Integrated transcriptome and DNA methylome analyses reveal the molecular regulation of drought stress in wild strawberry (Fragaria nilgerrensis)

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Abstract

Background

Fragaria nilgerrensis is a diploid wild strawberry, which is densely covered with yellow-brown sericeous on the whole plant, giving them excellent drought resistance. Hitherto, the general mechanism of drought resistance of *F. nilgerrensis* is unknown. We therefore investigated the drought response regulatory networks of *F. nilgerrensis* based on the integrated analysis of DNA methylation, transcriptome and physiological traits during four continuous time points under drought stress.

Results

The most differentially expressed genes (DEGs) and the corresponding physiological changes were found at 8 days (T8) compared with 0 day (T0, control). Methylome analysis revealed slight dynamic changes in genome-wide mC levels under drought conditions, while the most hypomethylated and hypermethylated regions were identified at T4 and T8. Association analysis of methylome and transcriptome revealed that genes that were not expressed in mCHG and mCHH contexts exhibited the expected hypermethylation levels, and highly expressed genes exhibited corresponding hypomethylation levels in the gene body, but CG methylation showed the opposite trend. Notably, hypomethylation was found to contribute to the increase in gene expression levels during the drought-critical period (T8). Then, the identified 835 differentially methylated and expressed genes were grouped into four clustering patterns to characterize their functions. The genes with either negative or positive correlation between methylation and gene expression were mainly associated with Kinases, ROS synthesis, scavenging, ABA signal pathway, etc. Consistently, weighted gene co-expression network analysis (WGCNA) revealed Hub genes including *NCED*, *CYP707A2*, *PP2Cs* and others that play important roles in the ABA signaling pathway.

Conclusions

F. nilgerrensis drought is dominated by ABA-dependent pathways, possibly accompanied by ABA-independent crosstalk. DNA methylation affects gene expression, although there is no common pattern or correlation between DNA methylation and gene expression on gene number. Maintaining the balance between ROS regeneration and scavenging is an important factor in drought resistance in *F. nilgerrensis*. These results would deepen our understanding of drought resistance and its application in breeding in strawberry.

Background

Drought stress is a key environmental factor affecting agricultural production since it usually constrains the photosynthesis rate and carbon dioxide assimilation [1]. In order to deal with drought stress, plants

enhance drought resistance by reducing water loss and improving plant osmoregulatory capacity [2]. The contents of proline, glycinebetaine, and soluble sugar in plants are increased to enhance the capacity of osmotic adjustment to adapt to drought stress [3–5]. A variety of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are produced to eliminate excess reactive oxygen species (ROS) and mitigate the effects of drought stress on plants [6, 7]. Plants also produce a series of hormones in response to drought stress, including abscisic acid (ABA), ethylene, and jasmonic acid (JA), in which ABA is the key hormone for plants to cope with adversity stress [8]. Under drought stress, the large accumulation of endogenous ABA in plants promotes stomatal closure and reduced transpiration [9, 10]. Although drought tolerance was reported mediated by ABA-independent pathways in some plants, more and more drought response genes were identified involving in ABA-signaling pathways or both [11].

In reality, plants response toward drought stress is a complicated process involving a range of physiological, biochemical, and genetic reactions [12]. These intricate processes are influenced by multiple genes expression, metabolic networks, and methylation of DNA. In plants, DNA methylation is important in regulating gene expression, silencing transposable elements (TEs), and maintaining genomic stability to cope with multiple stresses, such as drought, salt, and heavy metal stresses [13–15].

The pattern of DNA methylation changes in species specific, tissue specific ways under drought stress [16]. In the last few years, numerous studies have further explored the molecular mechanisms of plant response to abiotic stresses through combined analysis of transcriptome and DNA methylome. For example, in the DNA methylome and transcriptome analysis of sea buckthorn under drought condition, the expression level of hypermethylated genes is decreased, and that of hypomethylated genes is increased [17]. Another study has shown that drought stress increases the methylation level of sesame, which may induce a decrease in the transcription level of many drought-responsive genes [18]. These studies showed that plant gene transcription is closely associated with the changes in DNA methylation levels under drought stress.

Strawberry has large leaf surface and shallow root system, making it more sensitive to drought stress, which severely limits the yield and cultivation area of strawberry [19]. *F. nilgerrensis* is a wild diploid strawberry with wide distribution in southwestern China [20]. *F. nilgerrensis* not only has great disease resistance and waterlogging tolerance, but also possesses white fruits with special peach-like aroma, which can be used as a source of excellent traits for strawberry varieties improvement [21, 22]. More importantly, *F. nilgerrensis* has thick leaves, sturdy petioles, and dense yellow-brown sericeous on the whole plant, which give them strong drought-resistance and the ability to grow in arid areas [21, 23]. Thus it is worthwhile to study the molecular mechanism of *F. nilgerrensis* under drought stress. There are many studies on drought stress in cultivated strawberry (*Fragaria × ananassa*); Zahedi, et al. [24] found that drought treatment enhances the contents of anthocyanin, proline, CAT, and SOD in strawberry leaves. Gu, et al. [25] expressing *OsDREB1B* gene in cultivated strawberry reduced its stomatal aperture and significantly higher expression of plasma membrane intrinsic protein (PIP) in leaves under drought condition, giving the plant a stronger water retention capacity. However, there are no reports on the

drought tolerance mechanism of *F. nilgerrensis* and the methylation changes of the genome under drought stress.

In this research, we investigated gene expression profiles and whole genome DNA methylation of *F. nilgerrensis* in different time points of drought stress treatment. Through the integration of transcriptome and DNA methylome with physiological data, this work not only improves our understanding of the molecular mechanism of strawberry drought resistance, but also provides a foundation for further investigating and facilitating the breeding of drought resistant strawberry.

Results And Discussion

Physiological traits of F. nilgerrensis **under drought stress**

Harmful ROS content usually increases dramatically under drought stress and attacks membrane lipids, accompanied by peroxidative damage to DNA, proteins, and membrane lipids [26]. To resist oxidative damage, plants have gradually evolved many protectives scavenging or antioxidant defense mechanisms, such as SOD and POD antioxidant enzymes [27]. In this paper, the contents of malondialdehyde (MDA) and relative electrical conductivity (REC), which are indicators of oxidative damage [28], were significantly increased at T8 ($P < 0.05$), and continue to increase sharply at T12 ($P < 0.01$) (Fig. 1A, B), indicating that *F. nilgerrensis* were suffering from severe oxidative damage from T8. Correspondingly, the activities of POD and SOD were also displayed an increasing tread from T8 to T12, thereby alleviating the ROS damages. Besides these enzymes, we also found a significant rise in proline (Pro) content from T8 to T12 in *F. nilgerrensis*, which can modulate intracellular and extracellular osmotic potential to improve plant water retention [29]. The smallest increase of all the five parameters (Pro, MDA, SOD, POD, REC) were detected at T4. These drought inducible physiological changes indicated that multiple physiological processes, which were regulated by molecular alterations, were initialed near or at T8.

Changes of differentially expressed genes (DEGs) under drought stress

To explore the drought resistance mechanism of *F. nilgerrensis*, we sequenced the transcriptome and whole genome bisulfite sequencing of leaves at four time points (T0, T4, T8, and T12). Among them, transcriptome sequencing was completed in 11 samples with an average of ~ 6.4Gb per sample, which were subsequently mapped to the *F. nilgerrensis* genome. The mapping rate was as high as about 95%, and the unique mapping rate was greater than 92% on average (Supplementary Table S1). The Pearson correlation coefficients (R^2) among replicates mostly exceeded 0.9, indicating the high reproducibility between replicates of each time point (Supplementary Fig. S1A).

