De novo assembly of Amorpha fruticosa (Amorpha fruticosa L.) transcriptome in response to drought stress provides insight into the tolerance mechanisms

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Abstract

Background
Amorpha fruticosa (Amorpha fruticosa L.) is a deciduous shrub that is native to North America and has been introduced to China as an ornamental plant. In order to cultivate drought-tolerant Amorpha fruticosa varieties, it is important to understand the drought-tolerant mechanism of Amorpha fruticosa. Through the changes of the transcriptome of Amorpha fruticosa under drought stress, the mechanism of anti-stress of Amorpha fruticosa could be revealed. Different concentrations of polyethylene glycol-6000 (PEG-6000) was used to simulate drought stress, and transcriptomic analysis was used to reveal the changes of gene expression patterns in Amorpha fruticosa seedlings.

Results
Results showed that Amorpha fruticosa seedlings were seriously affected by PEG-6000. As for the differently expressed genes (DEGs), most of them were up-regulated. The additional Go and KEGG analysis results showed that DEGs were functionally enriched in cell wall, signal transduction and hormonal regulation related pathways. DEGs like AfSOD, AfHSP, AfTGA, AfbZIP and AfGRX play roles in response to drought stress.

Conclusion
In conclusion, Amorpha fruticosa seedlings were sensitive to drought, which was different from Amorpha fruticosa tree, and the genes functions in drought stress responses via ABA-independent pathways. The up-regulation of Salicylic acid signal related DEGs (AfTGA and AfPR-1) indicated that Amorpha fruticosa can resist drought stress through Salicylic acid.
1. Background

Amorpha fruticosa (Amorpha fruticosa L.) is a deciduous shrub that is native to North America and has been introduced to China as an ornamental plant [1, 2]. As a kind of urban greening and slope protection plant, Amorpha fruticosa has high ornamental value and is widely used in the construction of urban landscape and road slope protection. In addition, Amorpha fruticosa also has medicinal value, such as cytotoxic rotenoid glycosides, antibacterial and cytotoxic phenolic metabolite in seeds [3, 4]. What more, Amorpha fruticosa leaf was a kind of traditional Chinese medicine used for the treatment of fever, burns, pyogenic carbuncle and eczema [3, 5]. Amorpha fruticose can tolerate dry soils, but it is most abundant along river banks and roads and the edges of flooded forests, even is tolerant of occasional flooding [6]. The high tolerance of various habitat conditions and potent propagation ability promotes the aggressive invasive behavior of Amorpha fruticosa outside of its native range [6]. In order to breed more drought tolerant Amorpha fruticosa tree varieties, it is important to understand the drought tolerance mechanism in Amorpha fruticosa. Drought stress is one of the most prevalent environmental factors limiting plant growth [7]. Different plants adapt to drought stress in the environment through different mechanisms, but most plants could response to drought stress via hormonal regulation, such as abscisic acid (ABA), cytokinin (CK), gibberellic acid (GA), auxin, and ethylene, etc, which regulate diverse processes and enable plant adaptation to drought stress [8,9]. Many hormonal regulation related genes had been proved can improve plant resistance, including GH3, NAP, ABIs, AP37, PP2C, PP2C06, PYR/PYL, SIDP366, MYBs, RK1, hox22, SNAC2, OAT, bZIPs, SNAC1, EREBP1, DSM2, AREB2, SRO1c and ABA8OX3 [9]. When plants suffered from stress, a series of biological processes will be induced to
respond to stress signals, which will lead to the increase of reactive oxygen species (ROS) content in plant cells [10]. In the long evolutionary process, plants have evolved a series of anti-oxidative system to respond to drought, such as glutathione metabolism pathway, catalase system, peroxidase system, superoxide dismutase system, etc. As our knowledge, there were several researches found on drought resistance in Amorpha fruticosa. But few articles were focus on the expression pattern and molecular mechanisms of gene action response to drought. In this study, PEG-6000 was used to simulate drought stress, and transcriptomic analysis was used to reveal the changes of gene expression patterns in Amorpha fruticosa seedlings. The present study will provide theoretical basis and data support for Amorpha fruticosa drought resistant breeding.

