Transcriptional responses to drought stress in root and leaf of chickpea seedling

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Abstract Chickpea (Cicer arietinum L.) is an important pulse crop grown mainly in the arid and semi-arid regions of the world. Due to its taxonomic proximity with the model legume Medicago truncatula and its ability to grow in arid soil, chickpea has its unique advantage to understand how plant responds to drought stress. In this study, an oligonucleotide microarray was used for analyzing the transcriptomic profiles of unigenes in leaf and root of chickpea seedling under drought stress, respectively. Microarray data showed that 4,815 differentially expressed unigenes were either ≥2-fold up- or ≤0.5-fold down-regulated in at least one of the five time points during drought stress. 2,623 and 3,969 unigenes were time-dependent differentially expressed in root and leaf, respectively. 110 pathways in two tissues were found to respond to drought stress. Compared to control, 88 and 52 unigenes were expressed only in drought-stressed root and leaf, respectively, while nine unigenes were expressed in both the tissues. 1,922 function-unknown unigenes were found to be remarkably regulated by drought stress. The expression profiles of these time-dependent differentially expressed unigenes were useful in furthering our knowledge of molecular mechanism of plant in response to drought stress.

Keywords Chickpea · Oligonucleotide microarray · Drought · Transcriptional response · Root · Leaf

Introduction

Among abiotic environmental stresses, drought is one of the common adverse environmental conditions that have adverse effects on the growth of plants and the productivity of crops. Plants have adapted to respond to this stress at molecular and cellular levels as well as at physiological and biochemical levels, enhancing them to survive. To date, a large number of drought-induced genes in various plants have been identified, and their expression profiles in response to drought stress also have been revealed. And the products of some of these identified genes have been confirmed to function in drought tolerance or in signal transduction pathway [1–6]. Identification of drought-induced genes in various plants and revealing their expression profiles in response to drought stress have been not only of great interest for understanding their underlying molecular mechanisms in drought tolerance but also of great benefit to the breeding of drought-tolerant crops.

Chickpea is the third most important pulse crop in the world just behind dry bean (Phaseolus vulgaris L.) and field pea (Pisum sativum L.), and its cultivation area has been more than million hectares/year since 2006 year [7].
Chickpea is an annual, self-pollinating, diploid \((2n = 2x = 16)\) plant with a short life cycle of 3–5 months. It has a small genome size of 740 Mbp that is only 1.5 times larger than that of \textit{Medicago truncatula}. Due to its taxonomic proximity with the model legume \textit{M. truncatula} and its ability to grow in the arid and semi-arid regions, chickpea has been found rich in tolerance genes for a range of abiotic stresses such as drought, high salinity and cold, etc. and has been suggested as a model legume crop in study of crop agronomic responses to various stresses [8]. A lot of genes differentially expressed in response to drought stress in chickpea have been identified, and their expressions in response to drought stress have been analyzed. For example, Romo et al. [9] reported that one gene encoding lipid transfer protein (\textit{CapLTP}) and two genes encoding late embryogenesis abundant (\textit{CapLEA-1} and \textit{CapLEA-2}) were induced by water stress. Boominathan et al. [10] identified 101 dehydration-inducible transcripts in chickpea by repetitive rounds of cDNA subtraction. Mantri et al. [11] employed a 768-featured boutique microarray to compare the genes expressed by chickpea in response to drought, cold, high salinity and the fungal pathogen \textit{Ascochyta rabiei} and identified 46, 54, 266 and 51 differentially expressed transcripts, respectively. Molina et al. found that a total of 7,532 UniTags were more than 2.7-fold differentially expressed and 880 were regulated more than 8-fold in chickpea root upon drought stress. By means of constructing two cDNA libraries using the PEG-treated and -nontreated seedling leaves of chickpea [12], Gao et al. [13] have identified 36 up-regulated and 56 down-regulated genes in response to drought stress. Following the introduction of DNA microarray technology, massive changes in gene regulation in various plants can be assayed in a high-throughput manner [14]. DNA microarray analysis for the identification and characterization of large numbers of genes has been becoming one of the most powerful tools for dissecting environmental and developmental responses in plants. To date, some initial cDNA microarrays (or macroarray) have been utilized in study of drought tolerance of chickpea [10, 11, 15–18]. However, since their cDNA microarray scales were all very small (no more than 1,000 cDNA), even a part of their ESTs coming from grasspea and lentil, these researches’ information quantity and accuracy were very limited. Further more, there were no researches investigating gene expression profiles in root and leaf of chickpea at the same time to date [10, 11, 15, 16]. Therefore, the transcriptional profiling of genes differentially expressed in root and leaf of chickpea in response to drought stress has not been completely unveiled yet. In order to globally understand the molecular mechanism of chickpea in drought tolerance, it is necessary to detect the differential expressions of a variety of genes in chickpea in response to drought stress on a larger scale DNA microarray. To October of 2009, 34,177 ESTs of chickpea were available in the NCBI EST database (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), and this offered the condition for constructing a larger scale DNA microarray.