Compared with T0, the most DEGs (5,308) were detected at T8, of which significantly up-regulated and down-regulated genes were 2225 and 3083, respectively (Fig. 2A). Notably, the lowest number of DEGs

was found between T12 vs. T8 (100 DEGs), suggesting the gene expression patterns of T8 and T12 were similar. Consistently, Venn plot showed that T8 and T12 shared most DEGs (2,593) (Supplementary Fig. S1B) as well as the hierarchical cluster analysis of DEGs, which showed that T8 and T12 were clustered together (Supplementary Fig. S1C). These results further indicated that dramatic molecular and metabolic alterations of *F. nilgerrensis* occurred at T8. Then we searched common temporal expression patterns using the Short Time-series Expression Miner (STEM) program [30] and found 11 significant profiles (Supplementary Fig. S1D), among which two broad profiles exhibited up (1,549 genes) or down (1,274 genes) regulation at T8 (Fig. 2B). Notably, the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that up-regulated genes were enriched in glycerophospholipid metabolic pathway, MAPK signaling and ribosome biosynthesis etc., while down-regulated genes were enriched in carbon fixation and photosynthesis (Fig. 2B). Previous studies have shown that the MAPK cascade is a key signal mechanism in response to various external stimuli, including drought stress [31]. The Glycerophospholipid metabolic pathway has also been reported in drought tolerance studies on the tolerant diploid common wheat ancestor *Aegilops tauschii* [32]. In contrast, stomatal closure under water-deficient conditions allows plants to minimize water loss by transpiration, and with the inaccessibility of CO_2, the rate of photosynthesis decreases [33]. Thus, these genes maybe play crucial role in respondse to drought in *F. nilgerrensis.*

Consistently, if we focused on DEGs at T8 vs. T0, a larger number of pathways were enriched in drought resistance according to GO (Gene Ontology) enrichment analysis. The up-regulated genes were mostly enriched in response to water deprivation, oxidative stress, abscisic acid-activated signaling pathway, UDP-glucosyltransferase activity, calmodulin binding, and active transmembrane transporter activity (Fig. 2C). Whereas the down-regulated DEGs were enriched in photosynthesis, cell wall organization or biogenesis, transmembrane receptor protein kinase activity and water channel activity. The expression patterns of DEGs at T8 roughly delineated the processes involved in drought resistance of *F. nilgerrensis*.

Methylation landscape of F. nilgerrensis **under drought stress**

To explore the regulatory mechanisms of methylation level after drought, we simultaneously performed WGBS analysis. A total of ~ 126Gb clean data were produced, with an average of ~ 10.5Gb per sample, a sequencing depth of over 30x (305.9Mb for the genome), and a unique mapping rate of 67.75%-73.13%. In each library, the lowest BS conversion rate was 99.499%, while the lowest conversion rates were 96.78% and 90.18% for Q20 and Q30, respectively (Supplementary Table S2).

The average methylation level across the genome is methylated mC divided by the sum of mC and unmethylated umC at certain cytosine sites. Among three sequence contexts of *F. nilgerrensis*, the CG context exhibited highest methylation level, followed by the CHG and CHH contexts, with an average of 47.69%, 30.87% and 10.56%, respectively (Fig. 3A, Supplementary Table S3). In addition, we also calculated the percentage of three contexts (mCG, mCHG and mCHH) in the total mC sites and found they exhibited dynamic changes at various time points, of which mCHH not only account for the highest percentage but also showed biggest changes (Fig. 3B) (Supplementary Table S3). This was consistent

with previous findings in *Morus alba* and *Gossypium hirsutum* that overall methylation levels increased after drought stress and CG methylation has the highest levels [34, 35].

The sum of DMRs between each time point and the control (T0) were also determined and most hypo- and hypermethylated DMRs were detected in T4 and T8 respectively (Fig. 3D). These DMRs mainly occur in CHH context, and about 80% of them were in repeat elements (Supplementary Fig. S2A). Accordingly, the methylation levels for each element in different genomic regions exhibited that repeat elements showed highest methylation level in all the three contexts and all the time points, followed by promoters and introns (Fig. 3C). For the methylation of promoter and gene body usually affect transcriptional regulation differently, we classified differentially methylated genes (DMGs) between each time point and T0 as promoter methylated and gene body methylated genes. The results showed that promoter DMGs were more than gene body DMGs in *F. nilgerrenis* at all the time points of drought stress (Fig. 3D). Among the promoter DMGs, 317 hypo- and 21 hyper-methylated genes were shared across all the time points respectively (Fig. 3E), this suggests that these genes were may be constantly regulated by DNA methylation and may play a key role in drought resistance. KEGG enrichment showed that these genes were involved in plant hormone signaling transduction, MAPK signaling pathway and ubiquitin mediated proteolysis pathways (Supplementary Fig. S2B). These results were roughly consistent with KEGG enrichment of DEGs.

The relationship between methylation level and gene expression in response to drought

Dynamic changes in DNA methylation is crucial in regulating gene expression of plants under abiotic stresses [14, 36]. Take T8 (containing most DMEGs) for example we illustrated the relationship of DNA methylation and expression in different genomic regions and different contexts. The genes detected in transcriptome were divided into four categories according to their expression levels: no expression (FPKM < 1), low expression, medium expression and high expression level, of which the latter three corresponded to the lower, middle and upper quartiles of FPKM. We found that the unexpressed genes exhibited expectable high methylation levels and highly expressed genes showed low methylation levels correspondingly in gene body except for the CG methylation, which exhibited opposite trend (Fig. 4A). Conversely, genes with medium to high expression levels in all three contexts showed high methylation levels 2 kb upstream of the transcription start site (TSS), indicating that DNA methylation was positively correlated with gene expression in the promoter region (Fig. 4A). It is notable that all three contexts exhibited the lowest methylation levels near TSS and transcription termination sites (TES). This was consistent with previous report that the lack of methylation around transcription initiation and termination sites appears to be important for gene expression regulation [37, 38]. Further, we also divided the methylation levels of these genes into five groups by quintile, with the first group being the lowest and the fifth group the highest (Supplementary Fig. S3). This reverse verification, and analysis gave the same results as above.

To further characterize the relationship between DNA methylation and gene expression, we merged DMGs and DEGs of all the time points and identified 835 genes showed both differential methylation and alteration in gene expression, named as DMEGs (Fig. 4B, Supplementary Table S4). Analysis of the expression levels of DMEGs at different time points showed that the T8 time point showed significantly higher expression levels of hypomethylated DMGs than hypermethylated DMGs and all DEGs ($P < 0.001$) (Fig. 4C). This was consistent with the fact that most recognized hypomethylation usually promotes gene expression [14, 39]. On the contrary, DMEGs at T4 and T12 showed a slight increase in gene expression after hypermethylation, but it was not significant. In order to further explore the relationship of methylation and expression, these genes were systematically classified into four different clusters (C1, C2, C3 and C4, respectively) based on hyper- and hypo-methylation in promoter and gene body as well as gene expression patterns (Fig. 4D). Among promoter and gene body DMEGs, both positive and negative correlation were found in methylation and gene expression. In promoter DMEGs, the genes of C1 and C2 show typical methylation regulation patterns, i.e., hypermethylation represses the gene expression while hypomethylation promotes expression; in contrast, the genes of C3 and C4 showed positive correlation in methylation and gene expression (Fig. 4D). These gene clusters were found with different functions, such as many genes in C2 (Hypomethylation and high expression) and C3 (Hypermethylation and high expression) were related to hormone signaling, kinases, transcription factors, detydrin, and detoxificants etc., while C1 (Hypermethylation and low expression) and C4 (Hypomethylation and low expression) contained a large number of photosynthesis-related genes (Fig. 4E). Similar pattern was found in gene body DMEGs that genes with increased expression were involved in drought reaction, while the genes with decreased expression were mainly related to the normal physiologic metabolism no matter hypo- or hypermethylated. In rice, promoter DNA methylation has been reported to be associated with transcriptional repression, while gene body methylation usually upregulates gene expression [39]. Whereas, positive associations of DNA methylation to gene expression were found in both promoter and gene body in apple [40]. This may imply a deep relationship between DNA methylation and gene expression, and is not just satisfied with previous reports of maintaining genome stability and suppressing gene expression [35, 40].

