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Transcriptome analysis of genes associated with some phytohormones and phytohormone-like activities in drought-stressed tomato cultivar super strain B

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Phytohormones are crucial signaling mediators that, in trace amounts, modify growth and development-related genes in plants, enhancing their resilience to stress. In the present study, temporally expressed genes of the hormone synthesis pathways for salicylic acid (SA), abscisic acid (ABA), ethylene (ET), and jasmonic acid (JA) were studied in tomato super strain B under drought stress. This cultivar is widely cultivated in Saudi Arabia, so it is a suitable model for studying this stress. Their expressions were analyzed every 2-h interval over a 48-h period. Our findings revealed that the expression pattern of ABA-related genes was more responsive to drought stress than those of ET, SA, and JA. The stomatal-related genes were sensitive to drought stress, whereas probable protein phosphatase 2C_24 and protein phosphatase 2C_53 genes were downregulated during light exposure. In contrast, the serine/threonine-protein kinase gene was upregulated during high light intensity, demonstrating how they influence stomatal reflexes to preserve water. In addition, the guard cell S-type anion channel SLAC1 gene was found to have significantly fluctuated in monitoring the ion channel responsible for drought-induced stomatal closure. The study further revealed a distinct circadian gene expression pattern of the late elongated hypocotyl (LHY) transcription factor family that was upregulated from midnight to midday and then suppressed in the afternoon and the evening, demonstrating how stress reactions and circadian cycles interact to control hormonal pathways. In conclusion, the temporal gene expression dynamics of key hormones under drought stress provide insights into developing drought-resistant tomato varieties and improving stress management strategies.

Keywords: abscisic acid; gene expression; ethylene; jasmonic acid; salicylic acid

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INTRODUCTION

Due to the dynamic worldwide environmental changes, crops growing in an open field face various biotic/abiotic stress conditions(Lee and Yeom 2015; Kang and Yeom 2018), adversely affecting them. Crops, in turn, promote an array of defense strategies to respond to and cope with such stresses through physiological, biochemical, and molecular regulatory techniques (Hannachi and Van Labeke 2018), which activates the control of responsive genes that are influenced by multiple transcriptional cascades and different phytohormone signaling pathways (Lee et al. 2020).

Phytohormones are endogenous key signaling messengers that, in minute amounts, enhance, obstruct, or alter physiological operations in plants by modulating genes involved in growth and development. This strengthens plants' ability to adapt to and tolerate stresses (Pu et al. 2019; Arif et al. 2020).

Among the major phytohormones involved in plant defense responses to stressors are salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). These phytohormones react with each other through complex antagonistic or synergistic mechanisms (Shigenaga and Argueso 2016). Transcription factors transmit these signaling clues by activating or suppressing the expression of genes involved in immunological responses and metabolic processes. As a result, most plant immunological responses are transcriptionally regulated (Wise et al. 2008).

Several investigations have informed that JA and SA are two important small chemical compounds with hormonal-like activities that activate the plant self-protection system (Dempsey and Klessig 2012; Khan et al. 2012; Pieterse et al. 2012; Atamian and Harmer 2016; Druege et al. 2016; Campos-Rivero et al. 2017).

JAs are essential signaling molecules in plant development and defense processes and in plant response to abiotic stresses (Abouelsaad and Renault 2018; Ahmad et al. 2019). Compared to sensitive cultivars, salt-tolerant crops' leaves and roots have revealed the activation of JA biosynthesis-related genes and JA cumulation (Delgado et al. 2021).

Activating plant defense mechanisms is facilitated by SA, a phenolic compound that functions as an immunological signaling molecule (Dempsey and Klessig 2012). Also, it is easily transmitted from one organ to others (Kazemi et al. 2018) throughout the plant's inner body, giving it the potential to regulate and stimulate many physiological processes of plant

growth (Basit et al. 2018; Souri and Tohidloo 2019; Naeem et al. 2020), stress adaptation or tolerance (Fahad et al. 2015; Sofy et al. 2020; Hundare et al. 2022), and increases the antioxidant capacity of plants.

ET, a gaseous hormone, has various immunological responses due to disease, injury, and/or environmental stress in collaboration with other signaling networks. It also regulates final developmental processes like organ abscission, withering of leaves and flowers, and ripening of fleshy fruit and plays roles in germination and growth (Graham et al. 2012; Pech et al. 2012; Wang et al. 2013).

ABA regulates several stages of plant growth, such as dormancy and seed germination, and mediates an essential contribution to abiotic stress tolerance (Lamin-Samu et al. 2021). ABA can also affect plant-microbe interactions (Ton et al. 2009; Robert-Seilaniantz et al. 2011).

Generally, these hormone reactions require accurate control as plants have evolved coordinated mechanisms to withstand and fight off stresses. ABA reduces plant resistance processes by antagonizing SA- and JA/ET-dependent immuno-responses (Denancé et al. 2013), and the downstream responses revealed cellular SA/ABA ratios (Rico et al. 2010). JA/SA regulates how the balance between growth and defense is maintained in plants (Luo et al. 2019). However, ET signaling stimulates JA production, which reduces SA signals (Jia et al. 2013).

Solanum lycopersicum L. (Tomatoes) is one of the most cultivated horticultural crops worldwide. Meanwhile, its production quantity fluctuates globally due to its sensitivity to many biotic and abiotic stresses (Seng 2014). It has been used as a model plant in studies on the Solanaceae family plants to investigate plants' stress sensitivity/ tolerance (Yin et al. 2017). Polyethylene glycol 6000 (PEG 6000) treatments frequently mimic drought stress in tomatoes because they reduce water availability and cause osmotic stress. PEG-induced osmotic stress has impacted tomato germination, growth, and physiological responses, allowing researchers to examine drought resistance mechanisms. Studies highlight that PEG 6000 treatments can mimic drought stress to study biochemical and molecular changes, including antioxidant enzyme activities and stress-responsive gene expression (Basha et al. 2015; Kumar et al. 2021; Yadav et al. 2024).

