Research Article **Open Access**

Fuqiang Yin#, Ming Liu#, Jian Gao#, Wenyou Zhang#, Cheng Qin, Aiguo Yang, Chenggang Luo, Haobao Liu, Yaou Shen, Haijian Lin, Zhiming Zhang, Guangtang Pan*

Analysis of global gene expression profiles in tobacco roots under drought stress

DOI 10.1515/biol-2015-0035
Received November 11, 2014; accepted March 8, 2015

Abstract: Tobacco (*Nicotiana tabacum* L.) is an economically important and relatively drought-tolerant crop grown around the world. However, the molecular regulatory mechanisms involved in tobacco root development in response to drought stress are not well-known. To gain insight into the transcriptome dynamics associated with drought resistance, genome-wide gene expression profiling of roots from a tobacco cultivar (Honghua Dajinyuan, a major flue-cured tobacco cultivar in Southwest China) under 20% PEG6000 treatment for 0, 6 h and 48 h were conducted using Solexa sequencing (Illumina Inc., San Diego, CA, USA). Over five million tags were generated from tobacco roots, including 229,344, 221,248 and 242,065 clean tags in three libraries, respectively. The most differentially expressed tags, with either log2FC > 2.0 for up-regulated genes or log2FC < -2.0 for down regulated genes ($p < 0.001$), were analyzed further. In comparison to the control, 1476 up-regulated and 1574 down-regulated differentially expressed genes (DEGs) were identified, except for unknown transcripts, which were grouped into 43 functional categories involved in seven significant pathways. The most enriched categories were those that were populated by transcripts involved in metabolism, signal transduction and cellular transport. Many genes and/or biological pathways were found to be common among the three libraries, for example, genes participating in transport, stress response, auxin transport and signaling, etc. Next, the expression patterns of 12 genes were assessed with quantitative real-time PCR, the results of which agreed with the Solexa analysis. In conclusion, we revealed complex changes in the transcriptome during tobacco root development related to drought resistance, and provided a comprehensive set of data that is essential to understanding the molecular regulatory mechanisms involved. These data may prove valuable in future studies of the molecular mechanisms regulating root development in response to drought stress in tobacco and other plants.

Keywords: DGE (Illumina / Solexa digital gene expression); qRT-PCR (quantitative real-time PCR); Drought resistance; Tobacco

1 Introduction

Tobacco is an important cash crop in China as well as around the world, being cultivated in more than 100 countries. In addition, a recent study investigated the use of tobacco as a potential biofuel crop [1]. It is also recognized as an established model plant for physiological and genetic studies. Drought is one of the major abiotic stresses affecting crop growth, development, yield and quality, and in extreme cases, causes crop failure. In response to environmental extremes, plants have adapted several mechanisms allowing them to survive adverse conditions. Although much research has been dedicated to elucidating plant gene expression during exposure to dry conditions, the mechanisms underlying the regulation of gene expression largely remain unknown.

***Corresponding authors: Guangtang Pan,** Maize Research Institute, Sichuan Agricultural University, 211 Huimin Road, Wenjiang, Sichuan 611130, China, E-mail: pangt1956@aliyun.com
Fuqiang Yin, Ming Liu, Wenyou Zhang, School of Agricultural Sciences of Xichang College, Xichang, Sichuan, China
Cheng Qin, Yaou Shen, Haijian Lin, Zhiming Zhang, Key Laboratory of Biology and Genetic Improvement of Maize in Southwest Region, Ministry of Agriculture; Maize Research Institute of Sichuan Agricultural University, Wenjiang, Sichuan, China
Aiguo Yang, Chenggang Luo, Key Laboratory of Tobacco Genetics and Breeding, State Tobacco Monopoly Administration, Qingdao, Shandong, China
Haobao Liu, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, Shandong, China
Jian Gao, Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, and Key Laboratory of Tumor Immunopathology, Ministry of Education of China, Chongqing, China
# These authors contributed equally to this work

Recent studies have shown that drought stress induce the differential expression of thousands of genes [2,3]. In many cases, it has been shown that alteration of the expression of individual genes can significantly impact the responses to drought stress in plants [4]. In tobacco, it was reported that trehalose-6-phosphate synthase (TPS1) accumulated in plants exhibiting multiple phenotypic alterations including stunted growth, lancet-shaped leaves, reduced sucrose content, improved drought tolerance [5], and/or enhanced drought tolerance accomplished via water retention and enhanced root development [6]. N-acetyl-L-glutamate synthase (SINAGS1) improved germination ability and increased the accumulation of ornithine to improve drought resistance [7]. In addition, Guo et al. (2008) found that *Triticum aestivum* ubiquitin 2 (Ta-Ub2) accelerated the germination of seedlings under water deficit conditions and then improved the CO_2 assimilation rate of transgenic plants under both drought and non-drought conditions [8] Furthermore, a 3.8-fold higher level of ascorbate peroxidase (APX5) activity enhanced the net photosynthetic rate, and reduced toxicity of H_2O_2 in transgenic tobacco plants [9]. However, tocopherol cyclase (VTE1) decreased lipid peroxidation, electrolyte leakage and H_2O_2 content, and increased chlorophyll and tocopherol content in transgenic tobacco as compared to wild type plants in response to drought stress [10]. In addition, the *LTP* gene family, which includes at least five tightly regulated and differentially expressed genes, was linked to increased accumulation of cuticular wax in leaves of tree tobacco in response to periodic drought [11]. *LTP* is predominantly expressed in the epidermis and is induced under drought stress in tobacco [11]. Previously, drought stress response has been analyzed with microarray technology. A comprehensive analysis of H_2O_2-induced gene expression in tobacco showed that it induced photo-inhibition in *CAT1AS* plants, and that short-term H_2O_2 exposure triggered increased tolerance toward subsequent severe oxidative stress [12]. In another study, the relationship between the function of PIP-type water channels and water usage in tobacco plants (*Nicotiana tabacum* cv. Samsun) under drought stress was analyzed. It was found that drought stress led to reduction in stomatal conductance, transpiration, water potential and turgor pressure in leaves, and reduction in the sap flow rate and osmotic hydraulic conductance in roots [13]. Recently, a mitogen-activated protein kinase cascade in the signaling pathway for polyamine biosynthesis was discovered in tobacco. The NtMEK2–SIPK/WIPK cascade is involved in regulating polyamine synthesis, particularly putrescine synthesis, through transcriptional regulation of biosynthetic genes in tobacco [14].

The Illumina / Solexa digital gene expression (DGE) system is an improved tag-based method that can sequence, in parallel, millions of DNA molecules that are derived directly from mRNA [15]. The DGE system enables the sequencing of total cDNA for the derivation of an accurate estimate of gene expression, both individually and comprehensively, and the discovery of novel regions of transcription. This technology has dramatically changed the way in which functional complexity of transcriptomes is studied. Therefore, our objective in this study was to reveal important pathogenesis-associated genes involved in potential defense or resistance pathways by detecting differentially expressed genes (DEGs) in tobacco using high-throughput differential gene expression (DGE) technology. Our aim is to elucidate the molecular mechanisms of resistance to drought stress, and provide important information for breeding superior, drought-resistant tobacco varieties.

