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Integrated transcriptome analysis reveals ABA-dependent and ABA- independent regulatory networks underlying the early drought response in Poncirus trifoliata

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Abstract

Drought severely impacts plant growth and development. Plants have evolved multiple strategies to respond to drought stress, and ABA signaling plays a key role in this process; however, the precise mechanism by which ABA mediates the drought response in citrus plants requires further elucidation. In this study, we investigated the physiological characteristics and transcriptome landscape of leaves under dehydration treatment in trifoliate orange (*Poncirus trifoliata* (L.)) plants. Transcriptome analysis revealed 667 and 1,932 differentially expressed genes (DEGs) at 3 h and 6 h postdehydration, respectively, compared with the control (0 h). KEGG and GO enrichment analyses revealed that DEGs whose expression started to be upregulated at 6 h (cluster 1) were significantly enriched in pathways related to plant hormone signal transduction, MAPK signaling, and galactose metabolism. In contrast, genes in cluster 3, which started being induced at 3 h, were enriched in secondary metabolite biosynthesis pathways, suggesting that early drought stress promoted the accumulation of metabolites and hormones, contributing to the establishment of drought tolerance. Among the 227 consistently upregulated hub genes during dehydration treatment, critical regulators, such as NCED3, RBOHD, ABI1 and WRKY40, which play central roles in the drought response, were identified. Transcriptome analysis of ABA-treated trifoliate orange plants at 3 h and 6 h revealed that clustering analysis distinguished DEGs regulated through ABA-dependent pathways from those regulated independently of ABA. Protein–protein interaction (PPI) network analysis via STRING confirmed that genes such as CHS, OPR2, DUR3, and OMT were coinduced by both drought and ABA, whereas transcription factors such as WRKY50, WRKY53, and NAC029 were regulated independently of ABA. In summary, early drought stress in citrus plants triggers a coordinated response via both ABA-dependent and ABA-independent pathways, involving photosystem protection, membrane stability maintenance, ROS homeostasis, ABA signal regulation, and secondary metabolite accumulation. These findings provide novel insights into the molecular basis of the early drought response in citrus and identify promising genetic targets for improving drought tolerance.

Introduction

Drought is a major environmental stress that significantly impacts plant growth, development, and productivity worldwide (Dai 2013; Guo et al. 2019; Zhang et al. 2025). The frequency of drought events has increased in recent years due to adverse climate change, and understanding how plants respond to water scarcity is critical for improving their resilience. Upon exposure to drought stress, plants exhibit symptoms such as leaf curling, chlorosis, wilting, scorching-like lesions, retarded growth, and even trunk cracking and necrosis (Amede et al. 2004; Dong et al. 2020; Rane et al. 2022). As sessile organisms, plants have evolved complex regulatory networks to mitigate the damage caused by drought, including the accumulation of osmoprotectants (e.g., proline, betaine and sugar), the activation of antioxidant enzyme systems (e.g., SOD, POD, and CAT), and the modulation of hormone signaling pathways (McNeil et al. 1999; Nayer and Reza 2008; Hayat et al. 2012; Sperdouli and Moustakas 2012; Yang et al. 2013; Zanella et al. 2016; Zhu 2016; Ming et al. 2021; Zhang et al. 2023; Wang et al. 2025b).

Abscisic acid (ABA) serves as a pivotal phytohormone in plant growth, development and environmental adaptation(Nakashima and Yamaguchi-Shinozaki 2013; de Zelicourt et al. 2016; Collin et al. 2021; Yu et al. 2024). The ABA signaling pathway plays a central role in drought stress responses, transmitting water deficiency signals, facilitating stomatal closure, inducing stress-responsive gene expression and promoting the accumulation of osmoprotectants during drought stress (McNeil et al. 1999; Singh et al. 2015; Bharath et al. 2021). Plants have evolved both ABA-dependent and ABA-independent pathways to cope with drought stress (Kizis and Pagès 2002; Yoshida et al. 2014; Liu et al. 2019a, 2019b; Pardo-Hernández et al. 2024). The ABA-dependent pathway is initiated by the binding of ABA to PYR/PYL receptors, which inhibit PP2C phosphatases and activate SnRK2 kinases (Park et al. 2010; Gonzalez-Guzman et al. 2012). Activated ABF/AREB transcription factors drive the expression of ABA-responsive genes (Soon et al. 2012; Chen et al. 2020; Takahashi et al. 2020; Lin et al. 2021). The ABA-independent pathway is initiated by rapid activation of Raf-like kinases upon drought stress. These Raf kinases phosphorylate and activate ABA-unresponsive subclass I SnRK2s (e.g., SnRK2.1/4/5/9/10), which regulate downstream targets such as DREB2A transcription factors to induce stress-related gene expression (Dossa et al. 2016; Katsuta et al. 2020; Lin et al. 2020; Soma et al. 2020; Yu et al. 2021). Among them, SnRK2 kinases can integrate signals from both, and AREB/ABFs can also regulate DREB2A expression (Lee et al. 2010; Yu et al. 2021). However, whether citrus employs similar regulatory modules or unique adaptations remains unclear.

Citrus is one of the most widely cultivated crops worldwide, yet it faces substantial challenges from drought stress in arid and semiarid regions (Liu and Deng 2007; Geisseler and Horwath 2014). Trifoliate orange (*Poncirus trifoliata*), a commonly used rootstock for citrus grafting, exhibits notable tolerance to cold and multiple diseases, making it a valuable model for studying stress adaptation mechanisms in citrus (Santana-Vieira et al. 2016; Silva et al. 2021; da Silva Costa et al. 2025). However, its relative sensitivity to drought restricts its application and can compromise the drought resistance of grafted scions. Thus, the identification of drought resistance genes and their regulatory networks, followed by the development of drought-resistant rootstock resources via genetic engineering, represents an effective strategy.

When subjected to water stress, citrus plants mitigate dehydration through adaptive responses, including stomatal closure, reduced leaf water potential, and accumulation of compatible solutes (Wu et al. 2016; Meng et al. 2020; Dahro et al. 2023; Maleckova and Ponnu 2023; Zhang et al. 2023; Zhu et al. 2025). Moreover, hormone regulation plays a crucial role in the drought adaptability of citrus plants. Stress triggers dynamic changes in jasmonic acid (JA) and abscisic acid (ABA) levels in both the roots and leaves of citrus plants, with transient JA accumulation inducing ABA biosynthesis (Xiong et al. 2020). Polyamines, recognized as key regulators of plant stress responses, have been elucidated in multiple studies and mediate stress adaptation primarily via osmotic adjustment and increased reactive oxygen species (ROS) scavenging capacity in citrus (Liu et al. 2007; Wang et al. 2011; Huang et al. 2015; Wu et al. 2016). In addition, PtrCDPK10 enhances dehydration and drought tolerance by phosphorylating ascorbate peroxidase (PtrAPX), a key enzyme in ROS scavenging, which subsequently reduces the accumulation of cellular ROS (Meng et al. 2020). A ploidy-related study also revealed that, compared

with its diploid counterpart, tetraploid citrus has superior drought resistance because of its ability to maintain a more robust ROS scavenging system via increased antioxidant capacity and increased sugar accumulation (Wei et al. 2019). Moreover, stress-induced expression of PtrA/NINV enhances drought resistance through the modulation of ROS homeostasis (Dahro et al. 2016). The CsCYT75B1 gene contributes to drought tolerance via the biosynthesis of antioxidant flavonoids, thereby increasing ROS-scavenging activity (Rao et al. 2020).

Transcriptome sequencing (RNA-seq) is a powerful tool for revealing the molecular mechanisms involved in plant stress resistance and enables easy identification of differentially expressed genes and regulatory networks. Recent studies employing this technology have yielded significant insights into the response of plants to abiotic stresses. In maize, a total of 8477 drought-responsive genes were identified through high-resolution temporal transcriptome analysis under drought and well-watered treatments; among these genes, *ZmCPK35* and *ZmZIM23* were characterized to positively regulate drought stress (Kaderbek et al. 2025). Han et al. screened drought-tolerant and drought-sensitive varieties and elucidated the molecular network underlying drought adaptation in cotton via integrated transcriptomics. The functions of MdABI5 and MdOCP3, which are positively regulated by drought tolerance in apple, were verified via integrative multiomics analysis (Wang et al. 2025a). In addition, TtOTS1 was found to have a negative effect on drought stress by affecting root development via a combination of GWAS and transcriptomic analysis (Yang et al. 2024). This study aimed to characterize the transcriptome dynamics of *P. trifoliata* during the early drought response, with a specific focus on delineating ABA-dependent and ABA-independent signaling pathways. By integrating these insights, we seek to advance our understanding of drought tolerance in citrus and provide targets for molecular breeding.

