Improving Drought Tolerance in Tobacco By Application of Salicylic Acid

Yuqing Feng

Henan Agricultural University

Yuanyuan Zhao

Henan Agricultural University

Yafei Li

Henan Agricultural University

Jun Zhou

Shanghai Tobacco Group Co., Ltd

Hongzhi Shi (✉ shihongzhi88@163.com)

Henan Agricultural University

Research Article

Keywords: tobacco, drought, salicylic acid, photosynthesis

Posted Date: April 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-406971/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Drought causes not only the decrease of tobacco yield and quality, but also the lowering of net photosynthetic rate, leading to reactive oxygen species accumulation and even the death of plants. Salicylic Acid is involved in regulating many plant physiological processes and has increasingly been applied to improve tolerance in plants exposed to drought stress. To explore the regulating mechanism of SA, flue-cured tobacco K326 was used in the hydroponic experiments to design PEG drought stress. The photosynthetic characteristics, antioxidant enzymes activities and osmotic regulatory substances contents of tobacco seedlings under drought stress were investigated after 0.3 mmol L⁻¹ SA treatment. Transcriptome sequencing and GO/KEGG analysis were also performed. The main results showed that SA-applied greatly increased the activities of SOD, POD, CAT activity, Pn, proline and soluble protein by 44.27%, 50.18%, 26.23%, 45.74%, 34.67% and 24.91% while reduced the MDA content by 23.89%. GO and KEGG analysis showed that SA treatment was able to up-regulate the genes involved in photosynthesis, carbon metabolism, porphyrin and chlorophyll metabolism, photosynthesis-antenna proteins. The conclusion is that SA application would effectively improve the ability of pigment biosynthesis and photosystem repair of tobacco under drought conditions, thus enhance the photosynthesis, reduce the accumulation of ROS and increase drought resistance, which would provide a measure for alleviating the damage of tobacco caused by drought stress.

Introduction

Drought is one of the most severe environmental factors that adversely affects plant growth and development, as well as crop productivity and quality. Under drought stress conditions, many physiological and metabolic processes such as photosynthesis, are negatively affected. Drought causes the accumulation of reactive oxygen species (ROS) in plants, resulting in membrane lipid peroxidation, photosynthetic rate reduction and even the death of plants. Plants have evolved multifaceted adaptation mechanisms at morphological, physiological, biochemical, and molecular levels, to rapidly recognize and adapt to drought stress condition^[1-2]. These internal responses range from changes in photosynthetic activity to the development of antioxidant defenses to enhance drought tolerance^[3]. Tobacco (*Nicotiana tabacum* L.) is a model organism as well as a valuable economic crop. Recent years, drought stress has become a main limiting factor for the productivity and quality formation of tobacco due to the dramatic climate changes^[4]. Especially, drought occurred at the vigorous growing stage has the most impact on yield and quality of tobacco leaf. During this stage, drought causes the decrease of pigment contents, nitrate reductase activity and the net photosynthesis of tobacco leaves, thus leading to the decrease of yield and the increase of nitrate accumulation^[5-6]. Therefore, enhancement of tobacco drought resistance and improvement of tobacco physiological traits have become one of the main targets of tobacco breeders and agronomists.

Adding exogenous substances is one of the most important solution to abiotic stresses on plant damage. Melatonin, brassinolactone and methyl jasmonate have been widely applied to improve the plant stress

resistance^[7]. Salicylic acid (SA) is an essential plant growth regulator which plays an important role in regulating physiological processes such as growth, photosynthesis and some other metabolic processes^{[8-9[i]10]}. Prior research generally confirmed that plants treated with SA exhibited better resistance to drought stress^{[11-12[ii]13]}. Several studies agreed that SA alleviated drought stress by an increase in antioxidant enzyme activities^[13-14], proline and soluble carbohydrate content^[15]. A study reported that exogenous application of SA effectively improved Pn, Gs and Tr of *Atractylodes lancea* (Thumb.) DC., and protected its photosynthetic system and improved photosynthetic efficiency^[16]. Another revealed that foliar application of SA increased proline and soluble carbohydrate contents^[15]. However, there is currently no in-depth coverage about the drought tolerance mechanisms at the genome-wide transcriptional level in SA-regulated tobacco seedlings.

Transcriptome sequencing technology has been used to comprehensively study the species gene function and specific biological process at mRNA level. It has clear advantages over other existing methods and numerous studies have applied the RNA-seq method to assess the drought responses in plants and to explore the mechanism of plant response to drought. For example, the global transcriptomic profiles of drought responses have been surveyed in rice^[17], maize^[18], wheat^[19], and soybean^[20]. The objective of the present research is to comprehensively examine the effects of drought stress and SA treatment on the photosynthesis, secondary metabolites and transcriptome of flue-cured tobacco, aiming to reveal the regulating mechanism of SA and to provide effective methods to increase drought resistance.

