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Unraveling the adaptive mechanisms of Veronica nakaiana in response to drought stress: a transcriptome-based study

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Abstract

Drought is a severe environmental constraint to plant productivity. Being a multidimensional stress, it triggers a wide variety of plant responses at the physiological, biochemical, and molecular levels. Exploring stress-related genes in endemic plants such as *Veronica nakaiana* paves the way to identifying how these plants thrive in their respective locations. To identify genes related to the drought stress response in *V. nakaiana*, we compared the transcriptomes of plants under well-watered (WW) and drought-stressed (DS) conditions using RNA-sequencing. In DS plants, plant height, root length, leaf area, and stomatal width were significantly reduced, and stomatal density was increased. Interestingly, fresh, and dry weights did not significantly differ between WW and DS plants. To our knowledge, this was the first attempt to establish a *de novo* transcriptome-based sequence assembly of *V. nakaiana* under drought stress. We identified 2,261 upregulated and 3,315 downregulated differentially expressed genes in DS plants. In-depth data analysis revealed that a large number of these genes were associated with drought stress responses, including heat shock proteins, abiotic stress related transcription factors, and factors involved in abscisic acid signaling, lipid metabolism, and auxin binding. The results provide insights into the different mechanisms contributing to the drought response in *V. nakaiana*.

Introduction

Climate change has been an ongoing issue in all the sectors of life disrupting global economic systems, threatening the stability of nations and the prosperity of individuals. Projected increases in the frequency and intensity of extreme weather events by 2050 pose a significant threat to global food production[1]. One of the serious consequences of climate change is drought, which causes adverse effects in plants including decrease in photosynthesis and respiration, membrane damage, and osmotic imbalance. Plants cope with drought stress via several hormonal signaling pathways including auxin, salicylic acid, jasmonate, and brassinosteroid signaling. Abscisic acid (ABA) is a major phytohormone involved in the drought stress response. It mediates stomatal closure, which reduces water loss through transpiration, and regulates the expression of stress-responsive genes enabling the plants to adapt to water-deficit conditions [2]. Plants exhibit a multifaceted response to drought stress, encompassing transcriptional reprogramming and the modulation of protein activity. This intricate interplay of gene expression and protein function orchestrates the molecular mechanisms underlying drought tolerance. Drought stress signaling pathways include osmotic stress signaling, the calcium-dependent pathway, mitogen-activated kinase signaling, phospholipid signaling, and reactive oxygen species signaling [3].

Although research on drought-tolerance genes in major staple crops is growing rapidly, studies of less well-known plant species are still limited [4,5]. Plants native to specific regions are important gene pool resources because they are adapted to the changing environment specific to that region and often have undergone cycles of genetic evolution and selection in order to thrive in the most extreme environments [6]. *Veronica nakaiana* Ohwi is an endemic species that thrives on the island of Ullengdo in South Korea [7]. In

contrast to other species belonging to the genus *Veronica*, *V. nakaiana* has demonstrated a notable resilience in the face of drought stress [8].

Approximately 450 species within the genus *Veronica* predominantly inhabit regions across the Northern Hemisphere and are adapted to diverse ecological niches ranging from coastal shorelines to high-altitude alpine regions [9]. Various *Veronica* species have been traditionally employed in medicine to alleviate a range of ailments, including rheumatism, laryngopharyngitis, hemoptysis, hernia, and respiratory disorders, owing to their rich content of iridoid glycosides and phenolic compounds [10]. They are also widely used as ornamental garden plants, commercially available as potted plants or cut flowers [11]. So far, fourteen distinct *Veronica* species have been identified as native to South Korea.

The present study aimed to elucidate the physiological and molecular responses of *V. nakaiana* to drought stress by evaluating changes in plant growth parameters, stomatal density, and transcript abundance under drought conditions. RNA-sequencing analysis of drought-stressed (DS) in comparison to well-watered (WW) *V. nakaiana* plants revealed key genes associated with drought stress previously identified in various model plants. A total of 67,856 transcripts were identified. Further differential pattern analysis revealed key genes that were associated with drought stress response, which have been identified previously in various model plants. Our findings provide novel insights into the key genes and genetic mechanisms that allow *V. nakaiana* to survive in adverse conditions. Our study contributes to an otherwise scarce pool of data that is available on endemic plant species like *V. nakaiana*, offering a novel perspective and valuable resources for the molecular breeding of drought-tolerant crops.

Materials and methods

Plant material and experimental conditions

V. nakaiana seeds were provided by the Useful Plant Resources Center of the Korea National Arboretum and were sown in seedling trays (High, Punong, Gyeongju, Korea) on November 10, 2021. The Korea National Arboretum, an ex-situ conservation organization for endemic plants, supplied plants and seeds for joint research with Pai Chai University (Project No. KNA1-2-33). Experimental research and field studies, including the collection of plant materials, were conducted in accordance with guidelines laid down by The Korea National Arboretum and Pai Chai University.

The seedlings were planted in plastic pots with a 12-cm diameter filled with bedding soil mix on January 26, 2022. Plant height and width at this time were approximately 9.6 and 15.3 cm, respectively, and the plants had approximately 9 leaves. Until February 25, when the drought treatment was started, the plants were watered sufficiently daily to prevent drought stress.

The plants were subjected to two different irrigation treatments for a total of 3 weeks from February 25 to March 18, 2022. Plants in the DS group were not irrigated at all during the experimental period, whereas plants in the WW group received 200 mL of water per pot head-on when the volumetric water content

(VWC, v/v) fell to 0.30 $m^3 \cdot m^{-3}$. Soil moisture content was measured using a frequency-domain reflectometry (FDR)-type soil moisture sensor (EC-5; Decagon Devices, Pullman, WA, USA), and a correction formula suitable for the soil used in the experiment (VWC = 0.1546 × mV − 41.312, r = 0.96) was used. VWC values were measured and stored in real time using a data logger (CR1000; Campbell Scientific, Logan, UT, USA). Three FDR sensors were inserted into each of the control and drought treatment groups and the average soil moisture content value is presented. Nine plants per treatment were used for the measurements.

Phenotypic analysis

After 3 weeks of experimental treatments, plant height, plant width, leaf area, number of leaves, root length, fresh and dry weights of above- and below-ground parts, plant water content, and the shoot-to-root (S:R) ratio were measured. Dry weight was measured after complete drying in an oven at 80 ± 5°C for 72 h. Plant water content was calculated as the ratio of dry weight to total fresh weight, and the S:R ratio was calculated as the dry weight ratio between the aerial parts and roots. Leaf number, leaf area, and stomatal parameters were measured on newly developed leaves after the irrigation treatments were initiated. Leaf area was measured using an LI-3100C leaf area meter (HyScience, Korea). Stomata were photographed at 100× magnification using an optical microscope (HNB004; Tekpia, Korea), and the number of stomata was expressed per 1 mm^2. Stomatal area was calculated by multiplying the number of stomata by the leaf area of the corresponding leaf. Stomatal density and size on the abaxial leaf surface were observed. Leaves from the apical region, specifically, the 2nd to 4th leaves, were used for investigation.

RNA isolation, and RNA-seq

Total RNA was extracted from three WW control and DS whole plants each. To 1 g of tissue, 0.5 g of polyvinylpolypyrrolidone was added, and the tissue was finely grounded with liquid nitrogen using a mortal and a pestle. Total RNA was extracted using RNAxzol Reagent (Biomedic, Korea) per the manufacturer’s protocol. gDNA was removed using DNase 1 (Enzynomics, Seoul, Korea) and the RNA was purified using an RNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA). The RNA was qualitatively and quantitatively analyzed using a spectrophotometer and then run through a 1.5% agarose gel electrophoresis. RNA integrity was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were prepared from 5 µg of total RNA purified per sample using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. RNA sequencing was performed on the Illumina NovaSeq6000 platform.

Sequence processing and assembly

The sequencing raw data were pre-processed by removing the adapter sequences using Cutadapt [12]. The short transcriptome reads were pretreated using Dynamic Trim and LengthSort in the SolexaQA package [13]. Using DynamicTrim, low-quality bases were cut at both ends of the short reads to secure high-quality short reads and reads < 25 bp after the Dynamic Trim process were excluded from further analysis through length sorting. *De novo* read assembly was performed using Trinity (v.2.8.6) [14]. Bowtie2 was

used for read mapping, and HTseq (v.0.1.1.0) was used to measure gene expression by counting the total number of reads mapped to each gene [15,16]. Count normalization was done using DESeq library in R [17].

Sequence annotation and identification of differentially expressed genes

Unigenes were annotated using the NCBI Viridiplantae database, and BLASTX was used to determine gene functions. Using InterProScan, proteins were functionally classified, using default settings. Differentially expressed genes (DEGs) were identified based on |Log2Fold| > 1 and adjusted p (FDR) ≤ 0.1.

Functional annotation

The Gene Ontology (GO) database was used to functionally annotate the DEGs [18] and then Blast2Go was used for high quality functional annotation [19]. The significant threshold was set to 0.05. Gene annotations were divided into functional categories including biological process (BP), cellular component (CC), and molecular function (MF). Further annotation was performed using BLASTX on amino acid sequences from KEGG DB (filtering criteria: e-value ≤ 1e-10, best hits). Housekeeping genes were discovered through filtering (e-value ≤ 1e-10, identity > 440%) using TBLASTN (actin, tubulin, GADPH).