Materials and methods

Plant materials and growth conditions

The trifoliate orange (*P. trifoliata* (L.) Raf.) The samples used in the experiment were collected from the base of the Key Laboratory of Fruit Tree Germplasm Innovation and Utilization in Hubei Province. The seeds were harvested from the fresh fruits and disinfected with 1 M NaOH for 15 mins, followed by NaClO (20% v/v) for 15 mins. Sixty-day-old seedlings were grown in a growth chamber (light cycle: 16 h light, 8 h dark; temperature: 25°C) and used for further treatments. Dehydration treatment: Control (0 h) samples were collected immediately after the seedlings were gently removed from the soil, the roots were rinsed, and the samples were blotted dry. For dehydration treatment, the seedlings were then placed on filter paper at room temperature (temperature and humidity were specified if controlled) for 3 h or 6 h before leaf sampling. For the ABA treatment, the seedlings were removed from the soil, the surface soil was rinsed with clean water, the remaining moisture was gently absorbed, and the samples were placed in a 100 μM ABA solution. Samples were collected at 0 h (before treatment), 3 h, and 6 h. For each biological replicate, leaves were randomly sampled from at least ten healthy seedlings per treatment/time point and immediately pooled. The leaves were randomly sampled and mixed. The

samples were then quickly frozen in liquid nitrogen and stored at -80°C in an ultralow-temperature refrigerator for subsequent analysis.

Physiological measurements and histochemical staining

The MDA content was measured via a commercial kit (A003, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The maximum quantum efficiency of PSII photochemistry (*Fv/Fm*) was measured via a Pocket PEA Plant Efficiency Analyzer (Hansatech Instruments Ltd., UK). Electrolyte leakage (EL) was determined as described by Zhu et al. (2025) via a DSS-307 conductivity meter (SPSI, China). Histochemical detection of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) was performed via nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining, respectively.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT–PCR)

Total RNA was extracted via TRIzol reagent (RN33, Aidlab Biotechnologies Co., Ltd., Beijing, China) following the manufacturer's protocol, and first-strand complementary DNA (cDNA) was synthesized via the RevertAid First Strand cDNA Synthesis Kit (PC7002; Keep Biotechnologies Co. Ltd., Wuhan, China) according to the manufacturer's instructions. qRT–PCR was performed via an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with AceQ SYBR Green Master Mix (Vazyme, Nanjing, China). The thermocycling conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 58°C for 30 s, followed by a melt curve analysis step (95°C for 15 s, 60°C for 1 min, and then 95°C for 30 s). The 10 μl reaction mixture contained 5 μl of 2× AceQ SYBR Green Master Mix, X μl of diluted cDNA (equivalent to 200 ng total RNA input), and 0.2 μl of each 10 μM primer. The Actin gene was used as an internal reference control; the relative expression level was calculated via the 2^(-ΔΔCT) method. Three technical replicates were performed for each biological replicate. The sequences of primers used in this study are listed in Supplementary Table S1.

RNA-seq library preparation and data analysis

Total RNA was isolated from trifoliate orange leaves. The RNA concentration was measured via a NanoDrop spectrophotometer, and RNA integrity was assessed via an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Strand-specific cDNA libraries were constructed via a TREscript RT kit (PC7002; Keep Biotechnologies Co. Ltd., Wuhan, China). Sequencing was performed on the BGISEQ-500 platform (BGI-Shenzhen, Shenzhen, China), generating [read length, e.g., 150 bp] paired-end reads.

The raw sequencing data were subjected to quality control via Fastp software to obtain clean data. The clean data were aligned to the Citrus reference genome via HISAT2 software. The expression of the data was subsequently quantified via featureCounts. Differential expression analysis was conducted with DESeq2 software, with three biological replicates for each treatment group. The screening criteria for differentially expressed genes were |log2(fold change)| >1 and padj < 0.05. The functional annotation of the DEGs was performed on the basis of the reference genome annotation. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted via the clusterProfiler R package. Simultaneously, the online platform String was used for protein–protein interaction network analysis. Gene expression trend analysis was completed through the online plotting website Hiplot.

Statistical analysis

All experiments included at least three independent biological replicates. Statistical differences were evaluated via ANOVA with GraphPad Prism v9.5.1, with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicating significance.

Results

Physiological changes during early dehydration stress in trifoliate orange plants

To investigate the drought response mechanisms in citrus, trifoliate orange plants were subjected to dehydration treatment for accelerated drought simulation. Visible phenotypic changes were observed: young leaves wilted within 3 h of dehydration, followed by more severe wilting and severe wrinkling of tender leaves by 6 h (Fig. 1A). Concurrently, the maximum quantum efficiency of PSII photochemistry (Fv/Fm) significantly decreased under dehydration stress (Fig. 1B). The water loss content, a key indicator of hydration status, decreased rapidly within the first 3 h and then decreased gradually thereafter (Fig. 1C). Membrane integrity was compromised, as indicated by a steady increase in electrolyte leakage (EL) (Fig. 1D). Malondialdehyde (MDA) levels, reflecting lipid peroxidation, also progressively increased over time (Fig. 1E). Histochemical staining with DAB and NBT confirmed substantial accumulation of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$), respectively, in leaves during early dehydration treatment (Fig. 1F, G). These results indicate that dehydration disrupts the photosynthetic system, damages membrane integrity, and triggers oxidative stress.

Transcriptomic profiling of dehydrated trifoliate orange plants

RNA-seq analysis of P. trifoliata leaves at 3 h and 6 h postdehydration revealed dynamic transcriptional reprogramming. Sequencing yielded approximately 1 Gb of clean data per sample (Supplementary Table S2). Gene expression levels were quantified as transcripts per million (TPM) using uniquely mapped

reads. Sample correlation analysis revealed distinct clustering, with 6 h samples showing a low correlation with both the control (0 h) and 3 h samples (Fig. 2A), indicating that pronounced transcriptomic shifts occurred by 6 h. Differential expression analysis via DESeq2 identified a greater number of differentially expressed genes (DEGs) at 6 h than at 3 h (Fig. 2B). To characterize temporal expression patterns, DEGs were clustered into six distinct profiles via the Mfuzz algorithm on the basis of their expression dynamics across the time course (Fig. 2C). Among these, cluster 1 and cluster 3 were of particular interest. The genes in Cluster 1 presented delayed induction, with peak expression at 6 h, whereas those in Cluster 3 presented early upregulation, peaking at 3 h. These clusters likely represent genes activated in response to progressive dehydration stress.

Functional enrichment of differentially expressed genes

Functional annotation via KEGG and GO enrichment analyses provided insights into the roles of the distinct gene clusters (Fig. 3). KEGG analysis (Fig. 3A) revealed that Cluster 1 genes (induced at 6 h) were significantly enriched in pathways related to plant hormone signal transduction, MAPK signaling, galactose metabolism, and flavonoid biosynthesis. In contrast, Cluster 3 genes (induced at 3 h) were enriched in pathways including tryptophan metabolism, phenylpropanoid biosynthesis, and glutathione metabolism. GO analysis of biological processes (Fig. 3B) further revealed that Cluster 1 genes were overrepresented in response to water deprivation, ABA signaling, and stress signal transduction. The cluster 3 genes were associated primarily with secondary metabolite biosynthetic processes and hormone metabolic processes. These findings suggest that early drought stress triggers the accumulation of protective metabolites and phytohormones and that distinct response pathways are activated with different kinetics following stress onset.