Materials And Methods

Plant Material and Drought Treatment

A hydroponic experiment was carried out on substrate culture in the greenhouse located in National Tobacco Cultivation & Physiology & Biochemistry Research Center of Henan Agricultural University. Seeds of flue-cured tobacco variety K326 were sown in a floating system in green house that maintained 12/12 h light/dark cycle, 28/19°C light/dark temperature regime, average photosynthetic photon flux density of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity 65%~80%. Six weeks later, well-rooted and same size tobacco seedlings (with 5-6 leaves) were selected and cultivated in 25 cm×30 cm (diameter ×depth) plastic pots (plant/pot). Seedlings were cultivated with Hoagland solution. Nutrient solutions were refreshed every three days. The K326 seeds used in this study were provided by Yunnan Tobacco Company. The collection of the plant material complied with relevant institutional, national and international guidelines and legislation.

Flue-cured tobacco variety K326 seedlings were subjected to treatment after seven days (for recovery). One third of the tobacco seedlings were treated with SA. SA solutions were sprayed on leaves every day for 3 days. Then polyethylene glycol (PEG) simulated water stress method was applied. Moderate

drought stress was simulated using 15% (-0.388 MPa) PEG6000 treatment^[45]. The seedlings were grouped into three experimental test sets with 15 plants per treatment: CK (water + Hoagland nutrient solution), D (water +15% PEG6000-treated nutrient solutions), D+SA (0.3 mmol/L SA + 15% PEG6000). Sampling and analysis were carried out at 14h after the treatment. Fully expanded leaves (length > 5 cm, up to down, the fourth leaf from top) from the same position in three pots of each treatment were sampled and immediately frozen in liquid nitrogen and then stored at -80°C for RNA-sequencing.

Phenotypic observation and photosynthesis parameters measurement

The morphological changes of tobacco in different treatments were observed. Net photosynthesis rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO₂ concentration (Ci) were observed using a portable photosynthesis system (LI-COR Biotechnology, 6400XT, Lincoln, NE, USA) at 9:00 - 11:00 a.m..

Measurement of Enzymatic Antioxidant Activities and osmotic regulating substance

Leave lamina between the sixth and eighth side vein were sliced into small sections for determination of the physiological and biochemical indices. Every treatment had three biological replicates. Fresh lamina tissues were cut into 2 mm × 5 mm pieces. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) enzyme activity was respectively determined by NBT, Guaiacol and Hydrogen peroxide method^[46]. Proline and MDA (malondialdehyde) content were determined as described by Fan^[47]. About 1.0g of each samples was frozen in liquid N₂ and used to assay the soluble protein content according to Li^[48].

RNA Extraction, Preparation of cDNA Library, and Sequencing

The transcriptome sequencing and analysis were conducted by OE biotech Co., Ltd. (Shanghai, China). Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion, Waltham, MA, USA) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples with RNA Integrity Number (RIN) ≥7 were subjected to the subsequent analysis. The libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. These libraries were then sequenced on the Illumina sequencing platform (HiSeq™2500 or Illumina HiSeq X Ten) and 125 bp/150 bp paired-end reads were generated. Quality control was assessed on the remaining reads using NGS QC Toolkit^[49]. Raw data (raw reads) were processed using Trimmomatic^[50]. The reads containing ploy-N and the low quality reads were removed to obtain the clean reads. Then the clean reads were mapped to reference genome using hisat2^[51]. After removing low quality date, the clean reads were mapped to reference *P. trichocarpa* genome (ftp://ftp.solgenomics.net/genomes/Nicotiana_tabacum/assembly/Ntab-K326_AWOJ-SS.fa.gz) using bowtie2 or Tophat (<http://tophat.cbcb.umd.edu/>)^[52-53].

RNA-Seq Analysis, GO and KEGG Pathway Enrichment Analysis of Differentially Expressed Genes (DEGs)

Transcript profiles of RNA-seq data were analyzed by calculating the read fragments per kilo base per million mapped reads (FPKM). FPKM value of each gene was calculated using cufflinks^[54], and the read counts of each gene was obtained by htseq-count^[55]. DEGs were identified using the DESeq (2012) functions estimate Size Factors and nbinomTest^[56]. p-value < 0.05 and fold change > 2 or fold change < 0.5 was set as the threshold for significantly differential expression. GO enrichment and KEGG pathway enrichment analysis of DEGs were respectively performed using R based on the hypergeometric distribution^[57].

Statistical Analysis

Figures were processed using GraphPad Prism (v. 8.0.1, GraphPad Software Inc., CA, USA) and correlation analysis and variance between treatments were all processed using SPSS 20.0 (IBM, Palo Alto, CA, USA). Treatments were compared by LSD multiple range test. All presented data is mean of three biological replicates (n = 3).