In this work, we constructed a 6,164 oligonucleotide spotted microarray from 36,301 ESTs and 283 nucleotide sequences of chickpea. And this microarray was further employed to investigate the transcriptomic profiles of unigenes expressed in leaf and root of chickpea seedling under drought stress, respectively. Our results will provide insights into the expression profiles of the time-dependent differentially expressed unigenes in root and leaf of chickpea seedling under drought stress, and make a significant contribution to the understanding of the molecular mechanisms developed by the plant against drought stress.

**Materials and methods**

Plant growth and stress treatments

Seeds from a drought-tolerant chickpea cultivar Xj-209 were germinated in quartz sand in a growth chamber with a day/night cycle of 14/10 h at 28/20 °C, as described by Gao et al. [13]. When grown for 10 days, the seedlings were carefully transferred to one-off cups with water. 24 h later, the seedlings were separated into two groups: one group was exposed to 60 mM PEG 4000 as drought treatment, and the other was harvested immediately as control (0 h).

RNA isolation and preparation of fluorescent dye-labeled cDNA

For drought treated group, roots and leaves of chickpea seedlings were harvested separately at 0.5, 1.5, 6, 12, and 24 h during drought stress treatment, frozen in liquid nitrogen, and kept at −80 °C until use. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy mini spin column Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Residual genomic DNA was digested by RNasefree DNase I (Takara). cDNA was synthesized, linearly amplified and labeled using the cRNA Amplification and Labeling Kit (CapitalBio). In brief, total RNA was reverse transcribed with Cbcscript reverse transcriptase and T7 oligo dT primer. 2nd-strand cDNA was synthesized by DNA polymerase. The double-stranded cDNA was in vitro transcribed into cRNA and purified by Nucleospin RNA Clean-up kit (Macherey-Nagel, Germany). The resulting cRNA was reverse transcribed by MMLV and purified with PCR Nucleospin Extract II kit (Macherey-Nagel, Germany).
Germany). After reverse transcription, the cDNA was labeled by Cy5-dCTP or Cy3-dCTP (GE Healthcare, USA) with klenow enzyme at 37 °C for 60 min. The labeled cDNA was purified with PCR Nucleospin Extract II kit (Macherey-Nagel, Germany).

EST collection and annotation

All the ESTs and nucleotide sequences of chickpea in GenBank (to October of 2009) were downloaded. Vector sequences were screened against UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The resulting high quality sequences (>100 bp) were then assembled using Phrap software (http://www.phrap.org/). Default settings were used except 40 bp minimum overlap and 99% identity [19]. Assembled contigs and singletons (called clusters) were manually revised by Consed software (http://www.phrap.org/) [20]. All clusters were compared with the NCBI nucleotide (nt) and non-redundant protein (nr) databases by BLASTN (E-values ≤ 1 × 10^-10) and BLASTX (E-values ≤ 1 × 10^-3), respectively. The standards of exact choice of the most related entry in each group of alignments depended not only on the best hit values but also on the information of matched sequences in detail. All assembled sequences having the same annotation were further clustered into a unigene. In total, 6,164 unigenes were used in designing the oligonucleotide microarray. For each unigene, oligonucleotides were designed using the Agilent eArray Ver. 4.5 with the length of 60 bases.

Based on Gene Ontology (GO) classification, all unigenes of chickpea were analyzed for their functional characteristics under drought stressed and controlled conditions.