Characterization of key genes for drought response in F. nilgerrensis

The GO enrichment analysis of DEGs indicated the ABA signaling pathway was significantly involved in the drought resistance of *F. nilgerrensis* (Fig. 2C), which was reported responding to drought stress by regulating stomatal closure and the expression of genes [41, 42]. Notably, the expression of gene *NCED1* encoding 9-cis-epoxycarotenoid dioxygenase (a very important enzyme in the ABA biosynthetic pathway) and gene *CYP707A2* encoding ABA 8'-hydroxylase (a key enzyme in the oxidative catabolism of ABA) were significantly upregulated by more than 10-fold (Fig. 5A), in which *CYP707A2* was found to be accompanied by hypomethylation in the promoter region throughout drought stress. Significant upregulated expression of *NCED1* and *CYP707A2* and an increased ABA content was also detected in cultivated strawberries under drought stress (*F. × ananassa*) [43]. It suggested that drought stress on *F. nilgerrensis* promote ABA biosynthesis and activated ABA-dependent signaling pathways. It was known that under stress conditions, ABA levels increased and the PYRABACTIN RESISTANCE/PYR-

LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCAR) bounded to ABA to interact with and inhibit downstream target, the clade A type 2 C protein phosphatase (PP2Cs), thereby releasing the SNF1-related protein kinase 2 (SnRK2) [44]. The released SnRK2 (especially encoded by subclasses III and II of SnRK2) eventually leads to the phosphorylation of the downstream ABA-dependent signaling gene ABF/AREB to activate the stress response [45]. Our results showed that expression of five ABA receptor (*PYL6*, *PYR1*, *PYL8*, two *PYL2s*) genes were down-regulated, while expression of PP2Cs, genes in subclass II of SnRK2 and *ABF2* were significantly up-regulated in *F. nilgerrensis* under drought stress (Fig. 5A, Fig. 6, Supplementary Fig. S4). In contrast to the common expectation that ABA reduces the expression of PYR/PYLs receptors and induces the expression of PP2Cs, no common trend between ABA content and PYR/PYLs expression was detected in some species, which appears to be the mechanism for reducing persistent ABA damage [46]. This pattern was also observed in *Arabidopsis* that the expression levels of PYR/PYLs were down-regulated, while PP2Cs and ABFs were up-regulated under stress [47]. Consistently, most PYLs were down-regulated except for *ZmPYL4*, *ZmPYL7* and *ZmPYL8* in polyethylene glycol (PEG)-treated maize leaves, and PP2Cs and SnRK2s were either up-regulated or unchanged [48]. Furthermore, the lack of differential expression of SnRK2 subclass III genes in *F. nilgerrensis* may be caused by a missed right time point, and the activation of the downstream target gene *ABF2* also supported this speculation. It was reported that subclass III genes of SnRK2 were rapidly activated within minutes after exogenous ABA administration in *Arabidopsis* [49].

Through the hierarchical clustering method, we performed weighted gene co-expression network analysis (WGCNA) of physiological traits and RNA-seq data to characterize similar expression patterns, and 29 valid modules (grey is an invalid module) were obtained (Supplementary Fig. S5A, 5B). We found that the orange, lightcyan and turquoise modules were significantly positively correlated with the five physiological traits, with an average correlation of 0.76 ($P < 0.05$) (Fig. 5B). On the contrary, the blue module showed a significantly negative correlation with these physiological traits. The KEGG enrichment analysis showed that the positively correlated three modules were mainly enriched in ubiquitin mediated proteolysis, amino acid biosynthesis and MAPK signaling pathway, etc. While the negatively correlated blue module was mainly enriched in photosynthesis, phytohormone signal transduction and biosynthesis of secondary metabolites (Supplementary Fig. S5B). More importantly, a large number of important genes in the ABA signaling pathway were also identified in the turquoise color module, including *NCED1*, *CYP707A2*, and *PP2Cs*, which indicated ABA pathway play an important role in regulating physiological response in *F. nilgerrensis* under drought stress (Fig. 5C, 5D). It's notable that the *FaNCED* gene was reported to be expressed only in roots, but not in leaves in cultivated strawberry (Figaro and Flair) [50]. While in our study we detected the high expression of *NCED* in leaves of *F. nilgerrensis* under drought. Although the root-derived ABA theory has been widely accepted previously, several recent studies have shown that leaves are considered to be the initial source of ABA biosynthesis during water stress due to the presence of large amounts of endogenous carotenoid precursors required for ABA biosynthesis [51]. Besides, the hub genes of orange module, such as *WRKY28*, Zinc finger protein 7 *ZFP7*, and Peroxiredoxin-2B *PRXIIB* also has been shown to be highly related to drought resistance (Supplementary Fig. S5C). Among them, *ZEP7* was reported to regulate ABA signaling in *Arabidopsis*, and *WRKY28*, which

co-expressed with *AtbHLH17*, were known to be up-regulated under drought and oxidative stress, improving resistance to abiotic stress in *Arabidopsis* [52]. Meanwhile, *PRXIIBs* have been reported to play an significant role in cytoprotection against oxidative stress by eliminating peroxides and acting as sensors of hydrogen peroxide-mediated signaling events [53].

In addition, drought usually prevent the entering of CO_2 into leaves and finally result in the decrease of photosynthetic rate [54]. Consistent with this point, a large number of key photosynthesis related genes showed significantly down regulated expression and differentially methylation in our results, such as the genes encoding fructose 1,6-bisphosphatase (*FBPase*), chlorophyll-binding protein (*CBP*) and ribulose-1,5-bisphosphate carboxylase (*RuBisco*) (Fig. 6), which were important regulators of photosynthesis [55]. Furthermore, the drought-induced stomatal closure, reduced carbon dioxide uptake, reduced photosynthetic rate, and changes in chloroplast photochemical reactions also could cause overproduction of ROS [56]. Consistently, we observed that the expression of respiratory burst oxidase homologs (*RBOH*) was significantly unregulated with changed methylation (Fig. 6), which were reported to be the key genes for ROS synthesis and play a crucial role in their responses to biotic and abiotic stresses [57, 58]. Excessive ROS would cause oxidative stress to impair DNA, proteins and lipids, resulting in cell dysfunction and death [59]. Thus, ROS levels under stress conditions are associated with ROS production and ROS clearance maintenance, which also represents the ability of the stress response [60]. Our results showed that in response to ROS damage, ROS scavenging systems were activated and expression of genes such as *POD*, glutathione transferase (*GST*), glutathione reductase (*GR*) and glutathione peroxidase (*GPX*) were significantly increased, regulated by different methylation (Fig. 6). It indicated that rapid and effective antioxidant defense system involved in ROS detoxification in *F. nilgerrensis* under drought stress may provide them strong resistance.