Quantitative RT-PCR analysis

Total RNA was extracted from leaf, stem, and roots of control and drought-treated plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A; TaKaRa, Shiga, Japan). One microliter of four-fold diluted cDNA was used in a 12-μL qPCR mixture. Quantitative real-time PCRs (RT-qPCRs) were run using SYBR Green Realtime Master Mix (TOYOBO, Osaka, Japan) on a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The primers used are listed in Table S1. The thermal cycling conditions were as follows: denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 10 s and 55°C for 30 s. Fluorescence values were measured in the last step of each cycle. Transcript levels of the target genes were normalized to that of the actin gene *VnACT8-1* and quantified using the $2^{-\Delta\Delta CT}$ method. All measurements were performed in three technical and biological replicates.

Results and Discussion

Morpho-physiological response of Veronica nakaiana to drought stress

We evaluated morphological and physiological features of *V. nakaiana* plants grown under DS and WW conditions. The experiment began on February 25, 2022, with soil moisture levels of 0.5 $m^3{\cdot}m^{-3}$ in both the WW and DS plant groups (Fig. 1). Over time, the soil moisture content gradually declined to 0.3 $m^3{\cdot}m^{-3}$. At this point, WW plants were watered, whereas DS plants remained under drought conditions. The WW plants were automatically watered to maintain the soil moisture content, and the soil moisture content in the DS plants declined over time (Fig. 1). During the third week, DS plants showed a visually significant

decrease in overall size compared to WW plants (Fig. 2), whereas no significant differences were observed in fresh and dry weights between the two groups (Table 1). Drought stress negatively affects growth-related traits in many crops [20]. In our study, DS plants were shorter than WW plants (Fig. 3A), but there was no significant difference in plant width (data not shown). Reductions in plant height and leaf area have been primarily ascribed to decreases in cell expansion and elongation [21,22]. Leaf area is a crucial factor in radiation interception and photosynthesis and thus, overall biomass accumulation [23]. In our study, significant changes were observed in leaf development and stomatal density during the drought stress treatment. In the WW group, the average leaf area was 14.7 mm^2, whereas in DS plants, it was 5.4 mm^2, which was a 63% reduction (Fig. 3B). Plants' initial response to drought is to close their stomata to prevent water loss through transpiration. Compared to leaf water content, stomatal closure is more closely related to the soil moisture content [24,25]. Stomatal density and width were both significantly affected by drought stress in our study. DS plants had a higher stomatal density than WW plants (Fig. 3C), and stomatal width was significantly reduced in DS plants (Fig. 3D). Similarly, a drought stress-induced increase in stomatal density has been observed in rapeseed, although the change in stomatal width was reported to be insignificant [26]. Numerous studies have documented a reduction in stomatal length or width in drought-tolerant plants. This reduction primarily results in a decrease in the total stomatal area, which further contributes to the plant's adaptability to environmental stress [27,28].

Table 1
Effects of different irrigation treatment on the growth of *Veronica nakaiana* at 3 weeks after treatments (n = 9)

Treatments	Fresh weight (g)			Dry weight (g)			Water content (%)	S:R ratio	Root length (cm)
	Shoot	Root	Total	Shoot	Root	Total			
Well-watered	5.86	2.10	7.97	0.46	0.17	0.63	92.09	2.71	18.80 a [z]
Drought	5.26	2.61	7.86	0.37	0.19	0.56	92.88	1.95	15.42 b
Significance	NS	NS	NS	NS	NS	NS	NS	NS	*

Transcriptome sequencing, assembly, and annotation

Whole-genome sequencing of *V. nakaiana* has not yet been accomplished, limiting the establishment of a genetic database for studying endemic plants. We conducted six sequencing runs using whole plant parts from WW control plants (n = 3) and DS plants (n = 3), yielding a total of 286,863,688 raw reads with an average length of 101 bp. After filtering out low-quality reads and adapter trimming, 278,228,300 reads were retained. Because of the lack of a reference sequence, reads were assembled *de novo*, which resulted in total transcript number of 67,856 with an average length of 1,671 bp and N50 of 2,010 bp. We

obtained 25,798 representative transcripts with an average length of 1,464 and N50 of 1,799 bp (Table 2). The distribution of transcript lengths is shown in Fig. 4.

Table 2
Summary of *Veronica nakaiana de novo* transcriptome assembly

Data	Number of transcripts	Length of transcripts (bp)				
		Total length	Minimum	Maximum	Average	N50
Total transcripts	67,856	113,412,648	500	16,961	1,671	2,010
Representative transcripts	25,798	37,793,180	500	16,961	1,464	1,799

The availability of transcriptomes from a wide range of plant species, accessible through resources such as NCBI, represents a valuable asset in the field of functional genomics. Using the non-redundant (NR) Viridiplantae and InterProScan databases, 88.2% of total transcripts and 81.61% of representative transcripts were annotated (Table 2). All assembled transcripts were aligned against the public NCBI NR Viridiplantae and EMBL InterProScan databases. In total, 59,847 total transcripts and 21,055 representative transcripts were annotated in both databases (Table 3).

Table 3
Summary of the annotation results obtained from BlastX of the amino acid sequences against NR viridiplantae database and EMBL interproscan

Data	NR viridiplantae	Interproscan	Total annotation
Total transcripts	59,676 (87.95%)	48,254 (71.11%)	59,847 (88.2%)
Representative transcripts	20,977 (81.31%)	17,127 (66.39%)	21,055 (81.61%)

Functional annotation of assembled transcripts and identification of DEGs

Differential gene expressions between the WW and DS groups was investigated using the log2fold-change method. If the fold change value changed by twice, it was selected as DEGs. Principal component analysis (PCA) and hierarchical clustering analysis of the DEGs were conducted. Gene expression patterns could be clearly distinguished between the WW and DS groups (Fig. S1A and B). The DEGs were further categorized into subclusters based on the fold change in gene expression (Fig. S1C). Genes that exhibited a 32-fold increase in expression under DS conditions were selected for further analysis. We identified 2,265 upregulated DEGs, 2,038 of which were annotated, and 3,515 downregulated DEGs, 3,307 of which were annotated (Fig. 5). These DEGs, when searched against the KEGG database, were divided into five main categories and 18 subcategories. The main subcategory with the most DEGs was signal transduction (18.58%), followed by carbohydrate metabolism (15.02%), environmental adaptation (9.96%), lipid metabolism (8.25%), and other subcategories (48.16%) (Fig. 6). A significant proportion of the downregulated DEGs were classified in the category metabolism. This suggests that, in response to

drought stress, plants suppress metabolic processes related to activities such as the biosynthesis of secondary metabolites and amino acids, and lipid metabolism. This adaptive response reallocates plant energy resources towards mechanisms that enhance survival in drought conditions. These results are consistent with previous findings, reinforcing the understanding of the plant response to water scarcity [29,30].

The DEGs were further functionally grouped based on GO annotations in the BP, MF, and CC categories. Most upregulated DEGs were grouped in the BP and MF categories (Fig. 7). DEGs associated with drought responses were strongly represented. A total of 102 and 695 GO terms were significantly enriched in up- and downregulated DEGs, respectively. The top five BPs enriched in upregulated DEGs included response to stimulus (GO:0050896), regulation of cellular process (GO:0050794), response to chemical (GO:0042221), response to stress (GO:0006950), and response to abiotic stimulus (GO:0009628) (Fig. 7). Transcription factors (TFs) play a pivotal role in mediating plant responses to abiotic stresses, as has been extensively reported in many plant species [31,32]. The enrichment of GO terms including transcription (GO:0097659, GO:0006351), regulation of transcription (GO:1903506, GO:0006355), transcription regulator activity (GO:0140110), and DNA-binding transcription factor activity (GO:0003700) indicated enhanced drought tolerance activity during DS conditions. The top five BPs enriched in downregulated DEGs included metabolic process (GO:0008152), response to stimulus (GO:0050896), biosynthetic process (GO:0009058), biological regulation (GO:0065007), and organic substance biosynthetic process (GO:1901576). When plants encounter abiotic stresses such as drought, they initiate cellular signaling pathways to adapt and survive. In response, energy resources are allocated away from normal metabolic processes, such as growth and photosynthesis. The GO enrichment analysis results align with findings in various other plant species [33–35].

Validation of drought stress responsive DEGs

Fourteen DEGs were selected to validate the differential expression data by RT-qPCR (Table 4 and Fig. 8). All 14 genes showed similar expression patterns between RNA-seq and RT-qPCR.