Results

Phenotypic observation under PEG drought and natural drought stress

The growth and morphological changes of tobacco seedlings from different treatments were observed (Fig.1). Flue-cured tobacco seedlings were significant different between drought-only (D) and SA (D+S) treatments. It was worth noting that under drought stress (D), the bottom leaves of flue-cured tobacco turned yellow and all the leaves began wilting. In contrast, the leaves of flue-cured tobacco only had slighter symptoms of wilting under SA treatment (D+S).

Effect of SA on photosynthesis parameters

To evaluate the effects of SA on the photosynthesis of flue-cured tobacco seedlings, the net photosynthesis rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO₂ concentration (Ci) were measured (Fig.2a-d). Pn, Gs, Tr and Ci were influenced significantly by imposed drought stress. In comparison with control, Pn, Gs and Tr markedly decreased by 45.23%, 40.31% and 59.67% in 15% PEG-treated seedlings, while the Ci increased by 9.59%. Whereas, SA treated leaves had higher Pn, Gs, Tr of 45.74%±26.82%±61.37%, and lower Ci of 3.29%, compared with the seedlings of PEG treatment alone.

Reduction of MDA and increase of antioxidant enzyme activity and osmotic regulating substance by SA

Antioxidant enzymes play an important role in scavenging ROS which has been proven to hinder the synthesis of PSII core D1^[21]. An increase in POD, SOD, CAT activity, proline and soluble protein content was obtained in 15% PEG-treated tobacco seedlings (Fig.3a-c, e-f). In contrast, exogenous SA exerted a remarkable increase of POD, SOD and CAT by 50.18%, 44.27% and 26.23%, respectively compared with PEG treatment alone (Fig.3a-c, e-f). Moreover, a sharp increase in MDA (the product of lipid peroxidation)

was observed under drought stress (Fig.3d). The seedlings under 15% PEG drought stress caused 87.85% increase in MDA content compared to control (Fig.3d). However, a significant reduction of MDA and increase of proline and soluble protein were observed in SA supplemented drought-stressed seedlings compared to the seedlings exposed to drought only, indicating that SA could improve the cell osmotic function and alleviate the damage of drought stress.

Data processing

Nine cDNA libraries were established for RNA-seq analysis. A total of 85.88M, 88.96M, and 89.26M raw reads were identified in the CK, D and D+S samples, respectively. After data processing, 83.57M (97.31%), 86.43M (97.16%) and 86.91M (97.37%) million clean reads were obtained for further analysis (Table 1). In all, the mapping rates of all the samples to the reference genome were above 95%, the GC content of all samples was stable with the distribution ranging from 43.28~44.13% and the QC30 value of all sample was above 95%, implying successful library construction and RNA sequencing.

Gene expression and correlation analysis between samples

After filtering the raw reads, a high rate of clean reads from each sample was achieved. The FPKM expression levels for each sample were calculated (Fig.4a). In addition, Pearson's correlation coefficient and principle component analysis (PCA) of the data profiles from all 9 samples revealed high correlation among all the samples (Fig.4b-c). These analysis demonstrated that the sequencing data in this study was adequately representative and valid.

Differentially expressed gene selection, Go enrichment and KEGG pathway analysis of differentially expressed genes

Fold change (FC) > 2 or FC < 0.5, p-value < 0.05, was used as threshold to achieve the differentially expressed genes (DEGs).. A total of 6229 DEGs (2914 up-regulated and 3315 down-regulated) were identified between D and control groups, and 4840 DEGs (1944 up-regulated and 2896 down-regulated) were identified between D+S and D groups (Fig.5e).

Gene Ontology is a standardized system used for the functional classification of genes according to the 3 domains of biological process, cellular components, and molecular functions. The DEGs in D-vs-CK were further analyzed using GO and KEGG analyses (Figu.5a-d). We observed almost 5.97% of down-regulated genes correlated with biological process (GO-BP) of photosynthesis (GO:0015979), chlorophyll biosynthetic process (GO:0015995) and chloroplast organization (GO:0009658). In cellular component (GO-CC), the down-regulated genes were significantly enriched in thylakoid (GO:0009579), chloroplast (GO:0009507) and photosystem II (GO:0009523). The genes related to molecular function (GO-MF), such as pigment binding (GO:0031409), rRNA binding (GO:0019843), chlorophyll binding (GO:0016168) were also down-regulated. KEGG pathway analysis also showed that the down-regulated DEGs were mostly involved in photosynthesis (ko00195) and porphyrin and chlorophyll metabolism (ko00860). However, in

D+S-vs-D, these DEGs in the categories of molecular function, biological process, and cellular component were almost up-regulated, which were consistent with KEGG pathway analysis.