2 Materials and methods

2.1 Plant material and RNA extraction

A tobacco (*Nicotiana tabacum* L.) cultivar (Honghua Dajinyuan, a major flue-cured tobacco cultivars in Southwest China) was grown in a growth chamber for 8 weeks with a photoperiod of 16 h light/8 h dark at day/night temperature of 28°C /21°C and relative humidity of 70%. The light period started at 6 a.m. and ended at 8 p.m. daily. Drought stress treatments were conducted on young tobacco plants with 8 leaves. For drought treatment, plants in plastic pots were subjected to progressive drought stress by watering with 20% PEG6000 for 0, 6 h, 48 h. Following treatment, roots were collected. Each sample was independently derived from at least five healthy plants which were then pooled. All samples were snap frozen in liquid nitrogen. Total RNA were prepared from 0.1 g tissues and extracted with Trizol (Invitrogen, Shanghai, CHINA) in accordance with the manufacturer's instructions. Samples, including the control, were then separately submitted for digital gene expression profiling (DGE) based on Solexa sequencing and real-time PCR.

2.2 Tag Library construction and Solexa sequencing

For Solexa tag preparation and sequencing, 20 μg of total RNA and 6 μg of mRNA were purified by adsorption of biotin oligo magnetic beads. After mRNA binding, cDNA synthesis was performed. Then, double-stranded cDNA

was introduced into a cDNA fragment, which was digested using NlaIII endonuclease and these binging fragments with the sequences of CATG site and adjacent polyA tail in 3' end. After precipitation of 3' cDNA fragments, the Illumina adaptor 1 was ligated to the 5' end. Both adaptor 1 and CATG sequences can be recognized by MmeI, and cut downstream of CATG sites to produce 17 bp tags with adaptor 1. Adaptor 2 was ligated to the 3' end of these tags following removal of 3' end containing beads. Ligated fragments were then prepared for Solexa sequencing. After 15 cycles of linear PCR amplification, 95 bp fragments were purified by 6% TBE poly-acrylamide gel electrophoresis (PAGE). Following denaturation, the single-chain molecules were fixed onto the Illumina Sequencing Chip (flowcell). Through *in situ* amplification, each molecule develops as a single-molecule cluster-sequencing template. Unique nucleotides were then labeled by four colors, and used for sequencing by synthesis (SBS). Each capillary generates millions of raw reads with a sequencing length of 35 bp.

2.3 Sequence annotation

Experimental tags were first filtered to eliminate low-quality sequences (containing ambiguous bases) thereby leaving adaptor sequences for mapping to the reference tags derived from cDNA or EST sequences. For sequenced tags, copy numbers in their libraries showed the expression abundance in each library respectively. The expression level of each gene was estimated by the frequency of clean tags and then normalized to TPM (number of transcripts per million clean tags) [16] which is a standard method and extensively used in DGE analysis. The normalized number of matched clean tag were used to measure the expression standard. KOG functional classification, Gene Ontology (GO) and pathway annotation and enrichment analyses were based on the NCBI COG (http://www.ncbi.nlm.nih.gov/COG) [17], Gene Ontology Database (http://www.geneontology.org/) [18] and KEGG pathway (http://www.genome.jp/kegg/) respectively [19].

2.4 Identification of different expression genes

To compare DEGs (differentially expressed genes) between CK (Control) and treat samples, the probability that one gene G is equally expressed in two samples can be illustrated by a calculated p-value [20], which indicates the significance of differences between transcript accumulation. The threshold of the p-value in multiple test and analysis was determined by FDR (False Discovery Rate) [21] Additionally, we also applied the R package, DEGseq, to identify DEGs with the random sampling model based on the read count for each gene at different developmental stages [22]. We used a FDR ≤ 0.001 and the absolute value of log2FC ≥ 1 as a threshold for judging the significance of each gene expression difference. More stringent criteria with a smaller FDR and larger fold-change value can also be used to identify DEGs. GO functional enrichment analysis was carried out using Blast2GO (version 2.3.5) (http://www.blast2go.org/). KEGG pathway analyses of the DEGs were performed using Cytoscape software (version 2.6.2) (http://www.cytoscape.org/) with the ClueGO plugin (http://www.ici.upmc.fr/cluego/cluegoDownload.shtml) [23]. GO annotation was performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/) [24].

2.5 GO and pathway enrichment analysis of DEGs

We obtained the GO terms for each Tobacco gene using Blast2GO (version 2.3.5) (http://www.blast2go.org/) with default parameters. Blast2GO was also used for a GO functional enrichment analysis of certain genes, by performing Fisher's exact test with a robust FDR correction to obtain an adjusted p-value between specific test gene groups and whole genome annotation [25].

2.6 Real-time PCR

To validate DEGs obtained from Solexa sequencing, 12 genes were subjected to quantitative real-time PCR analysis. Primers were designed using beacon designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA). Specific primers for the internal standard gene, Actin (GenBank No. X63603, Forward: 5'-CGCGAAAAGATGACTCAAATC -3', Reverse: 5'- AGATCCTTTCTGATATCCACG -3'), were used to normalize gene expression. PCR primers are shown in Table S1. RNA samples from the DGE experiments were used for qRT-PCR analysis. cDNA was synthesized from 1μg total RNA with Prime Script RT reagent kit (TaKaRa). qRT-PCR was performed using the SYBR Premix Ex TaqTM II protocol (TaKaRa) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture (20 μL) contained 0.5 μL of each primer and the appropriate amounts of enzymes, cDNA and fluorescent dyes. All runs included a negative control lacking target cDNA, thus resulting in the absence of detectable fluorescence signal. A range of five serial dilutions of total cDNA was tested under identical conditions as the samples. Amplification reactions were

initiated with a pre-denaturing step at 95°C for 10 s, followed by denaturing (95°C for 5 s), annealing (60°C for 10 s) and extension (72°C for 15 s) steps for 49 cycles during the second stage, and a final stage of 55°C to 95°C to measure dissociation curves of the amplified products. All reactions were performed with at least three replicates. Statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method.

3 Results

3.1 Characterization of the sequenced Solexa libraries

To identify tobacco genes involved in drought resistance, three tobacco Solexa libraries were constructed from root tissues after treatment with PEG6000. Sequencing depths of 507321, 542408 and 630739 tags were achieved in the three libraries, including 276125, 319400 and 386829 distinct tags, respectively. Considering the robustness of the subsequent data analysis, any tags recorded only once were eliminated, which left 229344, 221248 and 242065 distinct tags in each library that were detected multiple times (clean tags). The frequencies of these tags are shown in Table 1, which lists the copy numbers in the range 2–100 or higher; the majority of clean tags (62.0% from each library) were present in low copy numbers (< 10 copies). Approximately 35.9% tags from each library were recorded between 11 and 100 times, and only 2.1% of the tags were detected greater than 100 times.

To identify the genes corresponding to the 229344, 221248 and 242065 clean tags in each library, tags were mapped to a reference database [26]. In this study, we used BLASTN to map unique tags against respective tobacco reference gene sequences. A dataset containing 32540 reference genes expressed in Tobacco were generated from the PlantGDB database (http://www.plantgdb.org/search/misc/plantlistconstruction.php) through expressed gene analysis. Only those clean tags that matched perfectly or exhibited only one mismatch were analyzed further. Altogether, 182525 genes (85.24%) possessed CATG sites, resulting in a total number of 214135 reference tags. By assigning the experimental Solexa tags to the virtual reference ones (Table 2), we observed that 87607 (38.2%),

Table 1. Basic statistics of tags in two treatment stages and control samples.