Table 4
List of notable upregulated and downregulated DEGs in response to drought stress

Gene ID	Log2Fold change	Gene description
Vn4695	10.95	Transcription factor SPATULA like
Vn728	9.03	Transcription factor SPATULA like
VN235	3.99	transcription factor SPATULA
Vn2069	10.04	PREDICTED: aquaporin TIP3-1-like
Vn4624	9.87	cinnamoyl alcohol dehydrogenase 1 (CAD1)
Vn2361	9.28	Serine/threonine protein phosphatase
Vn4193	9.47	probable xyloglucan endotransglucosylase/hydrolase protein 25
Vn5354	4.03	protein PROTON GRADIENT REGULATION 5, chloroplast
Vn3185	-8.58	ammonium transporter 1 member 3-like
Vn3411	-8.02	auxin-binding protein ABP19a
Vn2631	-7.40	abscisic acid receptor PYL4
Vn2629	-7.11	abscisic acid receptor PYL4-like
Vn2630	-6.09	abscisic acid receptor PYL4

The most strongly upregulated DEG in our study was a SPATULA-like TF (Vn4695). SPATULA, a member of the basic helix-loop-helix (bHLH) TF family, plays a crucial role in regulating root growth by controlling root meristem size [36,37]. RT-qPCR analysis of Vn4695 confirmed that its expression was significantly increased in DS plant tissues (leaf, root, and stem) after two weeks of drought treatment (Fig. 8). In addition to Vn4695, two more upregulated unigenes were functionally annotated as SPATULA TFs (Vn728 and Vn235). SPATULA regulates cell proliferation in parallel to gibberellic acid and affects auxin transport and accumulation. SPATULA is also known to limit the size of leaves and meristems in response to stress signals, which may be linked to the reduced leaf area in DS *V. nakaiana* plants (Fig. 3B) [36–38].

Another highly upregulated DEG was Vn2069, which was predicted to encode an aquaporin, tonoplast intrinsic protein 2-like. Aquaporins are involved in drought stress tolerance in some plant species [39]; therefore, the contribution of Vn2069 to drought tolerance warrants further study. Vn2069 expression increased drastically in the roots of DS plants in the second week, whereas its expression in the stem and leaves increased only slightly (Fig. 8). Vn4624, another significantly upregulated DEG predicted to encode cinnamoyl alcohol dehydrogenase 1 (CAD1), was highly expressed in all plant tissues during the second week of drought stress. CAD1 belongs to defense-related CADs and is a lignin biosynthetic protein. In sweet potato, CAD1 is induced by biotic and abiotic stresses [40]. Other DEGs highly upregulated to similar

levels included a serine/threonine phosphatase (Vn2361), xyloglucan endotransglucosylase (Vn4193), and proton gradient regulation 5 (Vn5354).

Under stress conditions, heat shock proteins, also known as chaperones, are upregulated to facilitate protein folding, association, translocation, and degradation. This adaptive response contributes to the stabilization of cell membranes and protein structures during various abiotic stresses [41–45]. We identified 17 heat shock proteins and heat shock factors at were upregulated in DS plants (Table S2).

Nitrogen is one of the most important factors in plant growth and development. It is predominantly absorbed by the plant in the form of ammonium. Drought stress negatively affects root formation and photosynthesis, thus slowing down nitrogen acquisition and assimilation [46,47]. We identified five downregulated DEGs related to ammonia transport in DS plants, among which Vn3185, predicted to encode ammonium transporter 1 member 3-like, was the most strongly downregulated. Similarly, downregulation of ammonium transporter 1 genes has been observed in *Solanum lycopersicum* (*SlAMT1*) and *Brassica juncea* (*BjAMT1.2*) plants under drought stress, which indicates an adverse effect of water deficit on nitrogen assimilation [48,49].

Another DEG that was notably downregulated in DS plants was Vn3411, predicted to encode auxin-binding protein ABP19a, with a conserved oxalate oxidase (germin), cupin domain. Germin and germin-like proteins (GLPs) belong to the cupin superfamily, which is associated with several abiotic stresses in different plant species [50–52]. GLPs have distinct enzyme functions and include two hydrogen peroxide-generating enzymes: oxalate oxidase and superoxide dismutase. True germins are characterized by oxalate oxidase activity, whereas other germins and GLPs have superoxide dismutase. In *Arabidopsis*, two GLPs have been found to be downregulated under water stress [53]. The downregulation of Vn3411 in roots and leaves of DS plants was validated by RT-qPCR (Fig. 9).

Abscisic acid (ABA) is a key regulator in abiotic stress signaling in plants. During unfavorable conditions, ABA signaling induces dormancy and retards plant growth [54,55]. Pyrabactin resistance 1-like (PYL)-type 2C protein phosphatase (PP2C)-SNF1-related protein kinase 2 (SnRK2) family proteins are central components of the ABA signaling pathway. PYLs act as ABA receptors, whereas PP2Cs and SnRK2s play vital roles as negative and positive regulators of ABA signaling, respectively. PYL plays an important role in normal plant growth and development during stress [56]. The overexpression of certain PYL and PP2C family genes has been demonstrated to lead to significantly improved or reduced drought tolerance [57,58]. In our study, three predicted PYL4 unigenes (Vn2631, Vn2629, and Vn2630) were strongly downregulated (Fig. 9).

TFs responding to drought stress

Drought tolerance is eventually determined by the regulation of drought-responsive genes by TFs such as AP2/ERF, bZIP, bHLH, MYB, NAC, and WRKY [31,59,60]. Many TFs have been identified as crucial participants in coordinating various abiotic stress responses by regulating downstream stress-responsive genes. The

expression or silencing of target genes serves as a stress response trigger. The AP2/ERF superfamily of TFs includes ethylene response factor (ERF), which plays diverse functions in plant responses to biotic and abiotic stresses [61,62]. Many studies in different plants species have reported the role of ERFs in drought stress tolerance [63–65]. We identified 11 predicted ERFs that were upregulated in DS plants. The role of MYB TFs in drought stress has been widely reported [66]. In rice, 65% of MYB TFs are differentially regulated under drought stress [67]. We identified 16 MYB TFs that were upregulated in DS plants (Table S3). Basic leucine zipper (bZIP) is another diverse family of TFs involved in drought stress tolerance in plants [31,68,69]. In our study, six bZIP TFs were upregulated in DS plants (Table S3). Furthermore, seven bHLH family genes, including the three SPATULA genes mentioned above, were upregulated, suggesting their potential involvement in the drought stress mechanism, as reported in other plant species [70–73]. Among the other upregulated TFs, one belonged to the NAC family, six to WRKY family, and seven to the GATA family (Table S3). The roles of these TF families in the drought stress response have been extensively reported [60,74–76].

Conclusions

In conclusion, our study demonstrates that *V. nakaiana* plants modulate stomatal density and aperture to cope with drought stress, thereby maintaining a relatively stable biomass. However, drought stress does induce moderate stress responses in *V. nakaiana*, manifesting as reductions in root size, leaf area, and overall plant height. At the transcriptome level, a number of TF-encoding and other genes known to play key roles in drought stress signaling and adaptation were upregulated. Further research is required to identify the interaction networks among these genes. Our findings provide a basis for further analysis of the drought stress response mechanism in *V. nakaiana*.

Declarations

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

Y.H.R. and G.J.L. conceived and designed the experiments. C.K., Y.H.K., H.Y.S., and M.G. performed the experiments. Y.H.R., G.J.L., C.K., Y.H.K. and H.Y.S. analyzed the data. C.K., Y.H.K. and H.Y.S. contributed the reagents/materials/analysis tools. C.K. wrote the paper.

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Data availability

The supporting data for the findings of this study has already been incorporated within the manuscript and supplementary file. Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) with BioProject accession number PRJNA1049910. For additional information, please contact the corresponding author.

References

1. Yerlikaya, B. A., Ömezli, S. & Aydoğan, N. in *Environment, Climate, Plant and Vegetation Growth* (eds Shah Fahad *et al.*) 109–122 (Springer International Publishing, 2020).
2. Muhammad Aslam, M. *et al.* Mechanisms of abscisic acid-mediated drought stress responses in plants. International journal of molecular sciences 23, 1084 (2022).
3. Yang, X. *et al.* Response mechanism of plants to drought stress. Horticulturae 7, 50 (2021).
4. Fuentes-Merlos, M. I., Bamba, M., Sato, S. & Higashitani, A. Comparative transcriptome analysis of grafted tomato with drought tolerance. Plants 11, 1947 (2022).
5. Xi, W., Hao, C., Li, T., Wang, H. & Zhang, X. Transcriptome analysis of roots from wheat (*Triticum aestivum* L.) varieties in response to drought stress. International Journal of Molecular Sciences 24, 7245 (2023).
6. Lamoreux, J. F. *et al.* Global tests of biodiversity concordance and the importance of endemism. Nature 440, 212–214 (2006).
7. Lee, Y.-E., Lee, Y. & Kim, S. A report of the second chloroplast genome sequence in *Veronica nakaiana* (Plantaginaceae), an endemic species in Korea. Korean Journal of Plant Taxonomy 51, 109–114, doi:10.11110/kjpt.2021.51.1.109 (2021).
8. Gil, M. *Soil moisture control through automatic irrigation system for cultivating Korean native plants in open field.* Master thesis, Pai Chai University, Daejeon, Korea, (2020).
9. Albach, D. *et al.* Chromosome Numbers in Veroniceae (Plantaginaceae): Review and Several New Counts1. Annals of the Missouri Botanical Garden 95, 543–566 (2008).
10. Salehi, B. *et al.* Veronica plants—Drifting from farm to traditional healing, food application, and phytopharmacology. Molecules 24, 2454 (2019).
11. Nau, J. Ball Redbook. Ball Publ, West Chicago 2, 720–721 (2011).

12. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. journal 17, 10–12 (2011).
13. Cox, M. P., Peterson, D. A. & Biggs, P. J. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics 11, 485, doi:10.1186/1471-2105-11-485 (2010).
14. Grabherr, M. G. *et al.* Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nature biotechnology 29, 644 (2011).
15. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. Nature Methods 9, 357–359, doi:10.1038/nmeth.1923 (2012).
16. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *bioinformatics* 31, 166–169 (2015).
17. Anders, S. & Huber, W. Differential expression analysis for sequence count data. Nature Precedings, 1–1 (2010).
18. Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. Nature genetics 25, 25–29 (2000).
19. Conesa, A. *et al.* Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676 (2005).
20. Anjum, S. A. *et al.* Morphological, physiological and biochemical responses of plants to drought stress. African journal of agricultural research 6, 2026–2032 (2011).
21. Jaleel, C. A. *et al.* Drought stress in plants: a review on morphological characteristics and pigments composition. Int. J. Agric. Biol 11, 100–105 (2009).
22. Farooq, M., Hussain, M., Wahid, A. & Siddique, K. Drought stress in plants: an overview. *Plant responses to drought stress: From morphological to molecular features*, 1–33 (2012).
23. Karademir, C., Karademir, E., Copur, O. & Gencer, O. in *11th Meeting of Inter-Regional Cooperative Research Network on Cotton for the Mediterranean and Middle East Regions.* 5–7.
24. Mahajan, S. & Tuteja, N. Cold, salinity and drought stresses: an overview. Archives of biochemistry and biophysics 444, 139–158 (2005).
25. Hoshika, Y., Omasa, K. & Paoletti, E. Both ozone exposure and soil water stress are able to induce stomatal sluggishness. Environmental and Experimental Botany 88, 19–23 (2013).
26. Shekari, F., Soltaniband, V., Javanmard, A. & Abbasi, A. The impact of drought stress at different stages of development on water relations, stomatal density and quality changes of rapeseed (*Brassica napus* L.). Iran Agricultural Research 34, 81–90 (2016).
27. Mehri, N., Fotovat, R., Saba, J. & Jabbari, F. Variation of stomata dimensions and densities in tolerant and susceptible wheat cultivars under drought stress. Journal of Food Agriculture and Environment 7, 167–170 (2009).
28. Wang, S.-g. *et al.* Mapping QTLs for stomatal density and size under drought stress in wheat (*Triticum aestivum* L.). Journal of integrative agriculture 15, 1955–1967 (2016).

29. Ghirardo, A. *et al.* Protein expression plasticity contributes to heat and drought tolerance of date palm. Oecologia 197, 903–919 (2021).
30. Nakagawa, A. C. *et al.* Drought stress during soybean seed filling affects storage compounds through regulation of lipid and protein metabolism. Acta Physiologiae Plantarum 40, 1–8 (2018).
31. Manna, M. *et al.* Transcription factors as key molecular target to strengthen the drought stress tolerance in plants. Physiologia Plantarum 172, 847–868 (2021).
32. Hussain, S. S., Kayani, M. A. & Amjad, M. Transcription factors as tools to engineer enhanced drought stress tolerance in plants. Biotechnology progress 27, 297–306 (2011).
33. Jiao, P. *et al.* Short-term transcriptomic responses of *Populus euphratica* roots and leaves to drought stress. Journal of Forestry Research 32, 841–853 (2021).
34. Rasheed, S., Bashir, K., Matsui, A., Tanaka, M. & Seki, M. Transcriptomic analysis of soil-grown Arabidopsis thaliana roots and shoots in response to a drought stress. Front Plant Sci 7, 180 (2016).
35. Takahashi, F., Kuromori, T., Urano, K., Yamaguchi-Shinozaki, K. & Shinozaki, K. Drought stress responses and resistance in plants: From cellular responses to long-distance intercellular communication. Front Plant Sci 11, 1407 (2020).
36. Makkena, S. & Lamb, R. S. The bHLH transcription factor SPATULA regulates root growth by controlling the size of the root meristem. BMC Plant Biology 13, 1–15 (2013).
37. Castilhos, G., Lazzarotto, F., Spagnolo-Fonini, L., Bodanese-Zanettini, M. H. & Margis-Pinheiro, M. Possible roles of basic helix-loop-helix transcription factors in adaptation to drought. Plant Sci. 223, 1–7 (2014).
38. Hao, Y., Zong, X., Ren, P., Qian, Y. & Fu, A. Basic Helix-Loop-Helix (bHLH) transcription factors regulate a wide range of functions in *Arabidopsis*. International Journal of Molecular Sciences 22, 7152 (2021).
39. Zargar, S. M. *et al.* Aquaporins as potential drought tolerance inducing proteins: Towards instigating stress tolerance. Journal of proteomics 169, 233–238 (2017).
40. Kim, Y.-H., Bae, J. M. & Huh, G.-H. Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweetpotato in response to plant developmental stage and environmental stress. Plant Cell Rep 29, 779–791 (2010).
41. Ohama, N., Sato, H., Shinozaki, K. & Yamaguchi-Shinozaki, K. Transcriptional regulatory network of plant heat stress response. Trends Plant Sci. 22, 53–65 (2017).
42. Aghaie, P. & Tafreshi, S. A. H. Central role of 70-kDa heat shock protein in adaptation of plants to drought stress. Cell Stress and Chaperones 25, 1071–1081 (2020).
43. Liu, X. *et al.* The chloroplastic small heat shock protein gene KvHSP26 is induced by various abiotic stresses in *Kosteletzkya virginica*. International Journal of Genomics 2021 (2021).
44. Ma, G. *et al.* Genome-wide identification and functional analyses of heat shock transcription factors involved in heat and drought stresses in ryegrass. Environmental and Experimental Botany 201, 104968 (2022).

45. Carmo, L. S. *et al.* Comparative proteomics and gene expression analysis in *Arachis duranensis* reveal stress response proteins associated to drought tolerance. Journal of proteomics 192, 299–310 (2019).
46. Du, Y., Zhao, Q., Chen, L., Yao, X. & Xie, F. Effect of drought stress at reproductive stages on growth and nitrogen metabolism in soybean. Agronomy 10, 302 (2020).
47. He, J. *et al.* Prolonged drought affects the interaction of carbon and nitrogen metabolism in root and shoot of cotton. Environmental and Experimental Botany 197, 104839 (2022).
48. Filiz, E. & Akbudak, M. A. Ammonium transporter 1 (AMT1) gene family in tomato (*Solanum lycopersicum* L.): Bioinformatics, physiological and expression analyses under drought and salt stresses. Genomics 112, 3773–3782 (2020).
49. Goel, P. & Singh, A. K. Abiotic stresses downregulate key genes involved in nitrogen uptake and assimilation in *Brassica juncea* L. Plos one 10, e0143645 (2015).
50. Anum, J. *et al.* Germin like protein genes exhibit modular expression during salt and drought stress in elite rice cultivars. Molecular Biology Reports, 1–10 (2022).
51. KESKİN, B. C. Quantitative mRNA expression profiles of germin-like and extensin-like proteins under drought stress in *Triticum aestivum*. International Journal of Life Sciences and Biotechnology 2, 95–107 (2019).
52. Liao, L., Hu, Z., Liu, S., Yang, Y. & Zhou, Y. Characterization of germin-like proteins (GLPs) and their expression in response to abiotic and biotic stresses in cucumber. Horticulturae 7, 412 (2021).
53. Bray, E. A. Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. Journal of experimental botany 55, 2331–2341 (2004).
54. Kim, T.-H. Mechanism of ABA signal transduction: agricultural highlights for improving drought tolerance. Journal of Plant Biology 57, 1–8 (2014).
55. Daszkowska-Golec, A. The role of abscisic acid in drought stress: how ABA helps plants to cope with drought stress. *Drought Stress Tolerance in Plants, Vol 2: Molecular and Genetic Perspectives*, 123–151 (2016).
56. Sun, Y., Pri-Tal, O., Michaeli, D. & Mosquna, A. Evolution of abscisic acid signaling module and its perception. Front Plant Sci 11, 934 (2020).
57. Liu, L. *et al.* Over-expression of a *Zea mays* L. protein phosphatase 2C gene (ZmPP2C) in *Arabidopsis thaliana* decreases tolerance to salt and drought. Journal of plant physiology 166, 531–542 (2009).
58. Kim, H. *et al.* Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. Journal of experimental botany 65, 453–464 (2014).
59. Joshi, R. *et al.* Transcription factors and plants response to drought stress: current understanding and future directions. Front Plant Sci 7, 1029 (2016).
60. Guo, Y. *et al.* Meta-analysis of the effects of overexpression of WRKY transcription factors on plant responses to drought stress. BMC genetics 20, 1–14 (2019).