Tomato plants utilize the interaction of the hormonal signaling pathways of SA, JA, ET, and ABA to adapt themselves to drought stress by optimizing water use, growth, and survival, regulating physiological and molecular processes (Raza et al. 2019; Ding et al. 2020). ABA controls stomatal closure to improve water intake under drought stress, reducing water loss and encouraging root growth (Chaves et al. 2003).

Also, (Finkelstein et al. 2002), ABA triggers the expression of cascade drought-responsive genes for osmotic adjustment and water conservation. However, JA strengthens plant defenses by modulating stress-responsive genes during drought (Wasternack and Strnad 2016) and promoting the synthesis of protective metabolites against oxidative damage (Bari and Jones 2009). Like JA, SA plays a key role in regulating the antioxidant defense system during drought, enhancing the expression of drought-responsive genes, and improving water retention (Khan et al. 2015).

Transcriptome profiling is an effective and popular technique for investigating gene transcription and regulation, which is essential in functional genomics analyses (Scharf et al. 2012). Despite various previously reported molecular investigations dedicated to phytohormones, their transcriptome information remains unclear, especially in major crops (Berens et al. 2017; Shigenaga et al. 2017) due to several cultivars and different stresses.

This investigation aimed to provide a transcriptome dataset to identify some controlling stress-specific key genes differently expressed and implicated in the hormonal biosynthetic pathways under drought stress in tomato cv. super strain B to enhance our understanding of their contributions to drought tolerance. The authors also studied these expressed genes related to photoperiod during the experiment under drought stress.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Emerald Seed Co., USA's Saudi agent, procured tomato cultivar seeds from “super strain B.” Many regions of Saudi Arabia commonly grow this cultivar. The seeds were repeatedly washed with distilled water to remove the antifungal agent (thiram). The germination percentage was used to test the viability of the seeds; after 2 and 6 days of cultivation, the seeds gave 80% and 100% germination, respectively. For 40 days, drip irrigation was used to irrigate all the seeds thoroughly to help them develop roots,

produce their first leaves, and become accustomed to the greenhouse-controlled conditions. The experiment started with seedlings receiving a daily 1 L watering at 6:00. To keep all seedlings well-watered as they grew, irrigation was raised daily to overall water of 1.5–4.5 L (given at 8:00, 13:00, and 17:00). The maximum temperature at noon, which lasted for six hours, was 30°C on average, with a diurnal 34°C (12:00–18:00). The daytime relative humidity ranged from 40% to 95%, causing a daily vapor pressure deficit (VPD) of 0.2 to 3.5 kPa. Above 2,000 mol $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR) was measured daily.

Drought Treatment

Forty-day-old seedlings were removed from the pots after being treated for 48 hours with 100 ml of 20% Polyethylene Glycol (PEG 6000) and 100 ml of water as controls. The leaves were then picked up and labeled separately. After labeling, the leaves were frozen in liquid nitrogen and preserved at -80°C until a subsequent RNA extraction experiment.

Extraction of RNA

After two days of cultivation in both PEG and regular sunlight (NL), tomato leaves were sampled every two hours. Leaf segments measuring 1.0 cm2 were cut and kept at -80°C. Total RNA was extracted from each sample using the manufacturer's instructions for the Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA), and the amount of RNA was evaluated.

RNA-Seq library preparation and sequence analyses

The cDNA library was constructed following the manufacturer's instructions using Illumina/Hiseq-2000 RNA seq provided by Macrogen (Seoul, Korea). Sera-mag Magnetic Oligo (dT) Beads were used to cleanse the poly (A)-mRNA molecules from the RNA samples. A fragmentation buffer was added to split mRNA into small fragments, which served as templates to synthesize the first cDNA strand. The sequencing adapters were supplemented to the purified and synthesized cDNA. A gel extraction kit was utilized to extract the cDNA fragments (200± 25 bp) from the gel. After that, the Illumina/Hiseq-2000 RNA-seq was used to sequence the library.

Transcriptome analysis

FastQC software was used to control the quality of clean reads. To map clean reads against the 1706 genome sequences of the reference tomato Heinz, the mapping data were quantified using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml.) and RNA-Seq by Expectation Maximization (RSEM) software (http://deweylab.biostat.wisc.edu /rsem/) (Pech et al. 2012). RSEM software uses the lengths of target contigs to compute expression values. EM methods and reading counts for each contig were applied to obtain the number of mismatches and isoform- or gene-level estimates. Consistently, even for data mapped at multiple points, precise high estimates of expression data were given. In Bowtie2, the mapping parameter was set to default. The RPKM value was used to normalize the expression data. The DDBJ database registered the expression data (http://trace.ddbj.nig.ac.jp/DRASearch, Accession numbers: DRA003530, DRA003529, and DRA003528).

Histogram analyses with RPKM values of <0.1 were set to 0.1, and RPKM values were log (2) transformed. The Gene Cycle and Samr packages of R software were used for statistical analyses. Fisher's exact g test was used to calculate the p-values, and sorting data were used for heatmaps: 24-h cycle; maximum value, 7 (time, 6:00); minimum value, 1 (time; 18:00), 6 changes every 12-h.

The Gene filter package of R software was used to sort transcriptome data. A heatmap was generated using the stats package and R software. Based on the TAIR10 data (http://www.arabidopsis.org) of *A. thaliana,* mapping analyses were achieved using MapMan software (Ver.3.5.1) with a BIN code assigned by Mercator (http://mapman.gabipd.org). Mercator's BLAST cutoff value was set at 50. The MapMan software was utilized to categorize temporally expressed genes with p- and FDR values of less than 0.05.

Search for homologous genes

The BLAST system searched for homologous tomato hormone-related genes from transcriptome data. The Panther (http://www.pantherdb.org/) and Pfam (http://pfam.xfam.org/) databases of predicted amino acid sequences were used to identify motifs and domains of homologous tomato genes.