2. Results

2.1 Effects of different concentrations of PEG-6000 on plant growth

After PEG-6000 treatment, the Amorpha fruticosa plants were under drought stress. The leaves of the drought stressed plants showed severe wilting and curling, which were aggravate with the increase of PEG-6000 concentration (Fig. 1). This indicated that Amorpha fruticosa seedlings were sensitive to drought stress, and 30% concentration PEG-6000 treatment was fatal for plant seedlings. The SOD, MDA, Pro and REC results showed that plants suffered from drought stress had significant higher values than that of CK (Fig. 2).

2.2 Sequence data summary and de novo assembly

Approximately 42.6 G raw data including 142 million reads from the six libraries were generated. QC results revealed the good quality of the sequence data with average Q20 of 96.71%, average Q30 of 92.46%, average sequencing error rate of
0.01% and average GC content of 57.08% (Table S1). PCA and sample to sample correlation analysis results showed that the biological replicates in each group are clustered together (Fig. 3a and b), which indicated that the variability between samples in a same group were small. After de novo assembly of clean reads with Trinity, the transcripts were clustering and de-redundant, and 96,594 Unigenes were obtained with average length of 864 bp and N50 of 1,430 bp (Table S2). The annotation results showed that 52,010 unigenes were successfully matched with at least one database, including 15,883 (30.54%), 28,297 (54.41%), 18,348 (35.28%), 29,472 (56.67%), 33,287 (64.00%), 28,188 (54.2%), 46,878 (90.13) and 48,186 (92.65%) that significantly matched with the COG, GO, KEGG, KOG, Pfam, Swiss-Prot, EggNOG and nr databases, respectively.

2.3 DEGs between CK and 20% PEG-6000 treatment group

There were 1084 and 603 up- and down-regulated DEGs found between CK and 20% PEG-6000 group. In order to better understand the function of DEGs, we performed GO and KEGG analysis. The GO results showed that regulation of catalytic activity, regulation of peptidase activity, oxidation-reduction process, plant-type cell wall loosening and cell redox homeostasis were the main BP terms respond to drought stress. In which, AfSOD, AfHSP70, AfCYPs, AfCSY4 and 60S ribosomal proteins had high frequency. Most of these DEGs were up-regulated (Fig. 4a). As for KEGG analysis, DEGs were significantly enriched in Starch and sucrose metabolism, Carbon fixation in photosynthetic organisms and 2-Oxocarboxylic acid metabolism pathway. In addition, 34 DEGs, including c144508.graph_c0, c164358.graph_c0, c167827.graph_c0, c171717.graph_c1 and c187107.graph_c0, were enriched in plant hormone signal transduction pathway, although the p value > 0.05. DEGs in plant hormone signal transduction showed that DEGs of salicylic acid downstream
were active and transcripts of PP2C were upregulated. Expression of DEGs in ABA and salicylic acid pathway were shown in Fig. 4b and c. What more, DEGs in Starch and sucrose metabolism including AfTPS, AfTREH, AfAMY, etc. Most DEGs in Carbon fixation in photosynthetic organisms pathway were up regulated.

2.4 Interaction network of DEGs and proteins

Interaction network was performed according to STRING database. There were 262 up-regulated DEGs with Confidence Score > 0.9 and the interaction network showed that c160801.graph_c0, c192497.graph_c0, c190485.graph_c1, c175551.graph_c1 were the core regulators in these DEGs (Fig. 5a). The expression heatmap showed the distinct gene expression pattern between CK and PEG-6000 treatment group (Fig. 5b). The down-regulated DEGs formed an interactive network of six nodes. In addition, we classified the genes according to their functions, and then construct the interaction network. Genes related to ROS showed a six nodes network (Fig. 5c), of which three genes were differently expressed (Fig. 5d). DEGs related to translation showed that 17 genes had interaction relationship (Fig. 5e) and the expression of which were shown in Fig. 5 f.

2.5 qRT-PCR verification

A total of 20 genes were verified by qRT-PCR, including AfADH_Zinc_N, AfNAC1, AfZAT10, AfWRKY25, AfPhospholipase A1, AfDREB4, AfRAP2-1, AfABRE3, AfbHLH-MYC, AfABP19a, AfPAP17-X2, AfTrx_2, AfWRKY20, AfMYB1, AfPAL 1, AfMYB2, AfAPX2, AfCAT, AfFe/Mn-SOD and AfGST. The primers used were listed in Table S3. The qRT-PCR results showed that most of the stress respond genes were up-regulated, especially in 20% and 30% PEG treatment. Also, some transcription factors were down regulated with the aggravation of stress. All the expression of all these genes
was shown in Fig. 6.

