https://doi.org/10.48048/tis.2024.7934

Transcriptome Analysis of Drought-Tolerant Mechanisms in Mutant Chili

Gadewara Matmarurat[1], Katharat Chutinanthakun[2], Piyada Juntawong[3] and Ornusa Khamsuk[1,*]

[1]*Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[2]*Department of Applied Radiation and Isotope, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[3]*Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

([*]Corresponding author's e-mail: fsciosk@ku.ac.th)

Received: 8 January 2024, Revised: 1 February 2024, Accepted: 8 February 2024, Published: 20 June 2024

Abstract

Drought is a natural disaster that negatively impacts agricultural crops. Understanding the mechanisms of water deficit response is key to improving drought-tolerant crops. This study focused on comprehending the drought-tolerant mechanisms of a mutant chili, CaWDT-2, by investigating its physiological phenotypes and gene expression under PEG-induced drought condition compared to Tavee 60 as the original cultivar. Chili physiological status was monitored by shoot height, root length, leaf number, leaf area, shoot mass and root mass at 10 d after drought initiation. Relative water content (RWC), malondialdehyde (MDA), proline, soluble sugar and sucrose were measured during drought and after recovery. Transcriptome analysis using sequencing of RNA (RNA-seq) was employed to examine gene expression profiling under drought for 24 h. Results revealed diverse changes in physiological mechanism in response to drought between CaWDT-2 and Tavee 60 cultivars. Comparative transcriptomic analysis is helpful in understanding the differences in drought tolerance mechanisms between 2 crop varieties. Major differences that emerged from the physiological and transcriptomic analyses included drought-stressed injury level, proline accumulation, root cell wall adaptation, ABA biosynthesis, stress detoxification, heat shock proteins and transcription factors. Our data will contribute to further research in developing drought-tolerant crops. An improved understanding of drought tolerance mechanisms will enhance agricultural resilience and mitigate the impact of drought on crop yield.

Keywords: CaWDT-2, Proline, Drought-tolerant chili, Gene expression

Introduction

Drought adversely impacts the growth and productivity of many plant species. Global warming is predicted to increase drought frequency and severity, with arid areas expanding and water shortages increasing. Chili (*Capsicum* sp.) is renowned for its health benefits and medicinal properties, leading to increasing demand but climate change and water shortages have reduced suitable cultivation areas. Breeding and selection of chili cultivars that can adapt to drought offers a promising solution to address this problem. CaWDT-2, a mutant chili, was developed from the original variety Tavee 60 through gamma ray irradiation and subsequent screening under drought. CaWDT-2 exhibited fewer negative effects compared to Tavee 60 including minimal reduction in stem size, large leaves and high proline accumulation. Importantly, while Tavee 60 yield decreased under drought, chili production of CaWDT-2 remained unaffected [1]. Drought-tolerant plants can adapt their morphological and physiological responses. Transcriptomic analysis is a useful tool for clarifying and confirming differences in stress tolerance between varieties or cultivars, and an effective strategy for analyzing molecular stress responses from physiological measurement data. It has determined that drought resulted in the up-regulation of genes related to plant hormone signaling and the MAPK pathway, leading to enhanced drought tolerance [2], while a transcriptomic comparison showed significantly increased transcript abundance in drought-sensitive wheat under drought condition [3]. The analysis of RNA sequencing (RNA-seq) revealed the response mechanisms to drought in eggplant, with the transcriptome profiles displaying differential expression patterns of drought-related genes. Plants subjected to more severe drought exhibited a higher number of differentially expressed genes (DEGs), while gene ontology enrichment analysis indicated that drought-tolerant eggplant triggered the regulation of numerous transcription factors. Consequently, RNA-

seq analysis has proven to be a suitable tool for studying the mechanisms of drought tolerance in plants [4]. The drought-tolerant mechanism of the CaWDT-2 cultivar was evaluated based on the physiological phenotype of the whole plant and the gene expression in its root under PEG-induced drought. Results showed changes in physiological mechanisms in response to drought stress, highlighting the differences between the CaWDT-2 and Tavee 60 chili cultivars. Comparative transcriptomic analysis is helpful in understanding the differences in drought tolerance mechanisms between CaWDT-2 and Tavee 60 varieties.

Materials and methods

Plant preparation and drought condition

The experiments were conducted at the Department of Botany, Kasetsart University, Bangkok, Thailand. Seeds of the drought-tolerant chili, CaWDT-2 and the drought-sensitive chili, Tavee 60 were planted in peat moss. The 3-week-old plants were transplanted into a hydroponic solution to estimate drought-tolerant characteristics. Each genotype was divided into control and drought treatments. The drought treatment was created by adding 15 % PEG at 4 weeks after planting [1]. Plants were allowed to recover by transferring them to the hydroponic solution without PEG at 10 d after drought. The water deficit was maintained for 24 h to study gene expression. All experiments were conducted using a randomized complete block design (RCBD) with 5 replications and 3 biological replications.

Evaluation of plant phenotype during drought stress and recovery

RWC and all substances including MDA, proline, soluble sugar, reducing sugar and sucrose were measured at 0, 5 and 10 d after PEG addition and at 5 d after recovery. Leaf number, total leaf area, shoot height, root length, shoot mass and root mass were measured at 10 d after PEG addition to investigate plant biomass.

The measurement of RWC in leaves was conducted following the method described by González and González-Vilar [5]. An aliquot of 0.1 g of fresh leaf was soaked in water until saturated weight and then incubated at 70 °C for 1 week to determine the dry weight. The RWC was calculated using the following formula.

$$RWC = ((\text{fresh weight} - \text{dry weight})/(\text{saturated weight} - \text{dry weight}))\times 100$$

MDA was estimated using the TBA method outlined in the work of Zhang and Kirkham [6]. A 0.1 g aliquot of ground leaf was added with 0.1 % TCA, incubated at room temperature for 10 min, and then centrifuged at 10,000× g for 20 min at 4 °C. Then, 0.5 mL of the supernatant was mixed with 1 mL of 0.5 % TBA (prepared in 20 % TCA). This mixture was incubated at 98 °C for 30 min and chilled on ice for 5 min. The specific absorbance of the product was recorded at 532 nm, with the nonspecific background absorbance at 600 nm subtracted from the reading. The concentration of MDA was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} of fresh weight.