Using the single-directional best hit (SBH) method as recommended by the KEGG Automatic Annotation Server (KAAS) [21] for sets of ESTs allowed us to assign unigene pathway annotations.

Microarray hybridizations and data analysis

Chickpea oligonucleotide microarray containing 6,164 chickpea oligonucleotide probes (supplementary Table 1) was used. The gene expression profiles in root and leaf after 0.5, 1.5, 6, 12, and 24 h drought stress and the corresponding non-stressed controls (0 h) were investigated by microarray analysis.

Each test sample was Cy5-labeled and the reference samples Cy3 labeled. The arrays were hybridized at 42 °C for 12–16 h in BioMixer II (CapitalBio). After hybridization, the slides were washed with 2× SSC plus 0.2% SDS at 42 °C for 5 min, with 0.2× SSC for 5 min at room temperature and spin-dried. Hybridized microarray slides were scanned with a LuxScan 10 K/A scanner (CapitalBio). Spot intensities were quantified using GenePix Pro 4.0 image analysis software (Axon Instruments). Data with signal intensity more than 1,200 were accepted as the standard for gene expression. Unigenes up- or down-regulated greater than a 2-fold or less than 0.5-fold ratio value (Cy5 intensity/Cy3 intensity) were taken as differentially expressed. Microarray expression data were MIAME compliant and have been deposited in a MIAME compliant database (GEO accession number GSE25705).

To point out the time course in the expression data, a further evaluation was based on clustering the time-dependent expression profiles of these significantly differentially expressed unigenes with an expression-level alteration criterion of ratio value(n time point)/ratio value(n-1 time point) ≥ 2 or ratio value(n time point)/ratio value(n-1 time point) ≤ 0.5 at n time point (0.5, 1.5, 12, and 24 h) during drought stress, via self organizing maps (SOM) module of the Multieperiment Viewer ver. 4.2 software (MeV, www.tm4.org).

Two independent biological replicates for each time point of two tissues were used to hybridize. The processes of labeling, hybridization, washing, and scanning were carried out at Beijing National Biochip Research and Engineering Center, China.

Quantitative real-time PCR and data analysis

To validate the array data, the expressions of 10 genes (Contig_1885: proline dehydrogenase; Contig_3134: lipoxygenase LOXN3; Contig_2853: peroxidase1B; Contig_5876: nitrate transporter; Contig_3259: homocysteine S-methyltransferase; Contig_1500: Photosystem I reaction center subunit N, chloroplast precursor; Contig_2780: beta-amylase; Contig_2823: glycine-rich protein 1; Contig_2895: glutathione peroxidase 1; Contig_1672: alanine: glyoxylate aminotransferase) were confirmed by qPCR with the same RNA samples used for microarray hybridization. The primers used for qPCR are listed in supplementary Table 2.

The cDNA from the total RNA samples, which were used for the microarrays at different time points (0, 0.5, 1.5, 6, 12, and 24 h) during PEG-treatment, was synthesized with M-MLV Reverse Transcriptase (Invitrogen), respectively. qPCR was performed using SYBR premix EX Taq (TaKaRa Biotech, Dalian, China) in the Bio-RAD iCycler iQ5 Machine. The actin gene (GenBank accession no. AJ012685) was assigned for control. All reactions were performed in triplicate. Expressed ratio was calculated using the Livak method [22]:

Expressed ratio = 2^-ΔΔC_T

where ΔΔC_T is the sum of: [C_T(gene) - C_T(actin)] (Drought stressed) - [C_T(gene) - C_T(actin)] (Unstressed).
For statistics, an analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