Among the 227 genes consistently upregulated during dehydration (Fig. 4A, B; Dataset 3), several candidate genes were identified on the basis of their known roles in stress responses. Protein–protein interaction (STRING) analysis highlighted key players, including NCED3 (ABA biosynthesis), RBOHD (ROS production), ABI1 (ABA insensitive protein 1), and WRKY40 (transcriptional repressor in ABA signaling). The proteins encoded by these genes orchestrate the molecular interaction network (Fig. 4C), and previous research results have shown that these genes play a key role in the response of plants to drought stress, which also confirms the reliability of the sequencing results(Merlot et al. 2001; Sato et al. 2018; Fraudentali et al. 2023; Han et al. 2023; Yu et al. 2023). qRT–PCR confirmed their significant upregulation under dehydration (Fig. 5), supporting the reliability of the RNA-seq results. Among them, NCED3, a key factor in the response of the ABA signaling pathway in response to stress, was upregulated nearly 800-fold after 3 h of dehydration, the PP2C gene has been reported to be widely involved in plant drought resistance in species, and its expression is increased nearly 100-fold after treatment. In addition, we also found that CML46 (calmodulin) and WRKY53 are significantly induced under dehydration stress, which may be involved in the drought stress response of citrus through transcriptional regulation; however, these findings need further verification.

ABA signaling in citrus drought stress

To directly assess the role of ABA in mediating early transcriptional responses, seedlings were treated with 100 μM ABA for 3 h or 6 h and subjected to RNA-seq. ABA treatment partially recapitulated the transcriptional reprogramming observed under dehydration. Differential expression analysis revealed 225 upregulated and 612 downregulated genes at 3 h post-ABA treatment and 399 upregulated and 932 downregulated genes at 6 h post-ABA treatment compared with the 0 h control (Fig. 6A). Time-series clustering of ABA-responsive genes revealed six expression clusters (Supplementary Figure S2). Clusters corresponding to early (Cluster 3, induced at 3 h) and late (Cluster 2, induced at 6 h) ABA responses showed substantial overlap with dehydration-induced clusters (Cluster 3 and Cluster 1, respectively, from Fig. 2C). KEGG enrichment analysis of ABA-responsive DEGs (Fig. 6B) revealed the upregulation of genes involved in α-linolenic acid metabolism, fatty acid degradation, unsaturated fatty acid biosynthesis, and starch and sucrose metabolism. Conversely, genes associated with circadian rhythm, pentose and glucuronate interconversions, phenylalanine metabolism, and the MAPK signaling pathway were predominantly downregulated.

ABA-dependent and ABA-independent pathways under drought stress

By integrating the dehydration and ABA transcriptome data, clustering analysis clearly distinguished genes regulated in an ABA-dependent manner from those regulated independently of ABA under drought stress (Fig. 7A, Supplementary Figure S3, Dataset 7). Specifically, genes such as CHS, OPR2, DUR3, and OMT were strongly upregulated by both dehydration and exogenous ABA treatment (Fig. 7B, Supplementary Table S3), supporting their regulation through ABA-dependent pathways. Validation by qRT–PCR confirmed that the expression of these genes and other putatively ABA-dependent genes (e.g., CLH1, GSTU8, and HPT1) was significantly induced by ABA treatment (Fig. 8).

Conversely, a set of transcription factors, including WRKY40, WRKY50, WRKY53, NAC036, and NAC029, were robustly induced by dehydration but showed little or no significant induction by exogenous ABA (Fig. 7C, Supplementary Table S4, Fig. 9). This pattern suggests their involvement in ABA-independent regulatory pathways during the drought response. qRT–PCR confirmed the minimal response of NAC029, WRKY50, and WRKY53 to ABA (Fig. 9).

Collectively, our results demonstrate that early dehydration stress in citrus plants triggers coordinated physiological and transcriptional responses characterized by photosynthetic impairment, membrane damage, ROS accumulation, and differential activation of both ABA-dependent and ABA-independent regulatory pathways. These findings provide a foundation for understanding the molecular mechanisms underlying drought tolerance in citrus.

Discussion

Plants live in complicated and constantly changing environments, which imposes unbearable stress, such as drought, cold, heat, salt, flooding, toxic metal and nutrient deficiency (abiotic stress), and insect pest and pathogen infection (biotic stress) (Knight and Knight 2001; Abuqamar et al. 2009; Redondo-

Gómez 2013; Ku et al. 2018; Gong et al. 2020; Bharath et al. 2021; Zhang et al. 2022a; Jiang et al. 2025), on plants. These stresses occur periodically and in stages or persist throughout the lifetime of the plant and have a significant effect on the entire life cycle of plants, including seed germination, plant growth, flowering and fruit development (Redondo-Gómez 2013; Zhang et al. 2022a). As sessile organisms, plants rarely escape adverse conditions and seek new habitats such as animals and humans; therefore, they can only protect themselves from stress damage through the coordinated regulation of growth, stomatal movement, hormone synthesis, signal transmission, gene expression, and osmoprotectant accumulation. A dry climate and insufficient soil moisture are usually the causes of drought. Especially in recent years, with global warming, drought has become an imperative factor that limits crop yields across the globe. Plants exhibit symptoms such as leaf curling, chlorosis, wilting, scorching-like lesions, retarded growth, and, in severe cases, trunk cracking and necrosis (Suriyagoda et al. 2014; Arbona et al. 2015; Zhang et al. 2015; Qi et al. 2018; Bharath et al. 2021; Seleiman et al. 2021). In our study, we observed obvious leaf wilting, curling, and crinkling under drought conditions, with a significant decrease in the Fv/Fm ratio of the leaves, indicating that the photosynthetic intensity of the leaves was impaired (Figure 1A). Additionally, the electrical conductivity and MDA content gradually increased, which suggested that the photosystem and cell membrane structure of citrus leaves were damaged after dehydration treatment, resulting in obvious leaf damage (Figure 1B-D).

Over the past two decades, researchers have explored the response pathways of plants to water stress from various perspectives and have achieved many significant results in the study of plant drought resistance mechanisms (Jensen et al. 1996; Ng et al. 2001; Taji et al. 2002; Dubouzet et al. 2003; Xu et al. 2010; Hatzig et al. 2014; Fang and Xiong 2015; Kaur and Asthir 2017; Kim et al. 2017; Kuromori et al. 2022; Liu et al. 2023). The plant response to drought stress is a complex regulatory mechanism that encompasses various physiological reactions, ranging from signal perception under water-deficient conditions to the acquisition of drought resistance at the whole-plant level, and involves numerous genes and signaling pathways, especially early responses, which are crucial (Velikova et al. 2012; Hatzig et al. 2014; Xie et al. 2019; Baek et al. 2020; Li et al. 2020; Zhang et al. 2022b; Gao et al. 2024; Yin et al. 2024). Given the critical importance of early responses, we employed transcriptome sequencing to characterize the transcriptional landscape in trifoliate orange leaves during the initial hours (3 h and 6 h) of dehydration stress. A total of 25681 genes were differentially expressed after 3 h and 6 h of dehydration treatment. Mfuzz analysis classified all the DEGs into 6 clusters, and genes in cluster 1 and cluster 3 were significantly induced by stress at an early stage. The KEGG enrichment analysis of cluster 1 and cluster 3 revealed that the DEGs at 3 h (cluster 3) were enriched in pathways such as tryptophan metabolism, phenylpropanoids, MAPK signaling, and glutathione signaling, whereas at 6 h (cluster 1), the DEGs were enriched in hormone signal transduction, MAPK signaling, galactose metabolism, the calcium signaling pathway, flavonoids, and sesquiterpene substance biosynthesis. GO enrichment analysis also revealed that the DEGs whose expression increased after 3 h (cluster 3) were enriched in secondary metabolites, hormone levels and metabolic responses; additionally, the DEGs whose expression increased after 6 h (cluster 1) were significantly enriched in pathways related to the injury response,

water distribution, ABA, signal transduction, the stress response, and intercellular signal transmission (Figure 2).