The proline content was detected using the colorimetric method with acidic ninhydrin reagent, as described in Koca *et al.* [7]. A 0.1 g aliquot of ground leaf was mixed with 2 mL of 3 % sulfosalycylic acid. Glacial acetic acid and ninhydrin reagent (1 mL each) were added. The mixture was heated in a boiling water bath for 30 min and then cooled in cold water. Toluene (2 mL) was added, and the solution was mixed by vortexing. The suspension was measured by a spectrophotometer at a wavelength of 520 nm. The proline content was determined from a standard curve.

The concentration of soluble sugar was determined using the anthrone method, following the procedure detailed in Yemm and Willis [8]. Soluble sugar was extracted from 0.1 g of ground fresh leaf using 2 mL of distilled water. One mL of sample solution was mixed with 9 mL of anthrone reagent, consisting of 72 mL 95 % sulfuric acid, 28 mL distilled water, and 50 mg anthrone. The sample solution was incubated in a water bath at 98 °C for 3 min and chilled on ice before the absorbance was read at 620 nm by a spectrophotometer.

The estimation of reducing sugar was conducted using the dinitrosalicylic acid method, as described by Miller [9]. Reducing sugar was extracted from 0.1 g of ground fresh leaf by 2 mL of distilled water. Then, a 1 mL sample was mixed with 1 mL dinitrosalicylate reagent, which was a mixture of 1 g 3,5-dinitrosalicylate, 0.2 g phenol, 0.05 g Na_2SO_3 and 1 g NaOH in 100 mL. The solution was incubated in a water bath at 90 °C for 12 min and added with 1 mL 40 % sodium potassium tartrate. The absorbance of the sample was read at 575 nm by a spectrophotometer. The concentration of reducing sugar was determined from a standard curve, and the sucrose content was determined by calculating the difference between the levels of soluble sugar and reducing sugar.

The total area of all expanded leaves was measured by taking a photo on graph paper and estimated the leaf area using the ImageJ program [10]. The shoot and root samples were incubated at 70 °C for 7 d, and their dry weights were measured.

Data analysis for phenotypic study

Comparisons of the measured parameters between CaWDT-2 and Tavee 60 were carried out by Duncan's test among phenotypes at $p \leq 0.05$. This analysis was performed using SPSS Statistics 17.0 program (SPSS Inc., Chicago, USA)

RNA extraction, library construction and sequencing

The roots of 5 samples from each treatment were harvested and frozen in liquid nitrogen at 24 h after adding PEG. Total RNA was extracted using the PureLink® RNA Mini Kit (Thermo Fisher Scientific). The integrity of the RNA samples was confirmed using a spectrophotometer. The RNA samples were precipitated by mixing the RNA solution, sodium acetate and ethanol at a ratio of 1:0.5:2. The precipitated RNA samples were stored at −20 °C until sequenced at Macrogen, Seoul, Korea.

After performing quality control, the qualified samples proceeded to the library construction and sequencing on Illumina SBS. The sequencing library was prepared by randomly fragmenting the cDNA sample, followed by 5' and 3' adapter ligation. Alternatively, "tagmentation" combined the fragmentation and ligation reactions into a single step and increased the efficiency of the library preparation process. The adapter-ligated fragments were then PCR amplified and gel purified. For cluster generation, the library was loaded into a flow cell, where the fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was subsequently amplified into distinct clonal clusters through bridge amplification. Once cluster generation was completed, the templates were ready for sequencing.

The sequencing data were converted into raw data for analysis. The Illumina sequencer generated raw images using sequencing control software for system control and base calling through an integrated primary analysis software called RTA (Real Time Analysis). The BCL (base calls) binary file was converted into FASTQ using the Illumina package bcl2fastq.

Analysis of differential gene expression

The analysis of differential gene expression was performed by the CyVerse Discovery Environment (https://de.cyverse.org/de/). Raw paired-end reads were uploaded using Cyberduck version 3. The FASTX clipper 0.0.14 was used to trim the adaptors in the raw reads. High-quality sequences (Phred quality score: $Q \geq 30$) were selected by FASTX fastq quality filter 0.0.14. The cleaned reads were detected by FastQC 0.11.5 (multi-file). The forward and reverse reads were aligned to the reference genome (*Capsicum annuum* CV. CM334 from https://solgenomics.net/organism/Capsicum annuum/ genome) by Tophat-2.1.1. Cuffdiff2 with the JS option was used to identify DEGs from the alignment output. The number of DEGs with $\log_2$ (fold change) ≤ -1 and ≥ 1 was visualized using a Venn diagram created by http://bioinformatics.psb.ugent.be/webtools/Venn/.

Identification of differentially expressed genes

The reference genome (*C. annuum* CV. CM334) was also annotated with *Arabidopsis thaliana* using Mercator 3.6 to construct a mapping file [11]. The mapping file was used for the visualization of biological pathways in MapMan 3.6.0RC1 (https://mapman.gabipd.org/web/guest/ mapman-version-3.6.0). The pathway analyses were corrected at $p \leq 0.05$.

Results and discussion

Chili mutant survived under PEG-induced drought

After 10 d of drought, both Tavee 60 and CaWDT-2 plants showed reduction in size without clear dehydration features (**Figures 1(A) - 1(D)**). The drought-treated plants were then recovered in the hydroponic nutrient solution. After 5 d, Tavee 60 plants showed brown leaves and rotted roots, while CaWDT-2 plants survived but began to show injury after 10 d of dehydration including necrotic leaves and partially rotted roots (**Figures 1(E)** and **1(F)**).