	Control (D0)	Stage I (D1)	Stage II (D2)
Total tag	507321	542408	630739
Distinct tag	276125	319400	386829
Unique tag copy number >2	229344	221248	242065
Unique tag copy number >5	74537	72351	79181
Unique tag copy number >10	45430	43754	47360
Unique tag copy number >20	25858	25036	26336
Unique tag copy number >50	10712	10385	10447
Unique tag copy number >100	4936	4874	4701

Table 2. Statistics of tag mapping against reference gene of tobacco.

Tag mapping	Distinct tags		
	Control (D0)	Stage I (D1)	Stage II (D2)
Sense			
Perfect match	54200 (23.63%)	50898 (23.01%)	55915(23.01%)
bp mismatch	11018 (4.08%)	11001 (4.97%)	11212(4.63%)
Antisense			
Perfect match	19065 (8.31%)	17535 (7.93%)	18846(7.78%)
1 bp mismatch	3324(1.45%)	3142 (1.42%)	3418(1.42%)
All tags mapping to gene	87607(38.2%)	82576(37.33%)	89391(36.93%)
No matched tags	141737(61.8%)	138672(62.67%)	152674(63.07%)
Total distinct tags (clean tags)	229344	221248	242065

82576 (37.33%) and 89391 (36.93%) tags were perfectly matched to the reference genes in the libraries D0, D1 and D2 respectively. Intriguingly, 22.61% of the tags matched to reference genes were mapped to multiple locations, including low complexity tags with poly (A) tails, and tags derived from repetitive sequences. In addition, approximately only 9.4% tags among the three libraries were mapped to the antisense strands, demonstrating that those regions might be bi-directionally transcribed. With respect to discrepancies between reference tags and experimental tags [27], 5.53% of mismatched tags of 1 bp were present in the three libraries. Further, the large proportion of non-matched clean tags revealed that the efficiency of annotation was low when the copy number was between two and five. Sequencing data saturation refers to a situation in which no more new unique tags can be detected with any increases in the total number of tags. In this study, all three libraries fully represented the transcripts under the three experimental conditions (D0, D1 and D2) (Figure 1). In other words, progressively fewer tags were identified as the number of sequencing tags increased. In conclusion, the relatively higher mapping efficiency of D1 and D2 compared with D0 indicates that for the first two libraries, a greater number of transcripts were expressed during the development of tobacco roots treated with 20% PEG6000.

3.2 Comparison and analysis of differentially expressed genes

After mapping the obtained tags against reference genes of tobacco, the number of tags corresponding to each gene was calculated in each of the three libraries. This was then used to estimate differences in gene expression levels between different samples. Transcripts detected with at least two-fold differences (FDR < 0.001 and absolute values of log2FC ≥ 1) in the D1 and D2 libraries compared to the control sample are shown in Figure 2. The statistical difference of accumulation of unique tags between them is shown in Figure 3. To study a subset of genes that was associated with the responses of tobacco roots to PEG6000, and to assess the molecular basis of drought-resistance, we analyzed the most differentially regulated tags with either log2FC > 2.0 for up-regulated genes or log2FC < -2.0 for down regulated genes using a statistically high significance value ($p < 0.001$) and a stringent cutoff for false discovery rates (FDR < 0.001). This analysis revealed 969 up-regulated and 1254 down-regulated transcripts. To investigate dynamic changes in gene expression, the gene expression levels of stage I (D1) and stage II (D2) samples were each compared with controls. Accordingly, 1742 and 1749 DEGs were identified in the two stages, respectively. Among the DEGs exhibiting five-fold (or greater) differences in expression, 270 and 393 up-regulated genes were discovered in D1 and D2

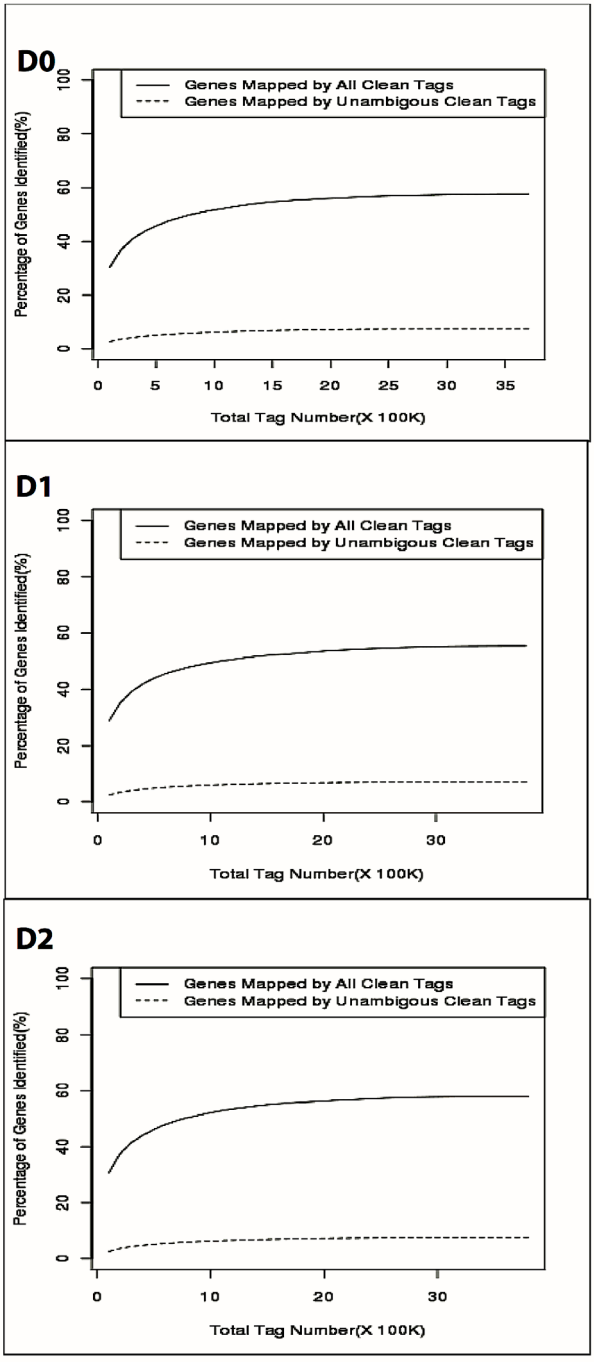

Figure 1. Accumulation of the genes mapped by all clean tags (solid line) and unambigous clean tags (broken line) in three libraries. D1 to D3 denote D0 (Ck), D1 (treatment with 20% PEG6000 for 6 h) and D2 (treatment with 20% PEG6000 for 48 h) respectively. The percentage of genes identified (ordinate) increases proportionally to the total tag number (abscissa). (From top to bottom is D0, D1, D2 respectively).

