CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Elucidating the roles of small open reading frames towards drought stress in *Solanum lycopersicum*

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Crop Breeding and Applied Biotechnology
25(1): e510625112, 2025
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332025v25n1a12

Abstract: *Solanum lycopersicum is highly susceptible to drought, which negatively impacts its production. While traditional genetic improvement focuses on coding genes, small open reading frames (sORFs) offer a new approach. This study aimed to identify and characterise drought-responsive sORFs in MT1 tomato plants exposed to 20 days of drought stress. Using the sORF finder pipeline, 676,611 coding sORFs were identified in the intergenic regions of the S. lycopersicum genome. Detailed analysis revealed 776 coding sORFs with translation potential, including 86 sORFs that shared homology to upstream sORFs. Drought transcriptome analysis revealed 862 differentially expressed genes, including 78 intergenic and 166 annotated sORFs. Gene ontology analysis highlighted the involvement of sORFs in "response to external stimulus" and "response to biotic stimulus" terms while KEGG pathway analysis revealed their involvement in "steroid biosynthesis" and "plant hormone signal transduction" pathways. This study provides insights into the role of sORFs in tomato drought responses.*

Keywords: *sORF, drought stress,* Solanum lycopersicum*, tomato, intergenic*

INTRODUCTION

Tomato (*Solanum lycopersicum*) is a globally significant crop whose yield and quality are increasingly threatened by climate change, particularly due to drought (Azzeddine et al. 2020). Drought stress adversely affects the plant's physiological processes (Lin et al. 2022), including photosynthesis, by disrupting both photochemical reactions and the enzymatic activities of the Calvin cycle (Waititu et al. 2021), ultimately leading to plant damage. Furthermore, studies in other plant species highlighted the involvement of canonical genes and transcription factors in the responses to drought stresses (Wang et al. 2021, Soviguidi et al. 2022). Thus, addressing these challenges is essential for sustaining tomato productivity under changing environmental conditions.

Advances in bioinformatics, like the sORF finder by Hanada et al. (2010), have facilitated sORF discovery and analysis. These sORFs encode small peptides, typically fewer than 100 amino acids in length, that regulate gene expression and signal transduction during stress responses (Bashir et al. 2019). Recent research underscores the role of small open reading frames (sORFs) in plant responses to abiotic stresses, including drought (Ong et al. 2023). Research has identified several unannotated translation events, such as small ORFs (sORFs) and upstream open reading frames (uORFs), that are involved in drought stress

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Received: 10 October 2024
Accepted: 26 January 2025
Published: 14 March 2025

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responses (Bashir et al. 2019). Some sORFs are conserved across species, while others are specific to certain plant families, such as Solanaceae (Wu et al. 2019), which includes tomatoes. In tomatoes, however, knowledge about sORF expression under drought stress remains limited. This study aimed to identify and characterize drought-responsive sORFs in tomatoes using RNA sequencing and bioinformatics. The findings could help develop drought-tolerant tomato varieties, ensuring stable yields and quality amid climate change.

MATERIAL AND METHODS

Plant materials and growth conditions

Solanum lycopersicum cv. MT1 seeds were purchased from Potters Garden Sdn. Bhd., Malaysia and germinated in a seedling tray. The mixture of soil was prepared based on the ratio of 1 peat: 1 perlite: 1 vermiculite. A seedling tray was prepared with three seeds in each cell and the tray was kept in the Plant Biotech Facility, Universiti Malaya, under greenhouse conditions under 12-hour photoperiod maintained at 25 °C ± 2. To ensure the tomato seedlings' healthy growth before the drought stress treatment, plants were watered once every 2 days. The plants at the two-leaf stage (39 days post-seed germination) were moved into pots (9 x 8 x 6 cm). Plants were grouped into two groups: well-watered treated and drought-stressed treated. Each group had three biological replicates.

Drought stress treatment

For the drought stress experiment, three replicates were used for each treatment, including both control and drought stress plants (n=3). Control plants received 50 mL of water every two days to maintain soil moisture between 60% and 70% throughout growth. Watering was stopped at the five-leaf vegetative stage to initiate the drought stress. The plants were subjected to 20 days of drought treatment until soil moisture content dropped below 10%. Compound leaves from the third to fifth positions were collected with liquid nitrogen and kept at -80 °C for subsequent RNA extraction.