RESULTS

Tables 1 and 2 illustrate the statistical analysis of some gene expressions of the ABA, ET, SA, and JA pathways in tomato super strain B under drought stress with a day/night photoperiod and normal light conditions. Among the 6 genes of the ABA pathway studied AAO3, NCED1, and ABA3 genes appeared to have nocturnal activities, while ABA2, ABI1, and RD22 had diurnal ones. The AAO3 gene was expressed at night more

Table 1. Statistical analysis of some abscisic acid (ABA) and ethylene (ET) pathway-related genes in tomato super strain B under drought stress with day/night photoperiod and normal light conditions.

Pathway	Name	Accession Number	Night (First Time)		Day (Second Time)	
			P Val	FDR	P Val	FDR
ABA	NCED1	NM_001247526	0.8804	0.9659	0.3151	0.9081
	ABA2	XM_004237780	0.0020	0.0593	0.0031	0.0874
	ABA3	NM_001247215	0.8247	0.9637	0.3515	0.9168
	AAO3	XM_004228420	0.0781	0.7155	0.0083	0.2067
	RD22	NM_001247592	0.2524	0.8904	0.6118	0.9505
	ABI1	XM_004253043	0.0000	0.0004	0.0001	0.0035
ET	ACO	NM_001247095	0.1059	0.7731	0.3022	0.9045
	ACS1	NM_001246993	0.0708	0.6950	0.5825	0.9481
	ERF1	NM_001247912	0.0203	0.3954	0.0002	0.0102
	EIN2	NM_001247589	0.0033	0.0963	0.2672	0.8934
	EIN3	NM_001247002	0.0010	0.0320	0.0324	0.5037
	PDF1	XM_004243183	0.8344	0.9641	0.8708	0.9647
	CTR1	NM_001247525	0.8410	0.9644	0.1004	0.7590
	ERF4	NM_001247384	0.2709	0.8971	0.0127	0.2845

ABA, Abscisic acid; NCED, 9-Cis-epoxycarotenoid dioxygenase; ABA2, abscisic acid deficient 2; ABA3, abscisic acid deficient 3; AAO3, Arabidopsis aldehyde oxidase 3; RD22, dehydration-responsive gene; ABI1, Abscisic acid insensitive 1; ET, Ethylene; ACO1, 1-aminocyclopropane-1-carboxylate oxidase; ACS1, Arabidopsis cysteine synthase 1; ERF1, ethylene response factor1 gene; EIN2, ethylene insensitive 2; EIN3, ethylene insensitive 3; PDF1, plant defensin 1; CTR1, Constitutive triple response 1; ERF4, ethylene response factor 4; P Val, p-values (0.05); FDR, false discovery rate values.

Table 2. Statistical analysis of some salicylic acid (SA) and jasmonic acid (JA) pathway-related genes in tomato super strain B under drought stress with day/night photoperiod and normal light conditions.

Pathway	Name	Accession Number	Night (First Time)		Day (Second Time)	
			P Val	FDR	P Val	FDR
SA	PAL5	NM_001320040	0.0297	0.4884	0.0047	0.1283
	PAD4	XM_004233289	0.0433	0.5820	0.1305	0.8036
	EDS1	NM_001320249	0.0037	0.1073	0.0014	0.0408
	NDR1	XM_004228677	0.4687	0.9378	0.2984	0.9034
	NPR1	NM_001247629	0.1756	0.8496	0.1615	0.8351
	EDR1	XM_004245317	0.9432	0.9681	0.0104	0.2454
JA	LOX	NM_001247944	0.8731	0.9656	0.9463	0.9674
	AOS2	NM_001287778	0.0039	0.1107	0.0016	0.0466
	AOC	NM_001247090	0.6605	0.9551	0.7167	0.9574
	OPR1	NM_001247852	0.0000	0.0001	0.0000	0.0000
	VSP	XM_004235589	0.4767	0.9388	0.7831	0.9609
	WRKY51	XM_004245017	0.0252	0.4480	0.1851	0.8530
	JAI1	NM_001324483	0.7250	0.9589	0.1880	0.8550

SA, Salicylic acid; JA, Jasmonic acid; PAL5, phenylalanine ammonia-lyase 5; PAD4, Phytoalexin Deficient 4; EDS1, Enhanced Disease Susceptibility 1; NDR1, Non-Race-Specific Disease Resistance1; NPR1, non-expressor of pathogenesis-related genes 1; EDR1, disease resistance 1; LOX, Lipoxygenase; AOC, allene oxide cyclase; VSP, vegetative storage protein; PDF1, plant defensin 1; WRKY51, transcription factor51; JAL1, jasmonate resistance long hypocotyl 1; AOS, Allene oxide synthase; OPR1, 12-oxo-phytodienoic acid reductase1; P Val, p-values; FDR, false discovery rate values.

than in the day by 3.46 folds, while NCED1 and ABA3 appeared slightly expressed at night compared to the day. On the day, the RD22 gene was slightly upregulated; ABA2 was moderately rose (1.47 folds) while ABI1 genes were highly upregulated (9 folds) (Table 1**)**.

Regarding the ET pathway, the expression of 8 genes was investigated. While 38.69 folds strongly expressed ERF1 throughout the night, the ERF4 and CTR1 genes were only slightly elevated (3.15 and 1.27 folds, respectively). During the day, there was a modest upregulation of ACO and ACS1. EIN2 and EIN3 were highly expressed in the day, recording increments of 9.28 and 15.73 folds, respectively. However, the PDF1 gene expression appeared to be not liable to stress (Table 1).

Six SA pathway genes were examined for expression in a drought-stressed environment with day/night

photo period and normal light conditions. Table 2 revealed that the expression of NDR1 and NPR1 genes was unaffected by drought stress during the night/day photoperiod; however, PAL5, EDS1, and EDR1 genes exhibited nocturnal activities, and the PAD4 gene had diurnal one. While the EDS1 gene was expressed 2.6 times more at night than during the day, the EDR1 and PAL5 genes were considerably elevated (4 times more) at night. The daytime expression of the PAD4 gene was 1.3 times higher than nighttime expression.

