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Comparison of transcriptomes of chilling- and drought-tolerant and intolerant *Nicotiana tabacum* varieties and identification of genes associated with stress tolerance

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Abstract

Environmental stresses like drought and low temperature lead to the decrease the arable fields. Therefore, investigation on mechanisms of stress tolerance in plants is important to agriculture and horticulture. The transcriptome changes during chilling or drought stress in model plant tobacco is not clear yet. In this study, transcriptomes of two *Nicotiana tabacum* cultivars, one from MSK326 (tolerance to chilling and drought) and the other from Yunyan203 (intolerance to chilling and drought) were investigated by Illumina Hiseq™ 2000 next-generation sequencing platform. Fourteen digital gene expression (DGE) libraries were sequenced by the same platform. From the sequencing results, 106 million 90-bp quality-reads were obtained from two transcriptomes. The reads were assembled as reference sequences into 97,921 non-redundant unigenes with mean length of 653 nt. After annotation to noted databank, all unigenes were used as references to annotate DGE sequences. Comparing expressed genes of these two cultivars, we found that 4,320 genes were up-regulated and 1,091 genes down-regulated in MSK326. On the other hand, all DGEs were sequenced, each contained more than 40 M clean tags. At least 70% of the clean tags in each DGEs could be mapped to the references. To investigate differential express pattern between stressed MSK326 and Yunyan203, all DEGs were compared. Sixty-three genes were found to be differentially expressed in the chilling- and drought-treated samples by comparing all DGE libraries. According to the results obtained from these commonly expressed genes and some other known stress tolerance increasing genes (i.e. P5CS, APX, SOD and so on), we suggest that the stress-tolerant variety might increase its tolerance by (1) rationally regulating membrane fluidity via changing saturation of lipids and membrane components; (2) synthesizing more macro-molecules stabilizers, and osmotic adjustment solutes such as osmolytes; (3) reducing reactive oxygen species production by adjusting photosynthesis components as well as eliminating reactive oxygen species by rapidly activating anti-oxidant enzymes. This study provides a global view of transcriptome response and gene expression profiling differences between two tobacco varieties of MSK326 and Yunyan203 in response to cold and drought stresses. The results can help us to improve our comprehension of the molecular mechanisms in plants.

Keywords digital gene expression, macro-molecules stabilizer, membrane fluidity, next-generation sequencing, osmolytes, ROS detoxification, tobacco.

Abbreviations: APX_ ascorbate peroxidase; BLAST_Basic Local Alignment Search Tool; CAT_catalase; CBF_ C-repeat binding factor; COG_Cluster of Orthologous Groups of proteins; CYP_cytochrome P450-dependent fatty acid hydroxylase; DGE_digital gene expression; DHN_dehydrin; DREB_dehydration responsive element binding factor; FAD_fatty acid desaturase; FPKM_fragments per kilobase of exon per million fragments mapped; GO_Gene Ontology; GR_ glutathione reductase; KEGG_Kyoto encyclopedia of gene and genomes; LEA_late embryogenesis abundant proteins; NAC_no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF), cup-shaped cotyledon (CUC) superfamily; Nr_non-redundant protein database of GenBank; Nt_nucleotide database of GenBank; P5CS_$\triangle^1$-pyrroline-5-carboxylate synthase; POD_peroxidase; ROS_reactive oxygen species; SOD_superoxide dismutase; T6P_trehalose-6-phosphate; TPS_trehalose-6-phosphate synthase.

Introduction

Plants are the most important food and fuel resources. The human population has been predicted to reach 9 billion by 2050 according to the United Nations Population Division (2008). However, optimal plantation areas for crops have been decreasing. Therefore, improving crop yields is necessary to meet the food and fuel demands. Abiotic stresses, including water, temperature, and nutrition, are dominant factors that affect crop production. In the future, drastic changes in global change with extreme temperature fluctuations have been predicted (Fitzpatrick, 2013). Moreover, water crisis might occur. It was estimated by UN that two-thirds of the global people would live in water crisis regions by 2025. Water is an important medium to change climate and to get agricultural yields. Water crisis will aggravate food and energy crisis.

Many crops are sown in the spring; however, many subtropic and temperate areas experience chilling and/or drought conditions in early spring. Hence, if seeds germinate in spring, the plantlets might encounter chilling and water scarcity

conditions; thereby, showing retarded growth and developmental abnormalities and even death. This might eventually adversely affect the yield.

Plants will adjust many parameters in physiology and morphology to respond and adapt abiotic stresses. Many researchers have investigated how plants respond to various stresses such as chilling, salinity and drought in rice (Zhang et al., 2012), maize (Shan et al., 2013; Uddin et al., 2013), and wheat (Fleury et al., 2010; Li et al., 2012), using genetic engineering (Han et al., 2013), plant physiological approaches (Uddin et al., 2013), and omics methods (Zhang et al., 2012). Zhang et al. (2012) compared transcriptomes of chilling tolerance and intolerance rice varieties and found that genes related to transcription regulation and signal transduction were up-regulated in early stressed stage and many other genes with diverse functions were differentially expressed during continuous stress. Investigating maize transcriptomes for salt, chilling and drought stresses illustrated that in many gene categories the expression levels changed, transcription factor DREBs were up-regulated both in cold and drought stresses, and CBF and MYC had similar expression patterns in these stresses (Shan et al., 2013). By utilizing gene chip, Li et al. (2012) studied two contrasting wheat varieties (drought tolerance and intolerance). The results indicated that annotated expressed genes were classified into ten function categories. Using Illumina Hiseq^{TM} 2000 RNA-seq and DGE, our lab undertook a global analysis of transcriptome response and gene expression profiles to chill-hardening at 12 ℃ in the energy plant *Jatropha curcas*, and found that 3,178 genes were significantly up-regulated and 1244 down-regulated during the chill-hardening. Then, these genes were functionally annotated based on the transcriptome data from RNA-seq analysis (Wang et al., 2013b). That is to say, abiotic stress will induce expression changes of large quantities of genes in plants.

Nicotiana tobacum is widely used as model plant for investigating plants response to various stresses (Dong et al., 2013; Hu et al., 2013; Schaeffer et al., 2012). We recently tested chilling and drought resistance in 20 tobacco varieties and found that the variety MSK326 had relatively better resistance to chilling and drought stress. On the contrary, the variety Yunyan203 showed more sensitivity to these two stresses (Sheng et al., 2013). The antioxidant system of these two varieties were also investigated, and results indicated good ROS scavenging systems is partly reason to resistance of MSK326 to stresses (Wang et al., 2014b).

In this study, to better understand the difference of chilling and drought resistance between these two tobacco varieties, we carefully investigated and compared transcriptome response and gene expression profiles of MSK326 and Yunyan203 under chilling and drought stresses using the newly developed Illumina Hiseq^{TM} 2000 RNA-seq and DGE technique. These results will be meaningful and helpful for us to better comprehend the molecular mechanism of response and adaptation of plants to chilling and drought stress.

Results and Discussion

Illumina sequencing and sequence assembly

A global overview of transcriptomes of Yunyan203 and MSK326 was obtained by generating cDNA libraries. The two cDNA libraries were pair-end sequenced by using Illumina Hiseq^{TM} 2000 platform. After the sequences were filtered and checked for quality, 54 million 90-bp reads of MSK326 and 52 million 90-bp reads of Yunyan203 were obtained. The Trinity software assembled these two groups of clean reads into 208,000 and 182,000 contigs, of which 111,000 and 99,000 were identified as unigenes, respectively. These data from the same species were compared by subjecting the unigenes from each sample to sequence splicing and redundancy removal by using sequence clustering software. Eventually, 97,921 non-redundant unigenes having a mean length of 653 nt were obtained (Table 1). They were used as reference sequences to following DGEs analysis. The size distributions of these unigenes are shown in Supplementary Fig 1.