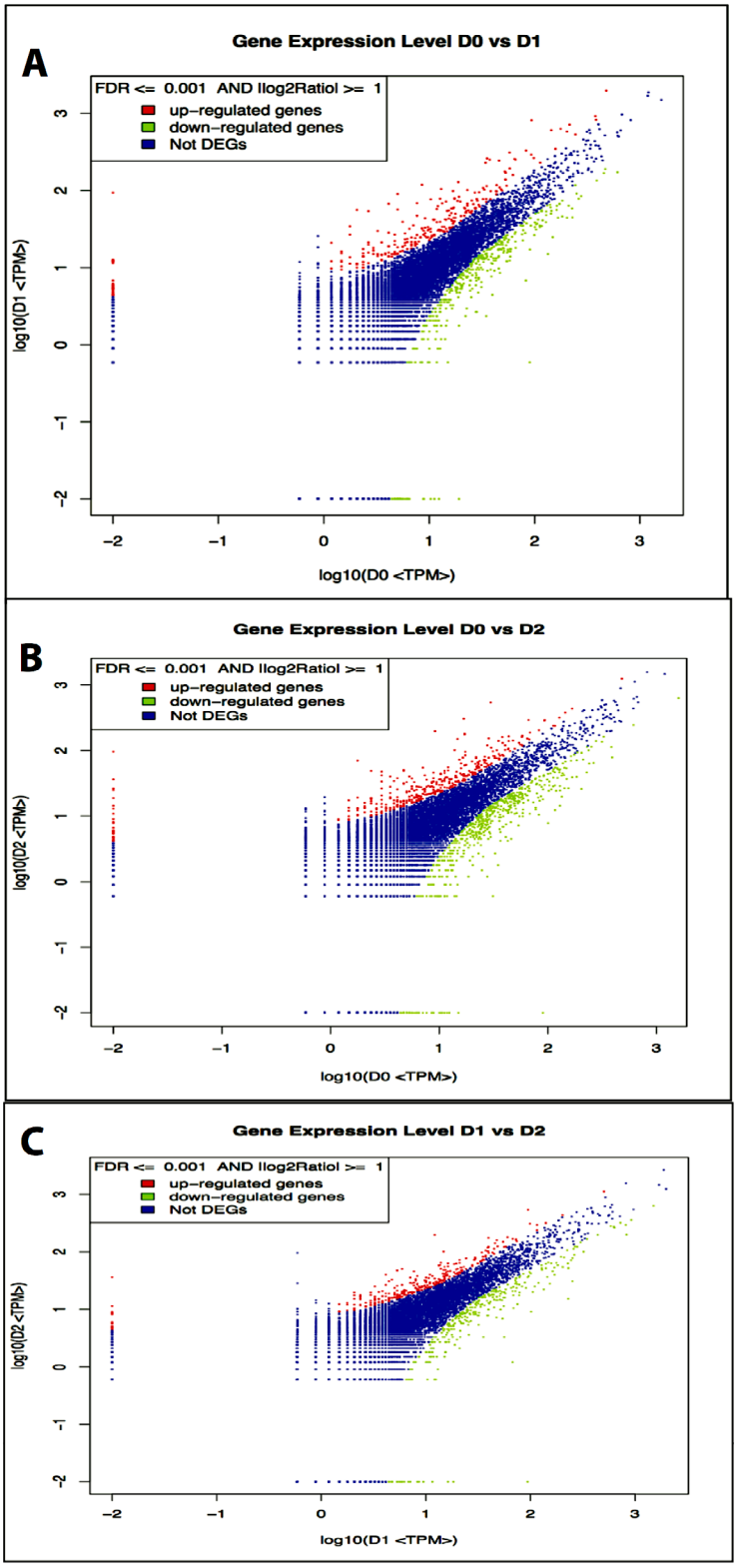

Figure 2. Comparison of gene expression between different libraries. Gene expression between D0 (Ck) and D1, D2 were compared. Blue dots represent the transcripts with no significant expression. Red and green dots represent transcripts more abundant from the stage samples and control, respectively. "FDR < 0.001" and "absolute value of log2 FC ≥ 1" were used as thresholds to judge the significance of differences in gene expression.

samples, respectively. In contrast, down-regulated DEGs were more abundant, with 371 and 532 genes in D1 and D2, respectively, showing five-fold (or greater) differences in expression levels when contrasted with controls (Figure 4). In addition, 15 up-regulated and 11 down-regulated genes were distinctly identified in the three libraries (Figure 5). Apart from unknown transcripts (61.8%), predicted or known genes were categorized according to their functions. GO Functional annotation of DEGs indicated that both the up-regulated and down-regulated genes could be classified into 43 categories, as indicated in Figure 6. These categories were based on predominant function, such as catalysis, electrical signal transport, transcription regulation, enzyme regulation, etc. Significant GO terms of DEGs in the annotation analysis of molecular functions showed that the most important function was the regulation of cellular processes. These DEGs were also involved in other biological processes such as responses to stimuli, responses to stress, signaling processes, and signal transmission (Table S2).

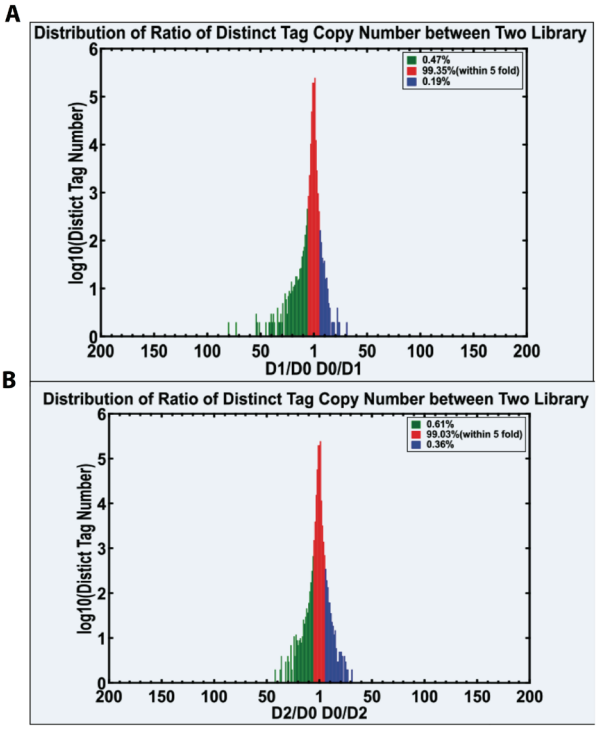

Figure 3. Tags with different expression in stage samples I (D1) and II (D2) compared to control sample (D0). The red region represents the expressed tags with differential expression less than 5-fold, and blue and green regions represent the up- and down-regulated tags for more than 5-fold, respectively.

3.3 Validation of DEGs by qRT-PCR

To validate DEGs obtained from Solexa sequencing, nine genes (gnl|UG|Nta#S33521750, gnl|UG|Nta#S50831789, gnl|UG|Nta#S40599160, gnl|UG|Nta#S33540206, gnl|UG|Nta#S33578357, gnl|UG|Nta#S33577608, gnl|UG|Nta#S57710991, gnl|UG|Nta#S45438006, and gnl),