The transduction of drought signals begins with the perception of drought signals, and drought stimuli are perceived and captured by sensors on the cell membrane and then transmitted downward through multiple signal transduction pathways (Jensen et al. 1996; Tardieu 1996; Zhu 2002; Bhargava and Sawant 2013). The ABA signaling pathway is one of the most important signal transduction pathways involved in drought stress (Bhargava and Sawant 2013; Mahmood et al. 2019; Wang et al. 2019). Drought-induced ABA is perceived by ABA receptors such as PYL to form a complex, which then binds to PP2C and SnRK2s and is subsequently released from the inhibition of the SnRK2/PP2C complex. (Fujii et al. 2009; Cutler et al. 2010; Gonzalez-Guzman et al. 2012; Fujita et al. 2013). The released SnRK2s further phosphorylate downstream AREBs/ABFs to bind to ABRE cis-elements in the ABA-dependent signaling pathway to regulate the transcription of downstream target genes. In addition, previous studies have shown that DREB2 transcription factors play central roles in the ABA-independent pathway. Crosstalk between the AREB-SnRK2 pathway and the ABA-dependent/ABA-independent pathway occurs, but knowledge of how the two signaling pathways regulate each other has been limited until recently. Generally, we believe that ABA-dependent genes and ABA-independent genes can be distinguished by their gene expression patterns under ABA treatment. The expression regulation of ABA-dependent genes directly or indirectly depends on ABA signals, whereas the expression regulation of ABA-independent genes does not depend on ABA signals, which may be initiated through other parallel signaling pathways. Under ABA treatment or ABA-related perturbations, the related genes present completely different expression characteristics. In our study, RNA-seq analysis was performed on *P. trifoliata* seedlings treated with 0.1 mmol ABA for 3 h or 6 h. In total, 225 DEGs were upregulated, and 612 were downregulated between before and after 3 h of ABA treatment; 399 DEGs were upregulated, and 932 were downregulated after 6 h of ABA treatment (Figure 5A). The number of DEGs that were simultaneously upregulated or downregulated at both 3 hours and 6 hours was 96 and 429, respectively. Gene clustering analysis revealed that under drought stress, the expression of 104 genes is induced by ABA, whereas the expression of 444 genes, which may be classified as ABA-independent genes, is not induced.

Interestingly, more than half of the DEGs under ABA treatment presented decreased expression. The observation that exogenous ABA initially downregulated a substantial number of genes, including some associated with ABA signaling, may seem counterintuitive. However, this likely reflects complex negative feedback regulation within the ABA signaling network, a mechanism crucial for preventing excessive responses and maintaining signaling homeostasis. The ABA pathway involves multilevel negative feedback to avoid excessive responses that cause growth inhibition or energy waste (Merlot et al. 2001; Wang et al. 2019, 2022; Waadt et al. 2022). When the amount of exogenous ABA increases suddenly, plants quickly inhibit ABA signaling via either short-term desensitization or the induction of negative regulatory transcription factors (Luo et al. 2014; Takatsuka and Umeda 2019; Waadt et al. 2022). This transient suppression could represent a mechanism for natural conditions, and the accumulation of ABA is usually accompanied by stress signals such as drought and high salinity. When exogenous ABA is

applied alone without actual stress, plants may inhibit the ABA signaling pathway to avoid excessive responses to "false stress", thereby maintaining normal growth (Duan et al. 2013; Collin et al. 2021; Gao et al. 2024). Plants preferentially activate short-term adaptation genes (such as ion transport proteins and osmotic regulatory factors) while temporarily inhibiting long-term response genes (such as ABA synthesis-related genes and certain transcription factors), which require more energy after ABA treatment (Duan et al. 2013; Gough 2014; Yang et al. 2014; Takatsuka and Umeda 2019; Wang et al. 2019; Ali et al. 2020). The inhibition of ABA-related gene expression in the early stage of exogenous ABA treatment is an adaptive strategy for plants to rapidly terminate oversignaling and balance the stress response and growth through negative feedback regulation.

Our integrated analysis revealed early molecular events. The identification of ABA-dependent effectors (e.g., OPR2, DUR3) and ABA-independent transcription factors (e.g., WRKY50, NAC029) provides specific candidates for future functional validation and genetic engineering aimed at enhancing drought tolerance in citrus rootstocks. Further investigation is warranted to elucidate the precise roles of these regulators and the potential crosstalk between the pathways they represent.

Declarations

Ethics declarations

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

YZ, LMW, and JHL conceived and designed the research. YZ and JZ performed the experiments. YZ and JZ wrote the first draft of the manuscript, and all the authors contributed to the manuscript revisions.

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Data Availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA028691) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

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Figures

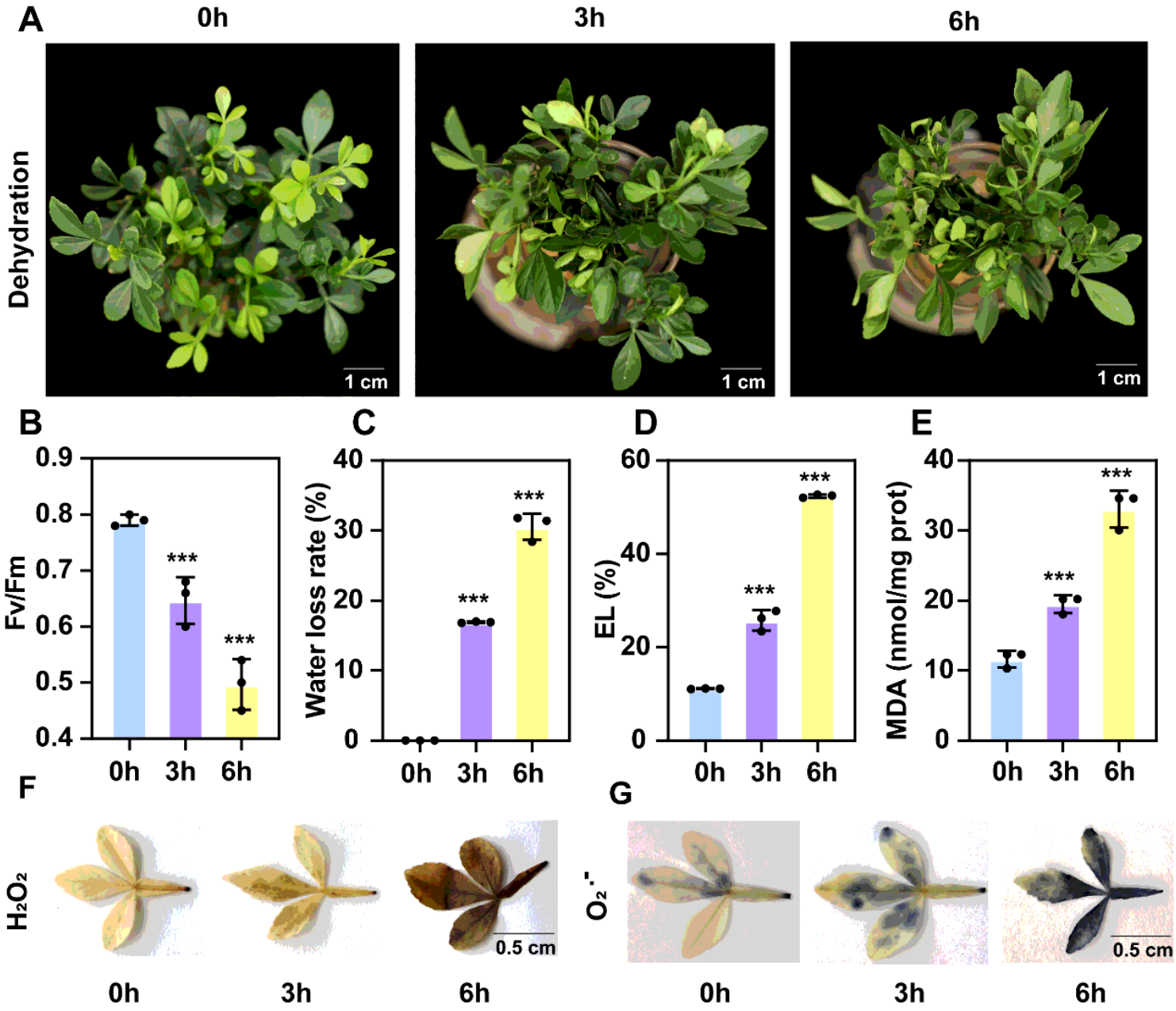


Figure 1

Physiological changes during early dehydration stress in trifoliate orange seedlings.