GO enrichment and KEGG pathway analysis of DEGs Response to SA

To explore the DEGs in tobacco seedlings improved by spraying SA under drought stress, venn digram analysis between D-vs-CK down and D+S-vs-D up was conducted (Fig.6a). GO and KEGG were then conducted to examine the potential gene function and metabolism pathway of DEGs (Fig.6b-c). Venn analyses showed that 1098 DEGs were enriched between D-vs-CK down and D+S-vs-D up. Genes involved in biological process mostly enriched in photosynthesis (GO:0015979), photosynthesis, light reaction (GO:0019684), photosynthesis, light harvesting (GO:0009765). we observed high values for categories involved in cellular component, such as chloroplast (GO:0009507), thylakoid (GO:0009579), photosynthetic membrane (GO:0034357), photosystem I (GO:0009522), photosystem II (GO:0009523). Genes involved in molecular function mostly enriched in chlorophyll binding (GO:0016168). KEGG pathways analysis presented that the genes were significantly enriched in the pathway of photosynthesis-antenna proteins (ko00196), photosynthesis (ko00195), carbon fixation in photosynthetic organisms (ko00710), carbon metabolism (ko01200), starch and sucrose metabolism (ko00500), porphyrin and chlorophyll metabolism (ko00860) and carotenoid biosynthesis (ko00906).

DEGs Involved in photosynthesis and carbon metabolism

Transcriptome sequencing technology provides a large amount of information regarding the DEGs that are involved in specific biological responses. The results showed that carbon metabolism, porphyrin and chlorophyll metabolism, photosynthesis were lower in PEG-treated tobacco than in well-watered tobacco (Fig.7a-e), which was consistent with the low Pn in PEG-treated tobacco seedlings. The expression levels of genes involved in carbon metabolism, such as FBP (Nitab4.5_0002152g0090), photosynthesis, such as PSBO (Nitab4.5_0000108g0110), PSBR (Nitab4.5_0002475g0030) and PSAO (Nitab4.5_0003068g0060), photosynthesis-antenna proteins, such as lhca-P4 (Nitab4.5_0001128g0010), LHC4.2 (Nitab4.5_0000476g0110), and CAB50 (Nitab4.5_0000592g0320) were consistently suppressed at drought stress in flue-cured tobacco while up-regulated after SA spraying. To explore the reason why SA can alleviate the damage of drought stress in flue-cured tobacco, we also analyzed the differences in gene expression levels of porphyrin and chlorophyll metabolism. The results showed that genes of POR1 (Nitab4.5_0002526g0100), hemC (Nitab4.5_0001791g0020), HEMA1 (Nitab4.5_0000377g0190) and CHLM (Nitab4.5_0000976g0010) were consistently and significantly down-regulated under drought stress while up-regulated in SA-treated tobacco (Fig.7d) , which might also be the cause for higher photosynthesis in SA-treated tobacco.

Discussion

Recent years, drought stress has become one of the most serious environmental factors for the productivity and quality formation of tobacco. Our investigation study revealed that the application of SA was able to protect the tobacco seedlings from damage of drought stress and the corresponding mechanism, which would have significant practical application. Under drought stress condition, a large amount of ROS would accumulate inside the plant, resulting in lipid peroxidation, MDA production, cell membrane damage, cell aging and death^[22-23]. Therefore, MDA levels was used as an indicator to report the damage of cellular components. Plants have evolved multifaceted adaptation mechanisms to rapidly recognize and adapt to drought stress conditions such as the increase of antioxidant enzymes activity and osmotic regulating substance^[24]. In wheat, exogenous SA had been reported to play an important role in improving the antioxidant enzymes activity and decreasing MDA content^[25-26]. As the important osmotic regulators in plants, proline and soluble protein have the ability to improve the cell osmotic function and alleviate the damage of drought stress^[27]. Positive effects of SA in triggering the synthesis of soluble protein and proline to improve the drought resistance of plants have been reported by Lu et al^[28]. In our study, an increase in the MDA, soluble protein, proline contents and antioxidant enzyme activities were obtained in PEG drought stress and natural drought-stressed tobacco treatments. However, less MDA content and higher soluble protein and proline contents were observed in SA-treated plants compared to no SA-treated drought stressed seedlings (Fig.3a-f). The decrease in MDA content in SA-treated plants may be associated with the induction of antioxidant responses which would protect the plant from damage. Zhang et al. have suggested a similar mechanism to be responsible for SA induced drought stress tolerance in eggplant plant^[29]. In ROS-scavenging systems, antioxidant enzymes have key roles to play, with SOD catalyzing the dismutation of O₂ to oxygen and H₂O₂, which is then scavenged by CAT and POD^[30]. The results proved that SA was able to enhance antioxidant defense systems and increase osmotic regulating substance level to eliminate ROS, which is in accordance with the fact that the ROS and MDA levels were markedly reduced in plants.