In silico prediction of sORF from the tomato reference genome

GFF-Ex (Rastogi and Gupta 2014) was used to extract sequences of introns, exons, and intergenic regions from the tomato reference genome (Tomato Genome version SL4.0, https://solgenomics.net/organism/Solanum_lycopersicum/genome). The intergenic coding sORFs of the tomato were identified according to the sORF finder pipeline reported by Hanada et al. (2010). CD-HIT Suite (Huang et al. 2010) was used to eliminate sORF duplication, and RepeatMasker (Smit et al. 2015) was used to mask the repetitive sequences from the intergenic coding sORFs. sORFs with translation potential (translated sORFs) were identified using BlastN algorithm against the translated uORFs and sORFs published by Wu et al. (2019) and PsORF database (http://psorf.whu.edu.cn/#/; Chen et al. 2020c). The intergenic coding sORFs were mapped to the tomato reference genome using the blat program (Sadak and Ramadan 2021). An updated genome annotation file was generated by incorporating the intergenic coding sORF annotation profile into the reference gene annotation file.

RNA isolation and sequencing

The total RNA of tomato cultivar MT1 was extracted from the leaf tissues using Total RNA Extractor (Sangon Biotech, China) according to the manufacturer's instructions, and the genomic DNA was removed using DNase I (Thermo Scientific, United States). The integrity and concentration of total RNA were evaluated using agarose gel electrophoresis and spectrophotometer (NanoDrop™ 2000, United States). The extracted RNA was subjected to RNA sequencing by Novogene Co. Ltd. RNA library preparation for transcriptome sequencing was done by Novogene Co., Ltd using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, United States). Qubit 3.0 (Thermo Fisher Scientific, United States) and QuantStudio 5 (Thermo Fisher Scientific, United States) were used to examine the library for quantification, and 2100 Bioanalyzer (Agilent Technologies, United States) was used to detect the size distribution. The RNA library was sequenced on NovaSeq 6000 sequencing system (Illumina, United States) to generate paired-end reads of 150 bp. The adapter sequences were removed using fastp (Chen et al. 2018).

Transcriptome analysis

RNA sequencing (RNA-seq) data for *Solanum lycopersicum* cultivar MT1 was obtained from Novogene Co. Ltd. The

raw sequencing reads were processed to ensure high-quality data for downstream analysis. For the alignment of clean reads to the tomato reference genome, HISAT2 (http://daehwankimlab.github.io/hisat2/) was employed. The aligned reads were then passed to the featureCounts tool (Liao et al. 2014), which was used to quantify gene expression by counting the number of reads mapping to each gene. To normalize the read counts, TPMcalculator (Alvarez et al. 2019) was utilized, providing a measure of gene expression that accounts for both sequencing depth and gene length. Differential expression (DE) analysis was performed using the DESeq2 package (Love et al. 2014), which employs a statistical model to identify genes with significant changes in expression between experimental conditions.

Functional annotation of differentially expressed genes and sORFs

Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analyses of the differentially expressed genes and sORFs were conducted using TBtools version 2.096 (Chen et al. 2020a).

Nanostring analysis

Three biological replicates from each drought-stressed and well-watered plant were analysed using the nCounter® Analysis System (NanoString Technologies, United States) to validate the drought-responsive gene expression patterns identified through RNA-Seq. For the nCounter® analysis, 500 ng of total RNA was extracted from tomato leaves. The analysis utilized seven CodeSets, including five differentially expressed genes and two housekeeping genes (actin and tubulin). Gene expression data were examined using the Advanced Analysis Module of nSolver™ Analysis Software version 4.0 (NanoString Technologies, United States). Correlation analysis of the nine differentially expressed genes between NanoString and RNA-seq data was performed using SRPplot (Tang et al. 2023) to determine the degree of correlation.