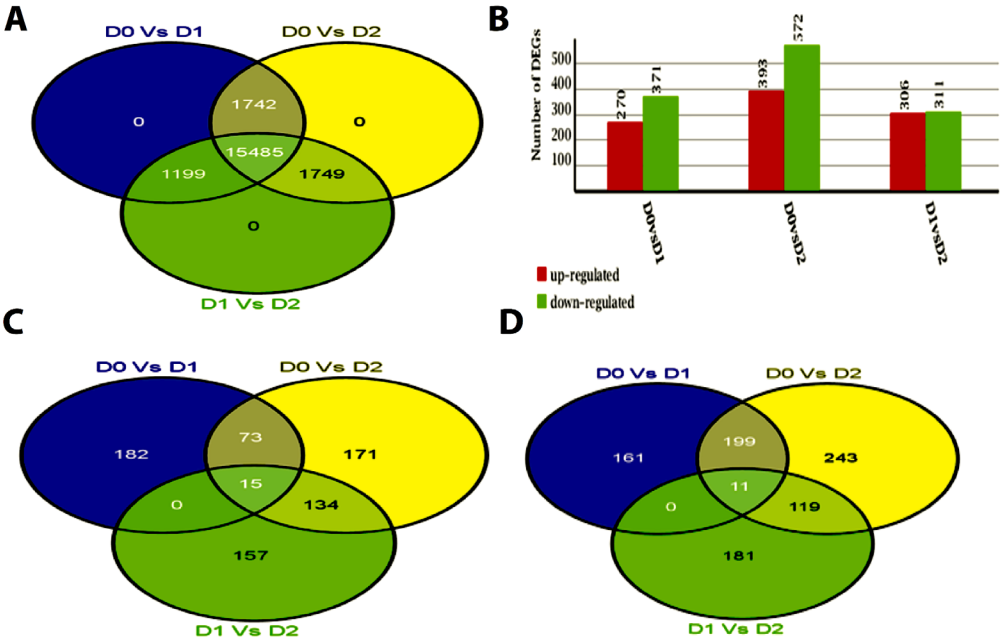

Figure 4. Differentially expressed genes in tobacco between D0 (CK) and D1, D2 (treated with 20% PEG6000). (A) Venn diagram, (B) statistical analysis of the differentially expressed genes between D0 and D1, D2, (C) the distinct up-regulated genes in D0, D1 and D2, (D) the distinct down-regulated genes in D0, D1 and D2.

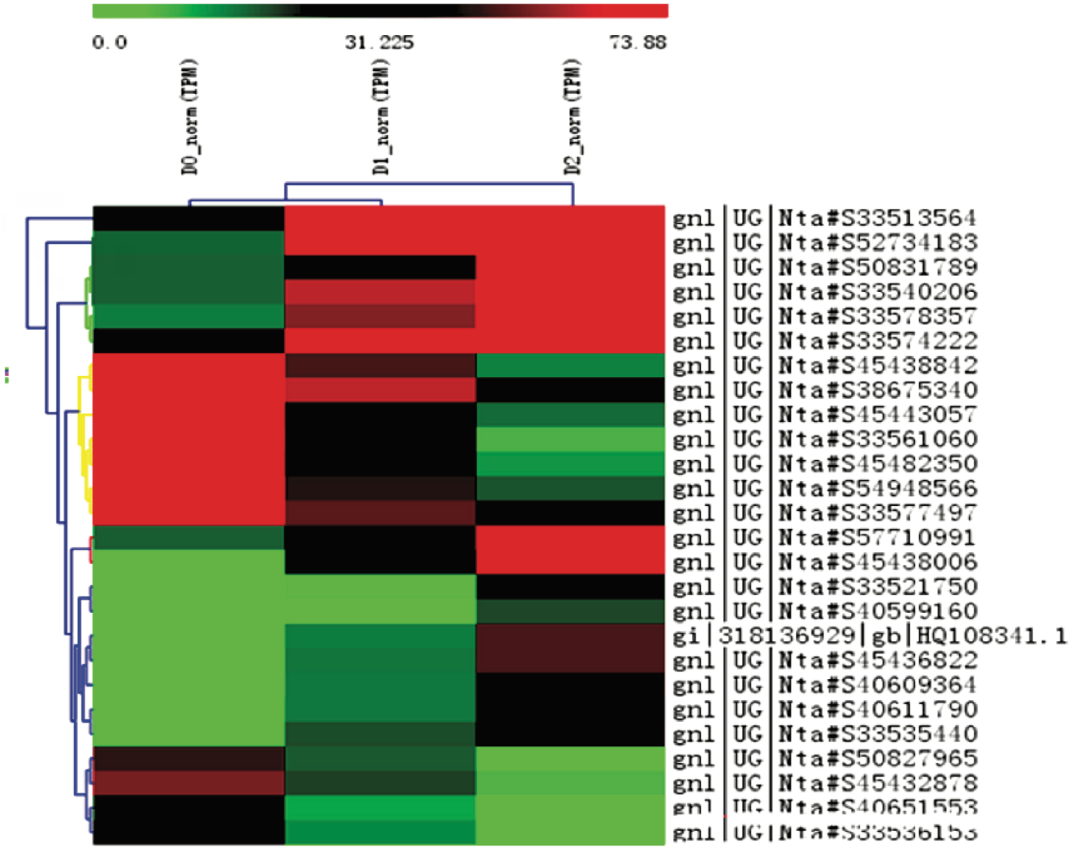

Figure 5. Heat map of distinct up- and down-regulated genes in D0, D1, and D2.

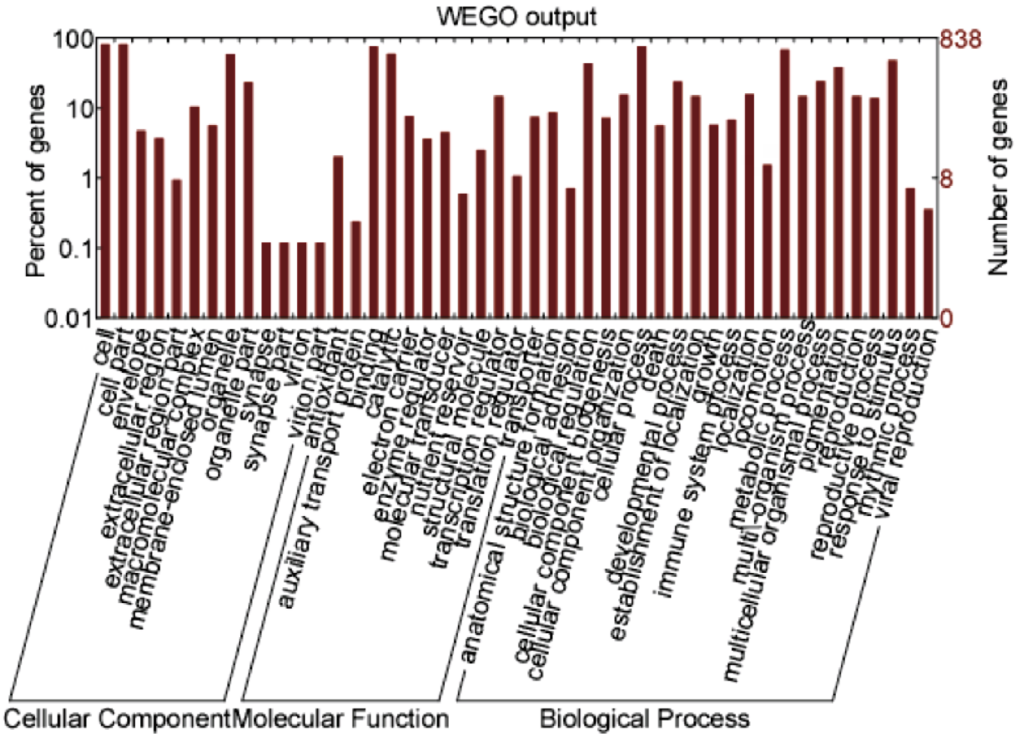

Figure 6. GO annotations of differentially expressed genes between D0, D1 and D2.

which were found to be up-regulated and four genes (gnl|UG|Nta#S40651553, gnl|UG|Nta#S54948566, gnl|UG|Nta#S45443057, and gnl|UG|Nta#S33577497), which were found to be down-regulated in the two stages of test samples and control (CK) samples (Table S3), were subjected to quantitative real-time PCR (qRT-PCR) analysis. Once again, gene expression levels in the samples were measured relative to those in the controls. As shown in Figure 7, qRT-PCR analysis was strongly consistent with DGE data, indicating excellent concordance between the two methods. Some of gene expression levels between DGE (digital gene expression profile) and qRT-PCR showed lower similar trends. This discrepancy may be attributed to fundamentally different algorithms employed by the two techniques. In the analysis of gene profiles, the deep sequencing method generates expression measurements that are absolute instead of relative.

