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ORIGINAL PAPER

Identification of differentially expressed genes preferably related to drought response in pigeon pea (*Cajanus cajan*) inoculated by arbuscular mycorrhizae fungi (AMF)

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Abstract Pigeon pea is an ideal crop for sustainable agriculture systems in Karst areas of southwest China, which frequently suffers from the formidable water deficit. Physiologically, arbuscular mycorrhizae (AM)-colonized pigeon pea (*Cajanus cajan*) demonstrated a further enhanced tolerance to drought stress. To elucidate the molecular mechanism underlying the elevated tolerance, suppression subtractive hybridization (SSH) were employed to dig up the differentially expressed genes using mixed cDNAs prepared from drought-stressed and unstressed pigeon pea seedlings inoculated by AM fungi (AMF) in the present work. Both forward and reverse SSH cDNA library were constructed and a total of 768 clones were obtained. Dot-blotting expression analysis identified that 142 clones were upregulated, and 49 were downregulated during water stress. After sequencing, 182 unique expressed sequence tags (ESTs) were obtained via blast analysis, among which 142 (78%) exhibited high homology to previously identified or putative proteins, however, 40 (22%) showed no homology in the database. The upregulated (102) and downregulated (40) ESTs with significant protein homology might be sorted into 16 and 12 functional categories respectively, which involved in a broad spectrum of biological pathways. Furthermore, semi-quantitative reverse transcription (RT)-PCR was carried out for the 35 differentially expressed genes whose putative functions implicated in abiotic stress tolerances in other species, and it was verified these differentially expressed genes highly involved in drought stress tolerance of AM-colonized pigeon pea.

Keywords Arbuscular mycorrhizae · Differentially expressed genes · Drought stress · Pigeon pea (*Cajanus cajan*) · Suppression subtractive hybridization (SSH)

Abbreviations

AM	Arbuscular mycorrhizae
AMF	AM fungi
FWC	Field water capacity
GM	*Glomus mosseea*
MDA	Malondialdehyde
PAR	Photosynthetic active radiation
RT	Reverse transcription
SSH	Suppression subtractive hybridization

Introduction

Coping with plant environment stress is the foundation of sustainable agriculture. Of all abiotic stresses, water deficit is regarded as the most adverse environmental factors limiting plant growth and crop productivity (Shinozaki et al. 2003; Bartels and Sunkar 2005; Gosal et al. 2009). Plants may actively respond and adapt to this stress through

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a series of morphological, physiological, cellular, and molecular processes (Reddy et al. 2004). These responses include stomatal closure, repression of cell growth and photosynthesis, activation of respiration, as well as accumulation of osmolytes and proteins specifically involved in stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2007; Nakashima et al. 2009; Saibo et al. 2009), among which the molecular mechanism had been the major subject of many studies in the past decade (Bartels and Sunkar 2005; Xiao et al. 2007; Nakashima et al. 2009). Various genes are involved in water stress response in plants. The products of these genes function both in the stress response and in the establishment of plant stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2007). The elucidation of molecular basis of plant responses to water stress might provide new strategies to improve the drought tolerance of agriculturally important plants (Xiong et al. 2002; Zheng et al. 2004; Rahaie et al. 2010). Up to date, a number of genes with diverse functions that responded to drought at the transcriptional level had been described in many annual plants (Shinozaki et al. 2003; Zheng et al. 2004; Wang et al. 2007), however, fewer cases had been obtained from trees (Liu et al. 2009). The existence of a variety of drought-inducible genes suggested that the responses of plants to water stress were extensively sophisticated.

Pigeon pea (*Cajanus cajan*), a perennial tree of the family Fabaceae, is an important grain legume of the semi-arid tropics and sub-tropics and is a hardy, widely adapted and drought-tolerant crop, which allow its cultivation in a wide range of environments and cropping systems (Varshney et al. 2011). Although compared with other grain legumes, pigeon pea ranks only the sixth in area and production; it is used in more diverse ways than others (Wu et al. 2009; Domoguen et al. 2010). In addition to its main use as protein-rich food, its immature green seeds and pods are also consumed fresh as a green vegetable (Ritchie et al. 2000). The crushed dry seeds are fed to animals while the green leaves form a quality fodder. The plant of pigeon pea also replenishes soil nutrients and controls soil erosion (ICRISAT 1998). With so many benefits at low cost, pigeon pea had become an ideal crop for sustainable agriculture systems in Karst areas of southwest China, where frequently suffers from the formidable water deficiency (Li et al. 2001; Gong et al. 2005).