After comparison of MSK326 and Yunyan203 transcriptomes, 4,320 unigenes were found to be up-regulated and 1,091 unigenes were down-regulated. The MSK326 variety is more resistant to chilling and drought than Yunyan203; the reason of this tolerance might be the ability of MSK326 to regulate transcription of more genes than Yunyan203 when encountering chilling and drought conditions.

Annotation of sequences

The unigene sequences were annotated by aligning to protein databases Nr, Swiss-Prot, KEGG, and COG by using blastx (e-value, <0.00001), and then to nucleotide databases Nt by using blastn (e-value, <0.00001), and proteins with the highest sequence similarity with the given unigenes along with their protein functional annotations were obtained. Of the 97,271 sequences, 70,031 showed significant sequence similarity (Table 2).

Only 19,411 unigenes (about 20% of all unigenes) were annotated by COG on the basis of sequence homologies. In the COG classification, these annotated sequences were classified into 25 function classifications (Fig. 1). Most genes were classified into "General function prediction", followed by "Transcription," "replication, recombination, and repair," and "Posttranslational modification, protein turnover, and chaperones." Only a few genes were classified into the terms "nuclear structure" and "extracellular structures." The COG analysis suggested that the identified genes were involved in various biological processes. These results indicate that plants subjected to chilling and/or drought stress increasingly regulate genes associated with tolerance and those involved in DNA repair and recombination.

GO classification for unigenes

The GO terms were used to classify the functions of the predicted *N. tabacum* unigenes. The 45,592 annotated unigenes were further categorized into 54 functional groups (biological process, 24; cellular component, 15; molecular function, 16; Fig. 2). Most of the genes were classified into the cellular (29,842 unigenes) and metabolic process (28,508 unigenes) classes. Cell, cell parts, and organelle were prominent groups in the "cellular component function" ontology. In the "molecular function" ontology, the highest percentage of genes was classified into the "binding" (41.7%) and "catalytic activity" (42.1%) groups. There were 293 unigenes that were classified into the "antioxidant activity" class (Fig. 2). This finding indicated that the function of the genes associated with antioxidant biosynthesis can be remarkably investigated in plants subjected to chilling and drought stress.

Metabolic pathway assignment by KEGG

The Kyoto Encyclopedia of Genes and Genomes (KEGG)

Table 1. Statistics of transcriptomes from MSK326 and Yunyan203 by sequencing and assembling data.

	MSK326	Yunyan203
Total number of raw reads	59,168,318	57,600,626
Total number of clean reads	54,454,722	52,221,914
Total clean nucleotides (nt)	4,900,924,980	4,699,972,260
Average read length	90	90
Total number of contigs	208,653	182,414
Mean length of contigs	267	268
Total number of unigenes	111,606	99,648
Mean length of unigenes	529	479
nNon-redundant (nr) Unigenes	111,606	99,648
Total number of nr Unigenes	97,921	
Mean length of nr Unigenes	653	

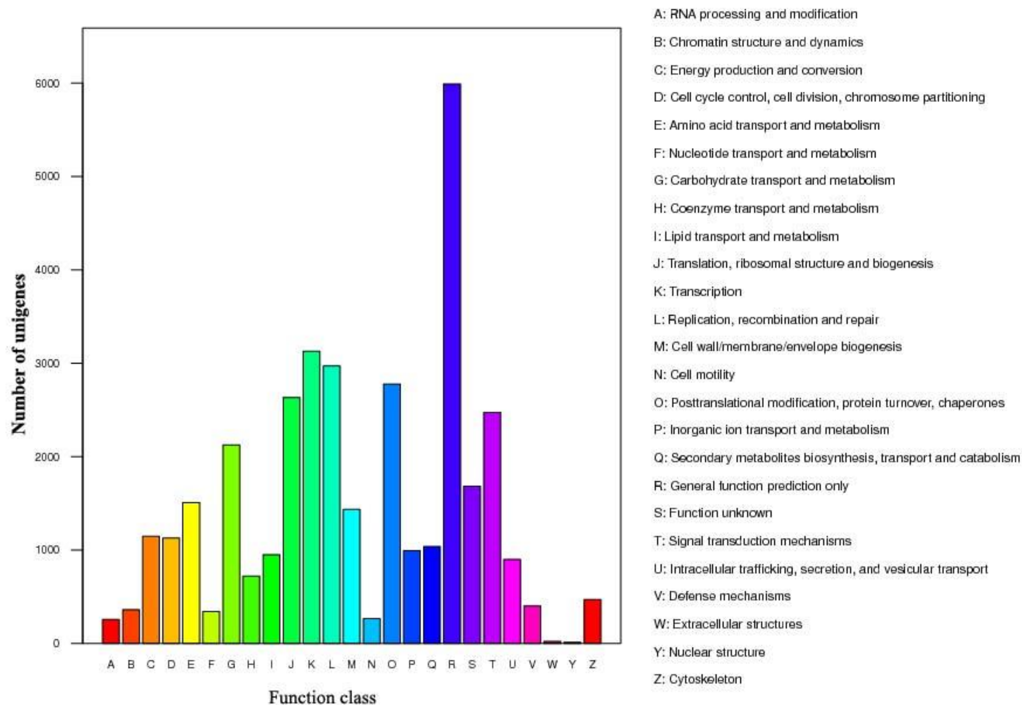

Fig 1. Clusters of orthologous group (COG) functional classification of *Nicotiana. tabacum* unigenes.

pathway maps are important bioinformatics resources to deal with high throughput sequence data (Kanehisa and Goto, 2000; Kanehisa et al., 2014). In this study, a total of 32,032 annotated sequences were mapped to 128 known metabolic or signaling pathways, including molecular metabolism, biosynthesis of secondary metabolites, and plant hormone signal transduction according to KEGG (Supplementary Table 1). Most unigenes were associated with metabolic pathways (7,149 members, 22.32%), biosynthesis of secondary metabolites (3,796 members, 11.85%), and plant hormone signal transduction (1,689 members, 5.27%). Among all metabolic pathways, the highest number of unigenes were involved in "starch and sucrose metabolism" (834, 2.60%), "glycerophospholipid metabolism" (754, 2.35%), "purine metabolism" (663, 2.07%), "pyrimidine metabolism" (560, 1.75%), and "ether lipid metabolism" (485, 1.51%). On the other hand, only a few of the unigenes were associated with "Arginine and proline metabolism", "Ascorbate and aldarate metabolism" and "Glutathione metabolism" pathways. These findings provide valuable information for investigating the specific processes, functions, and pathways regulated during chilling and drought stress.

Identification of genes associated with glutathione, ascorbate, and proline metabolism pathway

Glutathione, ascorbate, and proline are well-known antioxidants that are released in response to stress. Beside the genes associated with the glutathione, ascorbate, and proline metabolic pathways, some further genes were identified by KEGG. We selected genes that encoded enzymes catalyzing reactions involved with the synthesis or break down of these three amino acids. In all, 213 unigenes were identified by KEGG in the "Glutathione metabolism" (Supplementary Fig 2) pathway. For ascorbate and proline, 154 and 98 unigenes were separately assigned by KEGG to the "Ascorbate and aldarate metabolism" and "Arginine and proline metabolism", respectively.