3.4 Pathway enrichment analysis of DEGs

To elucidate the functional roles of DEGs responsible for drought resistance in tobacco roots, biological metabolic pathways were investigated by conducting enrichment analysis of DEGs between the two different stages of samples. The first ten key pathways for DEGs are listed in Table S4. In stage I (D1), it was revealed that the biosynthesis of stilbenoids, diarylheptanoids, and gingerol, biosynthesis of flavonoids, and the degradation of limonene and pinene were the three most significant pathways, which were indicated by both up- and down-regulated DEGs. In stage II (D2) samples, in addition to the three significant pathways found in D1 samples, seven other significant pathways were found, namely, plant hormone signal transduction, phenylpropanoid biosynthesis, circadian rhythm regulation, riboflavin metabolism, galactose metabolism, lysine biosynthesis, and phenylalanine metabolism. It is interesting that all significant DEGs were involved in stilbenoid, diarylheptanoid and gingerol biosynthesis, indicating the conserved and important roles of this pathway in response to drought stress.

3.5 Functional analysis of DEGs

In comparison to control samples, a set of significantly expressed transcripts in stage I and II samples were found to be derived from several genes that contribute to drought stress resistance. These transcripts were all grouped into the category of metabolism and signal transduction based on their functional annotations.

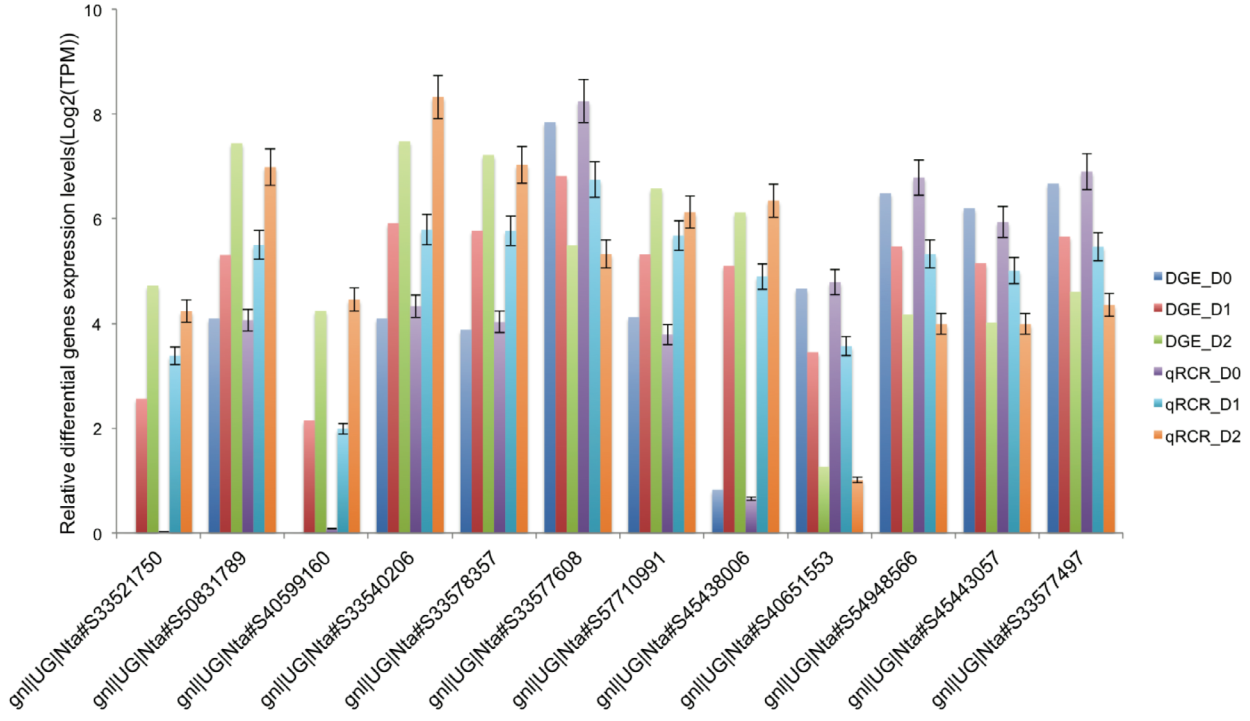

Figure 7. The differential expression genes levels randomly selected from differential expressed genes with high abundance enriched in D0, D1 and D2 libraries respectively and validation with qRT-PCR. The validation of selected differentially expressed genes enriched in three libraries indicated that the results from DGE sequencing were consistent with qRT-PCR results. Values are shown as mean (± SD).

3.6 Response to stimuli and stress

It is well-known that plants have developed defensive responses to biotic and abiotic stresses as a survival mechanism. Regulation of gene expression in response to a certain stress factor is key to such responses at the molecular level. In this study, 404 transcripts were expressed in the two analyzed stages under drought resistance as well as in CK samples. Among them, genes encoding circadian rhythm-associated FKF1 (gnl|UG|Nta#S33521750), diacylglycerol acyltransferase (gnl|UG|Nta#S50831789), and an aquaporin (gnl|UG|Nta#S33577497) contribute to modification-dependent protein catabolic processes, fatty acid metabolic processes and transport, and thus to responses to the stimuli of light, radiation and water stress, respectively.

3.7 Genes Related to Transporters

Regulated influx and efflux of substances across biological membranes is a vital component of cellular stress responses. In this study, a low-affinity nitrate transporter (gnl|UG|Nta#S40651553) was differentially down-regulated in the two stages of samples and in CK samples. Nitrate is a major nitrogen source for land plants and also acts as a signaling molecule that induces changes in growth and gene expression. Several recent studies have identified factors involved in the regulation of nitrate-responsive gene expression in plants.

3.8 Genes Related to Metabolism

The energy status of Plant Cells strongly depends on the energy metabolism in chloroplasts and mitochondria, which are capable of generating ATP by photosynthetic and oxidative phosphorylation, respectively. Another energy-rich metabolite inside plastids is the glycolytic intermediate phosphoenolpyruvate (PEP). However, chloroplasts and most other plastids lack the ability to generate PEP via a complete glycolytic pathway. Hence, PEP import mediated by the plastidic PEP/phosphate translocator or PEP provided by the plastidic enolase is vital for plant growth and development. In our study, low expression was found in the osmotically responsive gene 2 (LOS2), which encodes an enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) that converts 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP). Furthermore, GIGANTEA (gnl|UG|Nta#S45438006), a circadian-rhythm-controlled gene, showed a significant decrease in the two stages of samples. Cells are constantly exposed to external as well as internal stressors that threaten the integrity of their genome. In response to environmental and chemical genotoxic stressors, they induce an elaborate cellular response collectively known as the DNA damage response (DDR) to safeguard their genome [28,29]. Failure to repair damaged DNA can have dangerous or even fatal consequences to the cell or organism. Genomic instability can lead to neurodegenerative diseases and immune deficiency in animals. However, fewer reports of the consequences of genomic instability have been reported in plants. In our study, we found a putative RNA processing factor 1-like gene (gnl|UG|Nta#S45443057) with decreased expression at both stages which might cause recruitment to or exclusion from sites of DNA damage by DDR-induced phosphorylation, or generates resistance to DNA-damaging agents in response to drought stress.