An arbuscular mycorrhizae (AM) is a type of mycorrhiza in which the fungus penetrates the cortical cells of the roots of a vascular plant. The colonized plant is better nourished and better adapted to its environment. Recent evidence suggested that colonization of root systems by AM fungi (AMF) obviously afford host plants greater resistance to environmental stresses like drought stress (Augé 2001, 2004; Wu and Xia 2006). In our previous investigation, AMF colonization might substantially elevate the tolerance to drought of pigeon pea via cumulative effects (Qiao et al. 2011). However, in recent years, little has been known regarding the molecular mechanism of AMF colonization enhanced plant water deficit tolerance. Porcel et al. (2006) showed that AM plants responded to drought stress by down-regulating the expression of the plasma membrane genes and anticipating its down-regulation as compared to non-AM plants.

To isolate the drought-responded genes, as well as to unravel the molecular mechanism that AMF colonization further enhanced drought tolerance of pigeon pea plants, in the present study, suppression subtractive hybridization (SSH) and semi-quantitative reverse transcription (RT)-PCR strategies were used to identify differential expression genes implicating in drought stress from young pigeon pea seedlings inoculated by AMF in exposure to water deficit conditions.

Materials and methods

Plant material and stress treatment

Seeds of pigeon pea (cv. Guimu 1) that obtained from Guangxi Academy of Agricultural Sciences were surface sterilized in 0.1% (w/v) mercuric chloride for 5 min and washed five times (2 min each) in distilled water, then precultured at 30°C for 24 h in incubator. Subsequently, the seeds were germinated in plastic pots (15 cm × 12 cm) containing either sterilized loam (ca. 1,000 g) collected from Karst area alone (non-AM) or sterilized loam inoculated with 50 g *Glomus mosseea* (GM, one of the common AMF) inoculum, which was purchased from the Plant Nutrition and Resource Research Institute, Beijing Academy of Agriculture and Forestry (China). All seedlings were grown under 70–80% (w/w) of field water capacity (FWC) in the greenhouse with a maximum photosynthetic active radiation (PAR) of 1,200 μmol m^{-2} s^{-1} and temperature of 25 ± 2°C before experiment treatment. After 90 days of growth, the colonization status was detected using magnified intersection method (Mcgonigle et al. 1990). Then, both AM-colonized (AMD) and non-AM (NAMD) seedlings were randomly chosen to carry out drought stress, whose water content of soil was kept around 50% FWC. The unstressed plants, both mycorrhizal (AMC) and non-mycorrhizal (NAMC), were watered daily to maintain 70–80% FWC based on the loss of soil weight.

Isolation of total RNA and mRNA

Young leaves were harvested separately at the 5th, 10th, 15th, 20th, 25th and 30th day after stress treatment, and

kept at −80°C after being frozen in liquid nitrogen. Total RNA was extracted using PlantRNA reagent (Tiangen Biotech, China) according to the manufacturer's protocol. The quantity and quality of isolated total RNA was examined by spectrophotometry and gel electrophoresis, respectively. Equal amount of total RNA from the six time points were mixed for each four treatment (AMC, AMD, NAMC and NAMD), and the mRNA was purified from the mixed RNA using Oligotex^{TM} mRNA Midi Kit (Qiagen, USA) based on the manufacturer's instructions.

Suppressive subtraction hybridization

SSH was performed using the PCR-Select cDNA Subtraction Kit (Clontech, USA) followed the manufacture's instructions. The first-strand cDNA synthesis was carried out using 2 μg of the mixed mRNA which mentioned above. The forward library' tester was made using mRNA from the AMD, and the driver was prepared using that from the AMC. The final PCR product was designated as subtracted cDNA-forward, which was enriched in genes upregulated in the stressed sample. Similarly, the reverse subtraction library was constructed except that the mRNA used for the tester and driver was reversed, and the obtained genes were downregulated on stress. Actin gene (Qiao and Wen 2010) was amplified for 18, 23, 28, and 33 cycles to test the subtraction efficiency of the library before cloning.

Construction of the subtracted cDNA library

Products of the final PCR from both forward and reverse subtraction were purified using QIAquick PCR Purification Kit (Qiagen, Germany). The purified products were then ligated into a *pMD-18T* vector (Takara Biotechnology, China) to transform *Escherichia coli* DH-5α cells. Recombinant white colonies were selected and the length of inserted segments in the library was determined by PCR using nested primers. PCR products were analyzed by electrophoresis on 1.0% (w/v) agarose gel to confirm the amplification quality and quantity.

