem only in vascular plants.[16] Additionally, gene-LOC115714524 encodes MLP (major latex protein) like protein 28 (MLP28) responding to both biotic and abiotic stresses. Also, MLP28

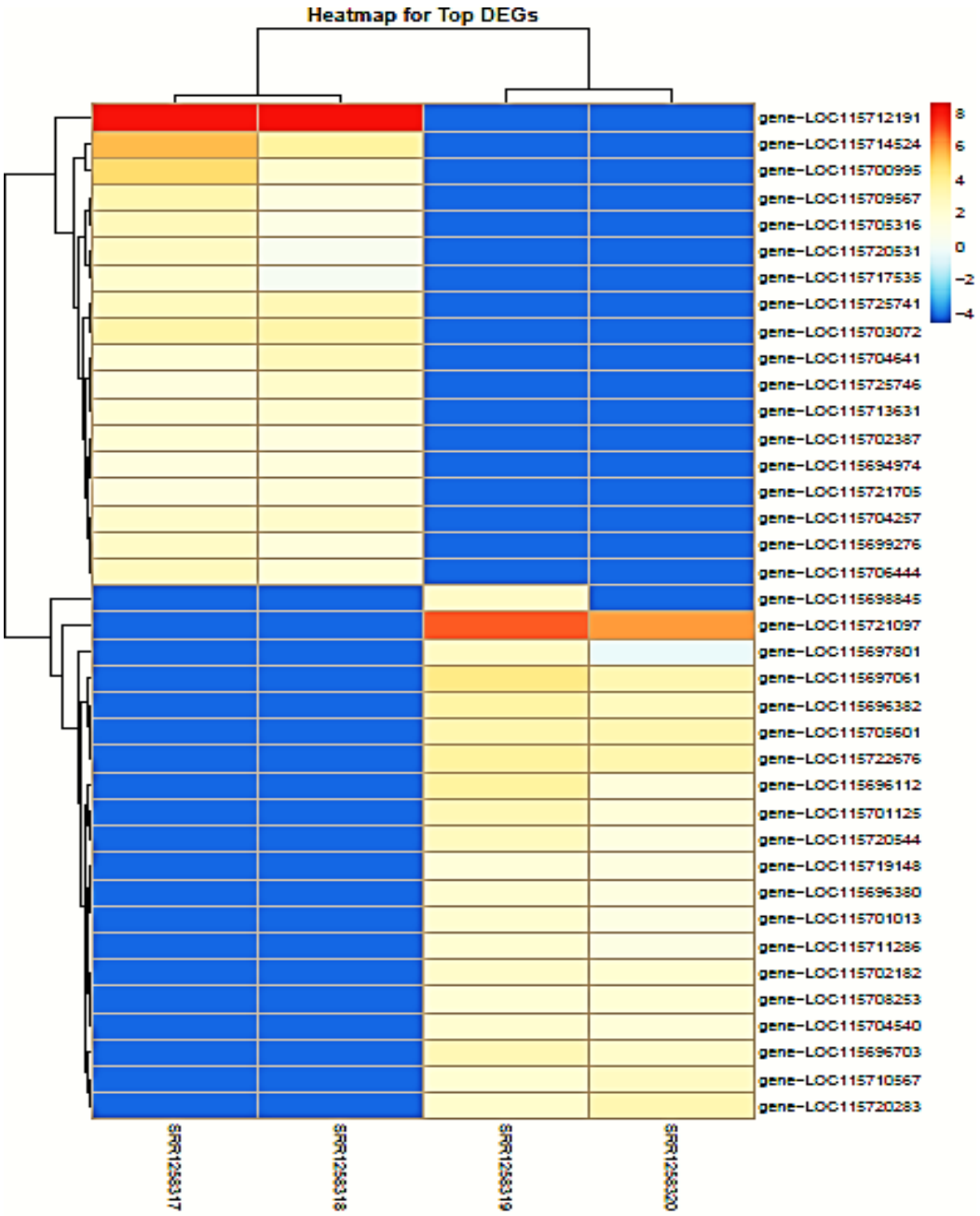


**Figure 3.** The Heatmap for Plot of DEGs.

could enhance resistance against Potato Virus Y (PVY) in tobacco plants. Moreover, the promoter of the gene contains MYB binding site involved in drought-inducibility.[17] However, there is no publication to report the function of gene-LOC 115700995 and the protein encoded by the locus.