3.9 Cell Signaling, Cell Structure and Cell Cycle Genes

Plants possess versatile detoxification systems to counter the phytotoxicity of a wide range of natural and synthetic compounds that are present in the environment. In our research, an increasing trend was found at both stages for the expression of genes encoding glutathione S-transferases (GSTs), which are enzymes that detoxify natural and exogenous toxic compounds through conjugation to glutathione. These enzymes also alleviate increased chemical toxicity in plants caused by drought stress. In addition, a decreasing trend was found for the expression of SR1 6, 7-dimethyl-8-ribityllumazine synthase (gnl|UG|Nta#S33577608), which is among a group of enzymes involved in riboflavin biosynthesis under physiological conditions that inhibit catalysis by other enzymes. This trend was observed at both stages. It was demonstrated in a previous study that this enzyme is also involved in the jasmonic acid signaling pathway, by restoring the sensitivity towards jasmonic acid with regards to root growth, senescence and plant defense [30]. The expression level of this gene may be associated with drought stress but we have not defined its function yet.

4 Discussion

Tobacco is one of the most economically important plants in the world, and is a model plant for studying drought stress, plant development and plant–pathogen interactions. The major goal of the present study was to preliminarily explore transcripts associated with drought-resistance in tobacco roots, as well as to provide a foundation for investigating their regulatory

mechanisms. To our knowledge, this is the first report that comprehensively shows the transcriptional changes during the development of tobacco roots in response to drought stress. We used the Illumina/Solexa DGE system to estimate gene expression and identify DEGs in libraries prepared from two growth stages of tobacco roots in response to drought stress, as well as in control samples. This system is essentially a serial analysis of gene expression-based tag profiling. Several previous studies using deep sequencing methods in the presence of biotic and abiotic stress to study plant transcript profiles have been reported. For example, root transcriptome responses to 2,4,6-trinitrotoluene exposure in Arabidopsis were investigated [27]. Another study determined the number and abundance of transcripts in root tips of maize seedlings under drought stress [31]. Solexa sequencing analysis has emerged as an efficient and economical method for screening transcript profiles under specific experimental conditions. However, there has not been an extensive report on the gene regulation mechanisms in tobacco roots in response to drought stress. About 45.2% of clean tags were mapped to 38.2% of the reference genes of tobacco in the two stages of libraries. There may be two main reasons for this. The genome of tobacco has not been sequenced and as a result, the reference gene annotations in the EST database may contain some mis-annotations. In addition, the *NlaIII* site that is required for detection by DGE technology could easily be contained by reference genes [26], indicating that some clean tags may not have been identified. Although this study represents only a preliminary analysis, the release of the tobacco genome sequence may reveal valuable information that can be exploited to identify those unmapped tags. Solexa sequencing provides a comprehensive and unbiased dataset in a global analysis of gene expression, and has greatly facilitated analysis of the expression levels of resistance genes in several studies. In the present study, 3050 differentially expressed annotated and novel transcripts ($p < 0.01$) were explored. The most differentially expressed genes with a log2 FC > 2 or log2 FC < -2 ($p < 0.001$) participate in various biological pathways, including those of metabolism, signal transduction, and cellular transport. Interestingly, many transcripts and/or biological pathways were found to be common among the three libraries, e.g. genes participating in nutrient transport, stress response, and auxin transport and signaling. For example, a low-affinity nitrate transporter (gnl|UG|Nta#S40651553) was differentially down regulated at both stages and in CK samples, indicating that tobacco roots undertake new functions with respect to N uptake or nutrient transport under drought stress. A dual-affinity nitrate transporter has been suggested to be one of the key components in Arabidopsis response to drought stress? [32]. Similarly, this has also been proposed to be a factor involved in nitrate signaling. The nitrate transporter AtNRT1.1 (CHL1) was shown to be involved in stomatal opening and contributes towards drought susceptibility in Arabidopsis [33,34]. Which was involved in stomatal opening with a reference to AtNRT1.1 [32]. Recent studies have suggested that a protein belonging to the NRT1 (PTR) family, encoded by the *M. truncatula* LATD/NIP gene (absence resulting in defective lateral root organs, numerous infections, and elevated polyphenolics contents), regulates legume root system architecture. LATD/NIP is expressed in the root apical meristem and elongation zones, as well as in the nodule meristem and infection zones [35,36]. Accordingly, LATD/NIP is required for the establishment and maintenance of primary roots, lateral roots, and symbiotic nodule meristems [36]. Therefore it can be speculated that a similar gene would result in enhanced drought tolerance in tobacco, through the reduction of stomatal opening and transpiration rates, and the regulation of the root system architecture under drought stress.

Previous studies have analyzed transcript profiles in Arabidopsis during drought, cold, high-salinity and ABA treatment conditions using a Tiling array [2] To our knowledge, this is the first study that comprehensively demonstrates transcriptional changes during root development under drought stress in tobacco. A large number of differentially expressed genes involved in critical pathways related to root development under drought stress were also found in this study, including genes participating in responses to stimuli and stresses, and auxin transport and signaling. In tobacco, it was reported that ELF4, FKF1, ZTL, GIGANTEA, and ELF3 are under circadian control in C3 and CAM leaves in Arabidopsis, and in particular, FKF1 shows an abrupt peak in transcript levels 3 h before subjective dusk. This is a photoreceptor important for the photoperiodic control of flowering [37]. Although overexpression or loss-of-function mutations of FKF1 have little effect on circadian function, double mutants of FKF1 and its homologue ZT1 were found to have circadian periods that were significantly longer than either single mutant, thus establishing a function for FKF1 in the regulation of the oscillator itself. It is similar to the gnl|UG|Nta#S33521750 transcript we examined under drought stress in this study, revealing that FKF1 may have a compensatory role in the plant circadian rhythm. In other words, it plays a role in development and resistance to abiotic stress by acting as a photoreceptor in the photoperiodic control of flowering