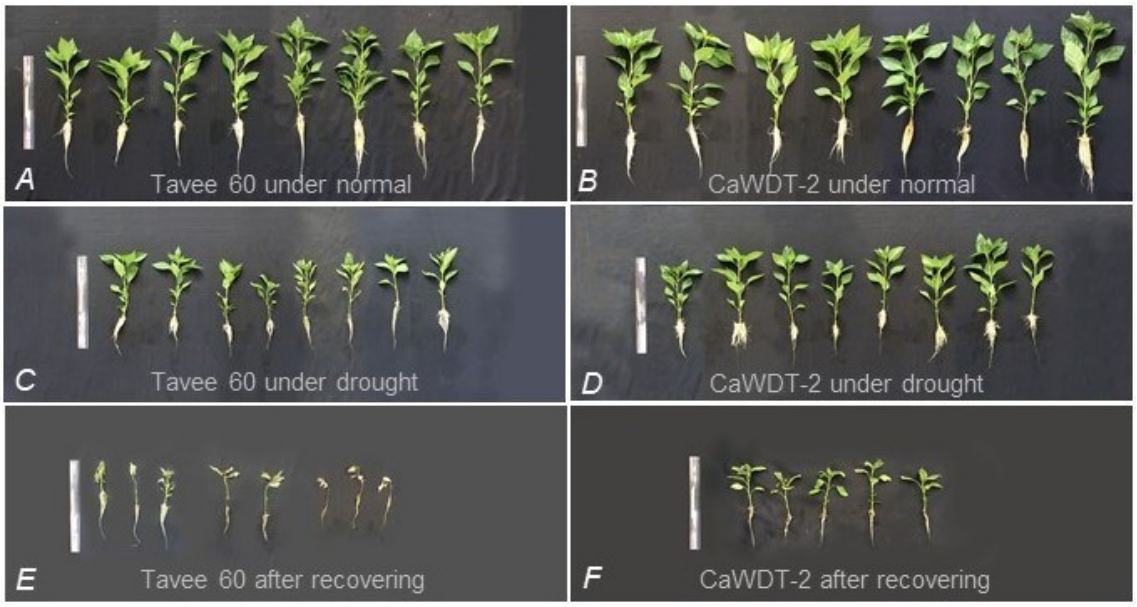

Figure 1 Tavee 60 (A, C and E) and CaWDT-2 (B, D and F) plants under normal, 10-d drought and 5-d recovery.

Root growth inhibition was less noticeable in drought-tolerant chili

Most plant growth parameters were unaffected in the 2 chili genotypes, except for root length, shoot and root mass (**Figure 2**). However, under drought, both Tavee 60 and CaWDT-2 exhibited reduced leaf number, total leaf area and plant height (**Figures 2(A)** - **2(C)**). Tavee 60 had longer roots under normal condition, while under drought, root length of Tavee 60 decreased by 20.54 % with CaWDT-2 experiencing a 16.01 % reduction (**Figure 2(D)**). After drought, both genotypes displayed reduced shoot and root mass, with a more pronounced reduction in shoot mass (**Figures 2(E)** and **2(F)**). The root mass of Tavee 60 (49.60 %) was more diminished than CaWDT-2 (47.97 %) under drought (**Figure 2(F)**).

Drought-tolerant chili exhibited less injury from drought

Tavee 60 did not survive after 10 d of drought exposure, with no recorded physiological parameters during the recovery phase. Water deficiency led to decreased RWC and increased accumulation of MDA, proline and soluble sugar in both Tavee 60 and CaWDT-2 (**Figure 3**). The RWC of both plants normally ranged from 89.23 to 93.53 %, with drought-stressed plants having low RWC from day 5 of drought. Our data showed that the mutant exhibited lower water loss than Tavee 60 (**Figure 3(A)**). After 10 d of dehydration, MDA level increased in CaWDT-2, coinciding with reduced RWC, while Tavee 60 exhibited 65.39 % higher MDA than CaWDT-2 (**Figures 3(A)** and **(B)**). In the recovery phase, the RWC of CaWDT-2 increased to 52.99 %, accompanied by a decrease in MDA content (**Figures 3(A)** and **(B)**).

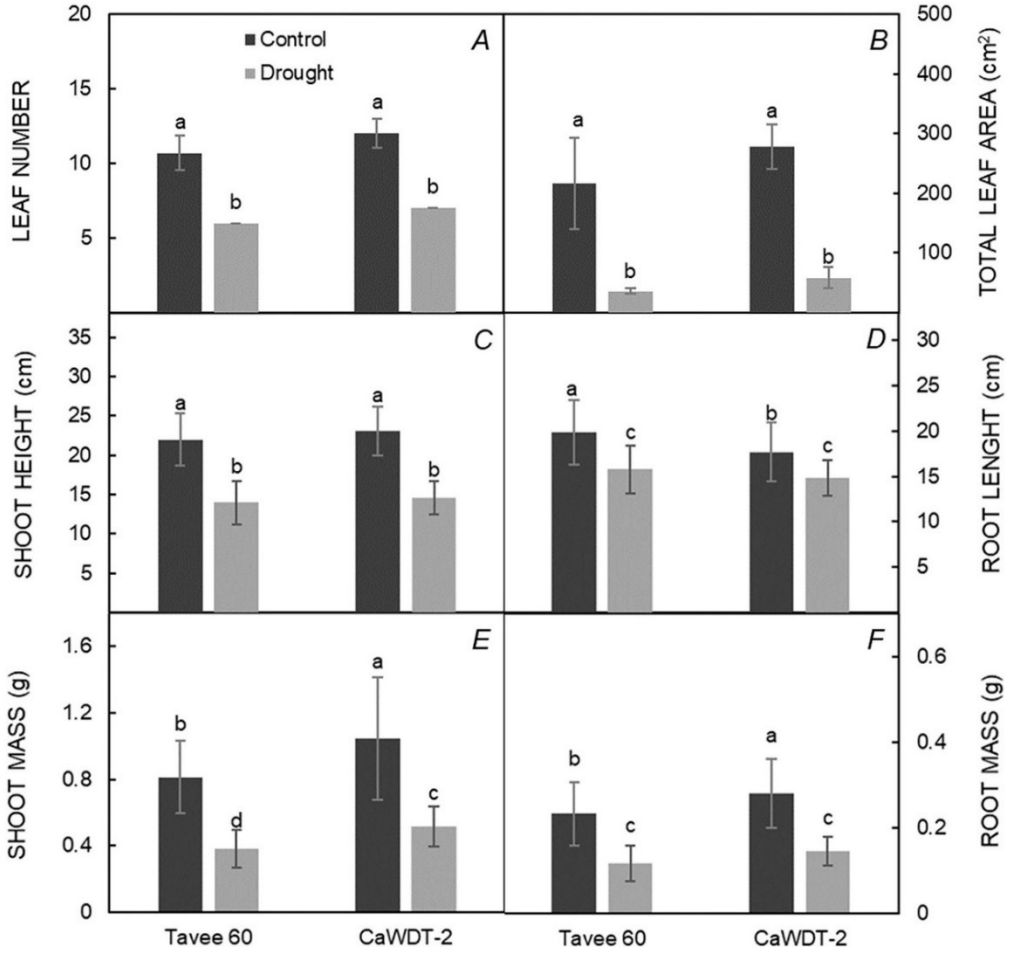

Figure 2 Leaf number (A), total leaf area (B), shoot height (C), root length (D), shoot mass (E) and root mass (F) of Tavee 60 and CaWDT-2 after growth under normal and 10-d drought. Data bars represent means ± SD. Bars with different lowercase letters are significantly different at $p \leq 0.01$ according to Duncan's test.