Leaf chlorophyll content is used as an indicator of photosynthetic capacity and plant vitality ^[31]. Photosynthesis plays an important role in crop growth, and is the ultimate source of yield formation. Besides, net photosynthesis rate (Pn) is regarded as an effective indicator to assess plants drought resistance ^[32]. Previous studies reported that the decrease in photosynthesis was usually caused by stomatal limitation when both Gs and Ci decline while non-stomatal limitation was the main reason for photosynthesis when Ci increased and Gs decreased ^[33-34]. In the present study, the data clearly showed a reduction in Pn, Tr, Gs accompanied with an increase in Ci under PEG-drought stress (Fig.2a-d). The results indicated that non-stomatal limitation primarily led to the drop of Pn in PEG-drought stress. Under PEG-drought stress, SA application significantly reversed the trend compared with the water stressed control. The results of this study were in the harmony with previous studies, demonstrating that drought stress inhibits carbon assimilation, chlorophyll biosynthesis and damages photosynthetic apparatus^[35-] ^{[a36a[i]a37a]}.

The decrease in photosynthesis could make plants absorb more light energy than the level necessary for photosynthetic carbon fixation. This excess energy has the potential to trigger an increase in reactive oxygen species (ROS) production, which has been proven to be capable of damaging the photosystem, decreasing the activity of photosynthetic electron transport chain and hindering pigment synthesis^[38]. For a better understanding of the mechanism of SA induced increase of photosynthesis rate, technologies of molecular biology were used in this study. Under drought stress, the down-regulated DEGs were most correlated with chlorophyll biosynthetic process, photosynthesis, light harvesting, chloroplast and photosystem II (Fig.5a). KEGG pathway analysis also showed that the down-regulated DEGs were mainly enriched in starch and sucrose metabolism, photosynthesis-antenna proteins and porphyrin and chlorophyll metabolism (Fig.5c). However, spraying SA has increased expression of genes involved in photosystem II, photosystem I, chloroplast thylakoid, photosynthetic electron transport chain, chloroplast thylakoid membrane, carbon fixation and synthesis of carotenoids (Fig.5b, d), indicating that spraying SA was effective in protecting the cell membrane and photosystem from damage, which then enhanced photosynthesis rate and pigment synthesis. Further investigation of down-regulated DEGs between watered-plants and drought treated plants and up-regulated DEGs between drought treated plants and SA-sprayed plants suggested SA promoted pigment biosynthesis, photosynthesis, carbon fixation and protect the photosystem from damage (Fig.6a-c). Besides, some key genes related to photosynthesis, photosynthesis-antenna proteins, porphyrin and chlorophyll metabolism and carbon metabolism were up-regulated after spraying SA (Fig.7a-e), including the RCA1 genes, which plays an important role in activation of the rubisco enzyme^[39], lhca-P4, which encode key subunit of light-harvesting complex and could convert light energy into chemical energy^[40], AGPS1 and SS1, which catalyzes the limiting steps in starch synthesis^[41], photosystem II genes (PSBO, PSBR, PSBS), photosystem I genes (PSAF, PSAG, PSAK, PSAO), POR1 and CHLP, which play crucial roles in the chlorophyll biosynthesis^[42]. Rubisco, a key enzyme in the calvin cycle, assimilates atmospheric CO₂ into the biosphere^[43]. Previous study has showed that overexpression of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase gene (RCA) was able to maintain photosynthesis under drought stress, which contributed to the higher drought tolerance in transgenic plants^[44]. Together, these gene products might contribute to the adaptation or resistance of tobacco to drought stress.

In conclusion, spraying SA decreased MDA accumulation while promoted photosynthesis, antioxidant enzyme activity and osmotic regulators in flue-cured tobacco seedlings under PEG drought stress. Drought stress inhibited the expression of genes involved in photosynthesis, carbon fixation and pigment biosynthesis. Spraying SA up-regulated the expression of genes involved in photosynthesis, photosynthesis-antenna proteins, photosystem II, photosystem I, carbon metabolism, porphyrin and chlorophyll metabolism. Spraying SA under drought stress was an effective method to alleviate the damage caused by drought stress.

[[i]] Gollack, D., Lüking, I., & Yang, O. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep.*, 30(8), 1383-1391 (2011).

Declarations

Acknowledgments

The study was financially supported by Shanghai Tobacco (Group) Corporation Research Program (grant number TP2019-C4).