RESULTS AND DISCUSSION

Small open reading frames (sORFs) are typically composed of 100 or fewer amino acids (aa) (Ladoukakis et al. 2011), although lengths of up to 120 aa have been reported (Matsubayashi 2011). In this study, 6,132 annotated sORFs (≤ 120 aa) including 4,649 with ≤ 100 aa were identified from the protein coding sequences of the tomato reference genome. Using the sORF finder pipeline (Hanada et al. 2010), 10,360,646 intergenic sORFs were identified, with 854,270 as coding sORFs. After repeatmasking and deduplication, 676,611 unique coding sORFs remained (Table 1). To identify sORF with translation potential, all unique coding sORfs were blast searched against ribo-seq datasets published by Wu et al. (2019) and PsORF database (Chen et al. 2020c). Our analysis revealed that 86 sORFs are homologous to translated uORFs, while 776 sORFs showed translation potential. Additionally, coding sORFs have been reported in different plant species such as *Arabidopsis thaliana* (7,159 sORFs; Hanada et al. 2007), *Oryza sativa* (48,620 sORFs; Feng et al. 2023), and *Cucumis sativus* (50,191 sORFs; Shiao et al. 2022).

Transcriptome analysis was conducted to study the drought response in cv. MT1, identifying gene expression changes. Figure 1A shows a heatmap of differentially expressed (DE) genes, grouped into two clusters. Cluster 1 includes genes with higher expression in well-watered samples (MCR1, MCR2 and MCR3) and lower expression in drought-treated samples (MDR1, MDR2 and MDR3), while Cluster 2 shows the opposite pattern (Figures 1A and 1B). In drought-treated MT1, 347 genes were downregulated, and 515 genes were upregulated (Figures 1B and 1C). Among 862 DE genes, 379 canonical genes, 41 intergenic sORFs, and 95 annotated sORFs were upregulated, while 289 canonical genes, 37 intergenic sORFs, and 21 annotated sORFs were downregulated (Figure 1D).

Table 1. Coding sORFs detected by sORF finder in the tomato reference genome and the summary of intergenic sORFs and uORFs found in *Solanum lycopersicum* ribosome profiling dataset

Type of sORF	Number of genes
sORF	10,360,646
Coding sORF	854,270
RepeatMasker	706,409
Deduplication	676,611
Coding sORF with translation potential	776
Coding sORF with translation potential and homolog to translated uORF	86

The drought-responsive sORF expression profiles in MT1 were validated with NanoString technology, showing a correlation with RNA sequencing data (Figure 1E). Strong correlations were observed, with a Pearson correlation coefficient (R) of 0.92 and a *p-value* of 0.03 (Figures 1E and 1F). This strong positive correlation supports the validity of the RNA sequencing data.

Gene ontology (GO) enrichment analysis was conducted to elucidate the function of MT1 DE sORFs under drought stress (Figure 2A). The DEGs in MT1 were categorized into 15 GO terms: 9 in biological processes (BP),