DGE library sequencing and evaluation

DGE analysis was performed to identify the changes in gene expressions caused by exposure to chilling or drought conditions. Leaf samples of Yunyan203 and MSK326 subjected to chilling or drought treatment for 0, 12, 24, and 48 h were collected to construct 14 DGE libraries, which were subsequently sequenced.

Table 2. Statistics of unigenes of *N. tabacum* annotation results.

Sequence	NR	NT	Swiss-Prot	KEGG	COG	GO	ALL
Unigenes	58,783	63,805	34,858	32,032	19,411	45,592	70,031

NR, non-redundant; NT, nucleotide; KEGG, Kyoto Encyclopedia of Genes and Genomes; COG, clusters of orthologous groups; GO, gene ontology; ALL, total number of annotated unigenes

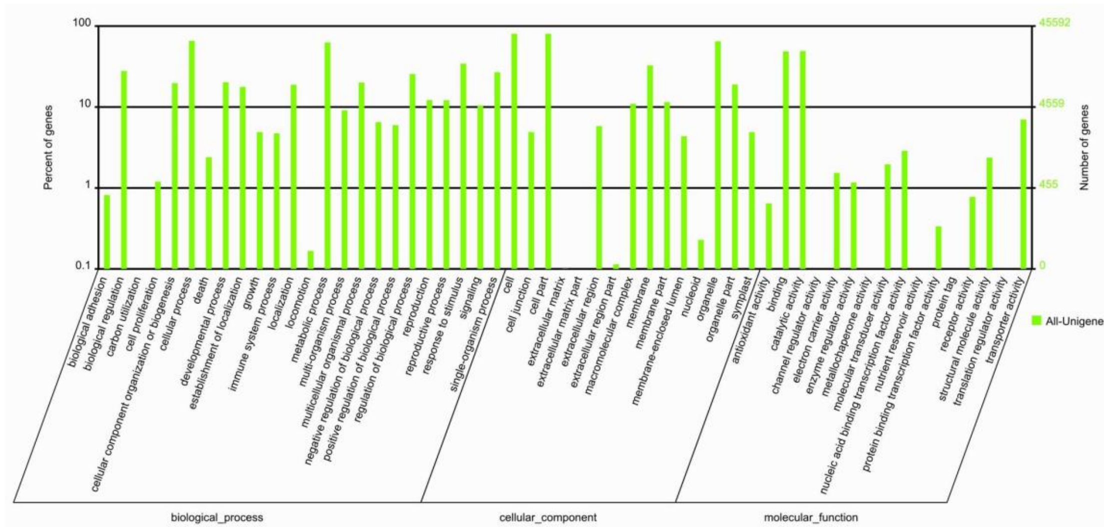

Fig 2. Gene ontology (GO) classification of *Nicotiana tabacum* unigenes.

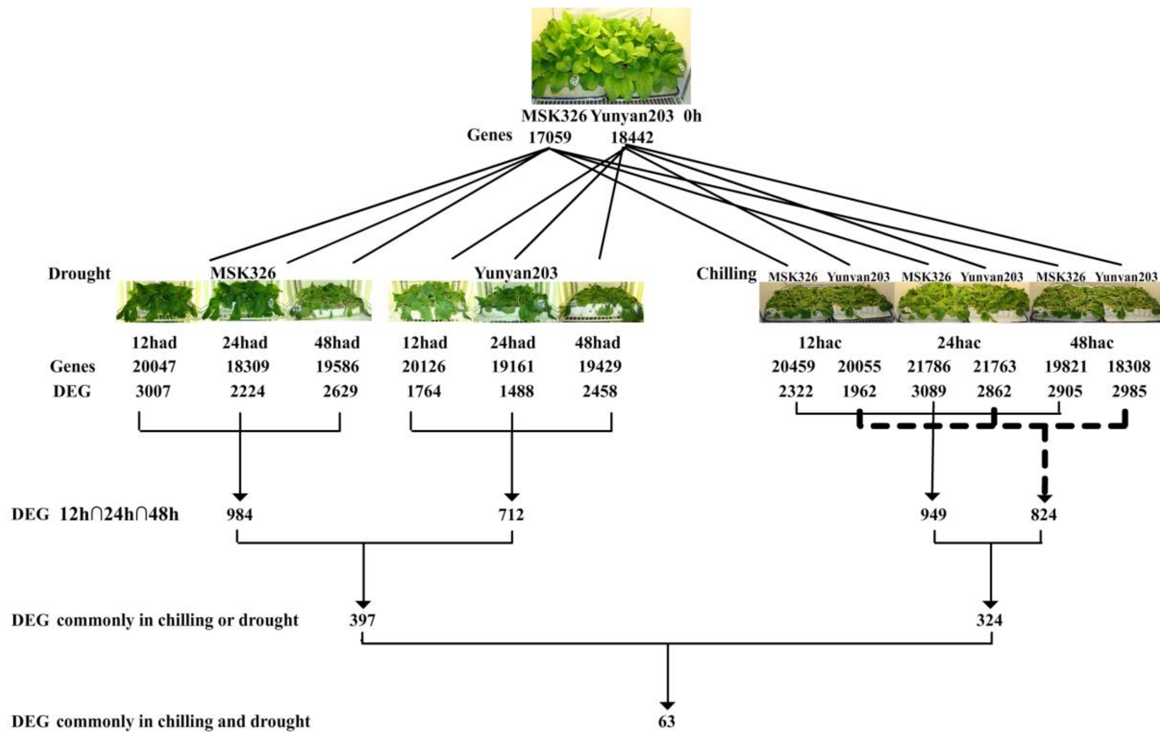

Fig 3. Comparisons of the 14 digital gene expressions (DGEs) profile libraries. The transcriptomes constructed in this paper were used as reference sequence, and all attained sequence tags were mapped to compare the differential gene expression profiles of the three chilling samples and three drought stress samples of MSK326 and Yunyan203 with those of the untreated samples. (Had: hours after drought treatment; hac: hours after chilling (2 ℃) treatment.)

The sequencing saturation analysis indicated that, when the sequencing amount reached 2 M or higher, the number of detected genes almost ceased to increase. Therefore, we sequenced 2 M reads for each DGE library. Further, the experimental repeatability analysis was performed to estimate the reliability of the experimental results as well as the operational stability. The results showed that the correlation value between two parallel experiments was 0.999. The sequencing quality evaluation and alignment statistics of the 14 DGE libraries is shown in Supplementary Table 2. More than 45 M raw tags and 40 M clean tags were obtained. The percentage of clean tags after filtering dirty tags was more than 90% in all the 14 libraries. For all the DGE libraries, at least 70% of the clean tags were mapped to sequences of our transcriptome. Tag annotation was performed by mapping tags to the aforementioned RNA-sequence-based transcriptome. Subsequently, the relative expression level for each unigene having DEG tag matches in each form of chilling treatment was computed and utilized for the identification of differentially expressed genes associated with chilling stress.

Comparison of the 14 DGE libraries

The annotation results of the 14 DGE libraries were compared to identify genes commonly expressed during drought and chilling stress (Fig. 3).