Drought stimulated osmolyte biosynthesis in both sensitive and tolerant genotypes, with CaWDT-2 accumulating proline and Tavee 60 accumulating soluble sugar to overcome water deprivation (**Figures 3(C)** and **3(D)**). The response of CaWDT-2 to drought was more rapid than Tavee 60, exhibiting higher proline level as early as day 5 of drought. After the recovery period, proline greatly reduced in stressed CaWDT-2 plants (**Figure 3(C)**). On the 10th day, Tavee 60 showed a 39.28 % higher increase in soluble sugar levels compared to CaWDT-2 (**Figure 3(D)**) mainly from different sucrose concentrations under stress. Drought caused an increase in reducing sugar and sucrose in both genotypes (**Figures 3(D) - 3(F)**). Results revealed a clear increase in reducing sugar at 5 d after drought, while sucrose levels of stressed plants were significantly higher than unstressed plants on the 10th day of dehydration (**Figures 3(E)** and **3(F)**). CaWDT-2 accumulated 40.58 % less sucrose content than Tavee 60. Our data indicated that water re-uptake did not influence the production of soluble sugar in CaWDT-2. By contrast, fluctuations of reducing sugar and sucrose levels were recorded in the recovered plants (**Figures 3(D) - 3(F)**). Reducing sugar exhibited a downward trend, while sucrose level remained elevated throughout the experiment.

Water deficiency negatively impacted the growth of CaDWT-2 and Tavee 60 plants. CaWDT-2 showed less drought damage than Tavee 60 due to efficient water conservation through proline accumulation (**Figures 3(A)** and **3(C)**). During early drought, CaWDT-2 lost less water than Tavee 60 and exhibited sensitivity to low water availability by promptly increasing proline level under early stress (**Figures 3(A)** and **3(C)**). This alleviated stress effects, as indicated by the low increase in MDA (**Figure 3(D)**). Tavee 60 and CaWDT-2 accumulated more soluble sugar compared to their controls, consistent with other studies showing massive proline and soluble sugar due to drought [12]. However, CaWDT-2 showed less impact than Tavee 60 in the aspect of soluble sugar accumulation (**Figures 3(C)** and **3(D)**). Previous

reports indicated that increased levels of soluble sugar and sucrose enhanced drought tolerance [13,14] while contrary findings indicated no correlation between soluble sugar level and drought tolerance [15]. In this study, the soluble sugar content of CaWDT-2 did not change after recovery, and the recovered plants displayed fluctuations between reducing sugar and sucrose levels. CaWDT-2 likely produced more sucrose instead of reducing sugar during the recovery period. Our previous report indicated decreased photosynthetic efficiency in chili plants under drought [16], leading to reduced soluble sugar supply to sink tissues, subsequently affecting growth (**Figures 2** and **3(D)**).

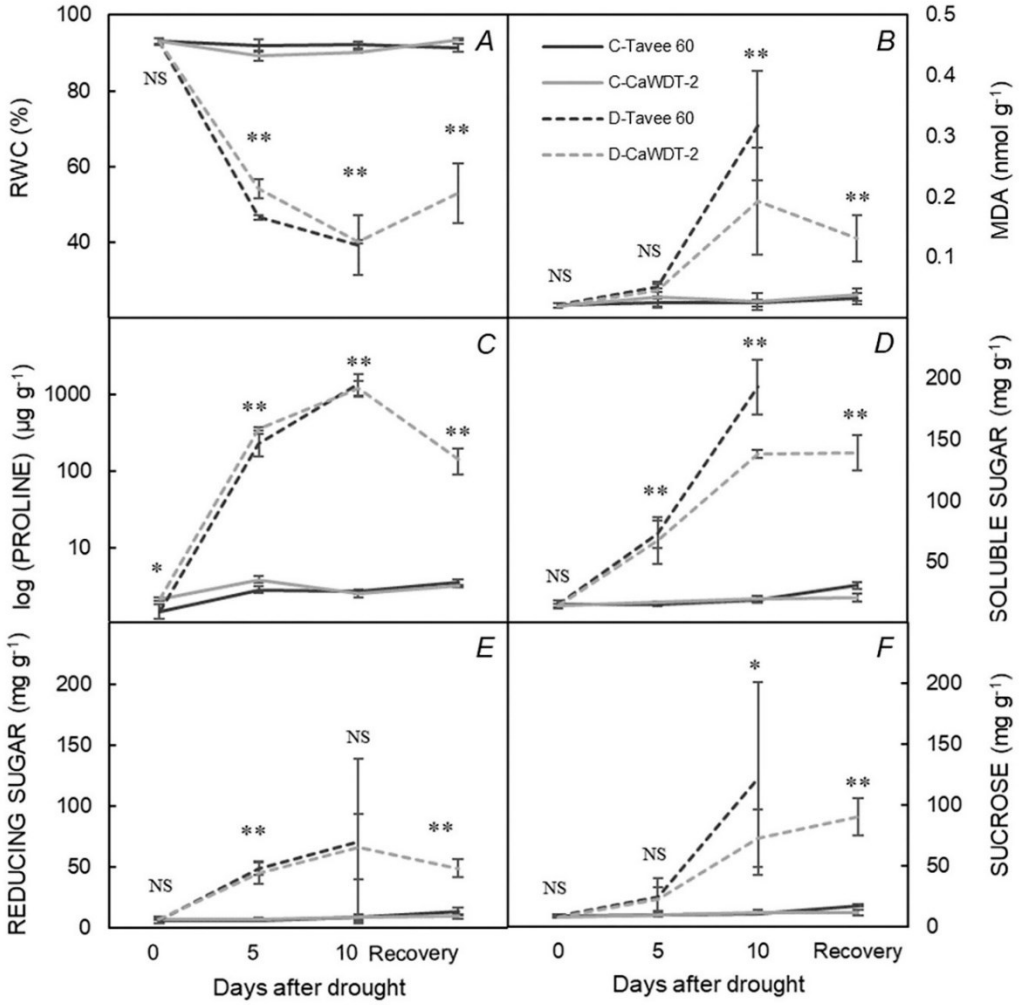

Figure 3 RWC (A), MDA (B), proline (C), soluble sugar (D), reducing sugar (E) and sucrose (F) of Tavee 60 and CaWDT-2 under normal (C-Tavee 60 and C-CaWDT-2) and drought condition (D-Tavee 60 and D-CaWDT-2) at day 0, 5, 10 and at 5 d after recovery. Data points are means ± SD. A single star (*) and double stars (**) indicate significant differences among treatments at $p \leq 0.05$ and $p \leq 0.01$, respectively according to Duncan's test. NS is non-significant difference.