Transcription factors (TFs) are inevitably activated in response to transduction of stress signals and subsequently trigger the expression of a large number of stress-responsive genes [61, 62]. To illustrate the changes in expression and methylation of TFs under drought stress, 1285 TFs in *F. nilgerrensis* genomes were identified according to the transcription factor database PlantTFDB (http://planttfdb.gao-lab.org), among which, 189 TFs differentially expressed under drought stress (Supplementary Table S5). These differentially expressed TFs were from 34 gene families, including NAC (19 members), WRKY (15 members), MYB (23 members), bZIP (14 members), ERF (13 members) and DREB (7 members). As the heat map showed that a large number of TFs showed significantly increased expression under drought stress, including NAC, WRKY, HSF and ERF ect., and some of them were accompanied by differential methylation (Fig. 6). Interestingly, we identified a significant upregulation of dehydration response element binding (*DREB*), which is a key stress TF in the regulation of the ABA-independent pathway. This may seem to imply that there are both ABA-dependent and -independent signals in *F. nilgerrensis* for drought response.

Among these differentially expressed TFs, 25 TFs were also showed methylation changes in the promoter region, including the heat stress transcription factor A-6b, the AP2/ERF family transcription factor *ERF4*, and the possible WRKY transcription factor 46 (Supplementary Table S6). Notably, we found that *ERF1*,

WRKY46, *B3* domain-containing and *Dof3.6* showed continuously increased expression and hypomethylation across all the time points. Among them, *ERF1* was reported to be able to integrate stress-specific gene regulation of multiple hormonal signals to play an active role in drought and heat stress tolerance in *Arabidopsis* [63]. Previous studies have shown that *WRKY64* is involved in stress osmotic and stomatal regulation [64]. Besides, *B3* domain-containing [65] and *Dof* [66] also have been reported to be involved in drought stress. These results suggested that these TFs should play a central role in drought stress tolerance in *F. nilgerrensis*. Finally, we randomly selected the above 12 genes (*NCED1*, *CYP707A2*, two each of *PYLs* and *PP2Cs*, *ABF2*, *WRKY46*, *WRKY28*, *ERF1*, *ERF4* and *B3*) for qRT-PCR verification, and obtained results consistent with the transcriptome trend (Fig. 5D).

Conclusion

Strawberry, as an extremely drought-sensitive plant (due to shallow root system and large leaf area), is of great significance for drought resistance studies involving complex mechanisms. *F. nilgerrensis* is an important source of drought resistant genes for genetic improvement of the cultivated strawberry. In this study, the drought response regulatory networks of *F. nilgerrensis* were deeply excavated based on the integrated analysis of DNA methylation, transcriptome and physiological traits. Our study demonstrated that ABA-dependent signaling pathway, are major part of the drought response regulatory networks in *F. nilgerrensis*, probably accompanied by crosstalk of ABA-independent signaling (One *DREB* gene was significantly up-regulated). Besides, the ability to maintain a balance between ROS regeneration and scavenging was also crucial to prevent metabolic dysfunction and greatly determined overall drought tolerance of *F. nilgerrensis*. Interestingly, the relationship between DNA methylation and gene expression was more subtle than expected, showing both positive and negative correlations at both promoters and gene bodies, with no visible common pattern or correlation in gene numbers. These results offer a model for a comprehensive explore of the drought resistance mechanism of plants, and also provide a reference for manipulation in breeding and crop management.

Materials And Methods

Plant material and drought stress treatment

F. nilgerrensis plants were grown in a greenhouse at Yunnan University, and all plants were from the same clone. Drought experiments were conducted in a conservatory at 20–26℃ and 55%-68% relative humidity. These plants were divided into control and treatment groups and treated as follows: (i) control group: plants were watered every 2d; (ii) drought treatment group: plants were not watered from 0d to 12d. Leaves of *F. nilgerrensis* were collected from four time points with three biological replicates during continuous drought stress: 0d (T0, CK), 4d (T4), 8d (T8) and 12d (T12) (Fig. 1). In the meantime, the materials utilized for DNA methylation, transcriptome analysis and qRT-PCR were obtained from three biological replicates at four time point, which were frozen in liquid nitrogen promptly and then stored at -80℃.

(We declare that the experimental research and field research on plants (cultivated or wild), including the collection of plant materials, comply with relevant institutions, national and international norms and legislation.)

Physiological traits measurement

Each physiological index was examined with 100mg fresh leaf samples respectively. Proline content was analyzed following the acid ninhydrin procedure modified by Bates, et al. [67]. Proline content was calculated as follows: (μg proline/ml × ml toluene) / (0.1g × ml sample) = μg proline/g of fresh weight material. The content of MDA was determined by a sulfubbadic acid measurement method, as described by Zhao, et al. [68]. The activity of POD was measured using the guaiacol oxidation method [69]. The total SOD activity was assayed according to the method described by Giannopolitis and Ries [70]. The measurement of REC was referred to the method described by Zhou, et al. [71]. Then one-way ANOVA of physiological parameters was based on SPSS 26.0.

Whole Genome Bisulfite Sequencing (WGBS)

We extracted total genomic DNA using the Hi-DNAsecure Plant Kit (Qiagen GmbH, Hilden, Germany). A total amount of 5.2 microgram genomic DNA spiked with 26 ng lambda DNA were fragmented by sonication to 200-300bp with Covaris S220, and then treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). Subsequently, BS_seq library concentrations were quantified using a Qubit® 2.0 fluorometer (Life Technologies, CA, USA) and insert sizes were determined on a Bioanalyzer 2100 system (Agilent). The prepared BS_seq libraries were then sequenced on the Illumina Hiseq2500/4000 or Novaseq platforms to generate 125bp of paired-end sequences.

Quality control of the raw data obtained from sequencing was performed using FastQC (fastqc_v0.11.5). The clean data were then mapped to the *F. nilgerrensis* genome [72] using Bismark software (0.16.3) for methylation site detection [73]. The *F. nilgerrensis* genome was transformed into bisulfite transformed versions (C-to-T and G-to-A transformations), indexed using bowtie2 [74] constructs, and subsequently compared to BS_seq reads against each other to determine the unique best alignment. Differential methylation regions (DMRs) were identified using the biological software package DSS(smoothing.span = 200, delta = 0, p.threshold = 1e-05, minlen = 50, minCG = 3, dis.merge = 100, pct.sig = 0.5) [75]. GO enrichment and KEGG enrichment analysis of DMGs were performed using clusterProfiler R package [76] and KOBAS software [77], respectively.

Transcriptome Sequencing

Four time points of leaf RNA extraction were performed using the Tenagen polysaccharide polyphenol kit (QIAGEN, Germany), according to the manufacturer's instructions. RNA was quality-checked using an Agilent 2100 bioanalyzer and qualified RNA was used for library construction, referring to the instructions of the kit NEBNext® Ultra™ RNA Library Prep Kit for Illumina® for library construction [78]. To ensure the quality of the RNA_seq library, cDNA fragments of 370–420 bp in length were selected using AMPure XP beads (Beckman Coulter, Beverly, USA) and the PCR products were purified, and finally the insert size of

the library was measured and quantified again using an Agilent 2100 bioanalyzer. High quality libraries were sequenced on the illumina NovaSeq 6000 (illumina, USA) platform to produce 150bp paired end reads.