Differential gene expression between the two varieties before the drought or chilling stress

The natural differences in gene expression profiles between MSK326 and Yunyan203 before exposure to chilling and drought stress was determined by conducting gene expression analysis under control conditions.Therefore, comparisons between DGEs of MSK326 control and those of Yunyan203 control were processed. In all, 250 and 524 genes in MSK326 showed higher and lower expression, respectively. Web gene ontology (WEGO) analysis suggested that these differentially expressed genes mostly belonged to three biological process terms: “metabolic process”, “cellular process”, and “response to stimulus”. Pearson chi-square test revealed that the differences in gene expression between the two samples in many biological processes, such as photosynthesis and metabolic process regulation, were significant (Supplementary Fig 3). Among all the GO terms with significant variations, “signal transduction” and “response to endogenous stimulus” were the most interesting. Precise investigation to these two terms suggested that these two classes included many hormone metabolism relative genes of almost all kinds of plant hormones. This indicated that MSK326 could tolerate stress better than Yunyan203 possibly because of the higher expression of these hormones metabolism relative genes.

Common differentially expressed genes at the three chilling time points

To determine genes regulated in response to chilling stress, the DGEs of samples obtained from plants exposed to chilling stress were compared respectively with those of control by variety (Fig. 3). For MSK326, after 12, 24, and 48 h of exposure to chilling stress, 2,322; 3,089; and 2,905 differentially expressed genes (DEGs, i.e., genes regulated in response to chilling stress), were regulated, respectively. Of these, 949 DEGs were commonly expressed at all three time points. For Yunyan203, 1,962; 2,862; and 2,985 DEGs were regulated after 12, 24, and 48 h of exposure to chilling stress, respectively, in which 824 DEGs were commonly regulated at all the three time points (Fig. 4). Among all commonly expressed DEGs in response to chilling stress in MSK326, 716 genes (75.4% of all DEGs) were up-regulated and 228 genes (24.0%) were down-regulated at all the three time points, and 5 genes were differentially regulated at least at one time point. Further, in Yunyan203, 417 genes (54.3%) were up-regulated, 337 genes (43.9%) were down-regulated, and 14 were neither up-regulated nor down-regulated consistently.

Transcriptome analysis revealed that 427 of the 716 up-regulated DEGs and 137 of the 228 down-regulated DEGs of MSK326 were annotated by GO terms. Similarly, for Yunyan203, 279 of the 417 up-regulated and 222 of the 337 down-regulated DEGs were annotated. Further, enrichment analysis of GO terms indicated that, when the up-regulated genes of MSK326 and Yunyan203 were compared, the variations of two GO terms were significant: one for GO:0044085 cellular component biogenesis, and the other for GO:0006950 response to stress. When the down-regulated genes of MSK326 and Yunyan203 were compared, the variations of various GO terms were significant: 13 cellular components, 5 molecular functions, and 17 biological processes (Supplementary Fig 4).

Under chilling stress, more genes related to membrane, chloroplast, and photosynthesis were down-regulated in MSK326 than in Yunyan203. The activation of these genes could probably reduce the production of reactive oxygen species (ROS) and protect plants. Furthermore, genes related to catabolic processes and water transport were also down-regulated in MSK326. This might allow the plants to economically utilize energy and resources.

Commonly expressed DEGs under chilling stress

The DEGs of the two varieties of tobacco exposed to three time points of chilling stress were compared and 397 genes were found to be commonly expressed. Of these genes, 305 (including 176 annotated by GO terms) were up-regulated, 87 genes (including 51 annotated by GO terms) were down-regulated, and 5 genes were neither down-regulated nor up-regulated accordingly.

The results of GO term enrichment analysis suggested that the top three child GO terms for cellular components were related to cytoplasm (125 genes, 54.1%), organelle (143 genes, 61.9%), and membrane (81 genes, 35.1%). The membranes were included plasma membrane, organelle membrane, and membrane in their child terms. In these DEGs, plasma membrane had 51 genes; organelle membrane, 34; and membrane part, 36. For the GO terms for biological process, the top three child GO terms were related to cellular process (154 genes, 66.7%), metabolic process (147 genes, 63.6%), and response to stimulus (107 genes, 46.3%).

Commonly expressed DEGs at the three drought time points

To determine genes regulated in response to drought stress, the DGEs of samples obtained from plants treated with drought stress were compared with those of control by variety (Fig. 3). For MSK326, 3,007; 2,224; and 2,629 DEGs were regulated at 12, 24, and 48 h after drought stress, respectively, whereas 984 DEGs were commonly expressed at all three time points. For Yunyan203, 1,764; 1,488; and 2,458 DEGs were regulated at 12, 24, and 48 h after drought stress, respectively, whereas 712 DEGs were commonly regulated (Fig. 5).

Of the 984 commonly expressed DEGs of MSK326, 658 (66.9%) were up-regulated and 302 genes (30.7%) were down-regulated at all three time points. Furthermore, 24 genes showed differential regulation at least at one time point. For

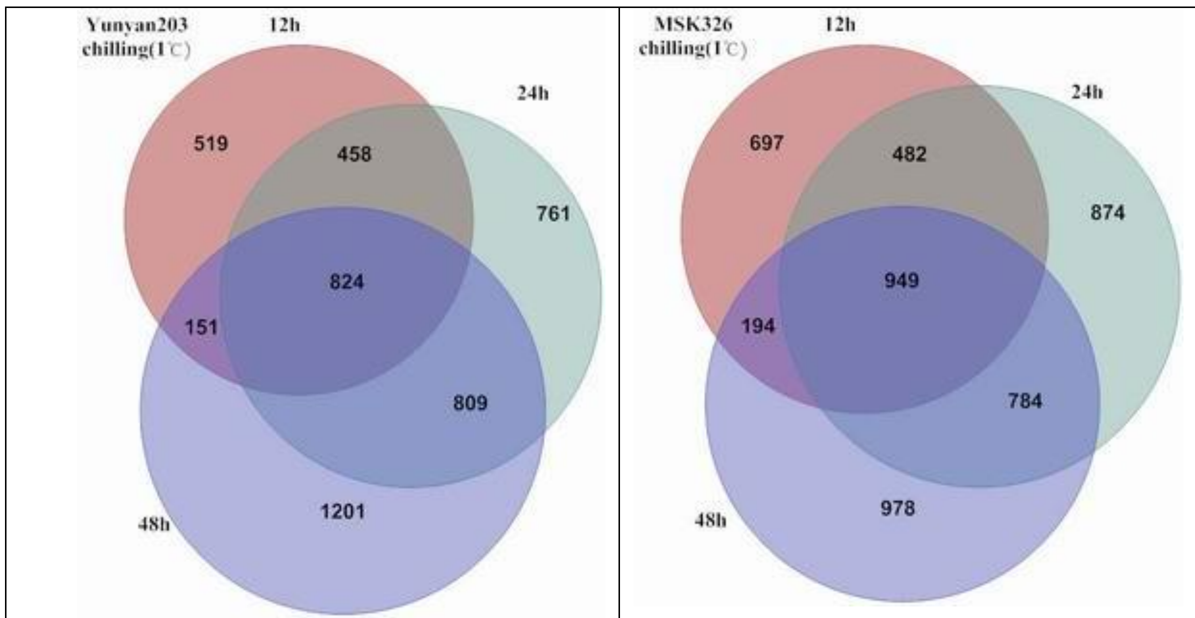

Fig 4. Relationships among differentially expressed genes (DEGs). Analysis conducted using the six samples from plants of the two varieties of *N. tabacum* exposed to chilling stress.