3. Methods

3.1 Plant material and PEG treatment

Amorpha fruticosa seeds were collected from our research test plot in November 2018 and identified by Seed Key Laboratory of Saline-alkali Vegetation Ecology Restoration, Ministry of Education (Northeast Forestry University). Seeds need to be disinfected before germination test. Amorpha fruticosa seeds were surface-sterilized with 70% alcohol and 5% sodium hypochlorite for 5 min followed by rinsing three times with distilled water. Seeds were then seeded into culture bowls full of fine sand (sterilized by high temperature) and cultured in a plant growth chamber (temperature 25 °C ± 2; relative humidity 60% ±5; light intensity 150µmol·m$^{-2}$·s$^{-1}$; light and darkness cycle: 16:8) with sufficient water supply for four weeks. Subsequently, the seedlings were randomly divided into four groups with three repetitions in each group. Osmotic stress was gradually applied with varying concentrations of polyethylene glycol-6000 (PEG-6000; w/v- 0%, group CK; 10%, group 10%; 20%, group 20%; 30%, group 30%) for 72 h. Whole seedlings of all groups were sampled, snap frozen in liquid nitrogen and then stored at -80 °C until testing. Superoxide dismutase (SOD), malondialdehyde (MDA), proline (Pro) and relative electrical conductivity (REC) were measured using samples in CK and 20% group according to previous reports (Guo Q et al.2016; Leonid V et al.2015). Transcriptome sequencing was performed to reveal the gene expression pattern using the six samples in CK and 20% group. Samples in CK, 10%, 20% and 30% were used for quantitative real-time PCR (qRT-PCR) detection.
3.2 RNA extraction, library preparation, and transcriptome sequencing

Total RNA was isolated using a RNAprep pure Plant Kit (Tiangen, China) according to the manufacturer’s instruction. RNA quality was tested using gel electrophoresis, Agilent 2100 (Agilent Technologies Inc., USA) and Nano Drop 2000 (Thermo Fisher Scientific Inc., USA). Then, total RNA was reverse transcribed to cDNA by a QuantScript RT Kit (Tiangen, China). After that, we started constructing sequencing libraries. An efficient mRNA-seq Library Prep Kit for Illumina (Vazyme, China) was used for the sequence libraries construction. Subsequently, the quality control (QC) was performed by an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR System to quantify the sample libraries. Finally, all the six mRNA-seq libraries were sequenced on an Illumina HiSeq 4000 sequencing platform with pair-end 2 × 150 bp mode to obtain sequencing data. The sequencing data are available at Bigsub database (https://bigd.big.ac.cn/gsub/) with accession number CRA002113.

3.3 De novo assembly, sequence annotation and differentially expressed genes (DEGs) screening

Raw reads were filtered to remove adapter and low-quality reads using FasqQC (version 0.11.5) with default parameter settings. De novo transcriptome assembly were performed by Trinity (version 2.2018) using the filtered clean data of the six libraries [13]. The assembled transcripts were hierarchically clustered using Corset (version 1.0.5) [14]. After hierarchical clustering, the longest sequence (unigene) of each cluster were used for further analyses, including length distribution statistics, gene annotation (Nr, Nt, Pfam, KOG/COG, Swiss-prot, KEGG, GO) and identification of DEGs. In addition, ESTScan (version 3.0.2) [15]was used for ORF predication of gene sequences that could not be aligned to any of the abovementioned databases.
To evaluate the correlation of biological repetition, principal component analysis (PCA) and Pearson’s correlation analysis were performed based on the FPKM of reads. Following this, read counts were normalized and DEGs in different comparisons were screened using DEseq2 (R package) methods [16] with the criteria of padj value < 0.05 by Negative binomial distribution test and |log₂(Fold Change, FC)| ≥ 1.5. Genes with identified as log₂FC > 1 and log₂FC < -1 were identified as up- and down-regulated DEGs, respectively. Hierarchical clustering based on the expression profiles of DEGs was presented by pheatmap (version 1.0.10).