Concerning the JA pathway, the expression pattern of 7 genes was assessed under drought with day/night photoperiod and normal light intensity (Table 2). Drought stress did not alter the LOX, AOC, or VSP genes throughout the day/night photo period. AOS2, OPR1, and JAI1 genes were more regulated at night than day, while the WRKY51 gene showed a 50% increase in activity during the day. So, under drought stress, the gene expression of ABI1 and ERF1 was selectively more activated through the ABA and ET pathways (Table 1), and EDS1 and OPR1 gene expression were highly responsive through the SA and JA pathways (Table 1)**.**

During 48 hours under day/night photo period with normal light circumstances, the drought-stressed tomato cultivar super strain B's expression pattern of several genes implicated in the ABA, ET, SA, and JA pathways was observed (Figures 1 and 2). ABA2, ABI1, FLACCA, and NCED1 were the genes studied in the ABA pathway; during the two study days, these genes' expression patterns varied during the day and night.

The expression of the ABA2 gene appeared almost constant during the experiment, except for the morning and midday (6:00 am to 12:00 pm) times when it was regulated. In the case of the expression of the ABI1 gene, for 48 hours, its afternoon down-regulated periods were crossed with its up-regulated expressions. However, the NCED1 and FLACCA genes showed fluctuation in their expressions during the experimental time under drought stress (Figure 1).

The EIN2, EIN3, EIL4, and ERF1 genes of the ET pathway were divergently expressed in drought-stressed-tomato super strain B for 48 hrs. (Figure 1). The EIN2 gene appeared to be slightly downregulated in the morning (6:00 to 10:00 am). The expression of the EIN3 gene started to down-regulate from midnight to mid-day and upregulated in the afternoons and evening periods. EIL4 gene expression began to diminish from late evening to midday, then upregulated again till the next late evening. However, ERF1 gene expressions were reduced during the late mornings (10:00 am) along with the afternoon, then gradually upregulated till the following late morning.

The JA pathway studied genes AOS2, OPR1, and AOC, which showed different expression patterns. The AOS2 gene was slightly downregulated from sunrise to midday (4:00 a.m. to 12:00 p.m.), the OPR1 gene was less active from 10:00 a.m. to 2:00 p.m., and the AOC gene slightly fluctuated, with the expression intensity being higher on the first day than on the second day (Figure 2).

The SA pathway was studied concerning the genes NPR1, EDS1, BURP, and BURP (Figure 2). Between 4:00 pm and 2:00 am, the EDS1 gene was elevated, while in the remaining times, it was moderately downregulated. NPR1 gene appeared to be downregulated from midday to afternoon (12:00 pm to 6:00 pm) and upregulated throughout the other periods, recording higher expression from sunrise to 6:00 am. The BURP gene appeared to have nearly unaffected expressions under drought stress during the experimental time. SAG101 gene was expressed differently through the 48 hours; on the first day, the gene was highly upregulated throughout the evening (10:00 pm to 2:00 am), while on the second day, the gene was aggressively downregulated and fluctuating.

By comparing the expression of genes involved in the ABA, ET, SA, and JA pathways along the experimental time under drought stress (Figures 1 and 2)**,** results revealed that early activation of the genes' expression in ABA and ET pathways than those of SA and JA pathways.

Figure 3 illustrates the gene expression of stomatal-related genes in tomato super strain B during day/night photo period, drought stress, and normal light conditions for 38 hours. The probable protein phosphatase 2C24 genes (XM_004241163.4) were up-regulated from sunset until 8 am; after that, they progressively began to down-regulate, reaching a minimum at 8:00 pm before rising again. Protein phosphatase 2C53 (XM_004253043.4) gene expression rose by evening, attaining its maximum by 4:00 am (sunset). The expression of the protein phosphatase 2C53 (XM_004253043.4) gene increased by the evening and reached its peak by 4:00 am (sunset), following which it steadily declined in the morning and afternoon. However, the serine/threonine-protein kinase gene (XM_004232007.4) showed a sudden jumping rise in its expression starting from midday, recording its maximum from 4:00 to 6:00 in the afternoon (106.675

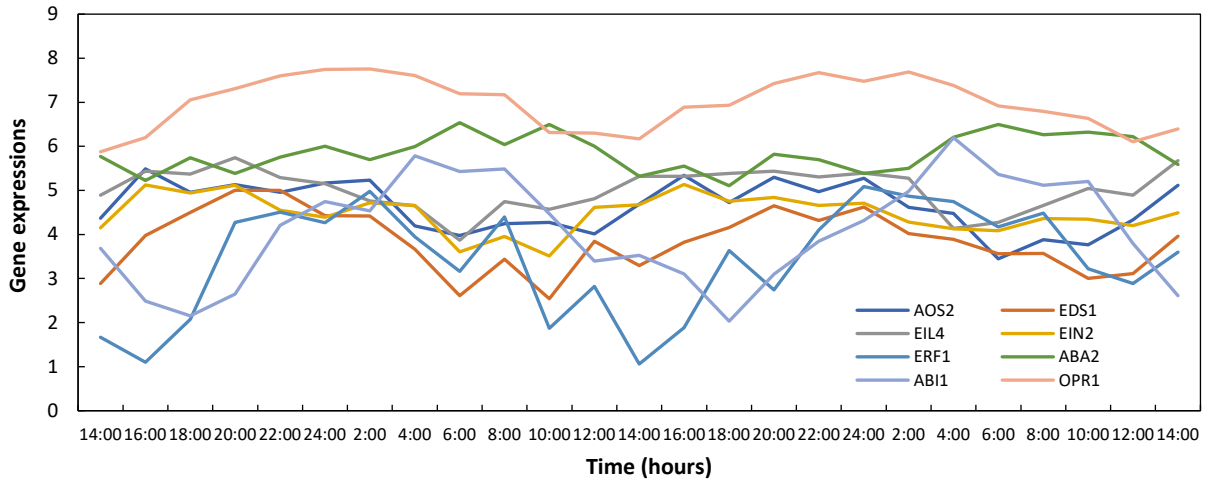

Figure 1. The expression pattern of genes associated with the abscisic acid (ABA) and ethylene (ET) pathways in the drought-stressed tomato cultivar super strain B over 48 hours under normal light conditions and day/night photoperiod.