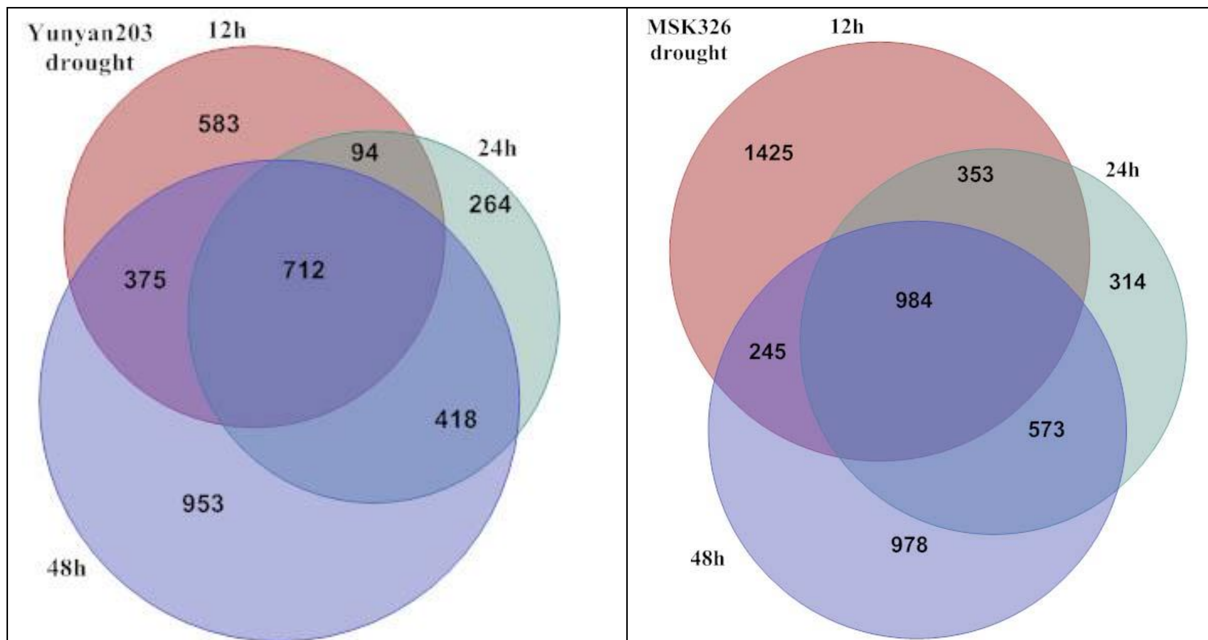

Fig 5. Relationships among differentially expressed genes (DEGs). The analysis was conducted using six samples from the two varieties of *N. tabacum* exposed to drought stress.

Yunyan203, of the 712 DEGs, 434 genes (61.0%) were up-regulated, 264 (37.1%) were down-regulated, where 14 genes were neither up-regulated nor down-regulated consistently. Transcriptome analysis revealed that 452 of the 658 up-regulated and 215 of the 302 down-regulated DEGs of MSK326 were annotated by GO terms. Similarly, 311 of the 434 up-regulated and 183 of the 264 down-regulated DEGs of Yunyan203 were annotated by GO terms. Enrichment analysis of GO terms indicated that, when the genes up-regulated in response to drought were compared between MSK326 and Yunyan203, variations of only 4 GO terms were significant, which were two for RNA metabolic process (GO: 0006350 transcription and GO: 0032774 RNA biosynthetic process), one for intrinsic hormone stimulus (response to hormone stimulus), and one for transport (GO: 0007034 vacuolar transport). When the down-regulated genes of MSK326 and Yunyan203 were compared, significant variances were noted for 12 cellular component, 4 molecular function, and 47 biological process GO terms (Supplementary Fig 5).

From the numbers and p-values of genes related to cell components that were associated with drought tolerance, it was evident that those mainly down-regulated in MSK326 were photosynthesis-related genes, including plasma membrane, chloroplast membranes, and envelops, and genes related to photosystem I. All these genes probably decreased energy and resource consumption as well as ROS production.

From the status of biological processes, the tolerant variety down-regulated the genes associated with the metabolism of many small molecules, such as carbohydrates, nucleobases, nucleosides, nucleotides, and alkaloids, and biopolymers such as polysaccharides, proteins, and RNA at prolonged drought endurance. Moreover, genes related to certain biological activities were down-regulated, including phosphorus utilization, transport, ribosome biogenesis, organelle membrane organization, and cell growth.

Commonly expressed DEGs under drought stress

When the DEGs regulated under different drought stress of the two varieties, 324 genes were found to be commonly expressed. Of these genes, 221 genes annotated by GO terms were up-regulated, with 156 annotated in the 6 samples of chilling stress of the two varieties, and 92 genes were down-regulated, with 51 genes annotated by GO terms; 11 genes were neither up-regulated nor down-regulated consistently.

The enrichment analysis of GO terms revealed that, under the term cell component, the common DEGs were mainly clustered in GO: 0043226 organelle (125 genes, 55.6%), GO: 0005737 cytoplasm (116 genes, 51.6%), and GO: 0016020 membrane (88 genes, 39.1%). Under the term organelle, the genes were mainly classified into plastid (61 genes), nucleus (40 genes), and mitochondrion (25 genes). For the term membrane, the genes could be divided into plasma membrane, organelle membrane, and membrane part.

Comparison between DEGs commonly regulated under chilling and drought stress

To identify the genes directly related to chilling and drought stress, we compared the commonly expressed DEGs under the stresses between the two groups. First, on the basis of the normalized expression quantities, they were separately compared between the up-regulated and down-regulated genes. When the commonly up-regulated DEGs under chilling and drought stress were compared, genes related to plastid were the most differentially regulated for the GO term cellular component. Further, for the term biological process, the variances of 10 child terms were significant, including cellular response to stress and pigmentation (Supplementary Fig 6).

Common DEGs regulated under chilling and drought stress

When all the DEGs from the 12 stressed samples were compared, we found that 63 genes were expressed in all the samples. Of these common DGEs, 28 were up-regulated and 32 were down-regulated. Cytochrome P450-dependent fatty acid hydroxylase (CYP; CL12658.Contig3_All) and myricetin *O*-methyltransferase 2 (MMT; Unigene25993_All) were up-regulated in all the 12 samples, but they showed higher expression in the tolerant MSK326 compared to intolerant Yunyan203 (Supplementary Fig 7), indicating that they played a role in plants stress tolerance.

The expression data of these genes was analyzed and nine of the 63 genes were selected as the most effective stress tolerance candidates. Of these nine genes, three were up-regulated, three down-regulated, and three either up- or down-regulated in all the samples obtained from plants exposed to chilling or drought stress.

CYP catalyzed the ω-position hydroxylation of fatty acids for synthesizing cutin and suberin under abiotic and biotic stress (Pollard et al., 2008). In the stress-tolerant variety, this gene was expressed 1,000–10,000 times higher than that in the control. However, in the intolerant variety, the expression level was only 4–120 higher that that in the control. Therefore, the expression of this gene might be one of the most important factors associated with drought and chilling stress.

Some DEGs involved in cold and drought stress

Under abiotic stress, plants often regulate various physiological, biochemical, and molecular processes. The examples are, changing membrane components (desaturated fatty acids related to fluidity and aquaporin to adjust water homeostasis), accumulation of compatible osmoprotection solutes (proline in the cytoplasm increases the levels of antioxidants and ROS detoxification enzymes such as superoxide dismutase and catalase), increasing the contents of chaperons and membrane stabilizers (late embryogenesis abundant (LEA) proteins), and expressing stress response transcription factors (dehydration-responsive transcription factors).