Results

Characterization of EST sequences

To capture the genes induced by drought stress in chickpea as many as possible, not only all the 34,177 EST sequences of chickpea in GenBank [supplementary Text 1, including our previously submitted 5,097 ESTs (GenBank accession no. FE668437–FE673533)] but also 283 nucleotide sequences (supplementary Text 2) were downloaded in October, 2009. After removing vector sequences and lower quality sequences, 36,301 ESTs (including our recently submitted 2,124 ESTs, GenBank accession no. HS107522–HS109645) resulted in the identification of 11,042 unigenes (7,770 singletons and 3,372 clusters). All unigenes were compared with the NCBI nucleotide (nt) and non-redundant protein (nr) databases by BLASTN (E-values $\leq 1 \times 10^{-10}$) and BLASTX (E-values $\leq 1 \times 10^{-5}$), respectively. The standards of exact choice of the most related entry in each group of alignments depended not only on the best hit values but also on the information of matched sequences in detail. In the case of the unigenes corresponded to different regions of the same gene, we kept the unigene with the longest sequence. The unigenes having annotation of non-plant genes were also taken out. Consequently, a total of 6,164 unigenes were obtained. The entire data, including all annotative attributes, are shown in supplementary Table 3. In this data, only 3,680 unigenes had specific function, while the others 2,484 unigenes showed similarities to proteins with function completely unknown or even having no homology.

The 6,164 unigenes were further analyzed by the GO assignments [23]. As a result, 4,674 unigenes (15,296 GO terms) were classified into three broad categories, namely, biological processes, cellular components and molecular functions (Fig. 1). The detailed GO assignment results are listed in supplementary Table 4.

Overall features of the drought stress-responsive expression profiles

To identify the differential expression of the genes induced by drought stress in root and leaf of chickpea seedling at different time points, an oligonucleotide microarray containing 6,164 unigenes was fabricated. RNA samples from the roots and leaves collected at 0.5, 1.5, 6, 12, and 24 h during drought treatment, as well as from that of the untreated sample at 0 h, were used for microarray hybridization, respectively.

The biological significance of transcriptional profiling can be revealed by grouping of differentially expressed genes. In this study, of 6,164 unigenes, there were 247 unexpressed at all six time points in root, while 320 were unexpressed in leaf. Nine unigenes were found to be unexpressed in both the tissues (Fig. 2; supplementary Table 5). Further, among the 6,155 expressed unigenes, only 4,815 were detected to be differentially expressed with expression ratio of $\geq 2$-fold up- or $\leq 0.5$-fold down-regulation at one or more time points; among these differentially expressed unigenes, 3,315 and 4,059 unigenes were differentially expressed at least one time point in root and leaf, respectively, while there were 2,559 differentially

![Fig. 1 The GO categories of 4,674 unigenes. The genes were functionally categorized according to the Gene Ontology Consortium and two levels of the assignment results were plotted here. In this ontology, “biological process”, “cellular component” and “molecular function” are categorized independently](image-url)
expressed in both the tissues (Fig. 3A; supplementary Table 6). Under the stricter criterion (expression ratio of ≥5-fold up- or ≤0.2-fold down-regulation), only 1,957 differentially expressed unigenes were identified at one or more time points. Among them, 1,003 and 1,480 unigenes were identified in at least one time point in root and leaf, respectively, while there were 526 differentially expressed in both the tissues (Fig. 3B; supplementary Table 7).

Changes in number of the unigenes differentially expressed in root and leaf of chickpea seedling over stress time are shown in Fig. 4 and supplementary Table 8. These diagrams provided an important overview showing the distribution of number of differentially expressed unigenes under different criteria into shared and tissue-specific responses. In the present study, whether under the lower or stricter criterion, the amount of the unigenes differentially expressed in leaf was first increased and then decreased over stress time, with the maximum occurring at 12 h, while the number of the unigenes differentially expressed in root showed the pattern of “increase–decrease–increase”, with the maximum occurring at 24 h (Fig. 4A, B; supplementary Tables 8, 9). The change in number of the unigenes differentially expressed in both leaf and root over time exhibited the same pattern as that in leaf. Compared to the root, the leaf was characterized by a relatively larger number of differentially expressed unigenes at all the investigated time points except at 0.5 and 6 h under the lower criterion (Fig. 4A, B; supplementary Tables 8, 9). Furthermore, whether in root or in leaf, the amount of the up-regulated differentially expressed unigenes were always less than that of the corresponding down-regulated ones at all the investigated time points except at 1.5 h in both the tissues and at 6 h in root under the stricter criterion. This situation also occurred in the unigenes differentially expressed in both the tissues (Fig. 4A, B; supplementary Table 8, 9). Additionally, a lot of differentially expressed unigenes were found to be always up- or down-regulated in one tissue or in both the tissues at all the investigated time points (Fig. 4C; supplementary Table 10), implying that these unigenes constantly play important roles in response to drought stress in root and leaf of chickpea seedling. It was noticeable that the number of these unigenes differentially expressed in leaf were much smaller than that in root under both the criteria (Fig. 4C), different from the results in Fig. 4A, B; moreover, whether in root or in leaf, the amount of these up-regulated differentially expressed unigenes were always less than that of the corresponding down-regulated ones.