The raw data generated by sequencing was quality controlled using fastp (version 0.19.7) to remove splices and low quality etc. to obtain high quality clean data. The clean reads were then mapped to the *F. nilgerrensis* genome using Hisat2v2.0.5, featureCounts (1.5.0-p3) was used to calculate the number of reads mapped to each gene, and FPKM (fragments per kilobase of transcript sequence per millions base pairs sequenced) was calculated for each gene based on gene length. New transcripts were also predicted using StringTie (1.3.3b) [79]. The DESeq2 R package (1.20.0) was used to perform DEGs analysis between two comparative combinations in four time points, and genes with adjusted *P*-value < 0.05 were considered as DEGs. GO and KEGG enrichment analysis of DEGs was performed using software consistent with the DNA methylation enrichment analysis.

STEM version 1.3.13 was used to analyze the temporal specificity of drought resistance gene expression by the STEM clustering method, up to 50 model profiles and all other parameters set to default values [30]. Based on five physiological traits, we performed a WGCNA (using R/WGCNA version 1.70.3) with the filtered (FPKM > 1) 18,542 genes [80]. Parameters were set up as follows: networkType was set to signed, minModuleSize to 30, power to 17, and MEDissThres to 0.25. The networks were visualized using Cytoscape v.3.7.1. The drawing software is mainly Graphpad and ImageGP [81]. The heat map is drawn with TBtools software [82].

qRT-PCR Analysis

High quality RNA was extracted from *F. nilgerrensis* leaves and reversely transcribed into cDNA and used as template for qRT-PCR. Consistent with the qRT-PCR method described in our previous study [83], *FaACTIN* was selected as an internal reference to normalize the relative expression of each gene. The primers sequences used for qRT-PCR validation were shown in Supplementary Table S7.

Abbreviations

DEGs
Differentially expressed genes
DMEGs
Differentially methylated expressed genes
DMGs
Differentially methylated genes
WGCNA
Weighted gene co-expression network analysis
SOD
Superoxide dismutase
POD

Peroxidase
CAT
Catalase
ROS
Reactive oxygen species
ABA
Abscisic acid
JA
Jasmonic acid
TEs
Transposable elements
PIP
Plasma membrane intrinsic protein
MDA
Malondialdehyde
REC
Relative electrical conductivity
KEGG
Kyoto Encyclopedia of Genes and Genomes
GO
Gene Ontology
TSS
Transcription start site
TES
Transcription termination site
PYR/PYL/RCAR
PYRABACTIN RESISTANCE/PYR-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS
PP2C
Clade A type 2 C protein phosphatase
SnRK2
SNF1-related protein kinase 2
PEG
Polyethylene glycol
FBPase
Fructose 1,6-bisphosphatase
CBP
Chlorophyll-binding protein
RuBisco
Ribulose-1,5-bisphosphate carboxylase
RBOH

Respiratory burst oxidase homologs
GST
Glutathione transferase
GR
Glutathione reductase
GPX
Glutathione peroxidase
TFs
Transcription factors
DREB
Dehydration response element binding.

Declarations

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Author contributions

Q. Q. and T.Z. conceived and designed the study; Q.C., L.H., J.L., P.Q., and P.T. conducted the experiments, analyzed data, and prepared the results; Q.Q., Q.C. and T.Z. wrote and improved the manuscript. All authors approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Availability of data and materials

Additional Supporting Information may be found online in the supporting information tab for this article: The raw data have been deposited in the NCBI Sequence Read Archive (SRA) (https://ncbi.nlm.nih.gov/subs/sra) with an accession number of PRJNA848242.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

We declare no competing interests

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Figures

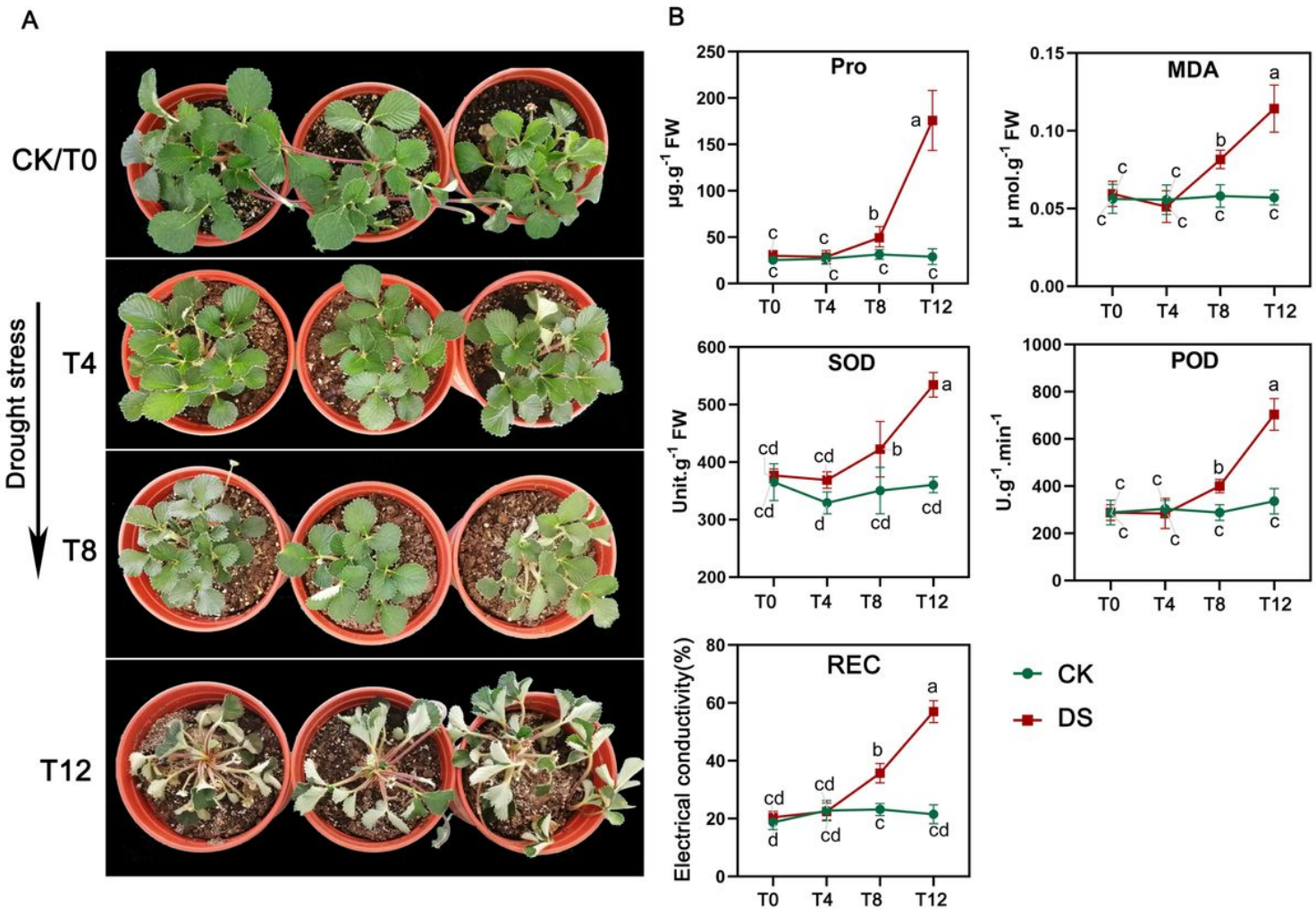
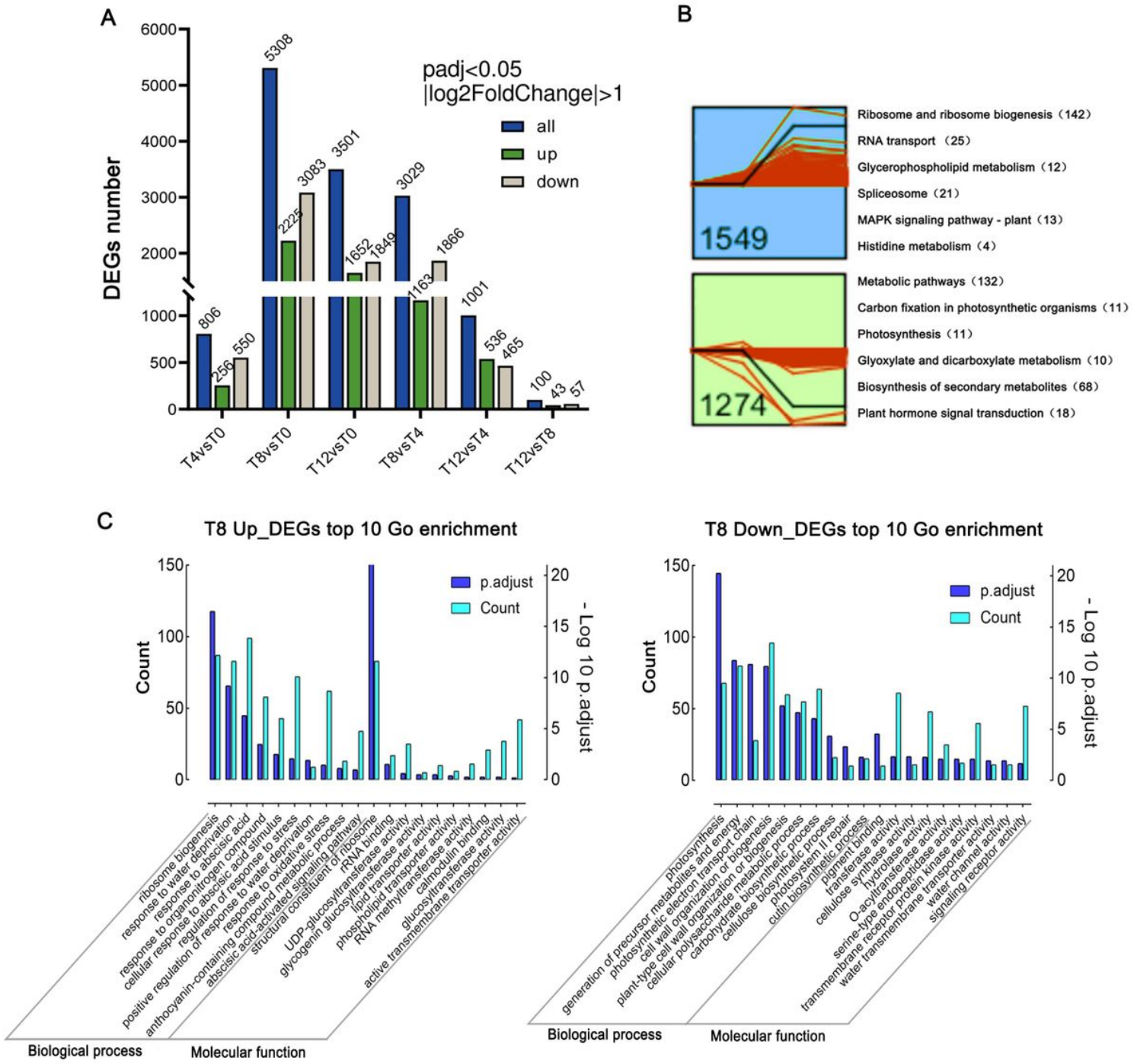