61. Dossa, K. *et al.* Insight into the AP2/ERF transcription factor superfamily in sesame and expression profiling of DREB subfamily under drought stress. BMC plant biology 16, 1–16 (2016).
62. Li, H. *et al.* Genome-wide identification of AP2/ERF transcription factors in cauliflower and expression profiling of the ERF family under salt and drought stresses. Front Plant Sci 8, 946 (2017).
63. Rong, W. *et al.* The ERF transcription factor Ta ERF 3 promotes tolerance to salt and drought stresses in wheat. Plant biotechnology journal 12, 468–479 (2014).
64. Yang, F. & Lv, G. Combined analysis of transcriptome and metabolome reveals the molecular mechanism and candidate genes of *Haloxylon* drought tolerance. Front Plant Sci 13, 1020367 (2022).
65. Zhang, Y. *et al.* Transcription factors ABF4 and ABR1 synergistically regulate amylase-mediated starch catabolism in drought tolerance. Plant physiology 191, 591–609 (2023).
66. Baldoni, E., Genga, A. & Cominelli, E. Plant MYB transcription factors: their role in drought response mechanisms. International Journal of Molecular Sciences 16, 15811–15851 (2015).
67. Katiyar, A. *et al.* Genome-wide classification and expression analysis of MYB transcription factor families in rice and *Arabidopsis*. BMC genomics 13, 1–19 (2012).
68. Ma, M., Chen, Q., Dong, H., Zhang, S. & Huang, X. Genome-wide identification and expression analysis of the bZIP transcription factors, and functional analysis in response to drought and cold stresses in pear (*Pyrus breschneideri*). BMC Plant Biology 21, 583 (2021).
69. Liu, Y. *et al.* Cgbzip1: a bzip transcription factor from chrysanthemum grandiflora confers plant tolerance to salinity and drought stress. Agronomy 12, 556 (2022).
70. Zhang, J., Li, J., Su, Y., Song, Z. & Wang, J. The Posttranscriptional Mechanism in *Salvia miltiorrhiza* Bunge Leaves in Response to Drought Stress Using Phosphoproteomics. *Agronomy* 12, 781 (2022).
71. Bhaskarla, V. *et al.* Comparative root transcriptomics provide insights into drought adaptation strategies in chickpea (*Cicer arietinum* L.). International journal of molecular sciences 21, 1781 (2020).
72. Chang, Y. *et al.* Genome-wise association study identified genomic regions associated with drought tolerance in mungbean (*Vigna radiata* (L.) R. Wilczek). Theoretical and Applied Genetics 136, 40 (2023).
73. Parmar, R. *et al.* Transcriptional profiling of contrasting genotypes revealed key candidates and nucleotide variations for drought dissection in *Camellia sinensis* (L.) O. Kuntze. Scientific reports 9, 7487 (2019).
74. Devitt, J. K., Chung, A. & Schenk, J. J. Inferring the genetic responses to acute drought stress across an ecological gradient. BMC genomics 23, 1–23 (2022).
75. Singh, D., Debnath, P., Sane, A. P. & Sane, V. A. Tomato (*Solanum lycopersicum*) WRKY23 enhances salt and osmotic stress tolerance by modulating the ethylene and auxin pathways in transgenic Arabidopsis. Plant Physiology and Biochemistry 195, 330–340 (2023).

76. Guo, J. *et al.* Identification of GATA transcription factors in *Brachypodium distachyon* and functional characterization of BdGATA13 in drought tolerance and response to gibberellins. Front Plant Sci 12, 763665 (2021).

Figures

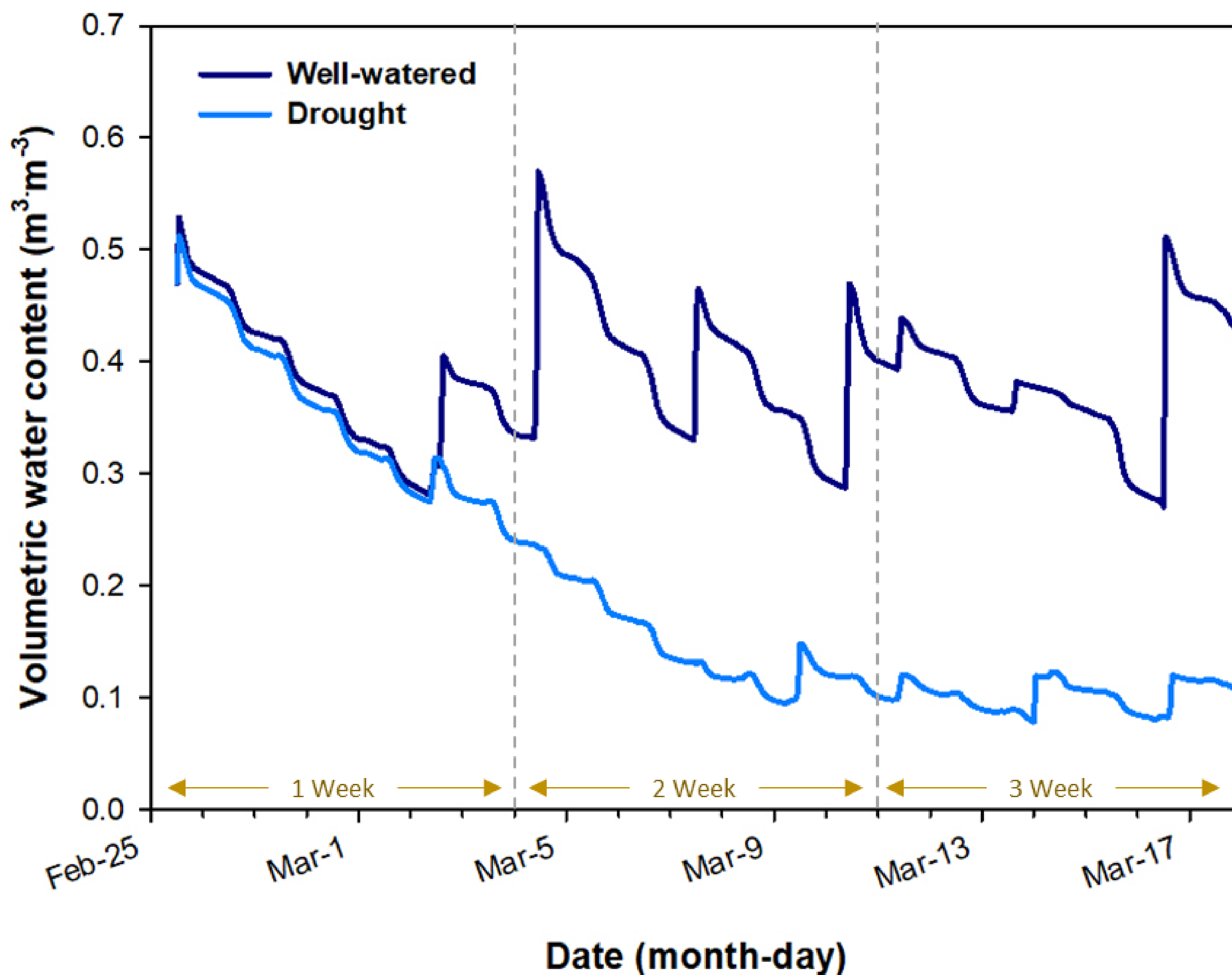

Figure 1

Volumetric Water Content (VWC) in the growth substrate under different irrigation treatments. The peaks in the graph for well-watered plants correspond to the days the plants were irrigated when the VWC fell to 0.30 $m^3 \cdot m^{-3}$. The dotted lines indicate one-week intervals.