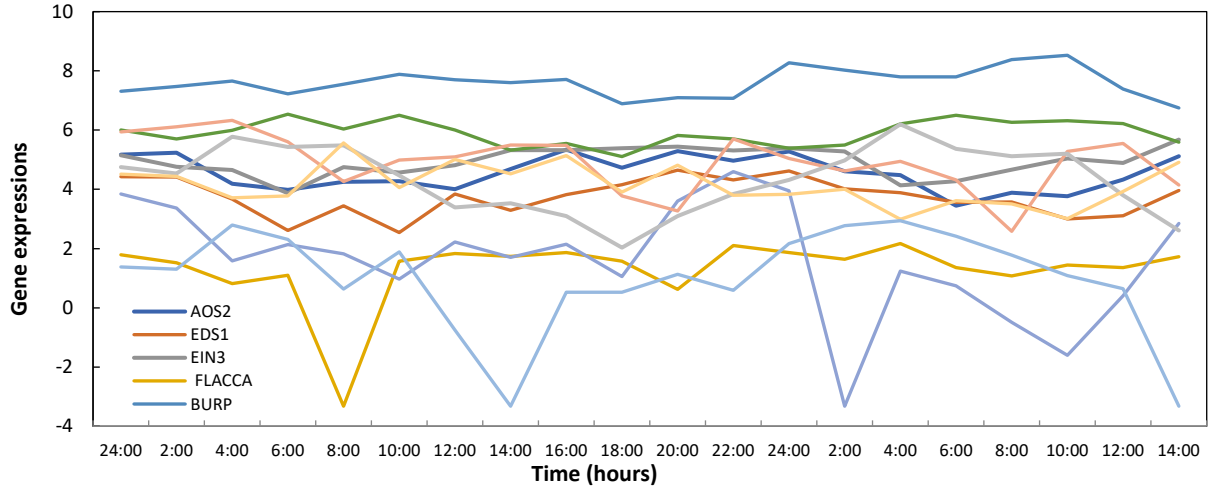

Figure 2. The expression pattern of genes associated with the salicylic acid (SA) and jasmonic acid (JA) pathways in the drought-stressed tomato cultivar super strain B over 48 hours under normal light conditions and day/night photoperiod.

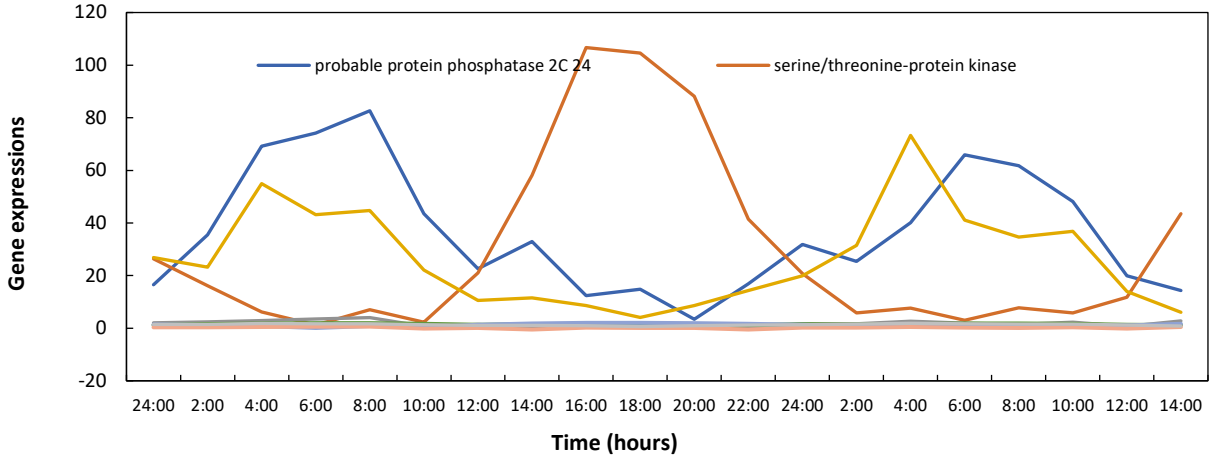

Figure 3. The expression of stomatal-related genes in tomato super strain B under day/night photo period, drought stress, and normal light conditions for 38 hours.

&104.600, respectively). After that, it gradually started down-regulating from evening to midnight, reaching its minimum value by sunrise and morning. The expression of the guard cell S-type anion channel SLAC1 gene (XM_004245638.4) appeared to fluctuate randomly.

By correlating the expression of genes involved in stomatal regulation (Figure 3) with those of hormonal pathways (Figures 1 and 2) during the experimental time under drought stress, results disclosed that the expression of stomatal-related genes had more drastic fold changes along with the gradual changes in genes' expression of hormonal pathways.

Figure 4 shows the gene expression pattern of the late elongated hypocotyl (LHY) transcription factor family of tomato super strain B under drought stress with a day/night photoperiod and normal light conditions for 48 hrs. LHY expression rose 12 hrs. from midnight to midday, with the highest values from sunrise and morning hours. However, its lower expression periods were in the afternoon and the evening. So, these genes were periodically expressed, with time-of-day-dependent peaks.

Analogizing the expression of stomatal-related genes (Figure 3) with LHY expression (Figure 4) revealed that the downregulation of LHY expression in the afternoon and at night aligns with a decrease in both Protein Phosphatase 2C24 and Serine/Threonine-Protein Kinase expressions and inversely with Protein phosphatase 2C53 gene expression at the same time.