Under the lower criterion, 10 differentially expressed unigenes were found to be always up-regulated during drought stress in both the tissues (Fig. 4C; supplementary Table 10). Of them, only two had specific function annotation. These two unigenes encoded Calcineurin B-like (CBL) interacting protein kinase (CIPK) (Contig_2886) and early light inducible protein (ELIP) (Contig_7860), respectively. One of the three important families of Ca²⁺ sensor proteins is referred to as CBL proteins. CBLs have been identified to specifically target a group of sucrose non-fermenting-related serine/threonine kinases (SnRK3), named CBL-interacting protein kinases (CIPK), to mediate the sensed calcium signal [24]. A role for ELIP in resistance to drought stress is indicated by several studies. Expression of an ELIP-like gene in Craterostigma plantagineum, a plant that can survive and recover from extreme dryness, is influenced by abscisic acid, light levels and drought. Constitutive expression of the ELIP gene in Helianthus annuus confers drought tolerance [25, 26]. And two ELIP genes in gametophytes of the moss Tortula ruralis were found to be up-regulated in response to...
desiccation, salt, high light, and rehydration stresses [27]. Contrarily, up to 69 differentially expressed unigenes were found maintaining down-regulation in both the tissues during drought stress (Fig. 4C; supplementary Table 10). Of them, only less than 50% (29 of 69) unigenes had unknown function annotation.

Whereas under the stricter criterion, only one differentially expressed unigene (Contig_3686) was found to be always down-regulated during drought stress in both the tissues, while there was no unigene showing up-regulation.

Time course analysis of differentially expressed unigenes induced by drought stress using SOM

In this study, 2,623 and 3,969 unigenes were time-dependent differentially expressed in root and leaf, respectively. To examine their changes in gene expression, the 2,623 and 3,969 differentially expressed unigenes were divided into 16 typical profiles via SOM clustering (Figs. 5, 6). The complete list of the 2,623 and 3,969 unigenes are shown in supplementary Tables 11 and 12.

For root (Fig. 5), the 2,623 differentially expressed unigenes were merged into three major groups: up-regulated (R1, R9, R11, R12, and R13), down-regulated (R2, R3, R5, R6, R7, R8, and R10) and fluctuating (R4, R14, R15, and R16). In the up-regulated group, the signal intensity of the unigenes in R1 was continuously increased during the investigated period, with a rapid increase period at 0.5–6 h; the unigenes in R9 and R13 had a rapidly increasing trend in signal intensity at 0–6 h followed by slight decreasing; and the signal intensity of the unigenes in R11 and R12 was maintained unchanged at early stage and increased thereafter, with a rapidly increasing period at mid-stage. In the down-regulated group, the signal intensity of the unigenes in R2, R7, and R8 was gradually decreased at 0–6 h and maintained unchanged thereafter; the unigenes in R3 and R6 had a similar decreasing trend in signal intensity after 0.5 h; the unigene expression in R5 was first decreased rapidly, and then increased at 0.5–1.5 h and maintained unchanged at 1.5–24 h; the unigenes in R10 were maintained almost unchanged in signal intensity at 0–6 h, and then rapidly decreased at 6–12 h and rapidly increased at 12–24 h. It was notable, in the fluctuating group, that the signal intensity of the unigenes in R14 showed an expression pattern completely contrary to that in R16.
For leaf (Fig. 6), the 3,969 differentially expressed unigenes were merged into three major groups: up-regulated (L2, L3, L5, L6, and L12), down-regulated (L1, L8, L13, L14, and L15) and fluctuating (L4, L7, L9, L10, L11, and L16). In the up-regulated group, the unigenes in L3 and L6 had a similar expression trend, whereas L2 and L5 had a similar expression trend; the unigenes in L12 showed a rapidly increasing expression in signal intensity at 0.5–1.5 h and a gradually decreasing expression thereafter. In the down-regulated group, the unigene expression in L1, L8, and L13 had a similar decreasing pattern; the unigenes in L14 was maintained unchanged for 0.5 h and then gradually decreased; in the case of the unigenes in L15, slight fluctuating in signal intensity was observed. In the fluctuating group, L4, L7, and L16 showed the strongest fluctuation in unigene expression while L10 had the slightest fluctuation.