(A) Representative phenotypes of trifoliate orange seedlings at 0, 3, and 6 hours (h) after dehydration treatment (scale bars = 1 cm).

(B) Fv/Fm values, (C) water loss rates, (D) electrolyte leakage rates and (E) malondialdehyde (MDA) contents of trifoliate orange leaves before and after 3 h and 6 h of dehydration treatment. Asterisks indicate significant differences between different groups under the same growth conditions ($^{***}P<$ 0.001). The error bars represent ±SDs (n=3).

(F) DAB and NBT staining (in situ detection of H_2O_2 and $O_2^{\cdot-}$) of trifoliate orange leaves before and after dehydration treatment (scale bars = 0.5 cm).

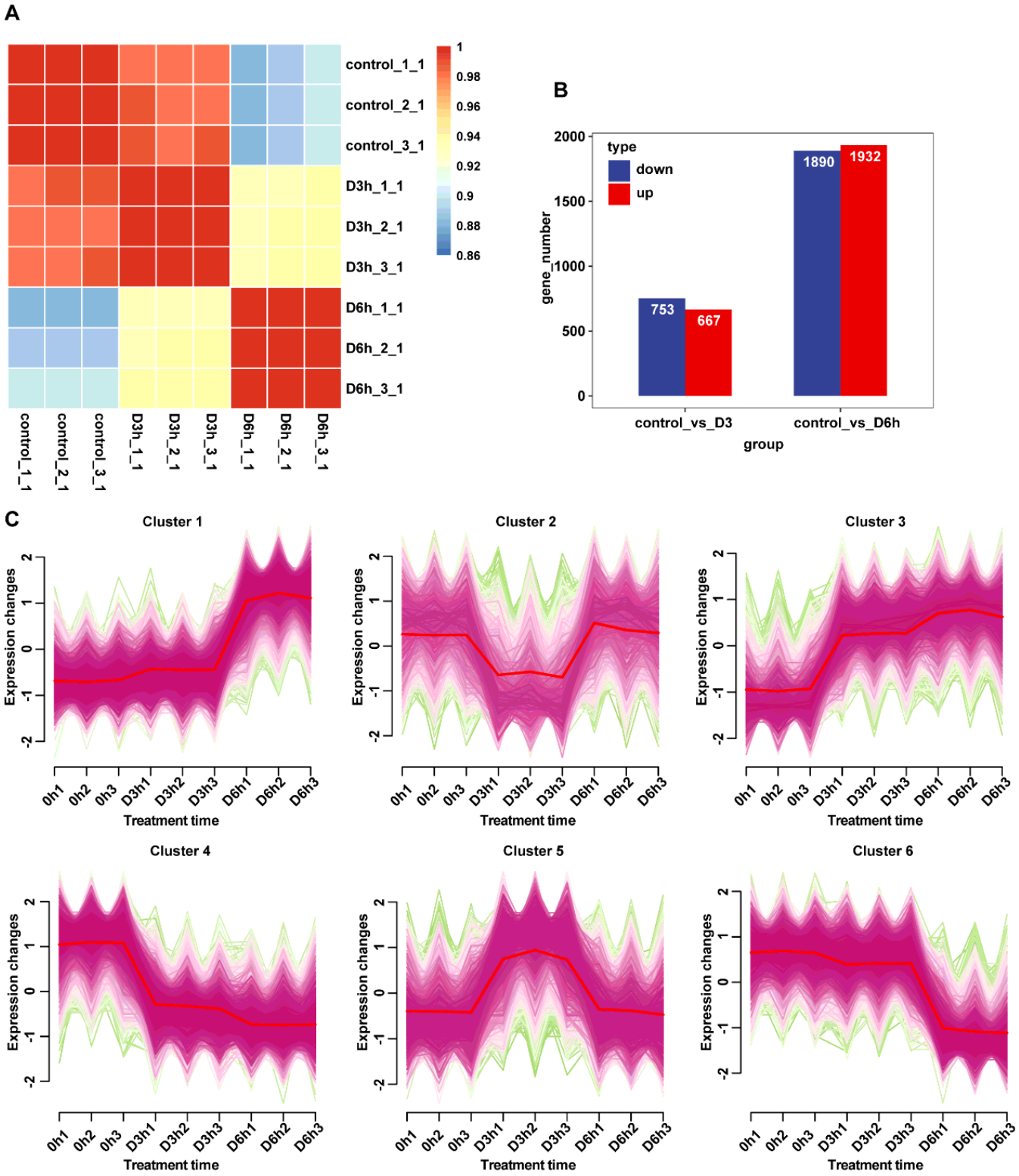


Figure 2

Transcriptome profiles of trifoliate orange seedlings under dehydration stress.

(A) Sample correlation heatmap based on TPM values (Pearson correlation coefficient).

(B) Number of differentially expressed genes (DEGs) (DESeq2, |log2FC|>1, p values<0.05). (C) Time series clustering analysis (Mfuzz) showing six clusters of gene expression patterns after 3 h and 6 h of dehydration treatment. Clusters 1 and 3 represent core drought-induced gene sets. “Control” represents “0 h” (before treatment), “D3h” represents “after dehydration for 3 h”, “D6h” represents “after dehydration for 6 h”, and “_1_1”, “_2_1”, and “_3_1” represent 3 biological replicates.

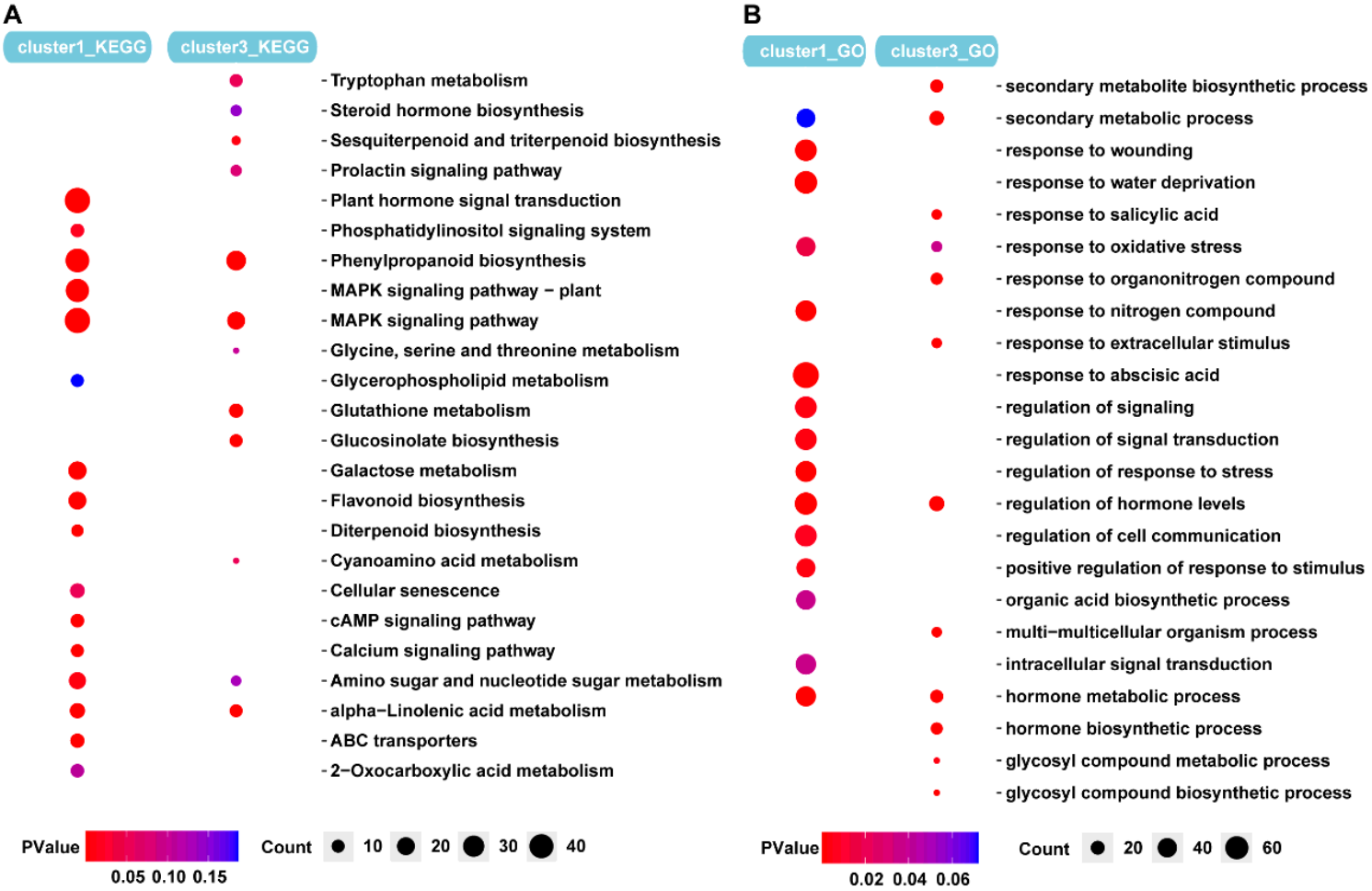


Figure 3

Functional enrichment analysis of differentially expressed genes.