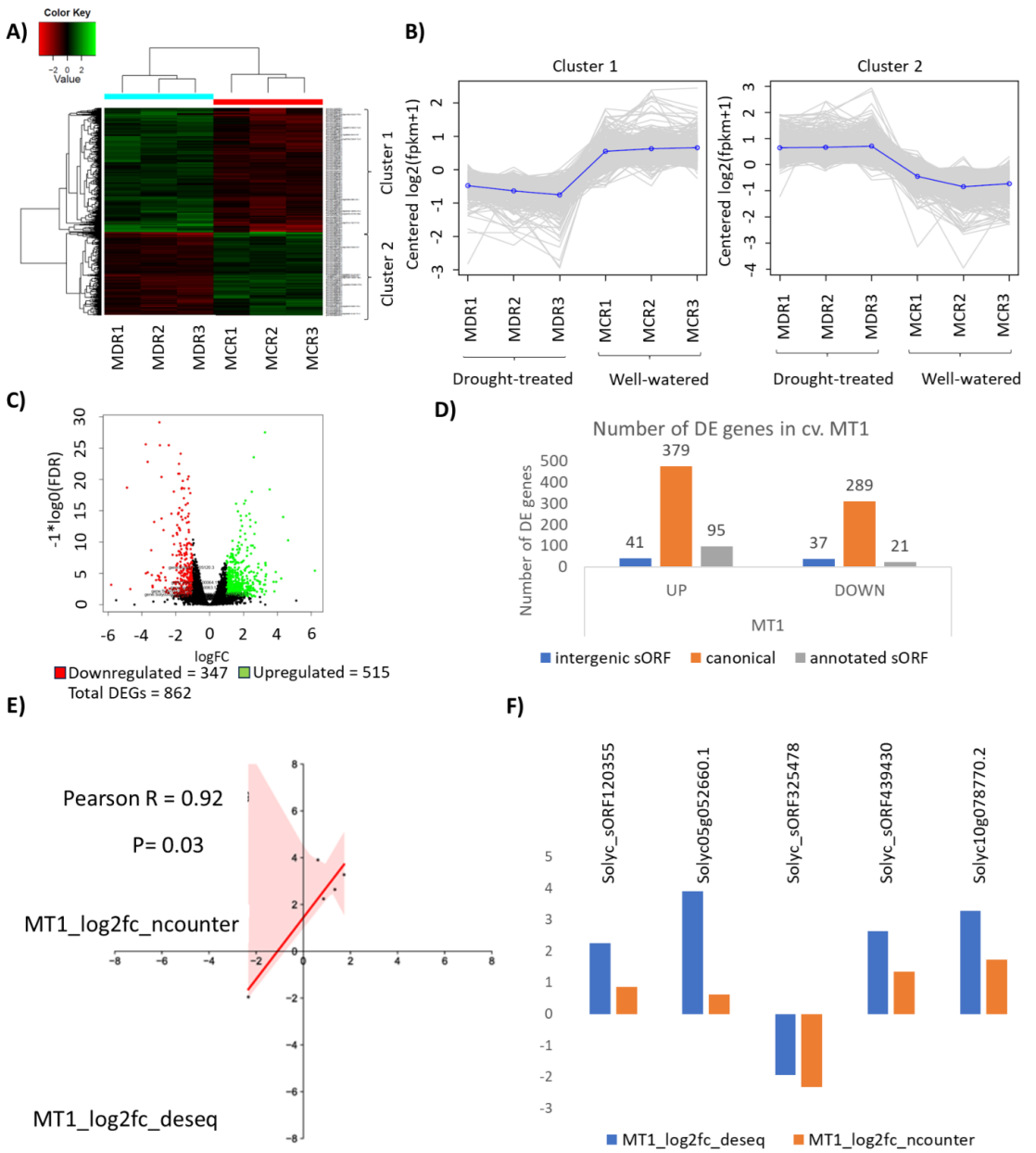

Figure 1. The results of MT1 expression analysis under drought stress were illustrated: (A) Heatmap, (B) gene cluster profiles, (C) volcano plot, (D) total number of DE canonical genes, intergenic sORFs and annotated sORFs, (E) correlation plot of the expression profile between RNA sequencing and NanoString, and (F) correlation bar plot between qPCR and RNA-sequencing data. The correlation coefficient (R) and p-value (P) are 0.92 and 0.03, p ≤0.05, respectively. MCR1, MCR2 and MCR3, well-watered samples; MDR1, MDR2 and MDR3, drought-treated samples.

5 in cellular components (CC), and 1 in molecular function (MF). In the BP subontology, "response to external stimulus" (GO:0009605) and "response to biotic stimulus" (GO:0009607) were the most enriched, reflecting key drought response pathways. The "response to external stimulus" term was enriched in DE annotated and intergenic sORFs of cv. MT1 (Figure 2A; Table 2), indicating complex biochemical processes that help plants adapt to environmental stressors like drought (Zhang et al. 2023). Similarly, the "response to biotic stimulus" term showed enrichment in DE sORFs (Figure 2A; Table 2), highlighting plant responses to drought through cellular adjustments, such as proline and soluble sugar regulation for osmotic balance, as observed in potatoes (Chen et al. 2020b). In MF, the term "transporter activity" was enriched, underscoring the importance of membrane transporters in drought tolerance (Figure 2A). For instance, overexpression of ABA transporter-like 1 (*AhATL1*) from the ABCG transporter family enhanced drought tolerance memory in Arabidopsis (Qin et al. 2021). In CC, the enriched terms "plasma membrane" (GO:0005886) and "membrane" (GO:0016020) reflected the impact of reactive oxygen species (ROS) on membrane integrity under drought conditions (Figure 2A), as seen in *Heimia myrtifolia* (Lin et al. 2022).