Author Contributions

H.S. performed the conception and design of the research. Y.F. and Y.Z. conducted the research and investigation process. J.Z. and Y.L. analyzed the data and prepared the figures1-6. J.Z. and Y.F. checked the data. Y.F. drafted the manuscript. H.S. completed the revision of manuscript for important intellectual content. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Shinozaki, K., & Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.*, 58(2), 221-227 (2007).
2. Qi, J. et al. Reactive oxygen species signaling and stomatal movement in plant responses to drought stress and pathogen attack. *J Integr Plant Biol*, 60(9), 805-826 (2018).
3. Gao, D., Gao, Q., Xu, H. Y., Ma, F., Zhao, C. M., & Liu, J. Q. Physiological responses to gradual drought stress in the diploid hybrid *Pinus densata* and its two parental species. *Trees*, 23(4), 717-728 (2009).
4. Yuan, Y., Li, J., Ding F., & Su, X. Effects of Drought Stress on Activity of Cell Defense Enzymes in Flue-cured Tobacco Leaves [J]. *Chin. Tob. Sci.*, 5 (2009).
5. Chen, Y., Yuan, L., & Xi, X. Y. Evolving of Research on Effects of Different Drought Stress on Tobacco [J]. *Journal of Gansu Lianhe University (Natural Science Edition)*, 2 (2008).
6. Correia, M. J. et al. Effects of water deficit on the activity of nitrate reductase and content of sugars, nitrate and free amino acids in the leaves and roots of sunflower and white lupin plants growing under two nutrient supply regimes. *Physiol Plant*, 124(1), 61-70 (2005).
7. Hashempour, A., Ghasemnezhad, M., Ghazvini, R. F., & Sohani, M. M. The physiological and biochemical responses to freezing stress of olive plants treated with salicylic acid. *Russ J Plant Physiol+*, 61(4), 443-450 (2014).

8. Syeed, S., Anjum, N. A., Nazar, R., Iqbal, N., Masood, A., & Khan, N. A. Salicylic acid-mediated changes in photosynthesis, nutrients content and antioxidant metabolism in two mustard (*Brassica juncea* L.) cultivars differing in salt tolerance. *Acta physiolo plant*, 33(3), 877-886 (2011).
9. Nazar, R., Iqbal, N., Syeed, S., & Khan, N. A. Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in two mungbean cultivars. *J. Plant Physiol.*, 168(8), 807-815 (2011).
10. Khan, M. I. R., Syeed, S., Nazar, R., & Anjum, N. A. An insight into the role of salicylic acid and jasmonic acid in salt stress tolerance. *Phytohormones and abiotic stress tolerance in plants*, 277-300 (2012).
11. Al-Hakimi, A. M. A., & Hamada, A. M. Counteraction of salinity stress on wheat plants by grain soaking in ascorbic acid, thiamin or sodium salicylate. *Biol. Plant.*, 44(2), 253-261 (2001).
12. Hayat, Q., Hayat, S., Irfan, M., & Ahmad, A. Effect of exogenous salicylic acid under changing environment: a review. *Environ. Exp. Bot*, 68(1), 14-25 (2010).
13. Kadioglu, A., Saruhan, N., Sağlam, A., Terzi, R., & Acet, T. Exogenous salicylic acid alleviates effects of long term drought stress and delays leaf rolling by inducing antioxidant system. *Plant Growth Regul.*, 64(1), 27-37 (2011).
14. Yao, X., Ma, J., Ji, J., Ou, C., & Gao, W. Effect of exogenous application of salicylic acid on the drought stress responses of *Gardenia jasminoides*. *Sciences in Cold and Arid Regions*, 8(1), 54-64 (2016).
15. Demiralay, M., SAĞLAM, A., & KADIOĞLU, A. Salicylic acid delays leaf rolling by inducing antioxidant enzymes and modulating osmoprotectant content in *Ctenanthe setosa* under osmotic stress. *Turk. J. Biol.*, 37(1), 49-59 (2013).
16. Wang, F., Sang X. H., Gu, W., Chao, J. G., Zhou, C., Tian, R. Effects of Salicylic Acid on Photosynthetic Characteristics and Physiological Indexes of *Atractylodes lancea* (Thumb.)DC. Under Drought Stress. *Molecular Plant Breeding*. 18(09):3060-3067 (2020).
17. Yoo, Y. H. et al. OsPhyB-mediating novel regulatory pathway for drought tolerance in rice root identified by a global RNA-Seq transcriptome analysis of rice genes in response to water deficiencies. *Front. Plant Sci.*, 8, 580 (2017).
18. Lu, X. et al. RNA-seq Analysis of Cold and Drought Responsive Transcriptomes of *Zea mays* ssp. *mexicana* L. *Front. Plant Sci.*, 8, 136 (2017).
19. Li, H. J., Yang, Y., Chang, P., Zheng, J. C., Chen, L., Hu, Y. G. Transcriptome and Photosynthetic Trait Analysis of TaER Over-expression Wheat under Drought Stress[J]. *Journal of Triticeae Crops*, 39(08):941-949 (2019).
20. Zhang, C. L., Cao, S., Man, L. L., Xiang, D. J., Liu, P. Analysis of PEG Stress on Drought Tolerance and Related Response Genes Expression in Soybean Seedlings of two varieties[J]. *Molecular Plant Breeding*, 17(18):5891-5898 (2019).
21. Takahashi, S., & Murata, N. How do environmental stresses accelerate photoinhibition?. *Trends Plant Sci.*, 13(4), 178-182 (2008).