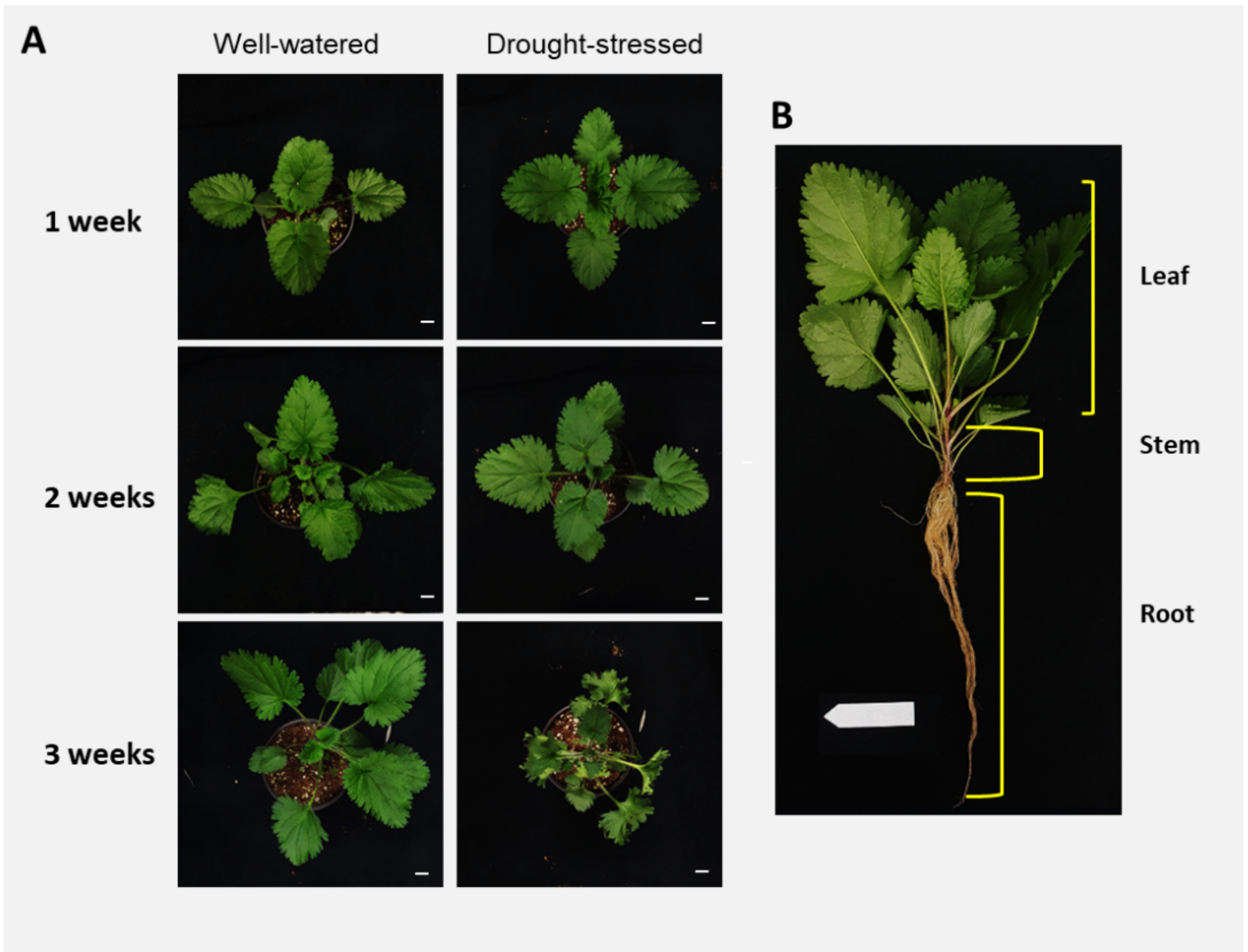

Figure 2

(**A**) *V. nakaiana* plants under WW and DS conditions at one, two, and three weeks of treatment. (**B**) Plant parts that were sampled for RT-qPCR. WW: Well-watered, DS: Drought-stressed

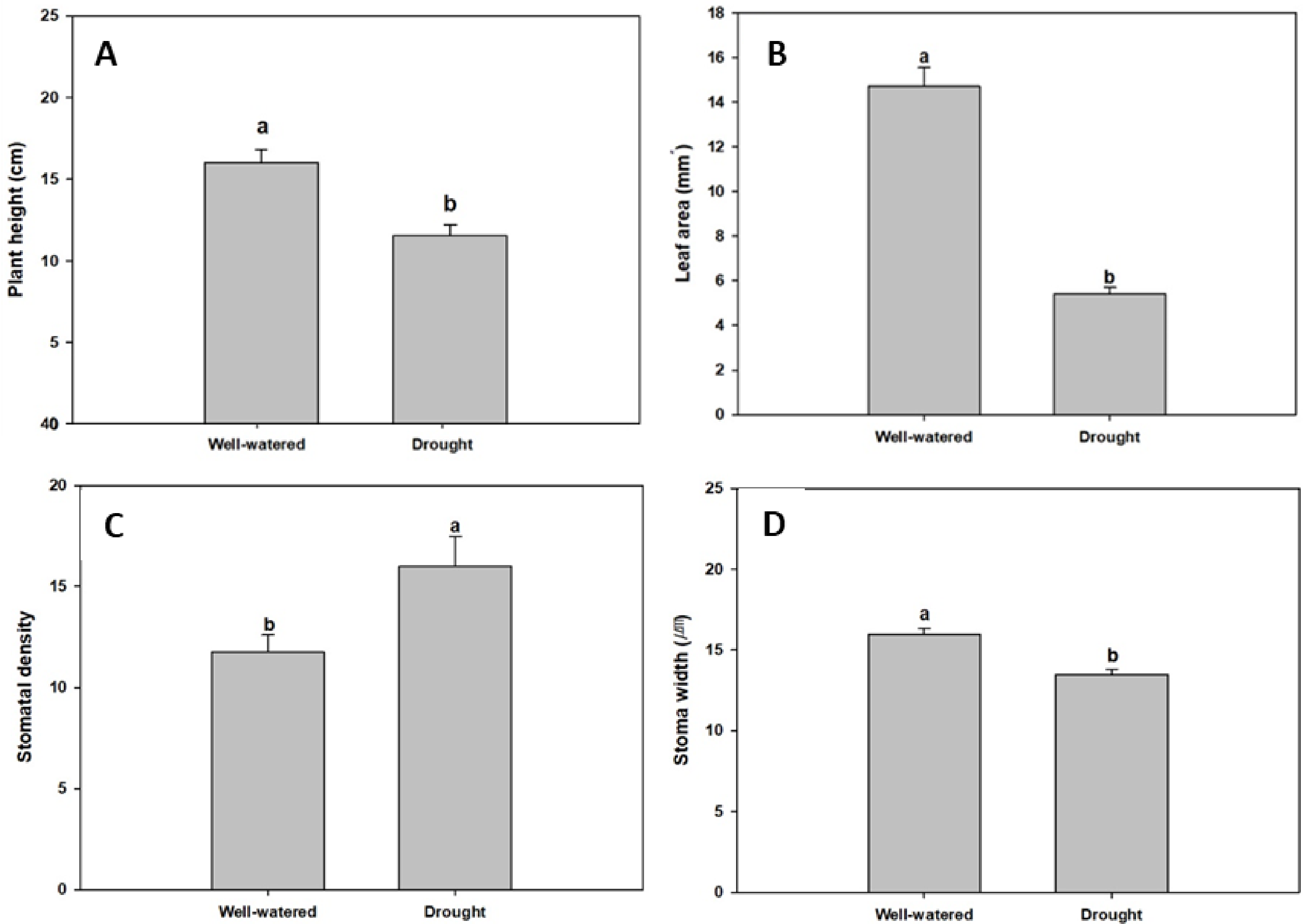

Figure 3

(**A**) Plant height, (**B**) leaf area, (**C**) stomatal density, and (**D**) stomatal width of *V. nakaiana* plants grown under WW and DS treatments. Different letters above bars indicate significant differences (Tukey's HSD test, $p < 0.05$). Vertical bars represent standard error (n = 9).

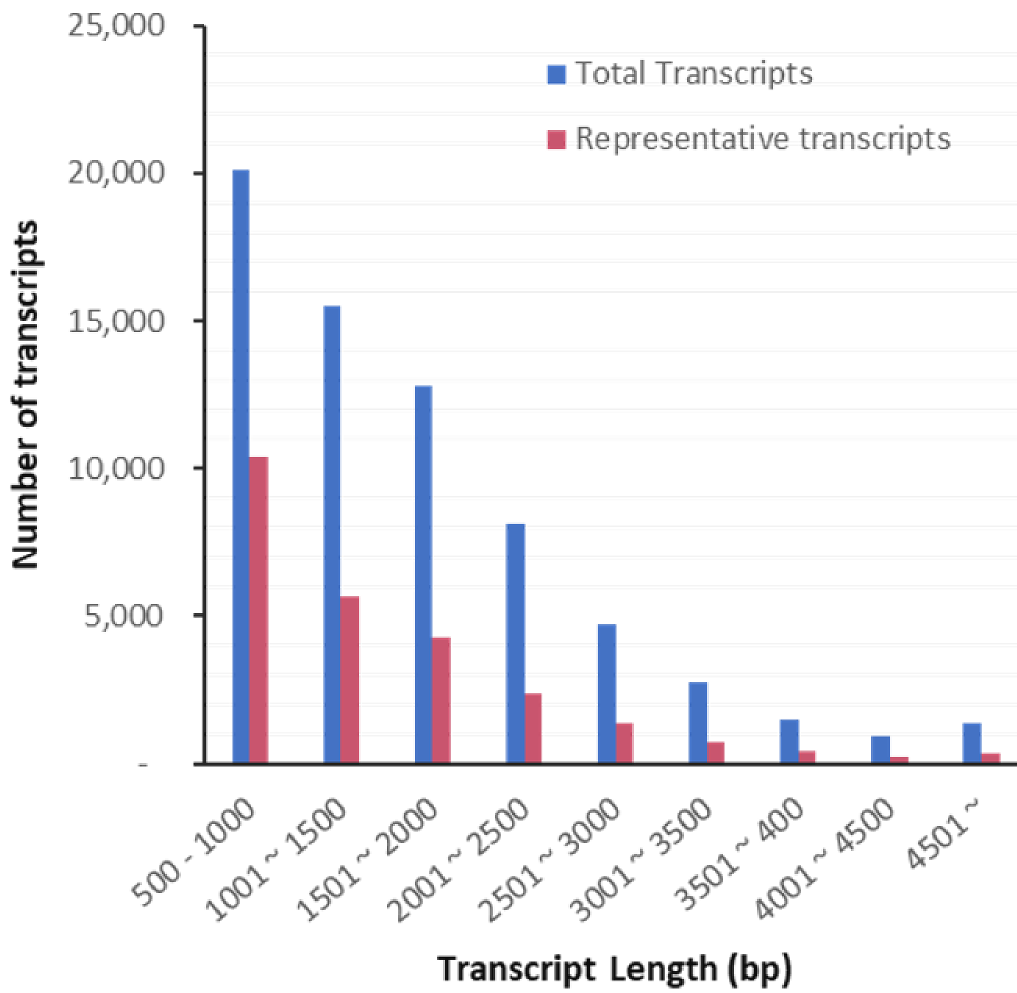

Figure 4

Length distributions of total and representative transcripts of *V. nakaiana* plants.