(A) KEGG pathway enrichment and (B) GO biological process enrichment for cluster 1 and cluster 3 genes.

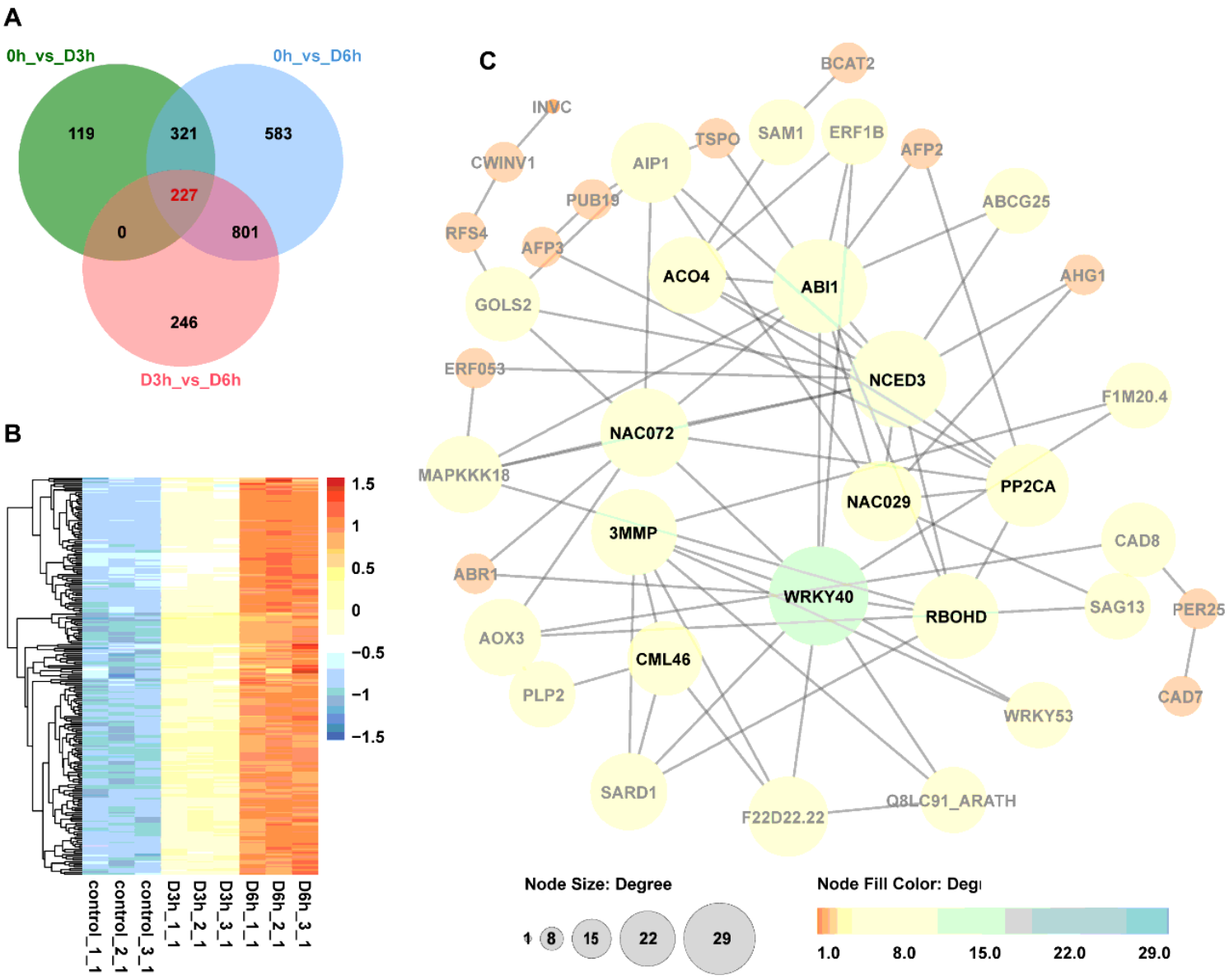


Figure 4

Core regulatory network of the upregulatedgenes.

(A) Expression trend clustering, (B) heatmap and (C) protein–protein interaction network (STRING, confidence score >0.4, degree as node size and fill color) of 227 hub upregulated genes from 3 h to 6 h.

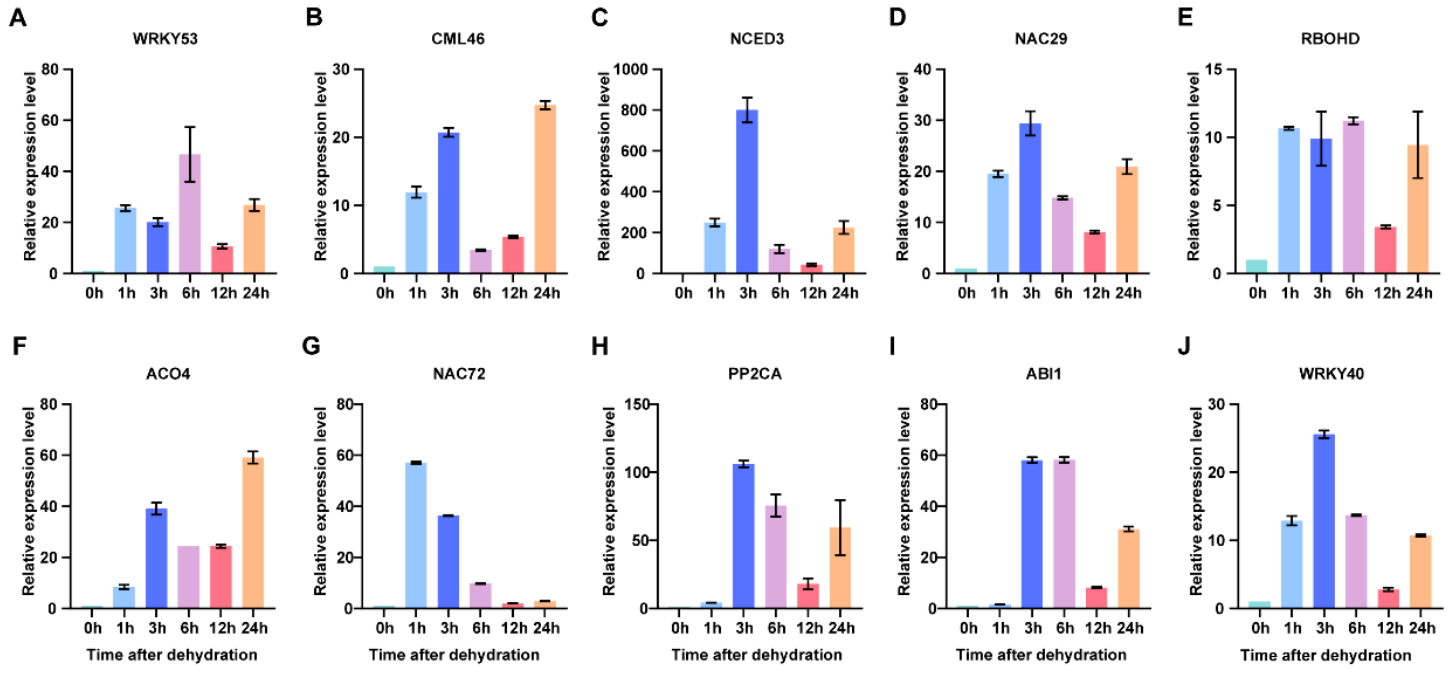


Figure 5

Expression of hub upregulatedgenes under dehydration stress.