3.4 DEGs functional analysis

The DEGs enriched into modules correlated with the phenotypes were separately subjected to the enrichment analysis for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [17]. Significant GO biological processes (BP) and KEGG pathways were identified with the criterion of p < 0.05. The candidate gene interaction analysis was performed using Cytoscape (version 3.7.2).

3.5 qRT-PCR verification of RNA-seq data

qRT-PCR analysis was performed on an Agilent Mx3000P QPCR system (Agilent, USA) using 2 × Brilliant III SYBR Green qPCR Master Mix (Agilent, USA). PCR amplification was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. Quantification of gene expression was performed by the comparative 2-ΔΔCT method. The validation analysis was performed with three independent biological replicates. The gene-specific primers for qRT-PCR were designed using Primer Premier 5.0 (http://www.PremierBiosoft.com) and were synthesized by Invitrogen.
3.6 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8. All experimental data were expressed as mean ± standard deviation (SD), and differences between groups or treatments were analyzed using the unpaired t-test. P < 0.05 was set as significant threshold for statistical differences.

4. Discussion

As an important tree species for highway and slope protection, Amorpha fruticosa is widely used in Northeast China. Amorpha fruticosa is vulnerable to drought stress, so revealing the biological process response to stress is important to Amorpha fruticosa breeding. When environmental conditions become adverse, plants can successfully deploy complex physiological and molecular strategies to cope with abiotic stress [11]. In this study, the curling and wilting of plant leaves prove that plants are under drought stress. The physiological indexes of Amorpha fruticosa against drought stress were consistent with that in citrus [18], Tibet Plateau [11] and some shrubs [19]. The tolerance of plants to drought resulted in increasing antioxidants activities which face the increased levels of free radicals [20,21]. The increased contents of SOD, MDA and Pro indicated the cellular defenses against ROS.

The statistical results of DEGs show that most of the DEGs were up-regulated, which demonstrated the response of gene expression pattern to drought stress. The DEGs functional analysis results showed that “regulation of catalytic activity”, “regulation of peptidase activity” and “oxidation-reduction process” were the main enriched GO terms and AfSOD, AfHSP70, AfCYPs and 60S ribosomal proteins were the key
regulators involved in these terms. SODs are key enzymes in many oxidation processes, and provide basic protection against ROS in plants [22]. The significantly increasing of AfSOD, AfHSP and AfCYPs after PEG-6000 treatment played an important role response to drought stress. Especially, various reports to date reveal that SODs overexpressing plants protect them from oxidative damage [23–26]. Similarly, HSP overexpressing plants also showed excellent stress tolerance [27–29], which act as core genes in our interaction network analysis results. In addition, CYP family members also act as key response genes to drought stress [30,31]. The mechanism of improving plant resistance to abiotic stress by SOD, HSP and CYP had been reviewed [32–35], and we will not discuss it in detail. As for KEGG enrichment analysis, we found 34 DEGs enriched in plant hormone signal transduction pathway, including ABA signal related DEGs (AfPP2C, AfPYR/PYL and AfSnRK2) and Salicylic acid signal related DEGs (AfTGA and AfPR-1). ABA is an important signal molecule, which act as a comprehensive factor in response to environmental changes. It play roles the regulation of seed germination and root growth, as well as the adaptive response to various abiotic stresses [36]. In our results, AfPP2C were up-regulated by PEG-6000 treatment. A significant amount of research on PP2C action is related to ABA signaling. The ABA INSENSITIVE1 (ABI1) and ABI2 genes encode homologous type-2C protein phosphatases with redundant yet distinct functions in ABA responses [37]. Genetic analysis of abi1 and abi2 mutants, their revertants, transient expression studies, and analysis of transgenic antisense plants showed that PP2Cs act as negative regulators of ABA signaling [38–41]. The increased expression level of AfPP2C revealed that Amorpha fruticosa seedlings were sensitive to drought and the genes functions in drought stress responses via ABA-independent pathways [42]. In addition, AfTGA and AfPR-1 were increased by high
concentration of PEG-6000. TGA factors constitute a conserved plant subfamily of basic domain/Leu zipper (bZIP) transcriptional regulators whose genomic targets are thought to include glutathione S-transferase and pathogenesis-related (PR) genes that are associated with detoxification and defense [43–44]. TGA factors contribute to protective gene responses that are mobilized by plants against stress. As for PR-1, of which the expression was promoted by TGA factors [45]. Johnson reported that the in vivo recruitment of TGAs to the PR-1 promoter precedes the SA (Salicylic Acid)-induced expression of a transcriptionally divergent XET gene, which encodes a putative xyloglucan endotransglycosylase [44]. These enzymes remodel the cell wall during development and in response to environmental cues, which is consistent with the enriched cell wall related GO terms in our results. Both AfTGA and AfPR-1 were up-regulated by drought stress, which indicated that Amorpha fruticosa can resist drought stress through SA. The possible mechanism is that when plant suffered from drought stress, NPR1 can transfer the SA-induced activation of PR-1 to the nucleus, where it interacts with the C-terminal domain of TGAs [46]. The interaction network analysis results showed that AfHSPs, AfCXXS1, AfGRXS11, AfGRXC6 and AfFAD play roles in gene interaction. Of which, GRX is a kind of small molecule redox protein, which plays an important role in antioxidation in the form of thiol reductase [47]. Previous study reported that environmental stresses such as cold, heat, drought and salt induce changes in fatty acid composition, mainly in the content of linolenic acid [48]. Zhang found that FAD3 and FAD8 overexpressing tobacco plants showed increased tolerance to drought and to osmotic stress [48]. The FADs induced drought tolerance can be related directly to the changes in membrane structure such as decreased membrane fluidity found in hyperosmotically stressed phospholipid vesicles and yeast cells, and the accumulation of unsaturated fatty
acids can attenuate rigidification of membranes leading to reduced damage by osmotic stress [49]. This view was also confirmed in our results, because many genes related to cell wall showed differential expression. The increased AfFAD in Amorpha fruticosa might played similar roles to against to the damage of drought stress.