Dot-blotting analysis

In order to screen positive clones, dot blotting was performed. The final PCR products were denatured by incubated at 95°C for 5 min and then cooled on ice immediately. Two microlitres of denatured PCR products were pipetted into the Hybond-N^{+} nylon membrane in a 96-well format and fixed by baking at 80°C for 2 h. Two forward-subtracted membranes were hybridized with two subtracted cDNA probes (forward- and reverse-subtracted), so were the two reverse-subtracted membranes. The probes were labeled with ^{32}P α-dATP using Strip-EZ DNA Kit (Ambion, USA). Then hybridization and washing were carried out according to the manufacturer's instructions. Result analysis and classification of differentially screened clones were performed according to the protocol recommended.

Sequencing and sequence analysis

Differentially expressed clones were sequenced by Shanghai Oebiotech in China. BLASTX of these sequences against protein database was performed at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The functional categorization of sequence was carried out based on the AMIGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) and MIP functional catalog (http://mips.gsf.de/proj/funcatDB/search_main_frame.html).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was employed to characterize 35 drought-induced ESTs, which probably implicated to abiotic stress tolerances based on the sequence analysis. In order to identify the contributions and genes affected by the presence of AMF to increased drought tolerance in pigeon pea, the NAMD and NAMC were used as reference. The actin gene was used as an internal standard. The mixed total RNA was reverse transcribed by M-MLV RTase. Then 0.5 μL of first strand cDNA were adopted as templates for PCR in 10 μL reaction volume. The parameters used for semi-quantitative RT-PCR analysis were listed in Table 1. Each semi-quantitative PCR was repeated at least twice. The PCR products were separated on 1% (w/v) agarose gel. Images of the electrophoresis gels were captured using BioRad Quantity One software, and the intensity of bands was quantified using Band Leader software (Magnitec) and normalized against actin gene band intensity. Expression profiles of these ESTs were also analyzed by the hierarchical average linkage clustering and *k* mean clustering in the Genesis software (Sturn et al. 2002).

Results

Qualitative analysis of RNA

Using the RNA extraction kit described above, total RNA was isolated from leaves of both stressed- and unstressed AM-colonized seedling. Electrophoresis of the extracted RNA on agarose gel showed distinct 28 s rRNA band which was more abundant than the 18 s rRNA band,