Figure 1


A
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all
up
down
T4vsT0
T8vsT0
T12vsT0
T8vsT4
T12vsT4
T12vsT8
B
Ribosome and ribosome biogenesis（142）
RNA transport （25）
Glycerophospholipid metabolism（12）
Spliceosome（21）
MAPK signaling pathway - plant（13）
Histidine metabolism（4）
1549
Metabolic pathways（132）
Carbon fixation in photosynthetic organisms（11）
Photosynthesis（11）
Glyoxylate and dicarboxylate metabolism（10）
Biosynthesis of secondary metabolites（68）
Plant hormone signal transduction（18）
1274
C
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Biological process
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Figure 2

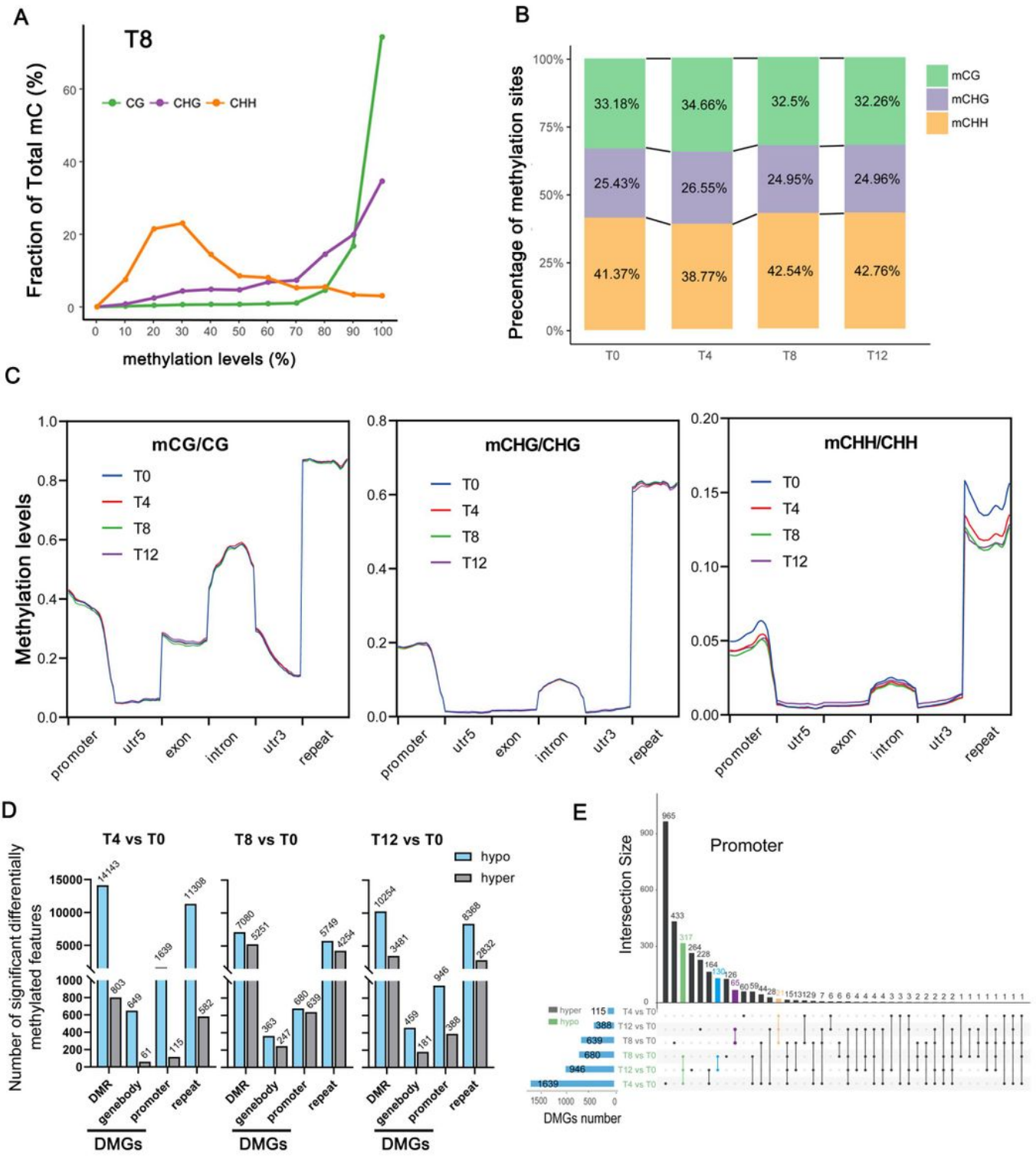

Figure 3

The dynamics of methylation levels at four time points of drought. (A) The distribution of methylation levels in different sequence contexts at T8; (B) Relative proportions of mCs in CG, CHG and CHH contexts at different time points of drought; (C) Dynamic changes in DNA methylation including gene components and repeats; (D) The number of differentially methylated regions (DMRs) and their corresponding genes

(DMGs) were counted for each comparison time point; (E) Upset Venn plot of promoter methylation-related genes in each comparation group.