Pathways in response to drought stress in root and leaf

To examine the changes of pathways in response to drought stress, the 6,164 differentially expressed unigenes of chickpea were annotated in KAAS. As a result, 113 pathways were obtained. These pathways can be classified into six groups based on their expression profiles in root and leaf (supplementary Table 13). In group I, 89 pathways with up- and down-regulated unigenes were involved in both the tissues. Among them, 75.3 % belonged to metabolism pathways including amino acid metabolism, carbohydrate metabolism, energy metabolism and lipid metabolism. In group II, 11 pathways with up-regulated unigenes were involved in both root and leaf. Contrarily, six pathways with down-regulated unigenes were involved in both root and leaf and classified into group III. Three pathways with down-regulated unigenes and one pathway with up-regulated ones were involved only in leaf and classified into group IV and group V, respectively (supplementary Table 13). In addition, three pathways with no significantly differentially expressed unigenes either in root or leaf were classified into group VI.

Unigenes unique and shared in root and leaf in response to drought stress

In this work, compared to the control, total 88 unigenes were found to be expressed only in drought-stressed root. Of them, only 18 unigenes were found to be unique for root, and the rest were also expressed in leaf (supplementary Table 14). Total 52 unigenes were found to be expressed only in drought-stressed leaf. Among them, only
13 unigenes were unique for leaf, and the rest were also expressed in root (supplementary Table 15). Nine unigenes were found to be expressed only in both the stressed tissues (supplementary Table 16).

Quantitative real time PCR analysis

To confirm the microarray results, we selected 10 unigenes that showed significant up- or down-regulation during drought stress, and analyzed their differential expressions by qPCR with the same RNA samples used for the microarray experiment. As shown in Fig. 7, some genes displayed significant higher fold-induction at certain time point in the microarray analysis, such as Contig_1672 at 12 and 24 h in root. On the contrary, some genes displayed significant higher fold-induction at certain time point in the qPCR analysis, such as Contig_5876 at 6 h in leaf. These differences probably reflected the different dynamic range of microarray compared to qPCR analysis. Though these differences existed, however, the qPCR results and microarray data showed similar expression kinetic patterns (up- or down-regulation) for all the unigenes tested, indicating the strong reliability of the microarray data. In addition, the qPCR results of CarNAC1 (Contig_3143) and CarNAC3 (Contig_795) in our previous research were also in agreement with those of the microarray analysis in this study [28, 29].