CaWDT-2 mutant sensitively transcriptionally responded to drought

Based on our previous studies, CaWDT-2 showed fewer negative effects in response to water deficit compared to the original Tavee 60 [16]. It minimized the impacts of drought and achieved high fruit yields, highlighting its potential as a drought-tolerant chili genotype. In this study, RNA-seq was employed to examine its gene expression profiling under drought. RNA samples from the roots of Tavee 60 and CaWDT-2 under normal and 24-h drought condition were used for sequencing. The assembled transcripts of both plants under drought were mapped to the reference genome (*C. annuum* CV. CM334), representing on average 81.0 and 79.45 % of the 33,673,212 and 35,751,327 average clean reads, respectively (**Table Supplement 1**). Comparison of DEGs exhibited 4 sets including CaDWT-2 Control vs CaWDT-2 Drought (CC:CD), Tavee 60 Control vs Tavee 60 Drought (TC:TD), CaWDT-2 Control vs Tavee 60 Control

(CC:CT) and CaWDT-2 Drought vs Tavee 60 Drought (CD:TD). For DEGs of CC:CD and TC:TD, drought altered the expression of 4,584 genes in CaWDT-2 roots compared to unstressed roots, while expression levels of 3,378 genes in Tavee 60 were changed by drought. Under normal condition, the expressions of 102 genes in CaWDT-2 were significantly different from Tavee 60, whereas 67 genes were differentially expressed between both chilies under drought (**Figure 4(A)**). Apart from their expression levels, unique DEGs influenced differences in phenotypic responses to drought between Tavee 60 and CaWDT-2. The four sets CC:CD, TC:TD, CC:CT and CD:TD showed 1,832, 633, 32 and 11 unique DEGs (**Figure 4(A)** and **Table Supplement 2**). DEGs of each set expressed 2 regulations including up- and down-regulated genes. Results showed that drought stimulated the expression of 1,774 and 2,417 genes in the roots of Tavee 60 and CaWDT-2, respectively. By contrast, 1,604 genes in Tavee 60 and 2,167 genes in CaWDT-2 were suppressed under drought (**Figures 4(B)** and **4(C)** and **Table Supplement 2**).

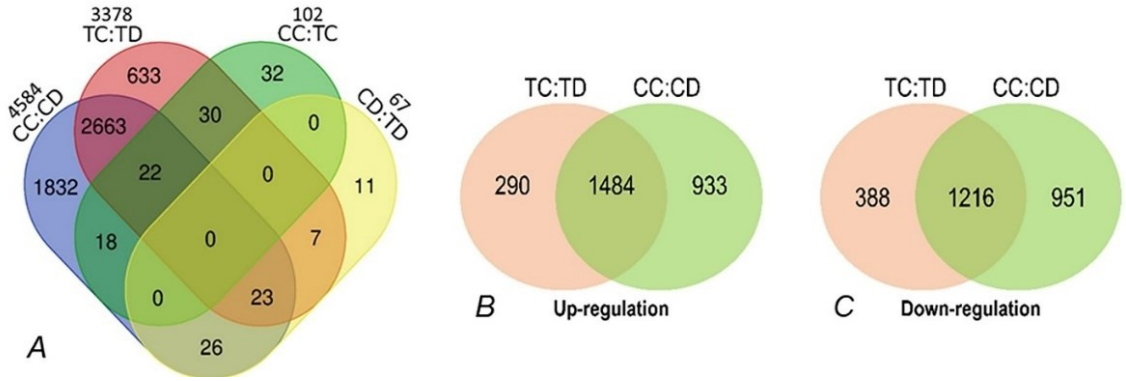

Figure 4 Comparison of DEGs. Venn diagram showing shared and unique DEGs from roots of 4 sets including CaWDT-2 Control vs CaWDT-2 Drought (CC:CD), Tavee 60 Control vs Tavee 60 Drought (TC:TD), CaWDT-2 Control vs Tavee 60 Control (CC:CT) and CaWDT-2 Drought vs Tavee 60 Drought (CD:TD) (A), number of up-regulated (B) and down-regulated (C) genes, respectively.

CaWDT-2 mutant showed a different drought response compared to Tavee 60

To better comprehend the drought-tolerant mechanism of CaWDT-2 compared to Tavee 60, the DEGs in the roots of both genotypes were visualized by MapMan (**Figure 5** and **Table Supplement 3**). The stress pathway showed that drought altered the expression of numerous genes in Tavee 60 in terms of proteolysis and cell wall. Some DEGs of Tavee 60 were also categorized into hormone signaling, peroxidases, glutathione-S-transferases and transcription factors (**Figure 5(A)**). By contrast, most DEGs in CaWDT-2 were grouped into abiotic stress, cell wall, protein degradation, heat shock proteins and secondary metabolism. Some DEGs of CaWDT-2 were also associated with peroxidases, transcription factors, hormone signaling and beta-glucanase (**Figure 5(B)**).