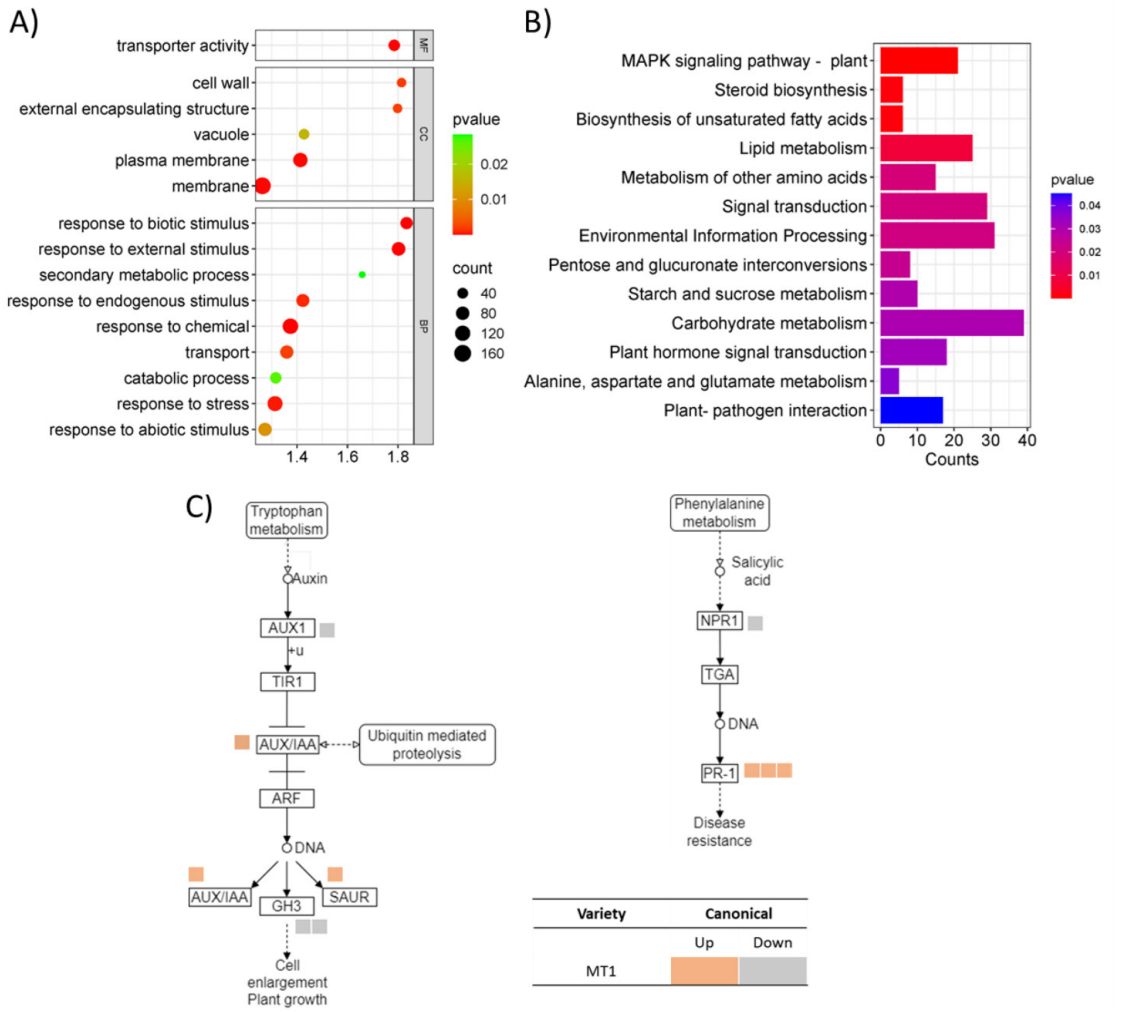

Figure 2. A) GO enrichment of the DEGs in MT1, X-axis indicates enrichment score; Y-axis indicates p-value. B) KEGG enrichment of the DEGs in MT1, X-axis indicates gene count; Y-axis indicates p-value. C) two pathways related to the "plant hormone signal transduction" pathway that is involved in tomato drought. The box's colour means the type of gene, and the number of boxes means the number of genes involved.