Table 1 Parameters used for semi-quantitative RT-PCR analysis

Gene[a]	Primer sequence		Annealing temperature (°C)	Cycles[b]	Product size (bp)
	Forward (5′–3′)	Reverse (5′–3′)			
ACT	AACCCAAAGGTCAAC GAG	TGAGCCACCACTAAGAACAA	55.0	30	574
AMT	GGTGTTGCTCGTCGTT	TTCCGCCTACAGTTGAT	59.5	28	164
AP2	GATACGCCGTCGCTTTC	CCCATCTTCCCGTCCTT	60.0	28	193
ABP	TGCTG GTTGG GAGTT AC	AGAAT GATGC GGAGG AA	59.0	32	181
CAIIB	CCTTGGGTTTCATTTAC	ATGCTTTCAGGTGGTG	54.4	30	308
CBP	CCGTGATGTGAATGG	TCTACCCGAAGGAAA	54.0	30	136
CDI	CGGAT AAGGA ATGAC GA	CCACC AGCAC TGATG AC	59.0	32	324
CK	CTCCCTCCATTATCTC	TCAAGTGGGTTCTCAT	50.0	34	178
CP	GGGGTATCATCTCACTCAA	GAAAGGCGGACAGGTAT	57.3	30	287
CPII	TAGTCTACCACCTCAGTCC	GCACAAACATCCTCGT	57.3	30	142
CYTB	GGACGCCAGACATTAGACG	CAAGCAGAGCCTCAGAACC	62.0	28	159
DTC	GCTCCATTGGTGTTTA	GTCTATGCCTTGAACG	54.3	26	377
FBP	CAAAT CTGGC AACCG	CGTCG CCTTT ACGC	56.0	28	268
GATA	TGTGATGTATCGGTTTG	GAAGATGAGTGGGAGTG	54.4	28	124
GDH	GTGGGATGAGGAGAA	GAAGAGTTGCCGAGA	53.0	30	223
GPAT	GTTCC CTCTG TTCCT G	CCAAA TTGTT AGACC CT	56.0	28	220
GST	AGTCCTTGCTCCATTT	CCTATCTTGCCTTCTG	50.8	32	316
H4	GTGCT CGGTG TAGGT GA	TGAAG ATGTC TGGTC GTG	59.0	28	233
HSP	ATCTACCCTTATCGTTGG	ATCCTGATGAGGCTGTG	58.6	28	438
LBP	GGGCAAGCATAGGAA	AAAGTTGGTGCGTTGA	56.4	28	357
LHCB	GCCTGAACGAAGAACCC	CCGACCCAATCTACCCT	57.0	26	139
MAP	TCTAACTCGGACCTCT	TTGGCAATAGCACA	52.5	34	316
MIP	TTGTTAGCACGGTCTC	TCACTGGTGGTGTTATT	55.0	30	357
OEE	GTTGCTGCTGGTTTGG	ATTCCTTTGCCCTTTGT	57.3	30	208
PDDA	AAAGTGACGCAACCAT	TTGAGAAAGAGCAGGAT	54.4	32	140
PG	ATGGT GTAGC CTTAT CTG	TTCTG TTTGT GCCTT TA	52.0	30	213
PGO	ATTTA TCAAC CCTCC C	AGCCA CCTCT TCAAC AC	60.5	28	499
PP2C	AATGG CGACG AACCT	CGCAG TGAGC AGCAA	59.0	28	276
PSS	ACTGCGATGATGTTGC	ACGGCTTGTATCCTAA	54.4	32	110
RBCS	CACCAGGAAGACCAACA	TCCAATGATACGGATGAA	59.5	26	370
RLK	AATAAACTGGGAGAAGG	CTGCCAAACTGAAACC	54.4	28	187
RPL	CCGTTTGAATGTAGCG	AGGTGTATTTGGGACTG	52.3	28	136
SAMS	GGAGT GGTGC TTACA TTG	CCCTT GAGTG GCTTG A	59.0	26	325
TBE	TGGCTCTTCCCTTGC	TCCTTGAACCACGACAC	58.9	30	384
UAE	CTCACCCAATCGCTTCC	GCAACACTTCTTACACCACC	61.5	30	207
ZFP	CAGTT CTGTC GCTAC CTT	TTGGG AGTTG ATTTGT CT	59.0	32	222

[a] The meaning of the abbreviated genes were given in Table 2

[b] The cycle number that the PCR amplification process reached the plateau

indicating little or no RNA degradation. Typical A_{260}/A_{280} absorbance ratios of the RNA ranged from 1.88 to 1.93. Yields were in the range of 0.60–1.19 mg/g fresh weight. The A_{260}/A_{280} ratios of purified mRNA were greater than 2.0. A clear smear greater than 0.5 kb was present on the 1% (w/v) agarose gel, justifying that the high quality mRNA was isolated from total RNA.

Evaluation of subtraction efficiency

Forward and reverse subtractions were conducted between stressed and unstressed seedlings infected by GM. The actin gene of pigeon pea was used to identify the subtraction efficiency. PCR amplification of the expected band showed that it appeared after 23 cycles when the

Table 2 Partial significantly changed transcripts in the suppression subtractive hybridization (SSH) library from pigeon pea (*Cajanus cajan*) inoculated by arbuscular mycorrhizae (AM)