Fatty acid desaturase

Many studies have suggested that cell membrane components and fluidity could be adjusted by temperature. Fatty acid desaturase FAD6 and FAD3 are known to separately introduce the second and third double bonds in all 16:1- or 18:1-containing chloroplast membrane lipids. Some researchers reported that FAD could improve the tolerance of abiotic stress (Wang et al., 2014a; Yu et al., 2009). In our study, FAD6 was remarkably down-regulated under drought treatment both in the tolerant and intolerant varieties. However, under chilling stress, FAD6 and FAD3 were up-regulated in MSK326 and up-regulated for 24 hours and then down-regulated in Yunyan203. Except in MSK326 samples exposed to drought stress, FAD3 were up-regulated more than twice of that in the control (Supplementary Fig 8). The results of FAD6 and FAD3 expression indicated that FAD was related to chilling and drought stress tolerance. Since the substrates of FAD3 were the products FAD6, in MSK326, the transcription of FAD6 was increased and the expression of FAD3 was remarkably increased. The products of FAD6 and FAD3 might affect membrane fluidity to increase stress tolerance. However, the expression pattern of FAD6 and FAD3 in Yunyan203 suggested that the products of FAD3 had more contribution to membrane fluidity that those of FAD6. This suggested that, for membrane fluidity, the tolerant variety possessed more elaborate regulation and control mechanisms than the intolerant variety.

Aquaporin

Aquaporins are a kind of proteins found in membranes that help maintaining water homeostasis of cells. Some of these proteins were reported to increase stress tolerance in cultured cells (Aroca et al., 2005; Zhou et al., 2012), whereas some did not (Aroca et al., 2005). In total, 14 aquaporins genes were investigated in this study. The FPKM analysis of the 14 samples showed that aquaporins were down-regulated in all the samples obtained from plants exposed to stress (Supplementary Fig 9). In plants exposed to chilling stress, low temperature decreases oxygen consumption and transpiration, and hence small quantity of aquaporin is needed to balance the water homeostasis.

Macro-molecule stabilizers or key enzymes of their synthesis

LEA proteins, proline, and trehalose are believed to be biological macro-molecular stabilizers. When water deficit and temperature stress are encountered, plants can synthesis LEA proteins to act with proteins and lipids to stabilize cell

membranes and enzymes (Gai et al., 2011; Hand et al., 2011; Tolleter et al., 2010).

LEA proteins

In our study, chilling- and drought-treated samples showed the transcription of 9 LEA unigenes, and 6 of these unigenes were LEA 5. The LEA proteins were constitutively over-expressed in all the stressed samples by varieties (Supplementary Fig 10). The LEA unigenes were transcribed more in the tolerant variety than in the intolerant one. We found that 60–98% of the LEA proteins were annotated as LEA 5 in MSK326; however, in Yunyan203, 38% to more than 98% LEA proteins were annotated as LEA 5. This indicated that LEA 5 was very important to overcome chilling and drought stress in this species.

Dehydrins (DHNs) are a sub-group of peptides, group II of LEA proteins (Dure, 1993). DHNs accumulate in plants exposed to drought and temperature stresses (Hanin et al., 2011). In our stressed samples, the expression of DHN genes was markedly increased after 48 h of exposure to stress, and the expression levels in samples from chilling stress were considerably higher than those in samples obtained from drought stress. Furthermore, the expression levels were higher in the stress-tolerant MSK326 than in the stress-intolerant Yunyan203 (Supplementary Fig 11).

Proline

Proline is one of the multi-functional molecules which known to decrease the water potential in cells, stabilize lipid and protein levels, and act as an antioxidant (Cuin and Shabala, 2007; Kaul et al., 2008; Szabados and Savouré, 2009). This amino acid is known to be adopted by plants to cope with abiotic and biotic stresses, such as primary and secondary water deficits, heavy metal stress, and *Agrobacterium tumefaciens* infection. The $\triangle^1$-pyrroline-5-carboxylate synthase (P5CS) is the key enzyme required for proline biosynthesis (Su et al., 2011). In our study, 4 unigenes annotated with P5CS were investigated. The P5CS was up-regulated in all the stressed samples except for the samples obtained from Yunyan203 subjected to 48 h of chilling stress. This indicated that P5CS was very important in plant stress response. In samples obtained from plants exposed to drought or chilling stress for 48 h, P5CS showed higher expression in MSK326 than in Yunyan203 (Supplementary Fig 12). This could be one of the reasons why MSK326 could endure more stress thanYunyan203.

Trehalose

Trehalose is an important disaccharide and had multiple functions, such as stabilizing folded proteins (Elbein et al., 2003; Jain and Roy, 2009) and decreasing water potential in the cytoplasm. Trehalose-6-phosphate (T6P), a derivative of trehalose, is also a multifunctional molecule that regulates sugar metabolites to control the development and growth of plants (Nunes et al., 2013; Paul, 2008; Schluepmann et al., 2012). Plants synthesize trehalose via a two-step pathway (Goddijn and Dun, 1999). Trehalose-6-phosphte synthase (TPS) catalyzes glucose-6-phosphate and uridine 5′-diphosphoglucose to produce T6P. In this study, the expressions of TPS were investigated in 14 DGEs; TPS mRNA levels were higher in all the stressed samples, and the levels were higher in drought-exposed samples than in the chilling-exposed ones (Supplementary Fig 13). This indicated that T6P and trehalose might be more effective in drought response than in chilling response.

Antioxidant enzymes

Many stresses are known to induce the overexpression of ROS in plants, and ROS in turn become a source of secondary stress and damage lipids, proteins, and nucleic acids. Plants have two scavenger systems, enzymatic [including SOD, peroxidase (POD), and CAT] and non-enzymatic (reductants such as ascorbate acid, glutathione, and proline) antioxidant system, to eliminate excessive ROS and protect cells from them (Miller et al., 2010; Mittler et al., 2004). In our study, we mainly investigated the expression of SOD, ascorbate peroxidase (APX), and CAT. The expression of SOD was up-regulated for 12 h after exposure to stress in MSK326, and decreased thereafter. In MSK326 exposed to 48 h of chilling stress, the expression of this enzyme was increased. The expression of APX was increased in all the samples after 12 h of treatment. Except in the drought-treated samples of MSK326, in which APX was continuously overexpressed, the expression levels of this enzyme decreased in all the other treated samples after 12 h. The maximum expression of CAT was noted at 12 h after exposure to chilling stress and decreased thereafter. In the drought-treated samples, although the expression of CAT was higher in the treated samples than that in the control, the level in 12-h drought-treated MSK326 was about twice that of the 12-h drought-treated Yunyan203. Wang et al. (2013a) reported that the differences between chilling tolerant and intolerant rice varieties were the differences in the antioxidant defense systems. Activities of SOD, APX, CAT, and GR and amounts of glutathione and ascorbate acid were increased in the tolerant rice species. Tian et al. (2012) found that the activities of antioxidant enzymes increased immediately after exposure to stress and decreased thereafter. Anjum et al. (2012) found that the activities of CAT, SOD, and POD were initially increased and then decreased progressively after exposure to drought in pepper, and that the constitutive activities of these enzymes were higher in the drought-tolerant variety than in the drought-intolerant one. Kim et al. (2013) suggested that APX precisely regulated the levels of ROS and CAT removed excess H_2O_2. In our study, after exposure to stress for 12 h, the expressions of antioxidant enzymes were higher in MSK326 than in Yunyan203 (Supplementary Fig 14). Recently, we treated tobacco seedlings of these two varieties for 6 days, and then antioxidant enzymes activities were measured. The results showed that composition of antioxidant systems of MSK326 were higher than those of Yunyan203 (Wang et al., 2014b). This indicated that plants need to highly activate antioxidant enzymes at the early stage of stress exposure. This may allow the enzymatic defense system to detoxify ROS before they can damage the cells.