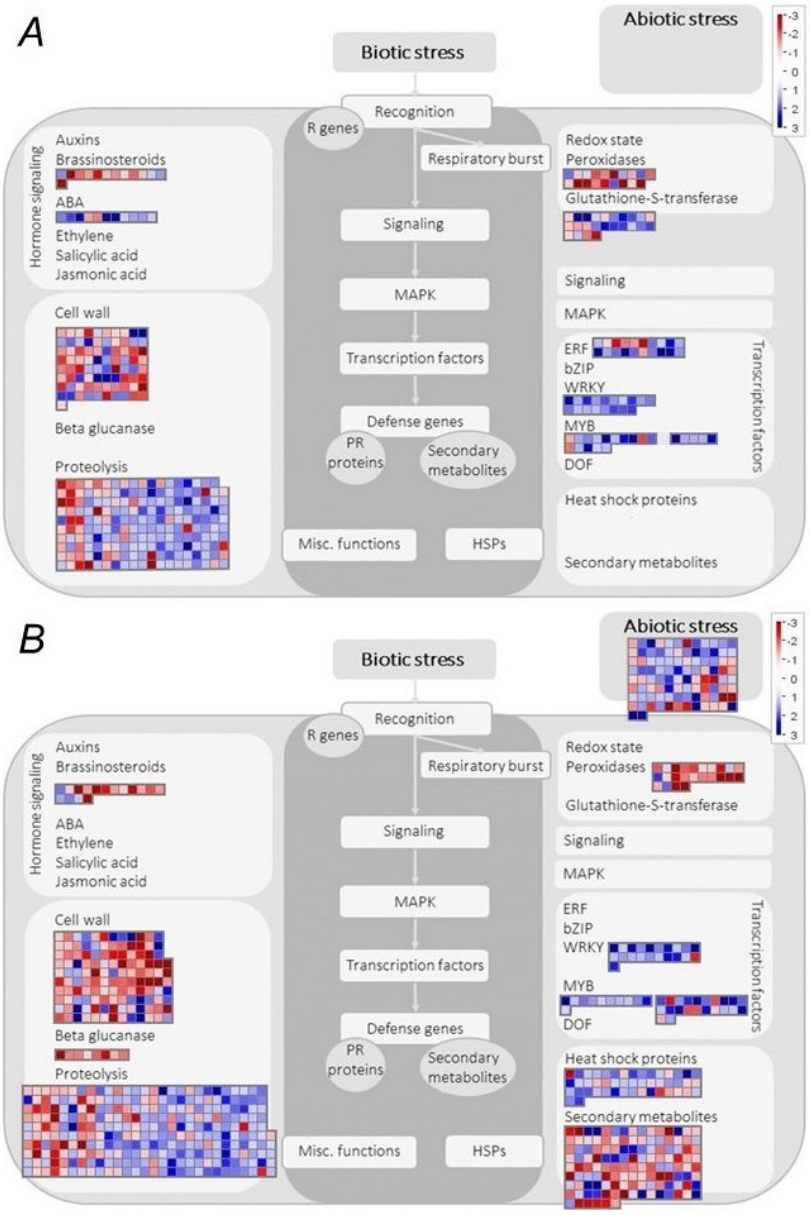

Figure 5 Drought stress response in the roots of Tavee 60 (A) and CaWDT-2 (B). Expression analysis was conducted by MapMan software from DEGs. Possible pathways are shown by colored squares in gradient shades of blue (up-regulated) and red (down-regulated). Each BIN is represented as a block and each DEG as a square.

Impaired root growth in both chilies correlated with active gene expression of aspartate proteases (AP) (**Figures 2(D)** and **2(F)**; **Table Supplement 4**). It has been found that increased expression of AP-related genes suppressed root development [17]. Altered nitrogen allocation in chili under low water availability was discovered, with more nitrogen content translocated to the aerial organs than accumulated in their roots, particularly for CaWDT-2, indicating a high level of gene expression involving proteolysis (**Table Supplement 4**). Similar findings in rice suggested that drought-induced proteases supported nitrogen uptake and mobilization for storage in the stem [18]. CaWDT-2 may convert root nitrogen to proline in aerial stems, accumulating high levels in shoots (**Figure 3(C)**).

Drought changed the expression level of genes involved in BRs and ABA signaling. Tavee 60 grown under drought for 24 h had DEGs associated with BRs and ABA metabolism, while the fluctuated expression level of genes in CaWDT-2 root occurred only in BR metabolism (**Figure 5** and **Table Supplement 4**). BRs are important plant hormones for development and responses to abiotic stresses [19,20]. Our results showed that reduced BR synthesis led to decreased root elongation and root mass in

both chilies (**Figures 2(D) - 2(F)** and **Table Supplement 4**). Under drought, the expressions of ABA genes reduced, and genes responsible for ABA degradation were highly active in Tavee 60 (**Table Supplement 4**), leading to decrease in ABA level in drought-stressed roots. Rapid water loss by low RWC in Tavee 60 likely resulted from low ABA levels, leading to late stomatal closure (**Figure 3(A)** and **Table Supplement 4**). The raised expression level of AP2/ERF also confirmed the low ABA level in Tavee 60 under drought (**Table Supplement 4**). AP2/ERF recognized DRE/CRT and ERE with GCC-box to confer abiotic stress tolerance [21]. It is regulated through the ABA-independent pathway to induce stress-responsive genes and displayed developmental defects such as senescence and growth retardation [22]. By contrast, the overexpressing genes of AP2/ERF improved stress tolerance in many species [23]. Expression of AP2/ERF in Tavee 60 changed under drought, consistent with reduction of ABA synthesis and the increment of ABA degradation (**Table Supplement 4**). However, DEGs of Tavee 60 were not categorized into abiotic stress-responsive genes (**Figure 5(A)**).

Up-regulation of transcription factors (TFs) in CaWDT-2 revealed its drought-tolerant strategies according to physiological characteristics. Drought expressions changed in TFs including WRKY, MYB and MYB-related (**Figure 6** and **Table Supplement 4**), supporting the role of TFs as key regulators of abiotic stress signaling pathways. The AP2/ERF responded to drought in Tavee 60 only (**Figure 5(A)** and **Table Supplement 4**), with WRKY regulated through the ABA-dependent pathway, while also supporting water retention, reducing electrolyte leakage and MDA, increasing antioxidant enzyme activities, facilitating osmolyte and biomass accumulation and mediating stomata movement [24-26]. Lower numbers of WRKY genes were found in Tavee 60 because WRKY expression was activated by ABA. In this case, CaWDT-2 retained high water content compared to Tavee 60 through stomatal closure by ABA and high proline accumulation. MYB regulates stomatal movement through ABA signaling and controls the expression of genes involving flavonoid, wax and cutin biosynthesis [27]. Drought negatively impacted the expression of most genes involved in the metabolism of flavonoids in CaWDT-2 but noticeably activated the expression of DFR genes, which encode the required enzymes for anthocyanin biosynthesis (**Table Supplement 4**). Anthocyanins scavenge radical species and mitigate oxidative stress, thus contributing to improved drought tolerance in CaWDT-2, while drought stimulated terpenoid accumulation in CaWDT-2 root (**Table Supplement 4**). Drought-tolerant sugarcane showed high expression levels of terpenoid biosynthesis genes that act as antioxidants to mitigate stress damage [28]. Therefore, CaWDT-2 defended against drought by increasing antioxidant production through anthocyanin and terpenoid gene levels.