22. Asada, K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol.*, 141(2), 391-396 (2006).
23. Hua, X. Changes of protective enzyme activity and MDA content in leaves of *Agropyron cristatum* under grazing stress. *Agricultural Basic Science and Technology*, 16(1) 22-24 (2015).
24. Apel, K., Hirt, H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction[J]. *Annu. Rev. Plant Biol.* 55(x): 373-399 (2004).
25. Chen, J. et al. Effects of Wheat Seed Soaking with SA on Protective Enzymetic Activities in Germination Stage under Drought Stress. *Molecular Plant Breeding*, 13(12) 2854-2857 (2015).
26. Ma, C., Wang, Z. Q., Zhang, L. T., Sun, M. M., & Lin, T. B. Photosynthetic responses of wheat (*Triticum aestivum* L.) to combined effects of drought and exogenous methyl jasmonate. *Photosynthetica*, 52(3), 377-385 (2014).
27. Liu, Q. et al. Effects of Exogenous Salicylic Acid on the Physiological and Biochemical Characteristics of Cotton Seedlings under Salt Stress[J]. *Journal of Soil and Water Conservation*, 28(2):16-174 (2014).
28. Lu, Y. Z., Li, N., Zhang, E. H., Yin, X., Zhao, K. T., Lan, X. Z. Effects of Soaking Seeds with Salicylic Acid on Seed Germination and Drought Resistance of *Dracocephalum tanguticum* [J/OL]. *Journal of Henan Agricultural Sciences*, 49(03):63-69 (2020).
29. Zhang, X. W., Wang, Y. P. Effects of salicylic acid on seed vigor and seedling drought resistance of eggplant under PEG-6000 stress[J]. *Journal of Gansu Agricultural University*, 54(01):103-109, (2019).
30. Miller, G. A. D., Suzuki, N., Ciftci-Yilmaz, S. U. L. T. A. N., & Mittler, R. O. N. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.*, 33(4), 453-467 (2010).
31. Carter, G. A., & Spiering, B. A. Optical properties of intact leaves for estimating chlorophyll concentration. *J. Environ. Qual.*, 31(5), 1424-1432 (2002).
32. Zhao, H. M. et al. Studies on evaluation indices for drought resistance capacity in wheat varieties. *J Plant Genet Resour*, 1, 76-81 (2007)..
33. Pérez-López, U., Robredo, A., Lacuesta, M., Mena-Petite, A., & Muñoz-Rueda, A. Elevated CO₂ reduces stomatal and metabolic limitations on photosynthesis caused by salinity in *Hordeum vulgare*. *Photosynth. Res.*, 111(3), 269-283 (2012).
34. Zhou, S., Duursma, R. A., Medlyn, B. E., Kelly, J. W., & Prentice, I. C. How should we model plant responses to drought? An analysis of stomatal and non-stomatal responses to water stress. *Agric. For. Meteorol.*, 182, 204-214 (2013).
35. Ali, Q., & Ashraf, M. Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defence mechanism. *J. Agron. Crop. Sci.*, 197(4), 258-271 (2011).
36. Golldack, D., Lüking, I., & Yang, O. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep.*, 30(8), 1383-1391 (2011).

37. Amini, H., Arzani, A., & Bahrami, F. Seed yield and some physiological traits of safflower as affected by water deficit stress. *Int. J. Plant Prod.*, 7(3), 597-614 (2013).
38. Wang, Z. et al. Effects of drought stress on photosynthesis and photosynthetic electron transport chain in young apple tree leaves. *Biol. open*, 7(11) (2018).
39. Li, H. X., Wang, Z. M., Zeng, H. L. The Research Progresses in Rubisco Activase in Plant[J]. *Plant Physiology communications*, 46(11):1092-1100 (2010).
40. Jansson, S. A guide to the Lhc genes and their relatives in Arabidopsis. *Trends Plant Sci.*, 4, 236–240 (1999).
41. Yuan, L., Pan, G. T., Zhang, Z. M., Tan, D. F. Research Progress of Starch Metabolism and its Regulation in Plants[J]. *Molecular Plant Breeding*, (S2):65-72 (2006).
42. Li, R. X. Screening, cloning and functional identification of POR gene in the Albino of Ananas comosus var.bracteatus leaves using VIGS technique [D]. Sichuan Agricultural University, 2018.
43. Mi, C. et al. Cloning of Rubisco sub-units genes rbcL and rbcS from winter rapeseed (Brassica ra-pa) and their expression under drought stress [J]. *Acta Ag-ronomica Sinica*, 44 (12): 1882-1890 (2018).
44. Wijewardene, I. et al. Improving drought-, salinity-, and heat-tolerance in transgenic plants by co-overexpressing Arabidopsis vacuolar pyrophosphatase gene AVP1 and Larrea Rubisco activase gene RCA. *Plant Sci.*, 296, 110499 (2020).
45. Michel, B. E., & Kaufmann, M. R. The osmotic potential of polyethylene glycol 6000. *Plant Physiol.*, 51(5), 914-916 (1973).
46. Liu, H. et al. Effect of Low Temperature Control in Greenhouse on Cold Tolerance of High Quality Rice at Seedling Stage[J]. *North Rice*, 50(06): 27-29 (2020).
47. FAN Shuqin, LIANG Shuwen. Guidance of modern plant physiology[M]. Shanghai: Science Press, 303-306 (1999).
48. Li, H. Principle and technology of plant physiological and biochemical experiments. (ed. Li, H) 123, 125, 130, 184 (2000).
49. Patel, R. K., & Jain, M. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. *PloS one*, 7(2), e30619 (2012).
50. Bolger, A. M., Lohse, M., & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120 (2014).
51. Kim, D., Langmead, B., & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. *Nat. methods*, 12(4), 357-360 (2015).
52. Langmead, B., & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. methods*, 9(4), 357 (2012).
53. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzberg, S. L. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.*, 14(4), 1-13 (2013).