Transcription factors

During plant response to stress, transcription factors are very important in modulating the expression of defense-related genes. Of the thousands of transcription factors, DREB and members of the NAC superfamily (no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF), cup-shaped cotyledon (CUC)) were paid more attention over the past years (Lata and Prasad 2011; Puranik et al., 2012).

Dehydration responsive element binding proteins

Dehydration responsive element binding (DREB) proteins are important plant transcription factors that regulate the expression of many stress-inducible genes. They can improve the tolerance of plants exposed to stresses such as water deficit (Jin et al., 2013b; Pino et al., 2013), salt stress (Bouaziz et al., 2013; Peng et al., 2013), and cold stress (Gupta et al., 2013).

In our study, the expressions of DREB proteins were up-regulated in all the stressed samples, and the expression levels of these genes in the chilling-exposed samples were considerably higher than those in the drought-exposed ones (Supplementary Fig 15). Of all the profiled DREB proteins, DREB2 was the most highly expressed. Many researches suggested that DREB2 was induced after exposure to dehydration, high salinity, and heat stresses and increased the tolerance of plants (Chen et al., 2009; Mizoi et al., 2013; Qin et al., 2007; Zhao et al., 2013). The expression patterns of DREB proteins, especially DREB2, suggested that MSK326 responded to stress more rapidly and actively than Yunyan203.

The NAC

The NAC superfamily transcription factors are involved in many biotic and abiotic stress responses (Aslam et al., 2012; Puranik et al., 2012; Saad et al., 2013; Wang and Dane, 2013e). Since this family includes many members, maybe tens to hundreds of members in plants (Hu et al., 2010; Jensen et al., 2010; Nuruzzaman et al., 2010; Wang et al., 2013c) belonging to 6 categories based on their structures (Puranik et al., 2012), the regulation mechanisms are very complicated. GmNAC2 overexpression in tobacco made the plants hypersensitive to drought and high salinity (Jin et al., 2013a). However, when SNAC1, a NAC of rice, was introduced into wheat, it increased the tolerance of transgenic wheat to drought and salinity by regulating type 2C protein phosphatases and components of abscisic acid receptor, among others (Saad et al., 2013).

In our study, like the DREBs, expressions of NAC were up-regulated in all the treated samples and the levels of these genes in the samples from chilling stress were considerably higher than those in the ones from drought stress (**Supplementary Fig 16**). In MSK326, the NAC genes showed rapid and pronounced increase in expression levels after exposure to 12 h of chilling stress and 24 h of drought stress, and decreased thereafter. However, in Yunyan203, the NAC genes were expressed much later after exposure to stress. This indicated that the stress-intolerant variety responded more slowly to stress than the stress-tolerant one. So, we can draw a conclusion that Yunyan203 is more sensitive to chilling and drought stress. This character is not only in 48 hours but also lasted even longer. These two varieties seedlings were treated with chilling and drought for 2 days, 4 days and 6 days, and then calculated comprehensive resistance to chilling and drought stresses of them. Data showed that MSK326 had more resistance than that of Yunyan203 (Wang et al., 2014b). The activities of all tested antioxidant enzymes and quantities of antioxidants of MSK326 samples are all higher than those of Yunyan203. To some extent, the higher resistance of MSK326 is due to its ability of alleviate stress induced ROS damage. This is similar to our DEG analysis.

As all the above, stress tolerant variety MSK326 is more tolerant to chilling and drought than Yunyan203. Compared to Yunyan203, MSK326 recruits more genes to reduce affection of chilling or drought stress, detoxifies ROS more timely with more antioxidants and antioxidant enzymes and decreases ROS production by lowering chlorophyll synthesis, sustains plasma membrane fluidity and integrity more effectively by altered membrane components and macro-molecules stabilizers.

Based on our comprehensive transcriptomes and DGEs analyses, it can be found that the two tobacco varieties with different stress tolerant levels utilized some similar mechanisms to deal with drought and chilling. When stress signals transducted into plants, transcription factors, such as DREB and NAC, are controlled to promote direct and indirect reactions (Supplementary Fig 17). (1) To maintain the integrity and fluidity of plasma membrane, sorts and quantities of lipids are adjusted. Sorts and quantities of desaturated lipids are relevant to fluidity and functions of plasma membranes (Nano et al., 2003). Lipids desaturases, such as ω-3 fatty acid desaturase are transcripted to change the composition of desaturated lipids in membrane in stressed plants. (2) To stabilize structures of biological macro-molecules, the molecule stabilizers are synthesized such as LEA proteins (Hand et al., 2011; Tolleter et al., 2010), proline and trehalose (Szabados and Savouré, 2009). The functions of biological macro-molecules are depended on their structures. So, it is very important to hold the right structures of them in stress. The transcription level of LEA goes high when plants encounter water deficit stresses. Moreover, to increase proline and trehalose, mRNA for key enzymes are up-regulated in biological synthesis pathway, P5CS for proline and trehalose-6-phosphate synthase for trehalose. (3) To conserve water in cell, cellular water potential is lowered by producing kinds of osmolytes including proline and trehalose and sorts and quantities of aquaporins are changed to attain water from environments. (4) To detoxify ROS, antioxidant enzymes are translated. ROS is a kind of very important signal molecules in living things (D'Autréaux and Toledano, 2007; Mittler et al., 2011). ROS together with plant hormones and other signal molecules compose a network to transduce *in vivo* and *in vitro* information into cells. But ROS can damage proteins, lipids and nucleic acids as well (Karuppanapandian et al., 2011). So, it is necessary to eliminate the toxicity of ROS. Plants utilize antioxidants and antioxidant enzymes to do so. In our analyzed data, transcription levels of SOD, POD and CAT are all up-regulated in stressed plants. Genes encoding key enzymes of proline and trehalose are transcribed much more than those of control. (5) To decrease extra ROS production, the transcription levels of chlorophyll a/b-binding proteins are down-regulated. ROS can be produced during photosynthesis in chloroplast, oxidative phosphorylation in mitochondria and β-oxidation in peroxisomes (Karuppanapandian et al., 2011). With function of absorption and transferring photo energy, chlorophyll a/b-binding proteins are important composition of photosystem I and II (Green et al., 1991), which are the main parts to generate ROS in chloroplast (Asada, 2006). Stressed plants down-regulate the transcription of these proteins to avoid energy leak to result more extra ROS.