Table 2. Summary of GO enrichment analysis of DE genes and DE sORFs in MT1

Pathway	Enrichment	P-value	Count	Class	Intergenic sORFs	Annotated sORFs	Canonical genes
transporter activity	1.785155984	3.74E-05	49	MF		✓	✓
plasma membrane	1.413399897	8.87E-05	99	CC		✓	✓
membrane	1.262662856	9.68E-05	160	CC	✓	✓	✓
cell wall	1.814189392	0.0019874	27	CC		✓	✓
external encapsulating structure	1.798310273	0.002251096	27	CC		✓	✓
vacuole	1.427958674	0.015856443	38	CC		✓	✓
response to external stimulus	1.802402838	2.82E-08	83	BP	✓	✓	✓
response to biotic stimulus	1.834038858	1.75E-06	61	BP	✓	✓	✓
response to chemical	1.37406744	2.09E-05	126	BP	✓	✓	✓
response to stress	1.31291795	4.46E-04	115	BP	✓	✓	✓
response to endogenous stimulus	1.423173669	9.29E-04	72	BP	✓	✓	✓
transport	1.359574197	0.002207297	77	BP		✓	✓
response to abiotic stimulus	1.273266808	0.010736352	79	BP		✓	✓
catabolic process	1.316010181	0.026333762	49	BP	✓	✓	✓
secondary metabolic process	1.658244564	0.028390408	17	BP			✓

A KEGG pathway enrichment analysis was conducted using TBtools to explore the biological functions of DE genes in MT1. Thirteen pathways were enriched in the DE genes, with the top three significantly enriched pathways being the "MAPK signaling pathway - plant" and "steroid biosynthesis" (Figure 2B). The "MAPK signaling pathway - plant" was the most enriched pathway, playing a crucial role in drought stress through ABA-mediated stomatal closure. In Arabidopsis, for example, the *AtMAPKKKK18-AtMAPKK3* gene positively regulates drought stress, and overexpression of *AtMAPKKKK18* enhances drought tolerance by mediating stomatal closure (Lin et al. 2021). The "steroid biosynthesis" pathway was also highly enriched under drought stress in MT1, functioning as an anabolic pathway for steroid production. Steroids like fecosterol and soyasaponin II, produced in soybeans, enhance drought tolerance (Yu et al. 2024).

The plant hormone signalling transduction pathway has been reported to be involved in drought response. Ong et al. (2023) reported tryptophan and phenylalanine related pathways of the plant hormone signalling transduction pathway were enriched in the DE canonical genes and sORFs of rice under drought stress. In contrast with Ong et al. (2023), only DE canonical genes associated with these two pathways were identified in drought stressed MT1 tomato plants (Figure 2C). In MT1, *Transport Inhibitor Response 1* (*TIR1*) and *Auxin Response Factor* (*ARF*) genes showed no differential expression (Figure 2C). Tryptophan plays a crucial role in enhancing plant tolerance to environmental stressors, such as drought. Auxin Resistant 1 (AUX1) is an auxin influx transporter that transports auxin to TIR1, facilitating the degradation of Auxin/Indole-3-Acetic Acid (Aux/IAA) proteins and regulating auxin-responsive transcription. In poplar, overexpression of *PtrFBL1* (homologs to TIR1) reduced drought tolerance (Shu et al. 2015). On the other hand, the downregulation of rice TIR1 leads to reduced tolerance to salt and drought (Xia et al. 2012). Downregulation of tomato *ARF4* improved salt and drought tolerance by increasing root growth and density (Bouzroud et al. 2020). Activation of TIR1 promotes the degradation of AUX/IAA proteins via ubiquitin proteolysis, which releases ARF to regulate the expression of *Gretchen Hagen 3* **(***GH3*), *AUX/IAA*, and *Small Auxin Up-Regulated RNA* (*SAUR*) genes (Rienstra et al. 2023). In this study, *AUX/IAA* and *SAUR* were upregulated, while *GH3* was downregulated in response to drought (Figure 2C). Overexpression of wheat *AUX/IAA* gene, *TaIAA15-1A*, in transgenic *Brachypodium* positively correlates with drought tolerance (Su et al. 2023). In potatoes, overexpression of *GH3* improved drought tolerance by increasing proline accumulation and reducing leaf water loss (Yao et al. 2023). *SAUR* enhances drought tolerance through both ABA-dependent and ABA-independent pathways (He et al. 2021).