The expression levels of hub upregulated genes under dehydration treatment in trifoliate orange leaves. The error bars represent ±SDs(n=3).

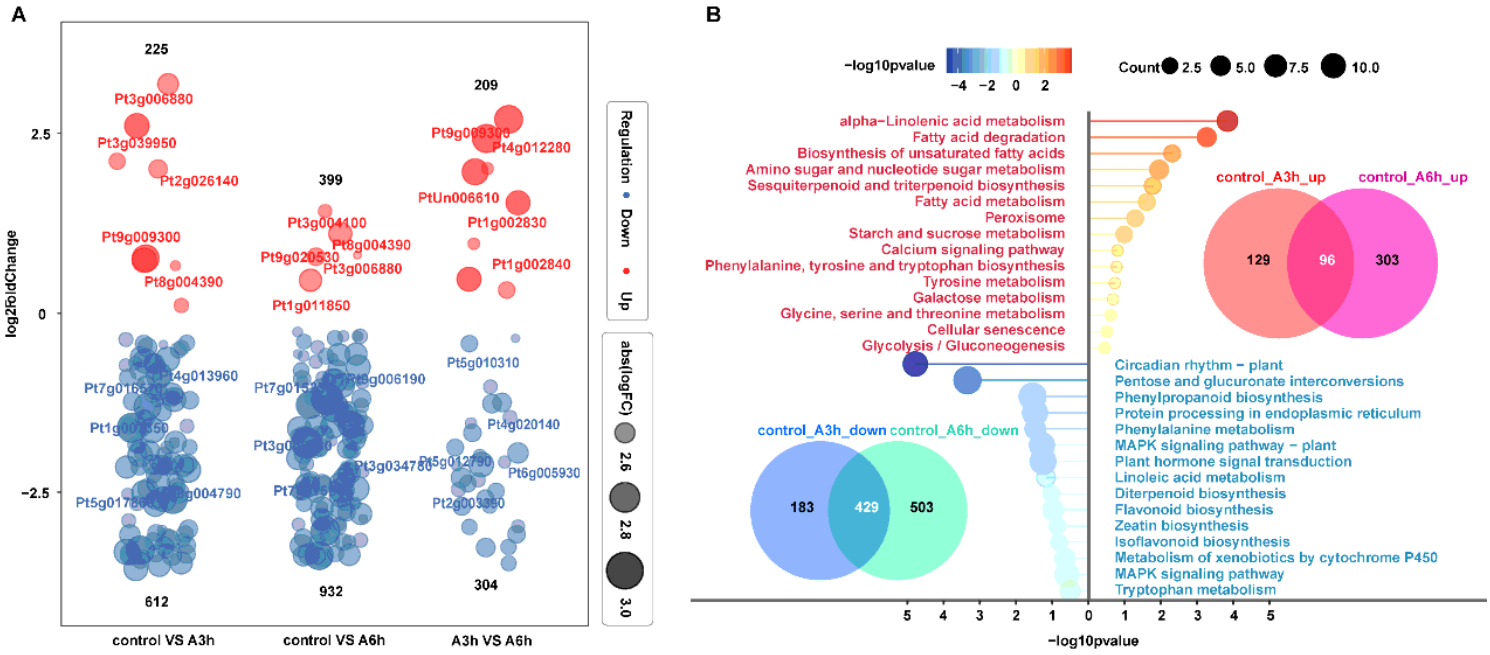


Figure 6

Differentially expressed genes induced by ABA treatment.

(A) Number of differentially expressed genes (DEGs) (DESeq2, |log2FC|>1, p values<0.05) after 3 h and 6 h of 0.1 mM ABA treatment compared with the control (0 h).

(B) KEGG pathway enrichment of DEGs. The red system represents the upregulated DEGs, and the blue series represents the downregulated DEGs.

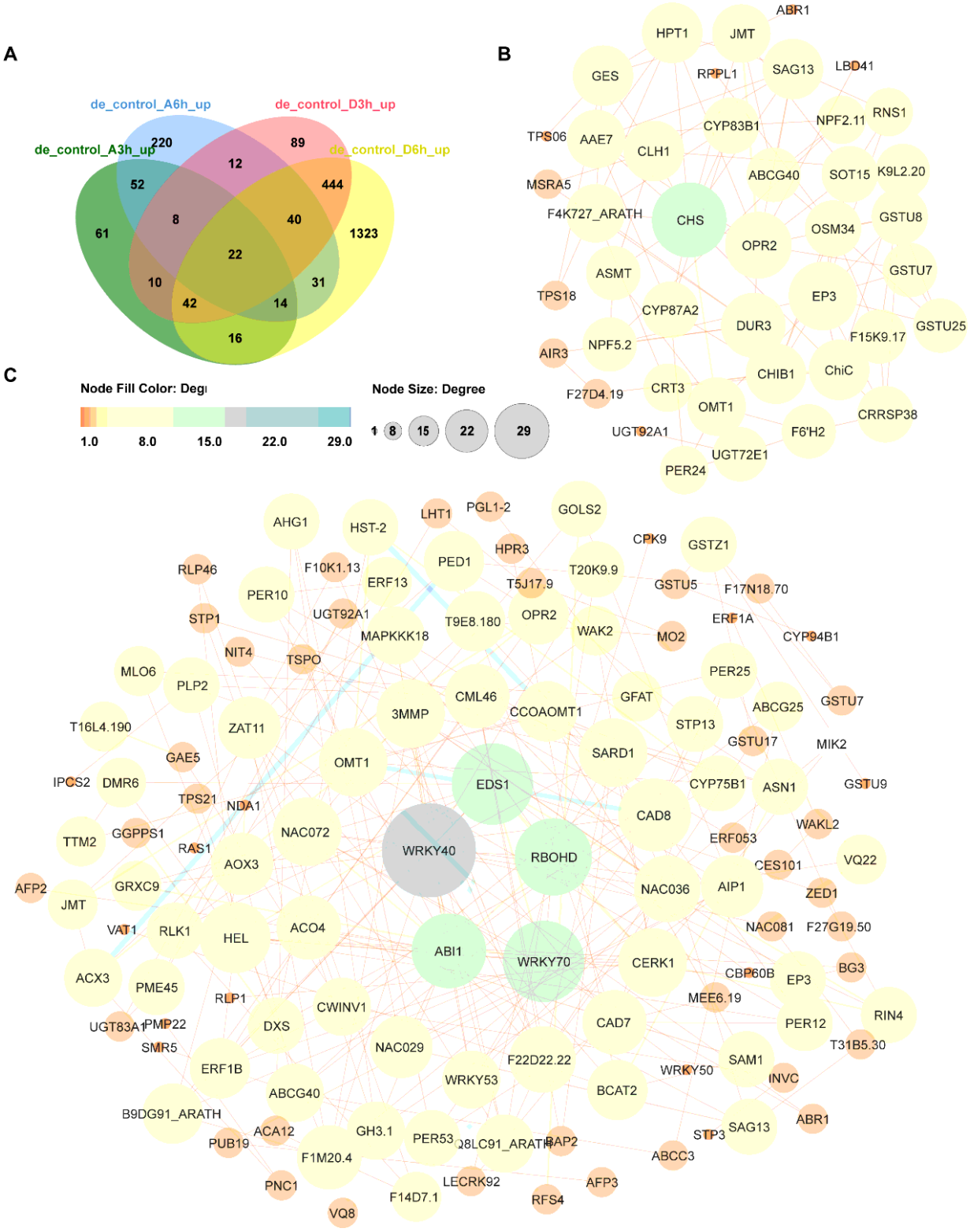


Figure 7

ABA-dependent and ABA-independent genesregulate networks under drought stress.