Materials and Methods

Plant materials and stress treatments

The methods are modified from Sheng et al. (2013) and Wang et al. (2014b). Seeds of *N. tabacum* cv. MSK326 and Yunyan203 from germplasm resources center of Yunnan Academy of Tobacco Agricultural Sciences were obtained and surface-sterilized with 1.5% $CuSO_4$ for 30 min, and rinsed thoroughly with sterile water, and then soaked in distilled water for 24 h. These seeds were sowed in polystyrene foam nursery plates containing sterilized soil with perlite, peat, and sand (1:2:1) and floating on water in a greenhouse. When plants had 4-6 leaves, they were moved into climate chambers with the parameters of having temperature of 26/20 ºC (day/night), 80% RH (relative humidity), and 16 h photoperiod, and sequentially grown for 7 d. Next, the temperature was adjusted to 1ºC for chilling stress. The nursery plates with plants were moved to

filter paper and in 28 ºC for drought stress (Sheng et al., 2013; Wang et al., 2014b). The leaves were removed and frozen in liquid nitrogen after the cold or drought treatment for 0, 12, 24, and 48 h.

RNA extraction and quality control

In total, 8 tissue samples were prepared for RNA extraction and sequencing by variety, including 3 for cold treatment, 3 for drought treatment, 1 control, and 1 for cold and drought treatment. The mixed sample was obtained by equally mixing samples from the 3 cold treatments, 3 drought treatments, and control seedlings. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The concentration of RNA was determined using a Qubit ® 2.0 fluorometer (Life Technologies), and the quality was assessed using Agilent 2100 Bioanalyzer and 1% (w/v) agarose gel electrophoresis. Only those RNA samples that had a 260 nm/280 nm ratio between 1.8 and 2.2; 260 nm/230 nm ratio, ≥2.0, 28S/18S ratio, >1.0; and RNA integrated number (RIN), ≥7.0 were processed further.

Magnetic beads with oligo (dT) were used to isolate mRNA. The mRNA sample was mixed with the 1 × fragmentation buffer (Ambion) to fragment the mRNA. The cDNA was synthesized using random hexamer primers and reverse transcriptase (Invitrogen), and the mRNA fragments were used as templates. The short DNA products were purified using the QiaQuick PCR extraction kit and ligated with sequencing adapters. The suitable fragments with 200–700 bp size were selected for PCR amplification and sequencing by using the Illumina HiSeq™ 2000 RNAseq system.

Data filtering and de novo sequence assembly into unigenes

The raw reads produced from sequencing were filtered by removing reads of low quality, sequences having ambiguous nucleotides larger than 5%, and fragments having adaptor sequences.

De novo sequence assembly was performed using the Trinity short reads assembly program (Grabherr et al., 2011). Trinity first combines the reads of certain overlapping lengths to form longer fragments as contigs. Next, contigs were consecutively connected to obtain sequences that cannot be extended on either ends. These sequences were defined as unigenes of tobacco.

Annotation of unigenes

Unigene sequences of tobacco were first aligned by blastx to protein databases non-redundant (Nr) and SwissProt and then by blastn to nucleotide database Nt (e value, $<1e^5$) in order to retrieve the proteins with the highest sequence similarity to the query unigenes along with their protein functional annotations. Similarly, the annotation information was obtained from GO, COG, and KEGG, and the expression level for each unigene was determined.

The deduced amino acid sequences of the proteins with the highest ranks retrieved from blast results were considered as references to determine the coding region sequences (CDSs) of unigenes, using the standard codon table. The unigenes that could not be aligned to any databases were predicted using the expressed sequence tag (EST) Scan program (Iseli et al., 1999), and the nucleotide and amino acid sequence information of their putative coding regions was obtained.

Functional classification of unigenes

The Nr annotation was used to derive the GO functional annotation of *N. tabacum* unigenes. The GO is known to have three ontologies: molecular function, cellular component, and biological process. First, Blast2GO program (Conesa et al., 2005) was used to obtain the GO annotation of all unigenes. Subsequently, WEGO software (Ye et al., 2006) was used for performing GO functional classification and systematically elucidating the gene function distribution in this species.

The COG is a database that is developed on the basis of coding genes from the complete genome of an organism and the evolutional relationships among prokaryote, archea, and eukaryote organisms. This database can be used to classify orthologous genes. In this study, all unigenes of tobacco were aligned to the COG database for function prediction and classification.

The KEGG database (Kanehisa and Goto, 2000; Kanehisa et al., 2014) can be used to determine the cellular pathways and biological complexity of unigenes. According to the KEGG annotated results, tobacco unigenes were classified into different metabolic pathways. In addition, unigenes encoding cold tolerance-related components such as enzymes for synthesis of unsaturated fatty acids and osmolytes (proline, trehalose), enzymes related to the antioxidant system, and transcription factors were retrieved.

DGE profile

Oligo(dT) magnetic beads were used to enrich mRNA from the total RNA of each of the 6 treatment and control samples, and then mRNAs were converted to double-stranded cDNA through reverse transcription. Enzyme *Nla*III and *Mme*I were used to digest the cDNA, and Illumina adaptors 1 and 2 were ligated. Linear PCR amplification was performed, and fragments were purified using polyacrylamide gel electrophoresis and then subjected to Illumina sequencing. After sequencing, raw tags were filtered to obtain clean tags by discarding empty tags and low-quality tags (longer or shorter than 21 bp, with unknown sequences, and tags with only one copy number).

Unigene differential expression analysis

Differences in gene expression among different varieties were determined by serially treating samples of different varieties and predicting genes that showed differential expression. Subsequently, these genes were subjected to GO functional and metabolic pathway analyses. The influence of different gene lengths and sequencing level on the calculation of gene expression was eliminated, using the fragments per kilobase per million fragments (FPKM) method (Mortazavi et al., 2008) to determine the expression level of unigenes in every sample.

Statistical analysis for differential expression genes

The statistical method developed by Audic and Claverie (1997) was used to calculate the probability of every unigene. When thousands of hypothetical tests are used, relying on the p-value for an individual test is not sufficient to reduce the rate of false-positive results. Therefore, multiple corrections for each hypothesis need to be applied. False discovery rate (FDR) control is a statistical method used in multiple hypotheses testing to correct the p-values. In practical terms, the FDR is the expected false discovery rate. For example, if 1,000 observations were experimentally predicted to be different, and a maximum FDR for these observations was 0.1, then 100 of these observations would be expected to be false (refer to Benjamini and Yekutieli (2001) for details). After the FDR is obtained, the ratio of FPKMs of two samples can be used

simultaneously.

A small FDR with a large FPKM ratio suggest that the difference in the expression level between the two samples is large. In this study, an FDR of ≤0.001 and a ratio of >2 were used.

Conclusion

Two transcriptomes for *N. tobacco* cv. MSK326 (chilling- and drought-tolerance) and Yunyan203 (chilling- and drought-intolerance) and 14 DGEs of control and chilling/drought stressed of these varieties were sequenced by Illumina Hiseq^{TM} 2000 next-generation sequencing platform. The findings suggest that, although the two tobacco varieties with different stress tolerant levels utilized some similar mechanisms to deal with drought and chilling, the stress-tolerant MSK326 increased its tolerance by (1) regulating membrane fluidity by rationally changing lipid acid saturation and components such as aquaporins; (2) synthesizing more macro-molecule stabilizers such as LEA and dehydrins; (3) reducing ROS production by adjusting photosynthesis components such as chlorophyll a/b-binding proteins as well as rapidly eliminating ROS by activating anti-oxidant enzymes. These mechanisms were almost different in stress-intolerant variety Yunyan203.

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Data archiving statement

All the clean reads have been submitted to the sequence read archive (SRA) at NCBI with accession SRR1258875. The Transcriptome Shotgun Assembly (TSA) project has been deposited at GenBank/EMBL/DDBJ under the accession GBEA00000000. The version described in this paper is the first version, GBEA01000000.

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