Phenylalanine metabolism has been linked to drought tolerance, with its expression enhancing plant responses to pests and improving resilience to drought (Ramzan et al. 2023). In MT1, *NPR1* gene was downregulated when the plants were under drought stress conditions (Figure 2C). Overexpression of *NPR1* has been shown to promote drought tolerance by regulating reactive oxygen species (ROS) levels and supporting cell survival against oxidative damage (Li et al. 2023b). Liu et al. (2013) reported that overexpression of *PR1* enhances drought tolerance in Arabidopsis, with *TGA*, a

Table 3. Summary of KEGG enrichment analysis of DE genes and DE sORFs in MT1

Term	Count	P-value	Group	Intergenic sORFs	Annotated sORFs	Canonical genes
MAPK signaling pathway - plant	21	8.54E-05	Environmental Information Processing			✓
Steroid biosynthesis	6	0.001005	Metabolism		✓	✓
Biosynthesis of unsaturated fatty acids	6	0.001162	Metabolism		✓	✓
Lipid metabolism	25	0.00729	Metabolism		✓	✓
Metabolism of other amino acids	15	0.019249	Metabolism			✓
Signal transduction	29	0.019841	Environmental Information Processing			✓
Environmental Information Processing	31	0.0209	Environmental Information Processing			✓
Pentose and glucuronate interconversions	8	0.023997	Metabolism			✓
Starch and sucrose metabolism	10	0.029532	Metabolism			✓
Carbohydrate metabolism	39	0.030294	Metabolism	✓		✓
Plant hormone signal transduction	18	0.032513	Environmental Information Processing			✓
Alanine, aspartate and glutamate metabolism	5	0.036764	Metabolism			✓
Plant-pathogen interaction	17	0.045256	Organismal Systems			✓

gene that synergistically activates *PR1*, playing a key role (Hussain et al. 2018). In this study, MT1 exhibited upregulation of the *PR1* gene under drought stress, similar to findings in rice (Ong et al. 2023); however, TGA showed no differential expression (Figure 2C).

Three differentially expressed (DE) annotated sORFs (one Δ7-sterol-5-desaturase and two Δ12-desaturase) were enriched in KEGG pathways. Δ7-sterol-5-desaturase, which was upregulated in the steroid biosynthesis pathway of MT1 (Figure 2B; Table 3), has been reported to enhance drought and pathogen resistance by increasing wax deposition in transgenic tomato and *Arabidopsis* (Kamthan et al. 2012). Li et al. (2023a) reported that some of the Δ12-desaturase genes (*CtFAD2* and *CtFAD6*) of safflower (*Carthamus tinctorius* L.) were significantly induced under cold, heat and salt stresses. However, the role of Δ12-desaturase in response to drought stress is lacking. In this study, Δ12-desaturase was upregulated in MT1 under severe drought stress (Figure 2B). This indicates that Δ12-desaturase may also play an important role in tomato drought tolerance.

Besides annotated sORFs, two DE intergenic sORFs that homolog to *ribulose-bisphosphate carboxylase small chain (RBCS)* and malate synthase of the "glyoxylate and dicarboxylate metabolism" pathway were identified (Figure 2B). Malate synthase has been reported to be crucial in the glyoxylate cycle and contributes to glucose accumulation for drought tolerance in rice (Todaka et al. 2015). Similarly, the downregulation of *RBCS* genes, which suppress CO_2 absorption and reduce photosynthetic rates, was reported in drought-susceptible maize (Waititu et al. 2021).

CONCLUSION

In this study, we identified 676,611 intergenic sORFs and 862 differentially expressed genes (DEGs) from *Solanum lycopersicum* genome, including 78 drought-responsive intergenic sORFs and 166 annotated sORFs that were differentially expressed under drought stress conditions. GO and KEGG enrichment analyses of DEGs revealed the involvement of sORFs in key terms and pathways of drought response. Validation of transcriptome data using NanoString technology revealed a high positive correlation between two different gene expression platforms. These findings suggest that sORFs might be crucial targets for advancing research on plant stress resilience.

ACKNOWLEDGEMENTS

This work was supported by the RU grant of Universiti Malaya (RU004F-2020). Supplementary files are available upon request to the corresponding author (cheehow.teo@um.edu.my).

DATA AVAILABILITY

Transcriptome datasets supporting the conclusions of this article are available in NCBI (PRJNA1164923).

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