Drought induced changes in cell wall components by reprogramming sugar utilization, reducing degradation, altering protein activity and modifying the cell wall. Sucrose partitioning was altered, increasing sucrose accumulation in leaves (**Figure 3(F)**). CaWDT-2 produced more cellulose due to restrained sucrose synthesis into hemicellulose and pectin (**Table Supplement 4**), with genes associated with cellulose synthesis showing stronger up-regulation in CaWDT-2 compared to Tavee 60. Conversely, drought resulted in decreased levels of hemicellulose and pectin. As a result, the cellulose microfibrils of CaWDT-2 roots were loosely attached under drought, facilitating cell expansion during recovery (**Figure 5(B)** and **Table Supplement 4**). Reduced pectin content hinders root cell elongation by affecting cell wall bonds [29], leading to reduced root elongation in chili (**Figure 2(D)**). Gene down-regulation reduces degradation of cellulose, hemicellulose and pectin. Decreased degradation of hemicellulose and pectin is due to reduced precursor synthesis. Conversely, drought promotes cellulose biosynthesis while reducing cellulose degradation. Drought reduced callose degradation in CaWDT-2, leading to its accumulation along with increased expression of the MP gene (**Figure 5(B)** and **Table Supplement 4**). MP gene overexpression enhances cellulose and callose deposition, improving oxidative stress tolerance by limiting tissue death [30]. Callose deposition in CaWDT-2 remodels the cell wall, limiting dead tissue from drought damage. Dead cells likely result from autophagy, with recycled products supporting root growth [31,32]. Drought-stimulated ubiquitination was higher in CaWDT-2, facilitating protein degradation and modification for post-drought recovery.

Callose regulates substance transport, closes sieve plate pores, increases lateral root density and facilitates cell elongation by maintaining high turgor pressure. Water deficit reduced the expression of genes involved in lignin biosynthesis (**Table Supplement 4**). Previous studies showed that lignin biosynthesis was initially suppressed but induced later during stress [29,33]. This result aligned with the finding of low lignin accumulation in CaWDT-2, potentially facilitating easier water uptake during early drought. Lignin also serves as a water barrier, preventing water loss from the plant to the environment. Drought suppressed the expression of cell wall proteins that are crucial for growth and development in both chilies (**Figure 5** and **Table Supplement 4**). Only a few genes of cell wall proteins were induced by drought, resulting in impaired root elongation. CaWDT-2 exhibited greater molecular adaptation in its root cell wall compared to Tavee 60, as evidenced by the larger number of DEGs related to cell wall

modification. Thus, Tavee 60 and CaWDT-2 roots responded differently to drought, with CaWDT-2 showing higher sensitivity to water deficit due to a greater number of DEGs and different expression levels.

Conclusions

Both CaWDT-2 and Tavee 60 chilies showed reduced shoot growth, leaf area and leaf number due to water deficit. Nevertheless, CaWDT-2 demonstrated diverse physiological responses compared to the original Tavee 60 under PEG-induced drought conditions. Comparative transcriptomic analysis revealed significant differences in drought tolerance mechanisms between CaWDT-2 and Tavee 60, providing insights into the genetic basis of drought resistance. In drought-stressed CaWDT-2, mechanisms such as osmolyte accumulation, cell wall flexibility, stress detoxification, heat shock proteins and transcriptional factors were activated. In contrast, Tavee 60 experienced ABA breakdown, resulting in the absence of signals to regulate stomatal closure. Therefore, water within Tavee 60 plants rapidly decreased, indicating lower tolerance to drought compared to CaWDT-2.

The comparative transcriptomic analysis aids in uncovering the specific molecular responses to drought stress, highlighting key pathways involved in drought tolerance. These findings have implications for future research aimed at developing drought-tolerant crops. Insights into the identified mechanisms, including the specific genes and pathways, can guide molecular breeding strategies for improved drought resilience in agricultural crops. It should be noted that the 24-h duration of the drought stress period in this study may not fully capture the long-term effects of sustained water deficit. Therefore, conducting a more extended study is imperative to comprehensively understand severe drought responses and adaptations over an extended period.

Acknowledgements

This paper is a portion of the Ph. D. Dissertation of the first author. This research was financially supported by the Biodiversity-based Economy Development Office and International SciKU Branding (ISB), Faculty of Science, Kasetsart University, Thailand.

References

[1] G Matmarurat. 2022, The physiological characteristics and gene expression of drought-tolerant chili from the mutant induction by gamma radiation. Ph. D. Dissertation. Kasetsart University, Bangkok, Thailand.

[2] A Dudhate, H Shinde, D Tsugama, S Liu and T Takano. Transcriptomic analysis reveals the differentially expressed genes and pathways involved in drought tolerance in pearl millet [*Pennisetum glaucum* (L.) R. Br]. *PloS One* 2018; **13**, e0195908.

[3] A Fracasso, LM Trindade and S Amaducci. Drought stress tolerance strategies revealed by RNA-Seq in two sorghum genotypes with contrasting WUE. *BMC Plant Biol*. 2016; **16**, 115.

[4] G Villanueva, S Vilanova, M Plazas, J Prohens and P Gramazio. Transcriptome profiles of eggplant (*Solanum melongena*) and its wild relative *S. dasyphyllum* under different levels of osmotic stress provide insights into response mechanisms to drought. *Curr. Plant Biol*. 2023; **33**, 100276.

[5] L González and M González-Vilar. *Determination of relative water content. In*: MJR Roger (Ed.). Handbook of plant ecophysiology techniques. Kluwer Academic Publishers, Dordrecht, Netherlands, 2001, p. 207-12.