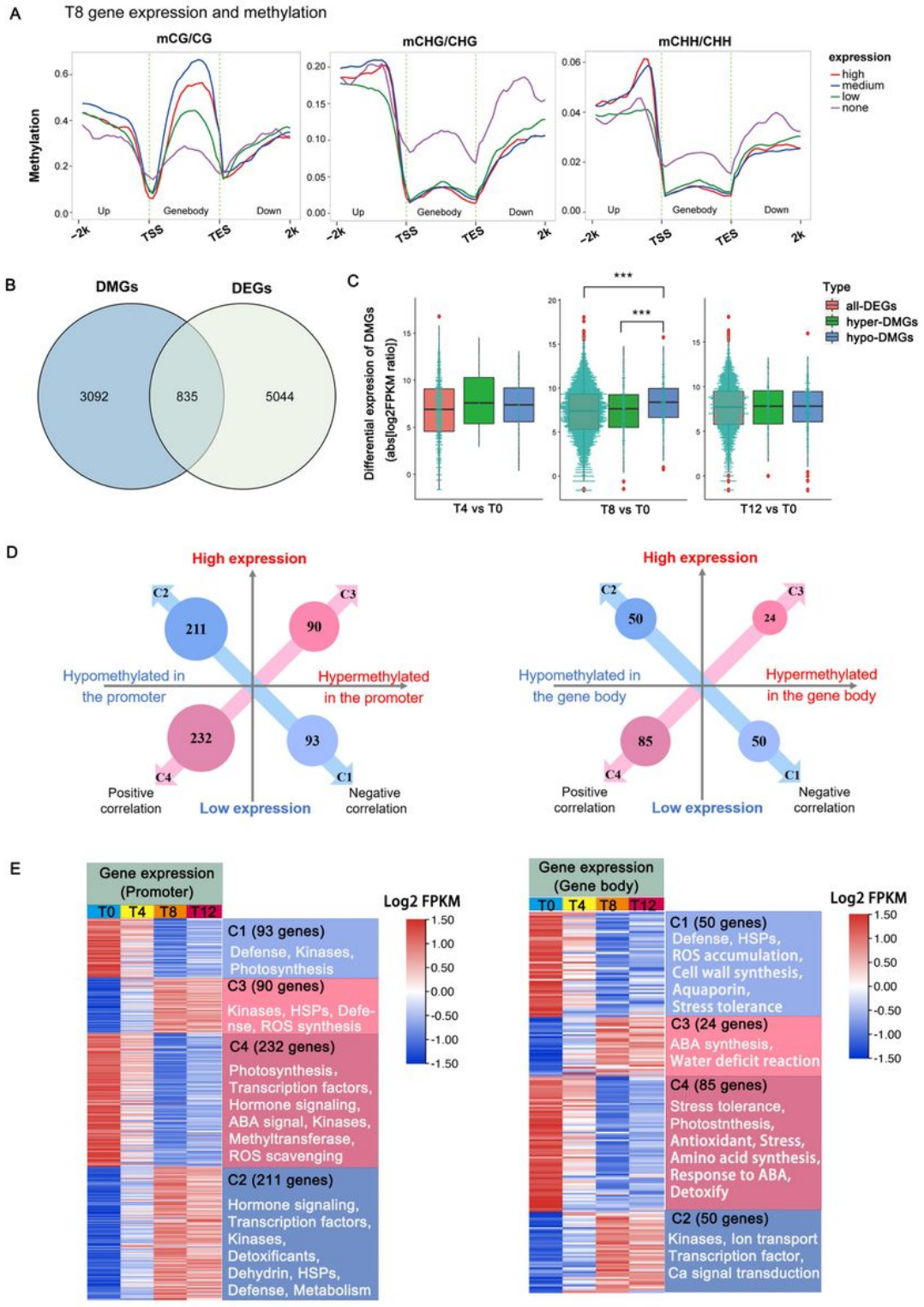

Figure 4

Integrative analysis of methylome and transcriptome revealed expression regulation of drought response genes. (A) Methylation levels of genomic fractions (gene bodies and their upstream and downstream 2 kb

regions) of different expression classes: low to high expression levels correspond to the lower, middle, and upper quartiles of FPKM, with FPKM < 1 indicating no expression (none); (B) The shared 835 genes were subjected to methylome-transcriptome association analysis; (C) Box plots of DEGs levels of all genes, hypermethylated and hypomethylation-related genes. The turquoise dotplot in the middle represent gene numbers and corresponding expression levels. *** *P*.adj < 0.001 (ANOVA, Tukey_HSD); (D, E) Identifies the association of promoter and gene body methylation with the expression of 835 genes. C1: Hypermethylation and low expression; C2: Hypomethylation and high expression; C3: Hypermethylation and high expression; C4: Hypomethylation and low expression. Detailed gene list were showed in supplementary data TableS4.