(A) Expression trend clustering and protein–proteininteraction network (STRING, confidence score >0.4, the degree as node size and fill color) of (B) ABA-dependent (104 genes, Dataset 6) and (C) ABA-independent (444 genes, Dataset 7) genes.

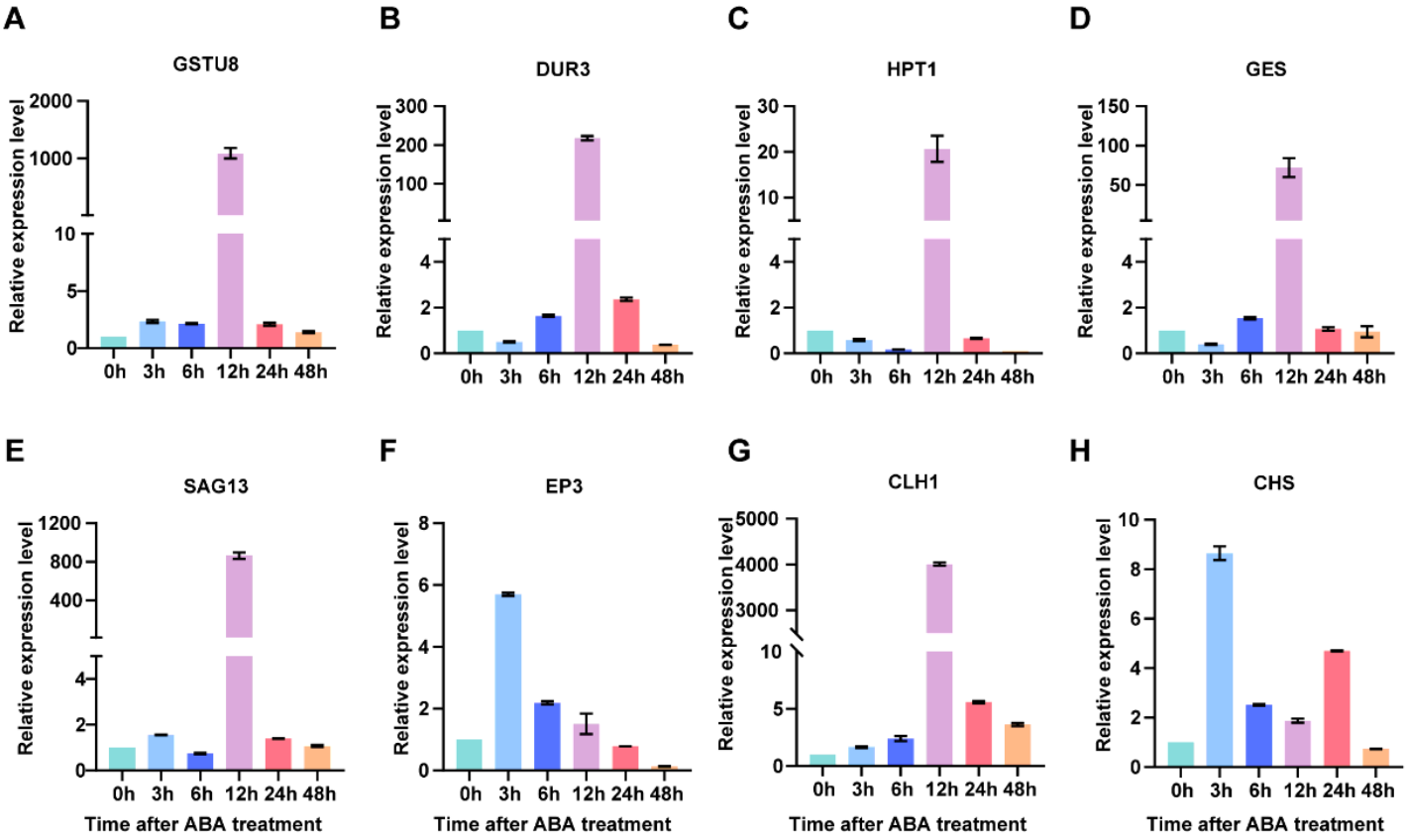


Figure 8

ABA-dependent gene expression pattern.

Expression levels of selected ABA-dependent genes intrifoliate orange leaves subjected toABA treatment (qPCR data represent the means ± SDs of three biological replicates).

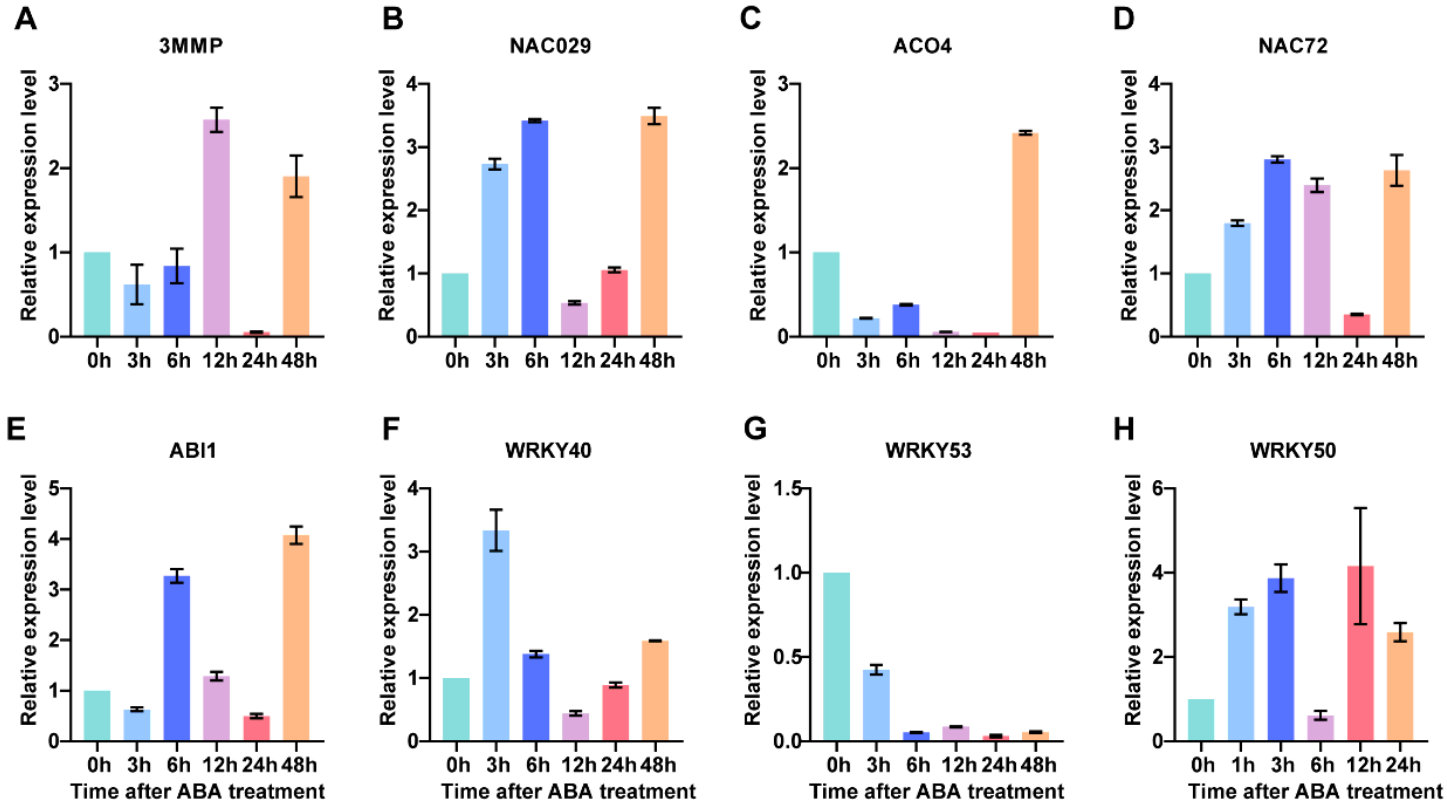


Figure 9

ABA-independent gene expression pattern.

Expression levels of selected ABA-independent genes intrifoliate orange leaves subjected toABA treatment (qPCR data represent the means ± SDs of three biological replicates).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiles.docx