[37, 38] and indirectly regulates the level of CONSTANS (CO) to control flowering times. Therefore, it may modulate the response to drought resistance through delay of the flowering time. Diacylglycerol (DAG) acyltransferase (DGAT; EC 2.3.1.20) mediates the final acylation step in the synthesis of triacylglycerol (TAG), and is present in most plant organs, including leaves, petals, fruits, anthers, and developing seeds [39]. DGAT1 has been extensively studied in Arabidopsis. Overexpression of DGAT1 in Arabidopsis seeds produces an increase in seed size and oil content, suggesting that DGAT catalyzes the rate-limiting step in TAG biosynthesis [40]. In addition, DGAT1 is up-regulated during the senescence of Arabidopsis leaves and this is temporally correlated with increased levels of TAG-containing fatty acids commonly found in chloroplast galactolipids [41]. In that study, a steep increase in DGAT1 transcript levels was evident in senescing leaves, coincident with the accumulation of TAG, which is similar to what we observed. The increase in DGAT1 transcript levels may enhance levels of the DGAT1 protein. This may play a role in senescence by sequestering fatty acids derived from galactolipids into TAG. This would appear to be an intermediate step in the conversion of thylakoid fatty acids to phloem-mobile sucrose during leaf senescence and thereby strengthen drought resistance. In addition, the family of O-glycosyl hydrolases (EC 3.2.1) (gnl|UG|Nta#S33540206) includes a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. In our study, a putative xylanase inhibitor and two putative O-glycosyl hydrolases exhibited preferential gene expression in shoots in response to changing levels of N supply. We found that the gene encoding a hydrolase that catalyzes the hydrolysis of O-glycosyl compounds was differentially up-regulated, as it appeared to respond primarily to extremes in water supply, suggesting it may influences response in tobacco xylem tissues under such conditions, thereby contributing to drought resistance. It may also be involved in general abiotic or biotic stress responses. Aquaporins are ubiquitous channel proteins that facilitate the transport of water across cell membranes. It was reported that salt treatment inhibited transpiration and hydraulic conductivity to a greater degree in salt-tolerant ('20-45') plants than in salt-sensitive plants ('T-1'). In '20-45', the effect was paralleled by a rapid (within a few hours) and persistent (3 days) down-regulation of aquaporins [42]. This is identical to our research in the drought tolerance of tobacco. It was also reported that aquaporins can facilitate water uptake in barley roots [43,44], and that their regulation is mediated by PIP aquaporins via phosphorylation events in the presence of salt stress. It is likely that root hydraulic characteristics are ultimately regulated by aquaporin activity and thus the rate of water uptake by a given plant's roots increases in response to adverse environmental stimuli, such as drought. In addition, a large number of transcripts involved in oxidative or wound defenses were significantly induced in our study, for example, Tau (GSTU)-tau class glutathione transferase, which showed an increasing trend in both stages under drought when compared to CK samples. This enzyme catalyzes the detoxification of natural and exogenous toxic compounds through their conjugation with glutathione. Plant glutathione transferases (GSTs) are classified as GSH-dependent enzymes of secondary metabolism, predominantly expressed in the cytosol, and their roles in catalyzing the conjugation and detoxification of herbicides, and the reduction of organic hydroperoxides formed during oxidative stress, are well studied [45]. In addition to these roles in herbicide detoxification, based on changes in their gene expression levels, plant GSTs have also long been associated with responses to biotic and abiotic stress, hormones, and developmental changes [46,47] Differential roles of tau class glutathione S-transferases have also been reported under oxidative stress [48]. GST expression is induced by a wide variety of stresses, such as oxidative stress [49], xenobiotic stresses [43], and dehydration [50]. Expression of TaGSTU1B in *Triticum aestivum* was induced by drought stress in four genotypes investigated, but high transcript levels were detected only in drought-tolerant genotypes [51]. George et al. reported the subcellular localization of a GST (PjGSTUi) in *Prosopis juliflora* (Fabaceae), a drought-tolerant woody species, and its ability to confer drought tolerance in transgenic tobacco as well [52]. In another study, tobacco plants overexpressing a GST gene from *Glycine soja* showed six-fold higher GST activity than wild-type plants, which was linked to enhanced drought tolerance [53]. Accordingly, we proposed that following drought stress, Tau (GSTU) increases levels of glutathione, which reacts with potentially harmful oxidizing agents and is itself oxidized. As is well-known, Glycine-rich proteins (GRPs), containing more than 60% glycine, have been found in different tissues from many eukaryotic species, and some of these proteins are components of the cell walls of many higher plants. In most cases, they accumulate in vascular tissues and their synthesis comprises part of a plant's defense mechanism. The presence of GRPs in a variety of plant species [54] and the regulation of their expression patterns by a number of external stimuli have been reported extensively. In our study, a glycine-rich protein precursor (GRP-CD2) (gnl|UG|Nta#S54948566) was

predicted and showed a decreasing trend of expression in both tobacco stages compared with CK samples. A decrease in the expression of GRP-CD2 genes under drought stress suggests that GRPs may be involved in the responses to drought stress. It was also reported that GRP superfamily proteins perform different functions in plants, such as processing, transport, localization, stability, and translation of mRNA molecules [55]. This is consistent with the literature on GRPs and biotic and abiotic stresses [56, 57]. Analysis of the transcriptome of *Malus prunifolia* (a Chinese crabapple with strong drought tolerance) revealed a GRP (MpGR-RBP1) that was expressed in roots and leaves, which played a role in the response to drought stress [57]. In addition, it was reported that AtGRP2, AtGRP4, and AtGRP7, three of the eight AtGRP members identified in Arabidopsis, have varying impacts on seed germination, seedling growth, and stress tolerance of plants under diverse stress conditions [58]. The osmotically responsive gene *LOS* encodes an enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) that converts 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway in Arabidopsis [59]. It was reported that *LOS2*, a genetic locus required for cold-responsive gene transcription, encodes a bi-functional enolase, and mutants of this locus characteristically displays low expression levels of this gene [59]. Molecular analysis of a LOS2 mutant of Arabidopsis revealed that another C2H2 zinc finger protein, ZAT10/STZ, may act as a negative regulator of CBF-target genes. LOS2 binds to the MYC recognition elements in the ZAT10 promoter *in vitro*, and LOS2 mutant plants exhibited enhanced and more sustained induction of ZAT10 under cold stress. Thus, LOS2 appears to be a negative regulator of ZAT10 expression during the process of cold acclimation. In this study, we hypothesized that LOS1, like LOS2, might play a role in negatively regulating ZAT genes under drought stress, accomplished through its binding to MYC recognition elements. Furthermore, GIGANTEA (circadian-clock-controlled gene) that regulates photoperiodic flowering, was show to encode a protein with several possible membrane-spanning domains [60]. In Arabidopsis, this gene is regulated by the circadian clock with highest expression 8 to 10 hours after dawn [61]. This can be compared with the high signal intensity for the tobacco GIGANTEA-like transcript in unstressed and drought-stressed tobacco (9 h light) in our study. However, salt stress reduced the amount of the putative GIGANTEA transcripts from 0.55 to 0.35. In our study, it is possible that the GIGANTEA gene is required for maintaining circadian amplitude and appropriate period lengths for these genes. Additionally, GIGANTEA may also control circadian rhythms and photoperiodic flowering by participating in a feedback loop of the plant circadian system in response to drought stress.

Although numerous unmatched or unknown tags were detected, the value of this tag collection will increase as more tobacco genomic sequences become available. Further functional analysis of the differentially expressed genes will provide deeper insight into the regulation of tobacco root development under drought stress. Our data provides valuable information for future studies on the molecular mechanisms underlying root responses to drought stress in tobacco and other plants.

5 Conclusion

In conclusion, in this study we describe a comprehensive set of transcriptome dynamics associated with tobacco drought resistance. Using Solexa analysis we identified 1476 up-regulated and 1574 down-regulated DEGs, of which, the expression patterns of 12 genes were validated by quantitative real-time PCR. Furthermore, a profile of the root transcriptome under drought resistance revealed substantial differences in the categories and abundance of expressed transcripts compared with control samples. In addition, complex changes in the transcriptome were revealed during the development of tobacco roots in response to drought stress. Taken altogether this comprehensive set of data will be useful for understanding the molecular regulation of drought resistance in tobacco and other plants, with future applications for research on this topic.

Acknowledgements: This work was supported by grants from the National Natural Science Foundation of China (No. 30900901), the Sichuan Province Special Sci-Tech Projects (No. 2006J13-039), the Ministry of Agriculture in China (No. 2011ZX08003-003). The Open Project Program of Key Laboratory of Tobacco Genetic and Breeding, State Tobacco Monopoly Administration (Molecular Mechanism of Drought Resistant in Tobacco).

Conflict of Interest: No conflict of interest exits in the submission of this manuscript, and all the authors approve it for publication.

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Supplemental Material: The online version of this article (DOI: 10.1515/biol-2015-0035) offers supplementary material.