Clone no.	Length	Regulation	Homologous sequence in Genbank Database	Abbreviation	*E* value[a]
Metabolism					
VC-CA6	662	Upregulated	Glutathione S-transferase GST 11 (*Glycine max*)	GST	4e−58
VC-AE12	423	Upregulated	Phosphatidylserine synthase, putative (*Ricinus communis*)	PSS	4e−34
VC-DC11	313	Upregulated	Phospho-2-dehydro-3-deoxyheptonate aldolase 1 chloroplast precursor,putative (*Ricinus communis*)	PDDA	1e−10
VC-BH7	527	Upregulated	Polygalacturonase, putative (*Ricinus communis*)	PG	3e−09
VD-DA11	631	Downregulated	Myo-inositol 1-phosphate synthase (*Phaseolus vulgaris*)	MIP	4e-90
VD-BF5	703	Downregulated	s-adenosylmethionine synthetase, putative (*Ricinus communis*)	SAMS	7e−76
VD-BE1	446	Downregulated	ER glycerol-phosphate acyltransferase (*Ricinus communis*)	GPAT	1e−23
VD-BG2	495	Downregulated	Histone H4 (*Eriobotrya japonica*)	H4	3e−39
VD-CA4	557	Downregulated	Peroxisomal glycolate oxidase (*Glycine max*)	PGO	5e−47
Energy					
VD-CG12	315	Downregulated	Rieske iron–sulfur protein precursor (*Glycine max*)		4e−37
VD-DE12	465	Downregulated	Chloroplast thioredoxin M-type (*Arachis hypogaea*)		3e−44
VC-AA11	308	Upregulated	Subunit IV of photosystem I (PSI-E) precursor (*Phillyrea latifolia*)		2e−04
VC-AH6	598	Upregulated	ATP synthase CF0 B subunit (*Glycine max*)		3e−11
VC-AB9	327	Upregulated	Light-harvesting complex II protein Lhcb1(*Populus trichocarpa*)	LHCB	3e−17
VC-DF10	803	Upregulated	Phosphoenolpyruvate carboxylase (*Glycine max*]		2e−133
VC-DE2	261	Upregulated	Cytochrome C oxidase polypeptide III, putative (*Ricinus communis*)		2e−24
Cell cycle and DNA processing					
VD-BG4	612	Downregulated	Cyclin-dependent kinase inhibitor 1;2 (*Glycine max*)	CDI	1e−29
Transcription					
VD-BD2	1091	Downregulated	Zinc finger protein, putative (*Ricinus communis*)	ZFP	5e−80
VC-DF3	737	Upregulated	AP2 domain-containing protein RAP2.7 (*Arabidopsis thaliana*]	AP2	6e−34
Protein synthesis					
VC-AE4	507	Upregulated	Ribosomal protein L14 (*Glycine max*)	RPL	2e−24
Protein fate					
VC-CB1	822	Upregulated	Receptor-like protein kinase homolog RK20-1 (*Phaseolus vulgaris*)	RLK	3e−100
VC-AA2	854	Upregulated	ckl3 (Casein Kinase I-like 3); ATP binding/kinase/protein kinase/protein serine/threonine kinase (*Arabidopsis thaliana*)	CK	2e−106
Protein with binding function or cofactor requirement					
VD-AC2	553	Downregulated	Chloroplast oxygen-evolving enhancer protein (*Manihot esculenta*]	OEE	7e−58
VD-CE12	662	Downregulated	ATP binding protein, putative (*Ricinus communis*)	ABP	5e−54
VC-CD7	533	Upregulated	F-box protein (*Gossypium hirsutum*)	FBP	3e−11
Cellular transort, transport facilities and transport routes					
VC-BH5	920	Upregulated	PREDICTED: similar to dicarboxylate/tricarboxylate carrier (*Vitis vinifera*)	DTC	5e−93
VC-CD4	400	Upregulated	Type IIB calcium ATPase (*Medicago truncatula*]	CAIIB	1e−38
Cell rescue defense, and virulence					
VC-DA8	459	Upregulated	Lipid binding protein, putative (*Ricinus communis*)	LBP	3e−28
Interaction with the environment					
VC-AH7	192	Upregulated	GAGA-binding transcriptional activator (*Medicago truncatula*)	GATA	4e−07
VC-AB10	898	Upregulated	Calmodulin-binding protein (*Arabidopsis thaliana*)	CBP	3e−99
VC-AB8	546	Upregulated	Glutamate dehydrogenase 1 (*Glycine max*)	GDH	7e−81