[6] J Zhang and MB Kirkham. Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytologist* 1996; **132**, 361-73.

[7] H Koca, M Bor, F Özdemir and İ Türkan. The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. *Environ. Exp. Bot.* 2007; **60**, 344-51.

[8] EW Yemm and AJ Willis. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* 1954; **57**, 508-14.

[9] GL Miller. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. 1959; **31**, 428-8.

[10] CA Schneider, WS Rasband and KW Eliceiri. NIH image to ImageJ: 25 years of image analysis. *Nat. Meth.* 2012; **9**, 671-5.

[11] M Lohse, A Nagel, T Herter, P May, M Schroda, R Zrenner, T Tohge, AR Fernie, M Stitt and B Usadel. Mercator: A fast and simple web server for genome scale functional annotation of plant sequence data. *Plant Cell Environ*. 2014; **37**, 1250-8.

[12] T Zaher-Ara, N Boroomand and M Sadat-Hosseini. Physiological and morphological response to drought stress in seedlings of ten citrus. *Trees* 2016; **30**, 985-93.
[13] Y Du, Q Zhao, L Chen, X Yao, W Zhang, B Zhang and F Xie. Effect of drought stress on sugar metabolism in leaves and roots of soybean seedlings. *Plant Physiol. Biochem*. 2020; **146**, 1-12.
[14] MJ O'Brien, A Valtat, S Abiven, MS Studer, R Ong and B Schmid. The role of soluble sugars during drought in tropical tree seedlings with contrasting tolerances. *J. Plant Ecol*. 2020; **13**, 389-97.
[15] DC Dien, T Mochizuki and T Yamakawa. Effect of various drought stresses and subsequent recovery on proline, total soluble sugar and starch metabolisms in rice (*Oryza sativa* L.) varieties. *Plant Prod. Sci*. 2019; **22**, 530-45.
[16] G Matmarurat, K Chutinanthakun, P Juntawong and O Khamsuk. Two distinct mechanisms of water and energy conservation confer drought tolerance in chili mutants. *Acta Physiologiae Plantarum* 2022; **44**, 7.
[17] A Soares, S Niedermaier, R Faro, A Loos, B Manadas, C Faro, PF Huesgen, AY Cheung and I Simões. An atypical aspartic protease modulates lateral root development in *Arabidopsis thaliana*. *J. Exp. Bot*. 2019; **70**, 2157-71.
[18] A Kohli, JO Narciso, B Miro and M Raorane. Root proteases: Reinforced links between nitrogen uptake and mobilization and drought tolerance. *Physiol. Plant*. 2012; **145**, 165-79.
[19] TM Nolan, N Vukasinovic, D Liu, E Russinova and Y Yin. Brassinosteroids: Multidimensional regulators of plant growth, development, and stress responses. *Plant Cell*. 2020; **32**, 295-318.
[20] GJ Ahammed, X Li, A Liu and S Chen. Brassinosteroids in plant tolerance to abiotic stress. *J. Plant Growth Regul*. 2020; **39**, 1451-64.
[21] K Shinozaki and K Yamaguchi-Shinozaki. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot*. 2007; **58**, 221-7.
[22] UJ Phukan, GS Jeena, V Tripathi and RK Shukla. Regulation of Apetala2/ethylene response factors in plants. *Front. Plant Sci*. 2017; **8**, 150.
[23] C Gu, ZH Guo, PP Hao, GM Wang, ZM Jin and SL Zhang. Multiple regulatory roles of AP2/ERF transcription factor in angiosperm. *Bot Stud*. 2017; **58**, 6.
[24] MY Shi, YT Du, J Ma, DH Min, LG Jin, J Chen, M Chen, YB Zhou, YZ Ma, ZS Xu and XH Zhang. The WRKY transcription factor GmWRKY12 confers drought and salt tolerance in soybean. *Int. J. Mol. Sci*. 2018; **19**, 4087.
[25] M Hrmova and SS Hussain. Plant transcription factors involved in drought and associated stresses. *Int. J. Mol. Sci*. 2021; **22**, 5662.
[26] M Wu, K Zhang, Y Xu, L Wang, H Liu, Z Qin and Y Xiang. The moso bamboo WRKY transcription factor, PheWRKY86, regulates drought tolerance in transgenic plants. *Plant Physiol. Biochem*. 2022; **170**, 180-91.
[27] X Wang, Y Niu and Y Zheng. Multiple functions of MYB transcription factors in abiotic stress responses. *Int. J. Mol. Sci*. 2021; **22**, 6125.
[28] W Nawae, JR Shearman, S Tangphatsornruang, P Punpee, T Yoocha, D Sangsrakru, C Naktang, C Sonthirod, W Wirojsirasak, K Ukoskit, K Sriroth, P Klomsa-ard and W Pootakham. Differential expression between drought-tolerant and drought-sensitive sugarcane under mild and moderate water stress as revealed by a comparative analysis of leaf transcriptome. *PeerJ* 2020; **8**, e9608.
[29] X Liu, H Cui, B Zhang, M Song, S Chen, C Xiao, Y Tang and J Liesche. Reduced pectin content of cell walls prevents stress-induced root cell elongation in Arabidopsis. *J. Exp. Bot*. 2021; **72**, 1073-84.
[30] PK Das, R Biswas, N Anjum, AK Das and MK Maiti. Rice matrix metalloproteinase OsMMP1 plays pleiotropic roles in plant development and symplastic-apoplastic transport by modulating cellulose and callose depositions. *Sci. Rep*. 2018; **8**, 2783.
[31] W Gou, X Li, S Guo, Y Liu, F Li and Q Xie. Autophagy in plant: A new orchestrator in the regulation of the phytohormones homeostasis. *Int. J. Mol. Sci*. 2019; **20**, 2900.
[32] HCJ van Rensburg, WV den Ende and S Signorelli. Autophagy in plants: Both a puppet and a puppet master of sugars. *Front. Plant Sci*. 2019; **10**, 14.
[33] G Reyt, P Ramakrishna, I Salas-Gonzalez, S Fujita, A Love, D Tiemessen, C Lapierre, K Morreel, M Calvo-Polanco, P Flis, N Geldner, Y Boursiac, W Boerjan, MW George, G Castrillo and DE Salt. Two chemically distinct root lignin barriers control solute and water balance. *Nat. Comm*. 2021; **12**, 2320.