Environmental stressors can greatly affect plants by activating a cascade of complex regulatory systems and activating plant hormone pathways. Using the Blast system, homologs of hormone-pathways-related genes of the ABA, ET, SA, and JA regulation mechanism (biosynthesis, signaling, and suppression) in tomato super strain B were predicted and included on the previously published schematic diagram of *A. thaliana* according to characteristic motifs and domains and changes in expression data (Figure 5).

Also highlighted several key genes necessary for the synthesis of ABA: 9-Cis-epoxy carotenoid dioxygenase (NCED), abscisic acid-deficient 2 (ABA2), abscisic acid-deficient 3 (ABA3), Arabidopsis aldehyde oxidase 3 (AAO3) and dehydration-responsive gene (RD22). ABA is crucial for managing plant responses to abiotic stress, particularly drought and salinity. The genes involved are responsible for converting precursor molecules into ABA, which then helps the plant conserve water by inducing stomatal closure and other protective measures. The gene Abscisic acid insensitive 1 (ABI1) temporally expressed itself in response to stimuli for adaptive responses inhibiting the ABA pathway.

Plants use the ET system to control growth and stress responses by showcasing the genes that produce ethylene and its signal transduction. Figure 5 emphasized genes like 1-aminocyclopropane-1-carboxylate oxidase (ACO1) and *Arabidopsis* cysteine synthase 1 (ACS1), which are pivotal in the ethylene biosynthesis process as well as ethylene insensitive 2 (EIN2), ethylene insensitive 3 (EIN3) and plant defensin 1 (PDF1), which are involved in ethylene signal transduction and modulation, coordinating the plant's adaptive responses to environmental changes. Constitutive triple response 1 (CTR1) and ethylene response factor 4 (ERF4) are genes that inhibit the ET pathway. In contrast, the ethylene response factor1 gene (ERF1) is temporally expressed in response to stimuli for the plant's adaptive responses.

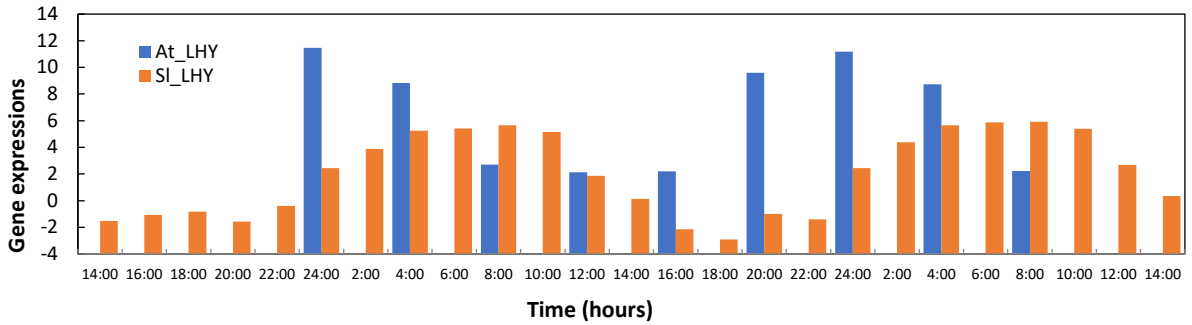

Figure 4. The gene expression pattern of the tomato super strain B late elongated hypocotyl (LHY) transcription factor family under drought stress with day/night photoperiod and normal light conditions for 48 hours compared with its homologous in *Arabidopsis salina*.

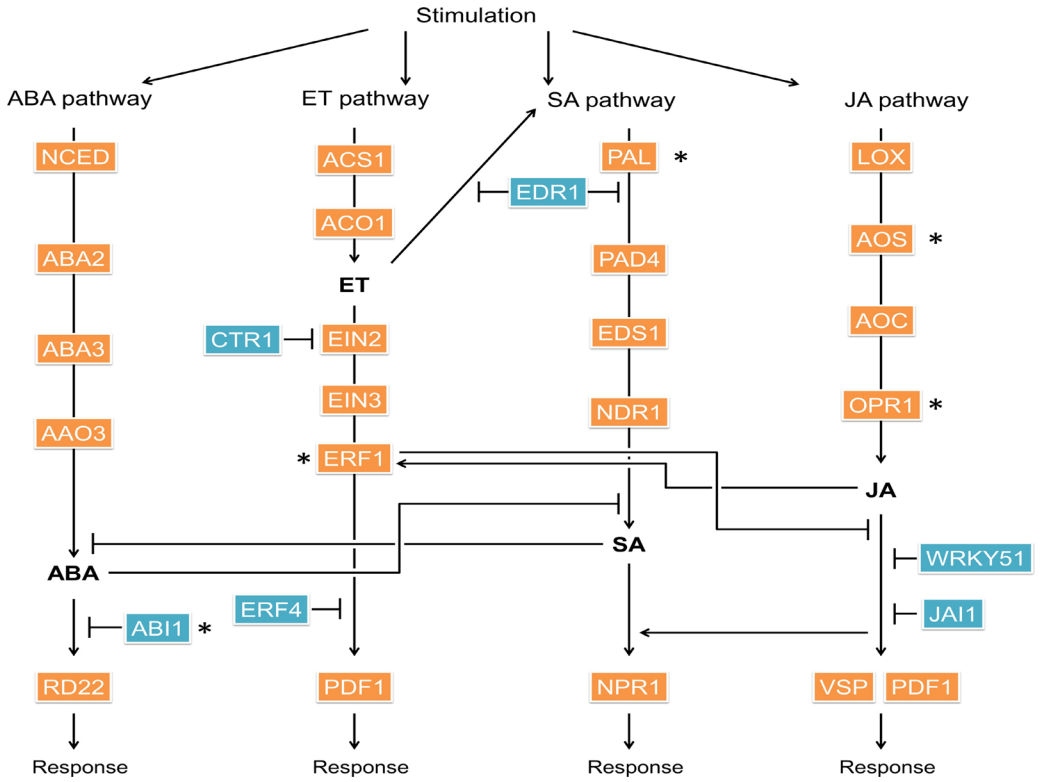

Figure 5. Schematic flow chat for the predicted Phytohormones [Abscisic acid (ABA), Ethylene (ET), Salicylic acid (SA), and Jasmonic acid (JA)] pathway-related genes. The homologous-related genes involved in the synthesis, signaling, and suppression of these hormones are visually distinguished by color-coded boxes: orange for genes responsible for the production and regulation of hormone levels and blue ones for suppressive genes, showing the temporally expressed genes (p-values $\leq$ 0.05) by asterisks (*) after stimuli responses.