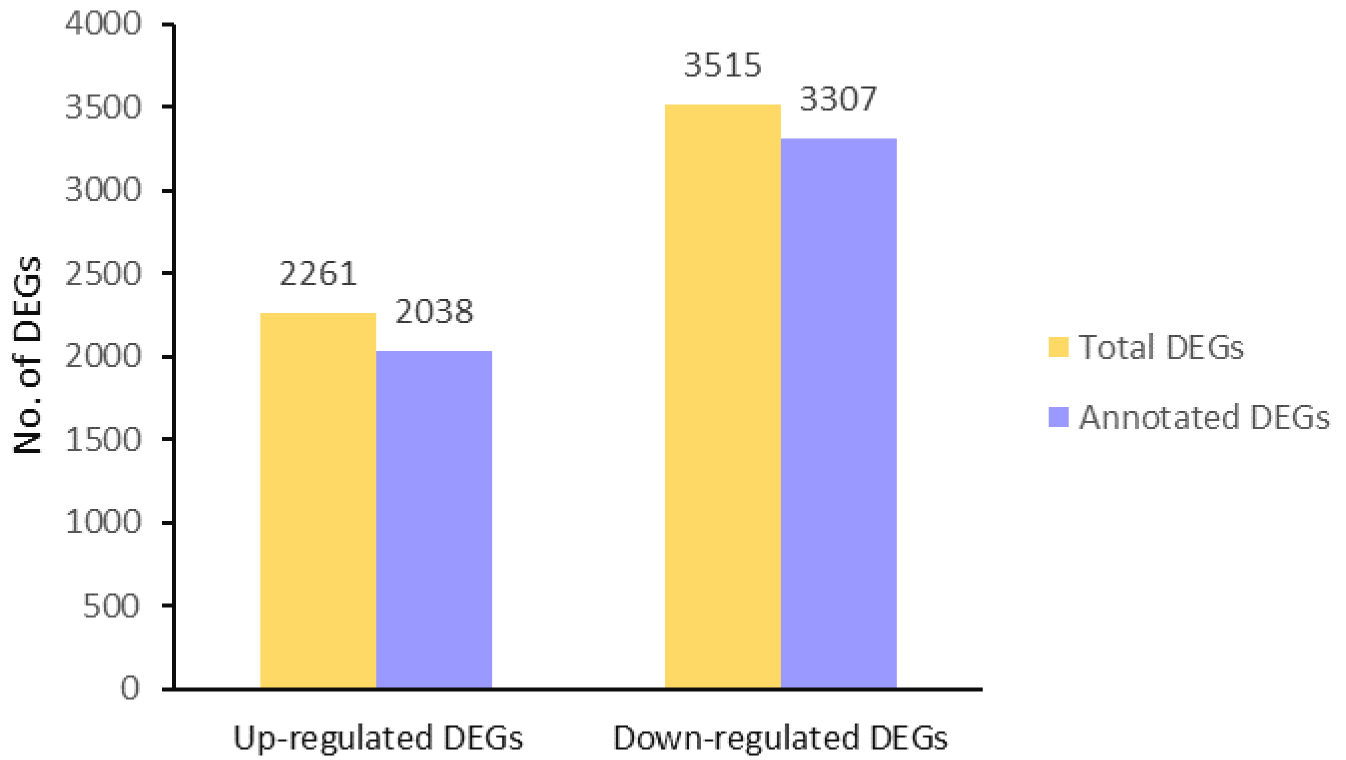

Figure 5

Summary of DEGs obtained from the transcriptome analysis of *V. nakaiana* plants. Annotation information for the DEGs was obtained from the NCBI NR database.

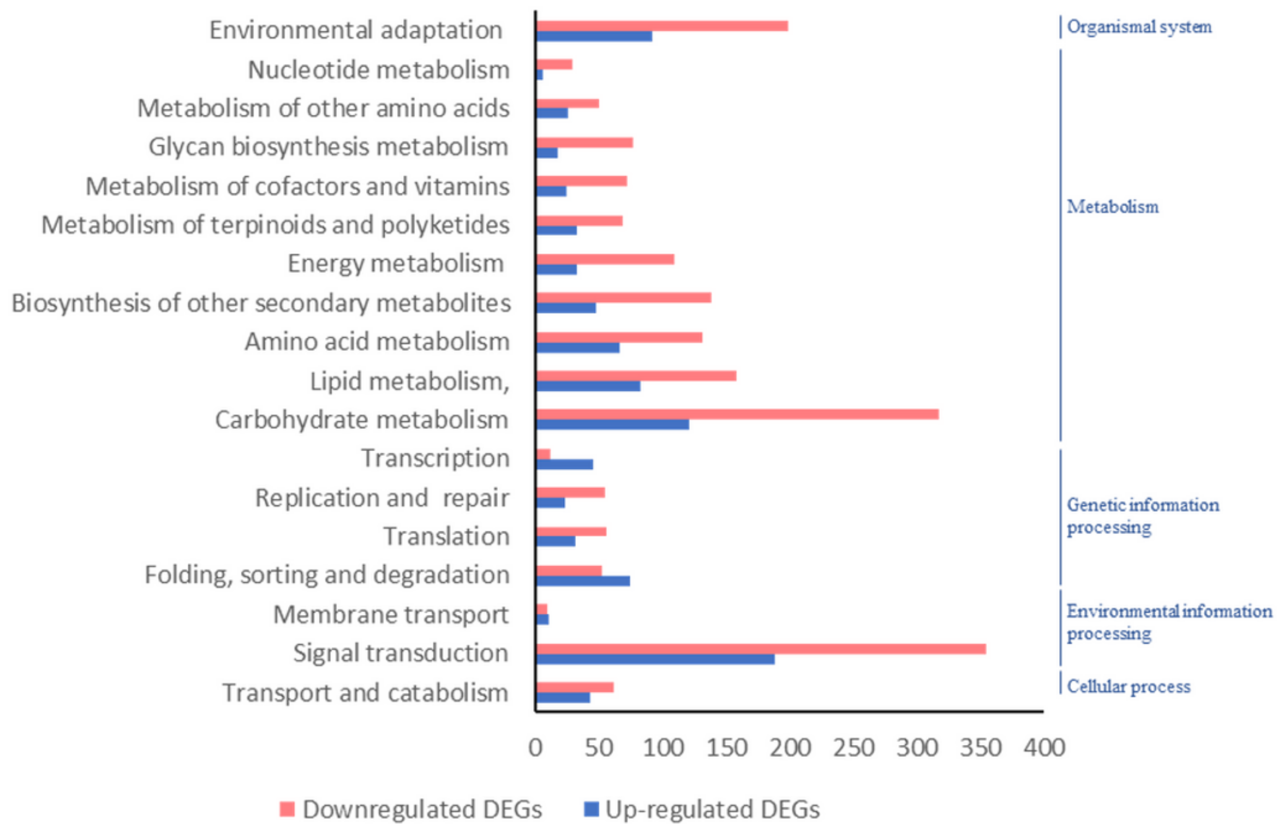

Figure 6

KEGG pathway analysis of DEGs in drought-stressed *V. nakaiana* plants. The y-axis corresponds to the pathways,and the x-axis corresponds to the DEG numbers associated with each GO pathway. The main KEGG categories are indicated in blue on the right.