5. Conclusions

Amorpha fruticosa can effectively respond to drought stress by increasing the intracellular content of SOD, MDA and Pro. Correspondingly, the expression of drought stress response genes and transcription factors increased. Some of the transcription factors played key roles in polygenic interaction against to stress. Amorpha fruticosa seedlings were sensitive to drought and the genes functions in drought stress responses via ABA-independent pathways. The up-regulation of Salicylic acid signal related DEGs (AfTGA and AfPR-1) indicated that Amorpha fruticosa can resist drought stress through Salicylic acid.

Additional Files

Additional files1: Table S1. sequence data summary
Additional files2: Table S2. Unigene assembly information
Additional files3: Table S3. Primers information.

Abbreviations

GO Gene Ontology
DEGs Differently expressed genes
KEGG Kyoto Encyclopedia of Genes and Genomes
ABA Abscisic acid
CK cytokinin
GA gibberellic acid
ROS reactive oxygen species
MDA Malondialdehyde
SOD Superoxide
REC Relative electrical conductivity
PRO Proline
BP Biological processes
PCA principal component analysis
SD standard deviation

Declarations

Ethics approval and consent to participate
The authors declare the compliance with institutional, national and international guidelines. The experiment was environmentally friendly and had no negative effects on the local ecosystem.

Consent for publication
Not Applicable.

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests. Each of the funding bodies took part in the design of the study and collection, analysis, and
interpretation of data, and the writing of the manuscript.

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**Authors’ contributions**

Q.G. and Y.Z. conceived and designed the study. Q.G., X.S., H.L., and H.S. performed the experiments. Q.G., Y.Z., and X.S. contributed to the sample measurement and data analysis. Q.G., S.X. and S.H. wrote the paper. All authors have read and approved the manuscript.

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Figures
Figure 1
Growth of Amorpha fruticosa seedlings under different drought stress conditions.

Figure 2
Contents of SOD, MDA, Pro and REC under drought stress. Abscissa shows the ind

Figure 3
Clustering results of all the samples. a. Principal component analysis (PCA) cluste

Figure 4
DEGs expression pattern and candidate pathways. a. DEGs expression patterns in
Figure 5

Interaction network of DEGs and proteins. a. interaction network of up-regulated DEGs under drought stress. b. interaction network of down-regulated DEGs under drought stress. c. interaction network of genes related to translation. d. Heatmap of core regulators in the interaction network.

Figure 6

qRT-PCR verification results.
Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Table S3 primers information.xlsx
Table S1 sequence data summary.xlsx
Table S2 Unigene assembly information.xlsx