As a result of the computational analysis, the gene-LOC 115698845 was increasingly expressed only in the presence of SRR1258319. The locus encodes Isocitrate Lyase (ICL) as a prominent enzyme in the glyoxylate cycle, as well as tolerating salt stress. Besides, ICL can facilitate shifting the metabolism of the energy to the glyoxylate cycle for modulating carbon balance to provide energy during salt stress.[18] Also, the results of *in silico* analysis indicated that SRR1258319 and SRR1258320 can enhance the expression of gene-LOC115721097. The locus encodes 60S ribosomal protein L39-1 participating as a particle of large ribonucleoprotein, translating messenger RNAs (mRNAs) into proteins. The ribonucleoprotein can be found as a free complex in the cytoplasm, attached to eukaryotic and prokaryotic cell membrane, plastids, and mitochondria.[19] It seems that the gene expression is positively induced by drought stress to enhance the production of other proteins which are vital for tolerating the stress.[13]

The results derived from experimental analysis demonstrated that LOC115695737 and LOC115713678 can be increasingly expressed by drought stress, more than other genes. There is

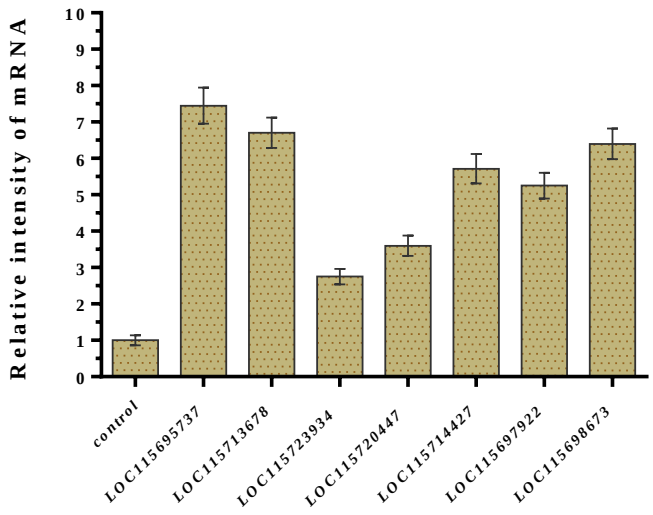


**Figure 4**. Gene Expression Under Drought Stress Condition, Assayed by Real-time PCR.

unfortunately no published data to report the function of LOC115695737 and LOC115713678. In addition, the protein encoded by the loci. LOC115723934 and LOC115720447 expression is enhanced lower than other loci by drought stress.

There is no reported function for LOC115723934 but LOC115720447 encodes bifunctional TENA-E protein also known as aminopyrimidine aminohydrolase or thiaminase II, playing a role in the biosynthesis of thiamine diphosphate (vitamin B1),[20] as cofactors of the enzymes contributing in environmental stress.[21] In many studies, it has been indicated that the gene, encoding thiaminase II, can be highly expressed by several abiotic stress such as osmotic and salinity stresses.[22] Also, LOC115714427 encodes transcriptional repressor OFP7 regulating multiple aspects of plant growth and development.[23] Wall-Associated receptor Kinase 1 (WAK1), as a signaling receptor of extracellular matrix component for responding to the infection of the pathogen and heavy metal toxicity, is encoded by LOC115697922 which is increasingly expressed by drought stress. In addition, the expression of the gene can be induced by stress-responding phytohormones including salicylic acid, methyl jasmonate, and ethylene.[24]

## Conclusion

Generally, it can be concluded that drought stress can induce overexpression of the genes encoding B3 domain-containing proteins, MLP28, MYB binding site, transcriptional repressor OFP7, and WAK1 as TFs play roles for responding to biotic and abiotic stresses. The overexpression of ICL, 60S ribosomal protein L39-1, and thiaminase II are required to provide energy, essential enzymes and proteins, and cofactors during a stress occurrence. However, many uncharacterized proteins can be found, encoded by genes such as LOC 115723934, LOC115695737, LOC115721097, LOC115700995, and LOC115713678. It is highly recommended that future studies concentrate on the roles of these genes, especially concerning resistance to drought stress.

## Authors' Contributions

SJD designed the study and HM wrote the first draft of the manuscript and carried out the R programming analysis. SJD revised and made appropriate changes to finalize the manuscript.

## Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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