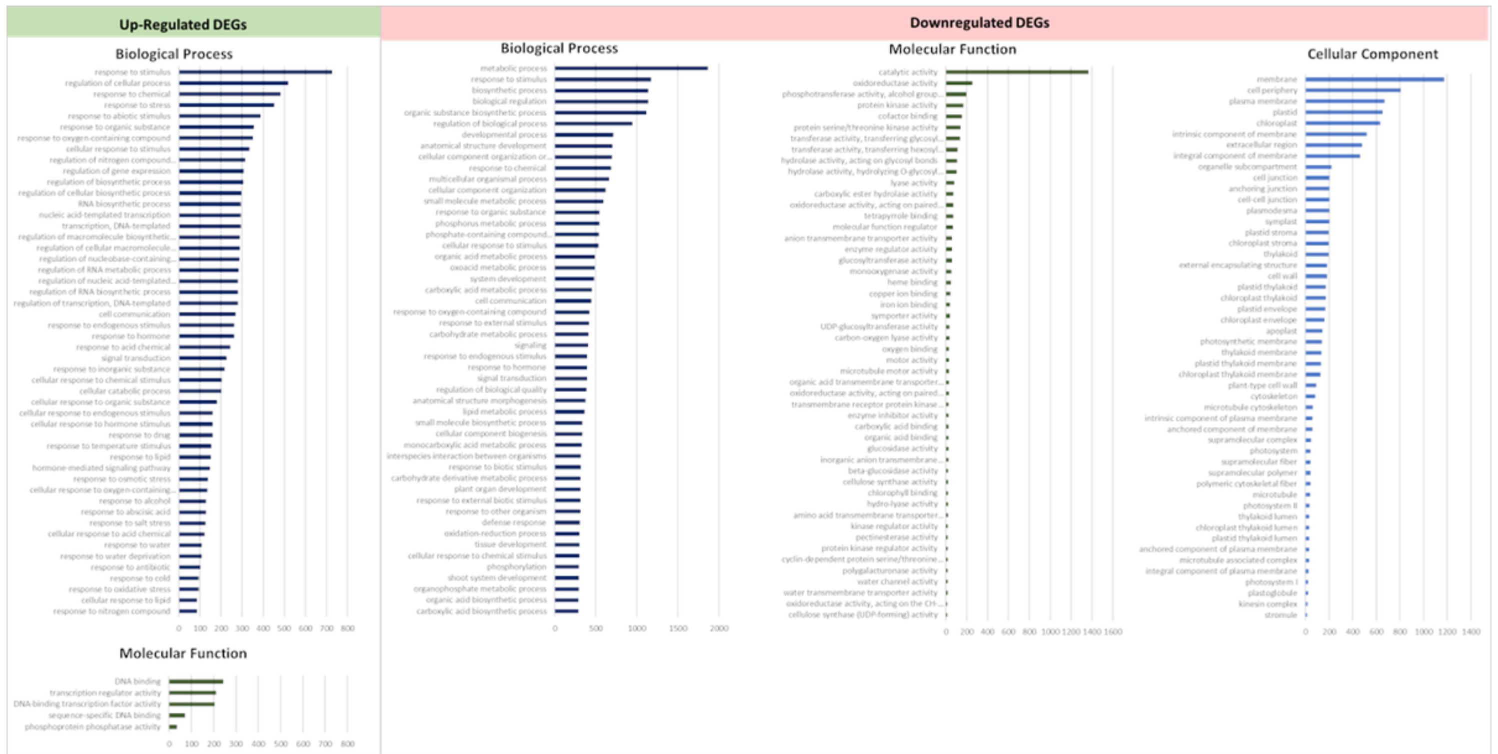

Figure 7

Gene Ontology (GO) classification. The results are summarized in three GO categories: Biological Process, Molecular Function, and Cellular Component. The y-axis indicates the subcategories, and the x-axis indicates DEG numbers associated with each GO term.

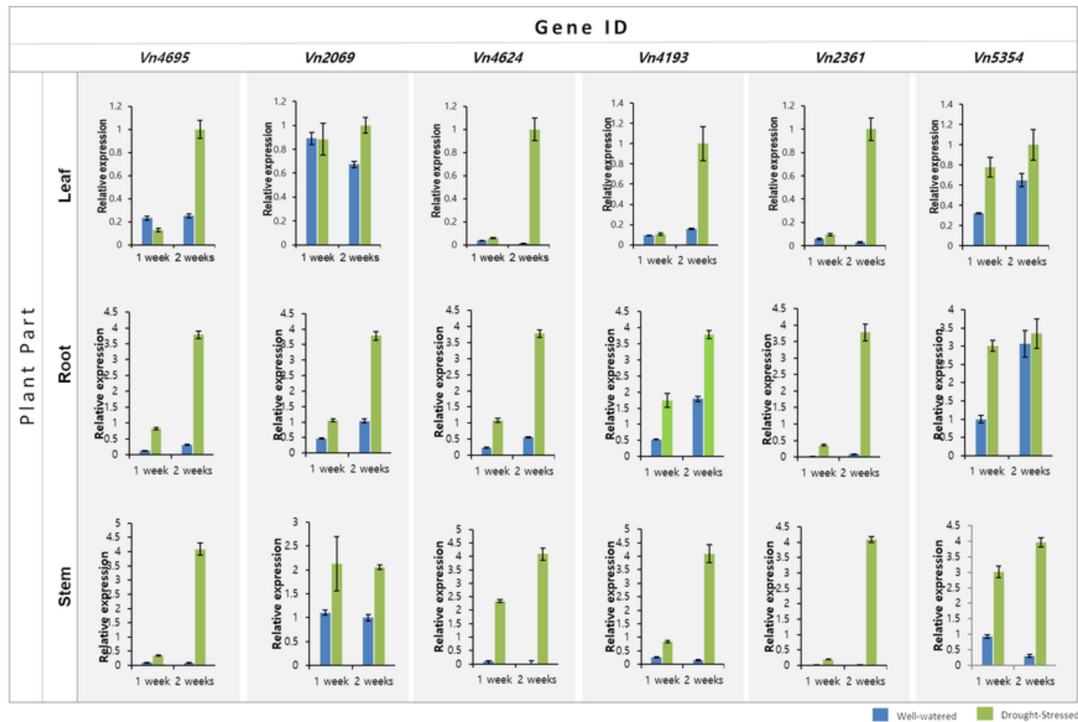

Figure 8

RT-qPCR analysis of the expression of the selected upregulated DEGs in WW and DS plants in the leaves, roots, and stems in weeks 1 and 2 of treatment. WW: Well-watered, DS: Drought-stressed

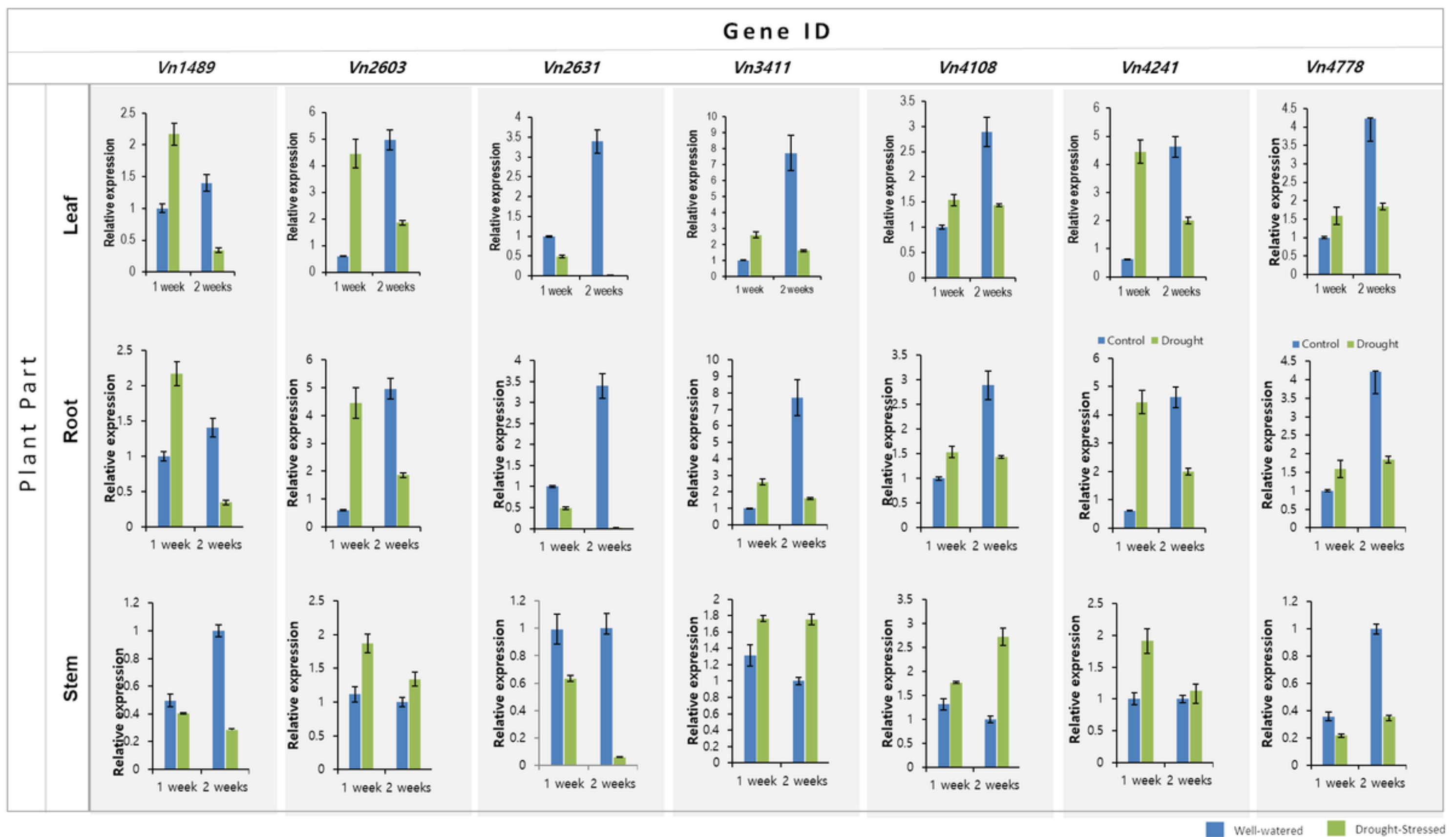

Figure 9

RT-qPCR analysis of the expression of selected downregulated DEGs in WW and DS plants in the leaves, roots, and stems in weeks 1 and 2 of treatment. WW: Well-watered, DS: Drought-stressed

Supplementary Files

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- SupplementarytablesV.nakaiana1.docx