Figure 5 highlights genes that contribute to the synthesis of SA and contribute to the defense against stress. Its pathway is monitored by Phytoalexin Deficient 4 (PAD4), Enhanced Disease Susceptibility 1 (EDS1), Non-Race-Specific Disease Resistance1 (NDR1) and non-expression of pathogenesis-related genes 1 (NPR1). The figure demonstrated that the enhanced disease resistance 1 (EDR1) gene can inhibit both ET and SA pathways, while the phenylalanine ammonia-lyase (PAL) gene is temporally expressed in response to specific stimuli for plant adaptive responses.

The JA pathway includes genes implicated in plant defense and stress responses, which are Lipoxygenase (LOX), allene oxide cyclase (AOC), vegetative storage protein (VSP), and plant defensin 1 (PDF1). Transcription factor51 (WRKY51) and jasmonate resistance long hypocotyl 1 (JAL1) genes inhibit JA hormone pathways. Also demonstrates that Allene oxide synthase (AOS) and 12-oxo-phytodienoic acid reductase1 (OPR1) genes had temporal expression in response to stimuli, underscoring adaptive responses (Figure 5).

DISCUSSION

Researchers found a link between the circadian clock and plant responses to drought, implying a close relationship between both pathways (Legnaioli et al. 2009; Wilkins et al. 2010; Marcolino-Gomes et al. 2014; Thanigai et al. 2015). The circadian clock is an endogenous timer that coordinates plant biological activities with day-night photo periods, providing organisms with adaptive advantages. Environmental cues such as light and temperature play an important role in entraining the circadian clock responses in this system (Marcolino-Gomes et al. 2014). The change in the temporal expression pattern of most of the ET, SA, and JA pathway-related genes and all ABA genes under drought stress along the regular night/day photoperiod and normal light condition, either by up or down-regulation, indicated that those biological clock genes were labile toward drought stress thus they try to stabilize and maintain the circadian rhythms either by diurnal or nocturnal reactions to provide tomato super strain B an adaptive way against the stress.

The significant activation of the ABI1 gene in ABA, the ERF1 gene in ET, the EDS1 gene in SA, and the OPR1 gene in JA pathways (Tables 1 and 2) appeared under drought, suggesting that these genes may be key genes in the stress response network for adaptation. The ABA pathway regulates stress-responsive genes and enhances drought tolerance in plants. The ET, SA, and JA pathways are critical for plant defense, influencing stress-responsive gene expression and integrating multiple stress signals for effective response (Munné-Bosch and Müller 2013). ABI1 encodes a protein phosphatase 2C (PP2C), a negative regulator of ABA signaling. Under drought stress, ABA accumulation promotes ABA receptor activation, inhibiting ABI1 (Ma et al. 2009). ERF1 is a transcription factor activated by ethylene and jasmonate signaling, which regulates stress-responsive genes (Cheng et al. 2013). Under drought stress, the EDS1 gene is critical in enhancing the plant's ability to cope with oxidative stress and accumulates SA (Lewandowska et al. 2013). The OPR1 gene is involved in the biosynthesis of JA (Wasternack and Strnad 2019).

The results indicated that the gene expression of ABA and ET pathways activate early during drought stress, while the gene expression of SA and JA pathways respond later. These may indicate that during drought periods, the early genes activation of ABA and ET pathways take precedence in drought response to conserve water and reduce oxidative stress damage (Cutler et al. 2010; Wilkinson and Davies 2010; Finkelstein 2013), while the delayed genes activation of SA and JA pathways focused on the downstream defense against prolonged stress exposure and recovery processes, not as immune responses (Pieterse et al. 2012; Wasternack and Song 2017).

In the present study, tomato super strain B was cultivated under drought stress and received regular sunlight from sunrise (4:00 am) to evening (6:00 pm). By tracking the gene expression of its stomatal-related genes, it can be observed that Probable protein phosphatase 2C 24 genes and Protein phosphatase 2C 53 were suppressed during light exposure. In contrast, the serine/threonine-protein kinase gene was upregulated in the period of high light intensity (midday to afternoon). Correlated results revealed that stomatal-related genes act as downstream effectors of hormonal pathways. This may be because, under drought stress, the genes of ABA and ET pathways drive stomatal closure to minimize water loss by triggering ion channel activity in guard cells and reducing turgor (Cutler et al., 2010; Wilkinson and Davies, 2010). However, the genes of SA and JA pathways indirectly influence stomatal behavior by modulating oxidative stress and ROS signaling in guard cells, ensuring long-term stress tolerance (Khan et al. 2015; Murata et al. 2015). By boosting antioxidant enzyme activity and preserving redox equilibrium, these pathways guarantee that guard cells are protected from oxidative damage during an extended period of stress (Wasternack and Song 2017).

Stomatal control is essential to plant water efficiently, especially during droughts. This may be a way for the stomata to try to open only in the dark and close in the daylight hours, avoiding loss of internally stored water under drought stress through the hormone-regulated genes. The process of ABA-induced stomatal closure to reduce water loss is crucial, and it is often associated with transcriptional changes in protein phosphatases. During drought, ABA levels rise and signal stomatal closure to prevent water loss. Research reported that 9-cis-epoxy carotenoid dioxygenase, abscisic acid-deficient 2 (ABA2), and abscisic aldehyde oxidase 3 (AAO3) are involved in the ABA pathway and are induced by and confer resistance to abiotic stresses such as drought (Seo and Koshiba 2002). Also, ABI1 and OST1 are the major genes in the ABA pathway implicated in the gating of stomata (Lee et al. 2009).