54. Trapnell, C. et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.*, 7, 562–578 (2012).

55. Anders, S., Pyl, P. T., & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, 31(2), 166-169 (2015).

56. Anders, S., & Huber, W. Differential expression of RNA-Seq data at the gene level—the DESeq package. Heidelberg, Germany: European Molecular Biology Laboratory (EMBL), 10, f1000research (2012).

57. Kanehisa, M. et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36(suppl_1), D480-D484 (2007).

Tables

Table 1. Statistics of transcriptome sequencing data

Sample	Raw reads	Clean reads	Total mapped	Uniquely mapped	Q30(%)	GC content(%)
CK_1	82.58M	80.33M	77436684(96.40%)	69524574(86.55%)	95.82%	44.13%
CK_2	85.00M	82.70M	81159252(98.14%)	72641490(87.84%)	95.83%	44.01%
CK_3	90.07M	87.69M	86015985(98.09%)	77229420(88.07%)	95.90%	44.06%
D_1	90.62M	88.00M	86474855(98.27%)	78005814(88.64%)	95.78%	43.35%
D_2	90.81M	88.38M	86833722(98.25%)	78540519(88.87%)	95.87%	43.46%
D_3	85.46M	82.92M	81407742(98.18%)	73611775(88.78%)	95.70%	43.28%
D+S_1	88.61M	86.17M	84647498(98.24%)	76454307(88.73%)	95.86%	43.59%
D+S_2	92.20M	89.85M	88071017(98.01%)	79263049(88.21%)	95.98%	43.67%
D+S_3	86.96M	84.70M	83238969(98.28%)	74704930(88.20%)	95.85%	43.92%

Samples for mRNA-Seq derived from the different treatments. Q30: DNA sequencing quality ≥ 30 . 1-3 represents three biological replications.

Figures

PEG Drought

Figure 1

The phenotype of flue-cured tobacco seedlings under PEG drought stress

Figure 2

(a) Pn. (b) Tr. (c) Gs. (d) Ci. Pn: net photosynthesis rate; Gs: stomatal conductance; Tr: transpiration rate; Ci: intercellular CO₂ concentration. CK: water +Hoagland nutrient solution; D: water+15% PEG6000-treated nutrient solutions; D+SA: 0.3 mmol/L SA + 15% PEG6000. Symbols little letters indicate that the signifcant differences between treatments are at 0.05. The same symbols as below.

Figure 3

(a) SOD. (b) POD. (c) CAT. (d) MDA. (e) soluble protein. (f) proline. SOD: superoxide dismutase; POD: peroxidase; CAT: catalase. MDA: malondialdehyde. Symbols small letters indicate that the significant differences between treatments at 0.05. The same symbol is used for other figures.

Figure 4

(a) Gene expression levels in samples. (b) Results of principal component analysis. (c) Correlation heatmap between samples based on gene expression abundance. The horizontal and vertical axes represent samples, and the numbers in each grid represents Pearson correlation coefficients.

Figure 5

(a, b) The top 10 GO terms in biological process (BP), cellular component (CC), and molecular function (MF) enriched by differentially expressed genes between different treatments. (c, d) Enrich analysis of DEGs. (e) Number of differentially expressed genes in each comparison group. Blue and red represent low expression and high expression.

Figure 6

(a). Venn diagram of DEGs between D-vs-CK down and D+S-vs-D up. (b). GO enrichment in DEGs response to SA. (c). KEGG pathway in DEGs response to SA.