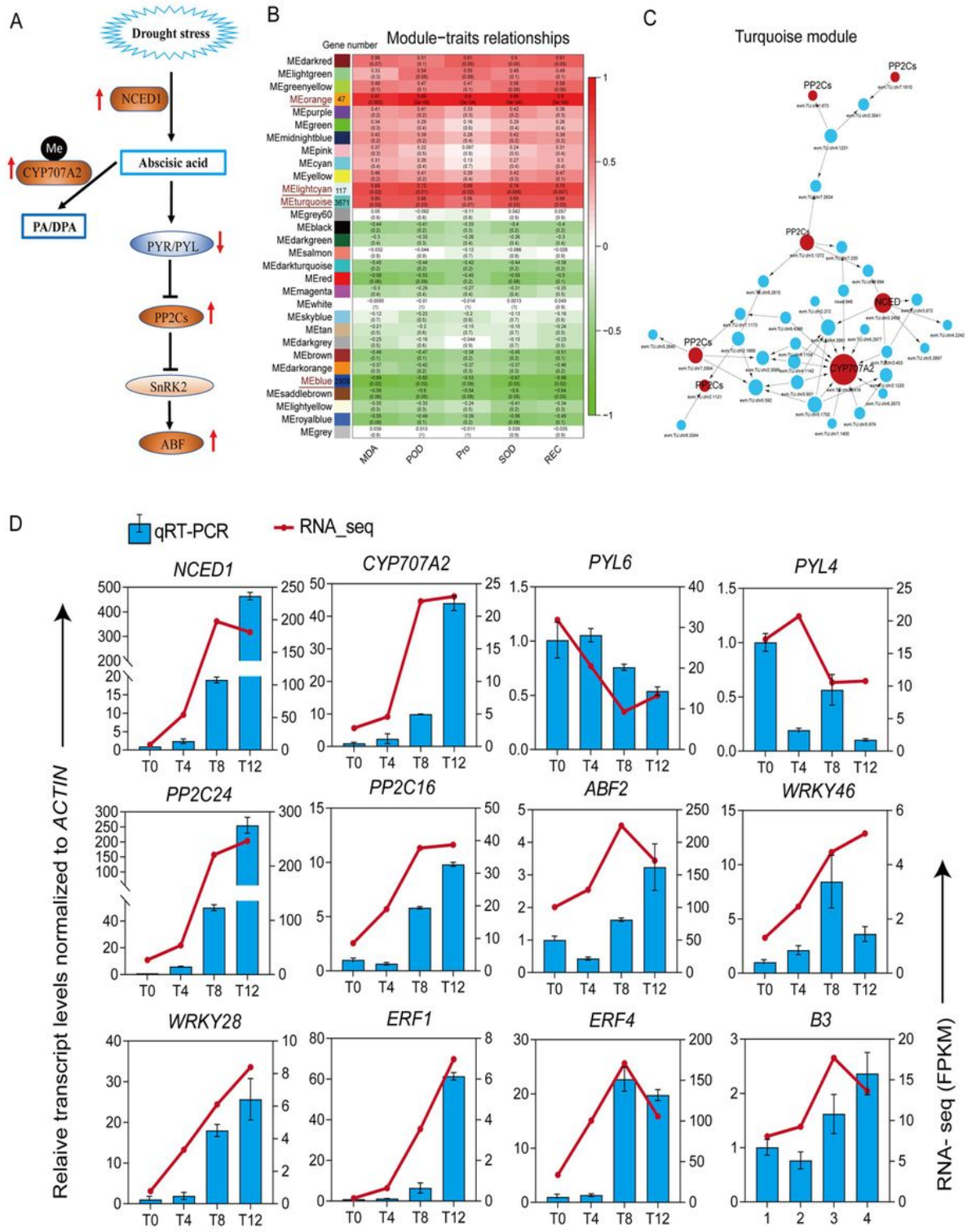

Figure 5

Weighted gene co-expression network analysis (WGCNA) reveals drought resistance modules and qRT-PCR validation of key drought resistance genes. (A) A model of ABA signaling regulation under drought in *F. nilgerrensis.* The upper and lower red arrows indicate up- and down-regulation of genes, respectively, and "Me" indicates differential methylation; PA/DPA: Phase acid and dihydro phase acid; (B) Module-trait correlation. Each column corresponds to a module indicated by a different color, each row corresponds to

a drought physiological characteristic, and the intersecting cell numbers indicate correlation and *P* value; (C) Correlation network of highly correlated turquoise modules. The core components genes of ABA signal are characterized by "red", and the weight is characterized by the size of the node, which reflects the number of genes related to it; (D) The expression level of response genes under drought stress was validated by real-time quantitative PCR. Bars represented means ± SD from three biological replicates.

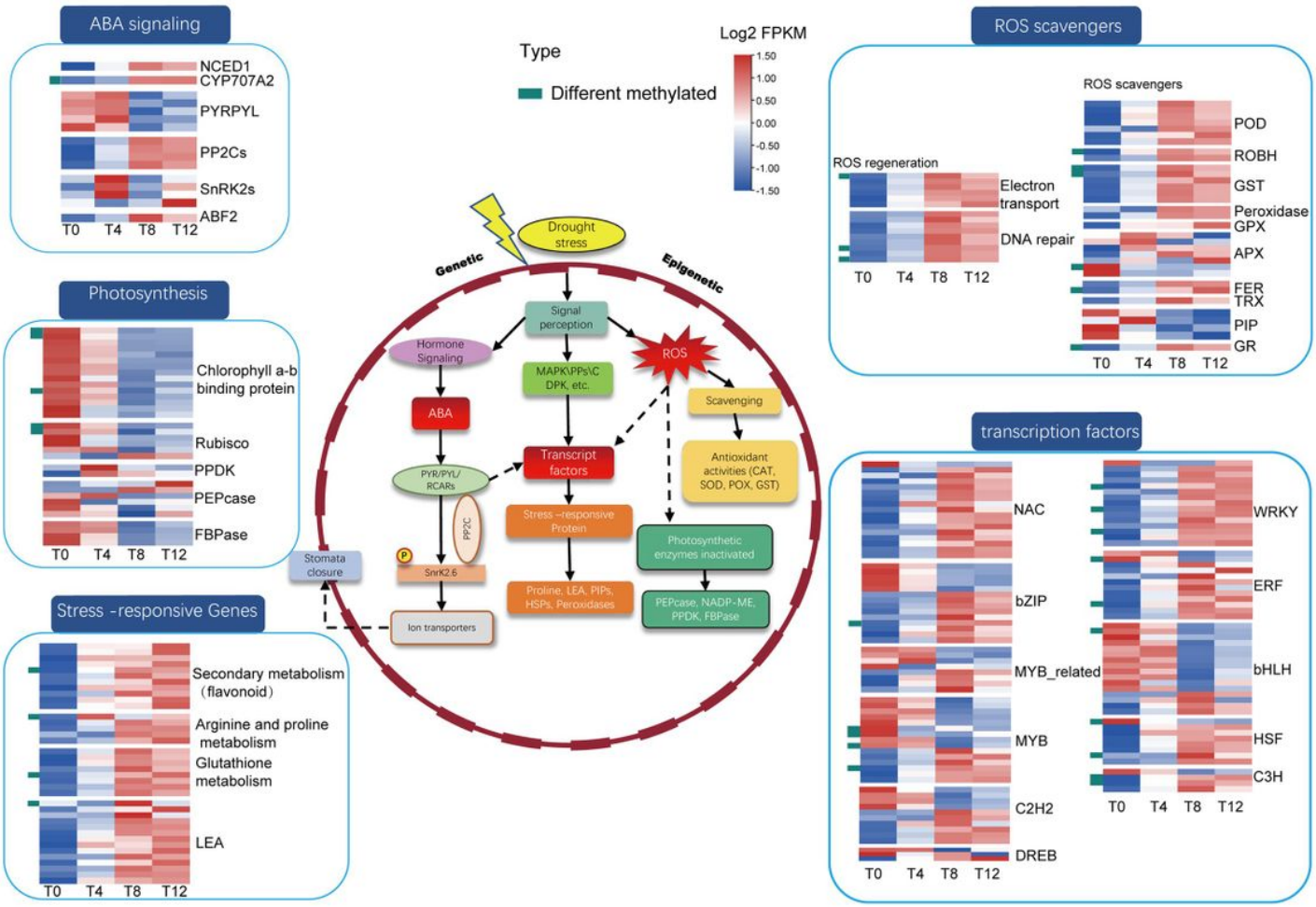

Figure 6

The drought response regulatory networks of *F. nilgerrensis*. The green bar on the left of heatmap indicates differential methylation occurred. *RuBisco*: Ribulose-1,5-bisphosphate carboxylase, *PPDK*: Pyruvate, phosphate dikinase, *PEPcase*: Phosphoenolpyruvate carboxykinase, *LEA*: Late embriogenesis abundant protein, *FBPase*: Fructose-1,6-bisphosphatase, *POD*: Peroxide Enzyme, *ROBH*: Respiratory burst oxidase homolog, *GST*: Glutathione transferase, *GPX*: Glutathione peroxidase, *FER*: Ferritin, *TRX*: Thioredoxin, *PIP*: Aquaporins, *APX*: Ascorbate peroxidase, *GR*: Glutathione reductase.

Supplementary Files

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