The present investigation also revealed that the random fluctuation in the expression of the guard cell S-type anion channel SLAC1 gene (XM_004245638.4) may be indicated by its high sensitivity to drought stress independent of light exposure and the hard effort of the guard cell S-type anion channel SLAC1 gene to monitor the ion channel responsible for the stomatal closure/opening. In this concern, some reports mentioned that tomato stomata open in the evening and close in the morning, manifesting decreased humidity during daylight hours. When the tomato plants were grown under dry conditions in the daytime (such as in drought), they suggested that tomato plants do not open stomata to suppress transpiration and produce ABA synchronously. The hydraulic signaling model suggests that root-derived chemical signals, like ABA, synchronize with stomatal rhythms to regulate transpiration under water-deficit conditions (Moldau et al. 2011; Tanigaki et al. 2015).

However, in unstressed tomato, the ABI1 gene and other proteins such as open stomata 1 (Sl OST1; XM 004232007), protein phosphatases type 2C from group A (PP2CA, Sl PP2CA; XM 004241163), and slow anion channel-associated 1 (SLAC1, SL_SLAC1;

XM_004245638) are involved in the opening and closure mechanism of stomata. It has also been revealed that ABI1 and OST1 are major genes associated with this mechanism (Lee et al. 2009). The ABI1 gene interacts with the OST1 gene, inhibiting its activity without ABA (Creelman et al. 1992). A related observation was also recorded by unstressed *Arabidopsis thaliana* (Lee et al. 2009).

The gene expression pattern of the late elongated hypocotyl (LHY) transcription factor family of tomato super strain B under drought stress with day/night photoperiod and normal light conditions for 48 hrs. was highly up-regulated throughout a 12-hour morning period and then down-regulated in the afternoon and evening. This trend can be explained in *Arabidopsis thaliana* as the clock's oscillatory mechanism is built on an interlocked transcriptional-translational feedback loop with three inhibitory steps: the inhibition of evening complex (EC) genes (ELF3, LUX, and ELF4) by the late-night rise of LHY/CCA1, the inhibition of PRR genes by EC, and the inhibition of LHY/CCA1 by PRRs during the day (Pokhilko et al. 2012). LHY is a circadian clock component that controls several physiological functions, including plants' reactions to stress. The transcription factors LHY and circadian clock associated 1 (CCA1) are expressed in the early morning, just after dawn (Genoud et al. 1998; Wang and Tobin 1998). They bind to Evening Element (EE) motifs (AAAATATCT) present in the promoters of timing cab2 expression1 (TOC1) to repress its transcription (Adams et al. 2015). However, the expression of TOC1 peaks in the early evening. As the levels of LHY and CCA1 proteins decrease in the afternoon, the PRR proteins are expressed in waves and repress LHY and CCA1 transcription until the next morning (Nakamichi et al. 2012). Late at night, an Evening Complex composed of early flowering 3 and 4 (ELF3 and ELF4) and lux arrhythmo (LUX, also signed as photo clock 1 or PCL1) lifts this repression, which allows LHY and CCA1 transcripts to be expressed at dawn and the cycle to restart (Nusinow et al. 2011).

The present investigation found that genes of LHY transcription factors **s**howed periodic expression with peaks from midnight to midday. LHY genes also regulated the timing of gene expression in all hormonal pathways. They influenced the responses of stomatal-related genes. The genes of ABA and ET pathways are pivotal in stress signaling, which might intersect with circadian regulation influenced by LHY genes (Hubbard et al. 2010; Carre 2015). Moreover, LHY has recently been found to play a role in ABA pathway regulation, as it can bind the promoters of many ABA biosynthesis, signal transduction genes, and responses. Furthermore, they discovered that LHY plays a role in regulating ABA accumulation as a new mechanism linking circadian regulation with drought and osmotic stress tolerance via ABA signaling. Also, LHY-mediated ABA signaling may interact with ROS detoxification pathways, providing an additional layer of drought adaptation (Adams et al. 2018). Also, LHY transcription factors may influence SA and JA signaling, as these pathways often interact with the circadian clock to modulate stress, defense responses, and drought adaptation (Belbin and Dodd 2018). There may be an indirect role for LHY in regulating stomatal behaviors through time-of-day signals, as control of stomatal opening and closing under drought stress is probably impacted by circadian rhythms (Dodd et al. 2004; Carre 2015).

Finally, the present investigation could reveal the presence of a strong connection between the circadian clock and plant responses to environmental stress, clarifying how temporal gene expressions are finely tuned to maintain circadian rhythms and adapt to drought. ABA pathway’s genes, ABI1, NCED1, and FLACCA, appeared to be critical for drought-induced stomatal closure, helping in water conservation. The antagonistic roles of ABA with both SA and JA produced a balance between growth and stress responses. Diurnal rhythms are exhibited by genes like LHY, which are key in connecting circadian control and stress tolerance. Temporal variations in gene expressions of ABA, ET, SA, and JA pathways underscore the plant's ability to effectively modulate the responses of tomato Super Strain B to drought signaling.

CONCLUSION

The study highlights the temporal regulation of phytohormonal pathways in tomato Super Strain B under drought stress, providing insights into adaptive mechanisms. It revealed that temporal gene expressions are precisely regulated to preserve circadian rhythms and adapt to drought, establishing a close relationship between circadian-regulated factors and key stress-responsive pathways of tomato plants to drought stress.

AUTHOR CONTRIBUTIONS

H.S.A.-Z., T.A.A.M., and H.A., idea, conceptualization, construction of the experiment, and writing the manuscript; H.S.A.-Z., T.A.A.M. and M.P.F., review and editing the manuscript; T.A.A.M. and R.M.H.,

interpreting the results; R.M.H., writing, review the manuscript. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY

The data used to support the findings of this study are included in the article.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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