Discussion

The advent of microarrays has enabled the screening of thousands of genes in parallel to assist in candidate gene identification. Ideally, one would like to scan the entire genome of a particular plant to obtain a more complete picture of transcriptional changes in response to various stresses. However, the whole genome sequences are not available for chickpea to date, leading to a dependence on collections of ESTs assembled from random cDNA libraries [11]. In this study, to construct an oligonucleotide microarray, we collected all ESTs and nucleotide sequences of chickpea in GenBank (to October 2009). This collection contained important ESTs of chickpea related to physiological acclimation and adaptation to biotic or abiotic stresses, such as 592 ESTs from chickpea infected by Ascochyta rabiei [30, 31], 6,273 related to immune-response of chickpea to Fusarium oxysporum [32], 24,419 related to salinity and drought stresses [15, 33]. Especially, this collection included 7,221 ESTs from two cDNA libraries constructed using the PEG-treated and -nontreated...
seedling leaves of chickpea by our research group [13]. As we expected, most genes, which have been identified to relate to drought stress by other researchers, were also observed in our result. Moreover, their up- or down-regulated expression patterns in this study were similar to those in other researchers’ investigations in response to drought stress. For example, the expression of two genes encoding ribosomal protein S2 and fructose-1,6-bisphosphatase in leaf in this study (Contig_2767, Contig_9775) showed the same down-regulation pattern with that by Romo et al. [9]
In the present study, 4,815 unigenes and 110 pathways significantly responded to drought stress. Some of them could be used as candidates for further study of drought tolerance. As far as we know, some unigenes in chickpea and other plants or crops have been extensively investigated by previous researchers, such as genes encoding LEA proteins, water channel proteins, various transcription factors (DREB, AREB, MYC, MYB, bZIP, and NAC, etc.), protein kinases, protein phosphatases, sugars (galactinol, and trehalose, etc.), amino acids (proline, methionine, and glycine, etc.), pathogenesis related proteins, and so on [3, 36–38]. However, many unigenes (such as genes encoding germin-like protein [39] and F-box protein [40]) and pathways (such as “Stilbenoid, diarylheptanoid and gingerol biosynthesis” [PATH: ko00945]) that responded to drought stress in chickpea were still poorly investigated. To select the genes and pathways that are candidates for drought tolerance, we should focus on the genes that were particularly high up- or down-regulated in response to drought stress and the pathways they involved in. Supplementary Table 17 listed 183 unigenes that were particularly up- or down-regulated (≥50-fold) in root or leaf under drought stress. Of the 183 unigenes, 110 had specific function annotation. The proteins encoded by these unigenes included ten transcription factors (9.1 % of 110 unigenes), nine LEA proteins (8.2 % of 110 unigenes), six heat shock proteins (5.5 % of 110 unigenes), five transporters (4.6 % of 110 unigenes), and so on. It is noteworthy that all genes encoding the heat shock proteins and LEA proteins showed to be up-regulated in leaf at 1.5–24 h of drought stress. Supplementary Table 18 showed the 17 pathways in which the 183 unigenes involved. These results would be helpful for researchers in the study of chickpea drought tolerance.

Through long-term evolution and adaptation to extreme conditions, chickpea has been found to be rich in resistance genes for a range of abiotic stresses, including drought, cold and high salinity [41]. Our study showed that a high proportion (1,922 unigenes, 39.92 %) of differentially expressed unigenes had no function annotation or function unknown. It is also noteworthy that many of these unigenes showed remarkable expression changes in response to drought stress, especially with some having a rapid increasing or decreasing expression. We daringly speculated that some of these differentially expressed unigenes might be specific for chickpea and play very important roles in response to drought stress. Therefore, it is possible to identify some drought responsive genes unique to chickpea. Further analysis of the functions and expression controlling mechanism of these genes in chickpea would not only supply the opportunity of isolation and identification of novel genes, but also enhance our further understanding of the diversity of responses to drought stress among plant organs, and more importantly, will benefit the investigation of mechanism of drought tolerance in chickpea.

In this study, the number of differentially expressed unigenes in chickpea root was almost always less than that in leaf at each investigation time point (Fig. 4A, B; supplementary Tables 8, 9), indicating that the biological processes involved in drought tolerance in chickpea leaf was more extensive and active than these in root. In addition, there were only nine unigenes with expression ratio of ≥50-fold up-regulation in root, while 44 unigenes in leaf (supplementary Tables 8, 9). It is noteworthy that the highest fold of the up-regulated unigenes in root (Contig 5431: unknown [G. max], 64.87-fold up-regulation at 24 h) was much lower than that in leaf (Contig 1183: PREDICTED: similar to Os12g0187800 [Vitis vinifera], 1048.80-fold up-regulation at 1.5 h). Os12g0187800 is a conserved hypothetical protein identified in rice [35]. This is the first evidence that Os12g0187800 may play an important role in plant drought tolerance, but more detailed expression, localization and functional analyses are needed.
understanding of specific mechanism of drought tolerance in chickpea.

**Acknowledgments** We gratefully acknowledge the partial financial support from the projects supported by the National Natural Science Foundation of China (30360201, 30960206, 30860152 and 31163036), from the project supported by the Xinjiang Science and Technology Department of China (2009K11254), from the projects supported by the National Science and Technology Ministry (2006BAD09A04, 2006BAD09A08), from the project supported by the National Science Foundation for Postdoctoral Scientists of China (20080431107), from the project supported by the Jiangsu Science Foundation for Postdoctoral Scientists of China (0801048B) for this research.

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