Table 2 continued

Clone no.	Length	Regulation	Homologous sequence in Genbank Database	Abbreviation	*E* value[a]
VD-DH6	565	Downregulated	Heat shock protein, putative (*Ricinus communis*)	HSP	3e−85
Systemic interaction with environment					
VC-CF1	505	Upregulated	Cationic peroxidase 2 (*Glycine max*)	CP	4e−22
Cell fate					
VC-AD12	238	Upregulated	Defender against apoptotic death 1 (*Plantago major*)		7e−06
VC-BD12	595	Upregulated	Rust resistance protein (*Glycine max*)		3e−16
Development					
VC-AE3	623	Upregulated	NMDA receptor-regulated protein, putative(*Ricinus communis*)		3e−44
VC-AA8	607	Upregulated	DUF26 domain-containing protein 2 precursor,putative(*Ricinus communis*)		8e−37
VC-CB9	652	Upregulated	Isopentenyl pyrophosphate isomerase (*Pueraria montana var. lobata*)		1e−73
Biogenesis of cellular components					
VC-BG1	396	Upregulated	Putative microtubule-associated protein (*Thellungiella halophila*)	MAP	1e−19
VC-BA4	494	Upregulated	Putative cytosolic factor (*Trifolium pratense*)		1e−34
VC-DC10	428	Upregulated	Ubiquitin-activating enzyme E1 domain-containing protein, putative (*Ricinus communis*)	UAE	7e−44
VC-AA7	607	Upregulated	Thiamin biosynthetic enzyme (*Glycine max*)	TBE	1e−69
VD-AD11	1110	Downregulated	Amino methyl transferase, putative (*Ricinus Communis*)	AMT	7e−106
VD-BC12	635	Downregulated	Ribulose-1,5-bisphosphate carboxylase small subunit rbcS (*Glycine max*)	RBCS	4e−93
Subcellular localization					
VC-BA8	317	Upregulated	Chloroplast photosystem II 10 kDa protein (*Arachis hypogaea*)	CPII	8e−34
Unclassified proteins					
VC-BB9	358	Upregulated	Cytochrome b561 (*Citrullus lanatus*)	CYTB	1e−40
VC-AA9	1078	Upregulated	Protein phosphatase-2c, putative (*Arabidopsis thaliana*)	PP2C	1e−16

The lower the *E* value, the more strongly the match is supported

[a] The expected (*E*) value refers to the number of matches expected by chance alone

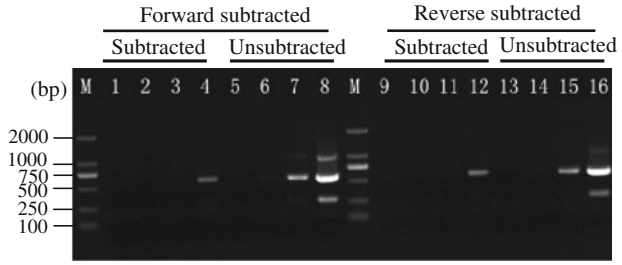

Fig. 1 Evaluation of the subtraction efficiency of the subtracted cDNAs. PCRs with pigeon pea actin gene-specific primers were performed using subtracted and unsubtracted cDNAs from 30 days water deficit stress-induced pigeon pea leaves. *Lane M*, DNA size markers. *Lane 1*, *5*, *9* and *13*: 18 cycles. *Lane 2*, *6*, *10* and *14*: 23 cycles. *Lane 3*, *7*, *11* and *15*: 28 cycles. Lane *4*, *8*, *12* and *16*: 33 cycles

unsubtracted tester cDNA was used as a template, conversely, this band did not appear until 28 cycles when the subtracted cDNA was taken as a template (Fig. 1), which indicated that cDNA homologous to both tester and driver had been considerably eliminated.

Differential screening of SSH library

The second PCR products of SSH were cloned into *pMD-18T* vectors after purification and preserved in *E. coli* DH-5α. Screening by blue-white spots demonstrated that approximately 98% of transformants contained inserts. A total of 768 clones were randomly picked from two SSH library. As the evidence of PCR screening, the average insert size of the SSH clones ranged from 250 to 1,000 bp, however, most were around 500 bp. Consequently, all the clones were further screened by dot-blotting analysis, among which 142 clones over expressed and 49 repressed were chosen to carry out the following analysis.

Sequence analysis of selected cDNA clones

A total of 191 clones were selected to have their inserts sequenced, and 182 differentially expressed cDNA section clones, including 133 upregulated and 49 downregulated,

were obtained (Table 2). The sequences of these EST clones were submitted to NCBI as dbEST IDs 74605977–74606158 and GenBank accession nos. JK086989–JK087170. These inserts were searched against GenBank using BLASTX program. In order to best identify the functions of these ESTs, a moderate *e* value cutoff (1e−8) was set up to assign the ESTs as "significant homology" or "no hit" based on the BLAST results. A total of 142 ESTs consisting of 102 upregulated and 40 downregulated exhibited high homology to previously identified or putative proteins in *Arabidopsis*, rice, maize, *Glycine max*, etc. However, 31 upregulated and 9 downregulated ESTs showed no homology in the database, which probably was short fragments of cDNA derived from the 3′ UTR or was more likely novel ESTs in AM-colonized pigeon pea.

According to the MIP standards, the forward library and reverse library cDNAs with significant protein homology were sorted into 16 (Fig. 2a) and 12 (Fig. 2b) functional categories, respectively. Functional categories showed that the genes represented by those ESTs involved in a broad spectrum of biological pathways. For the upregulated

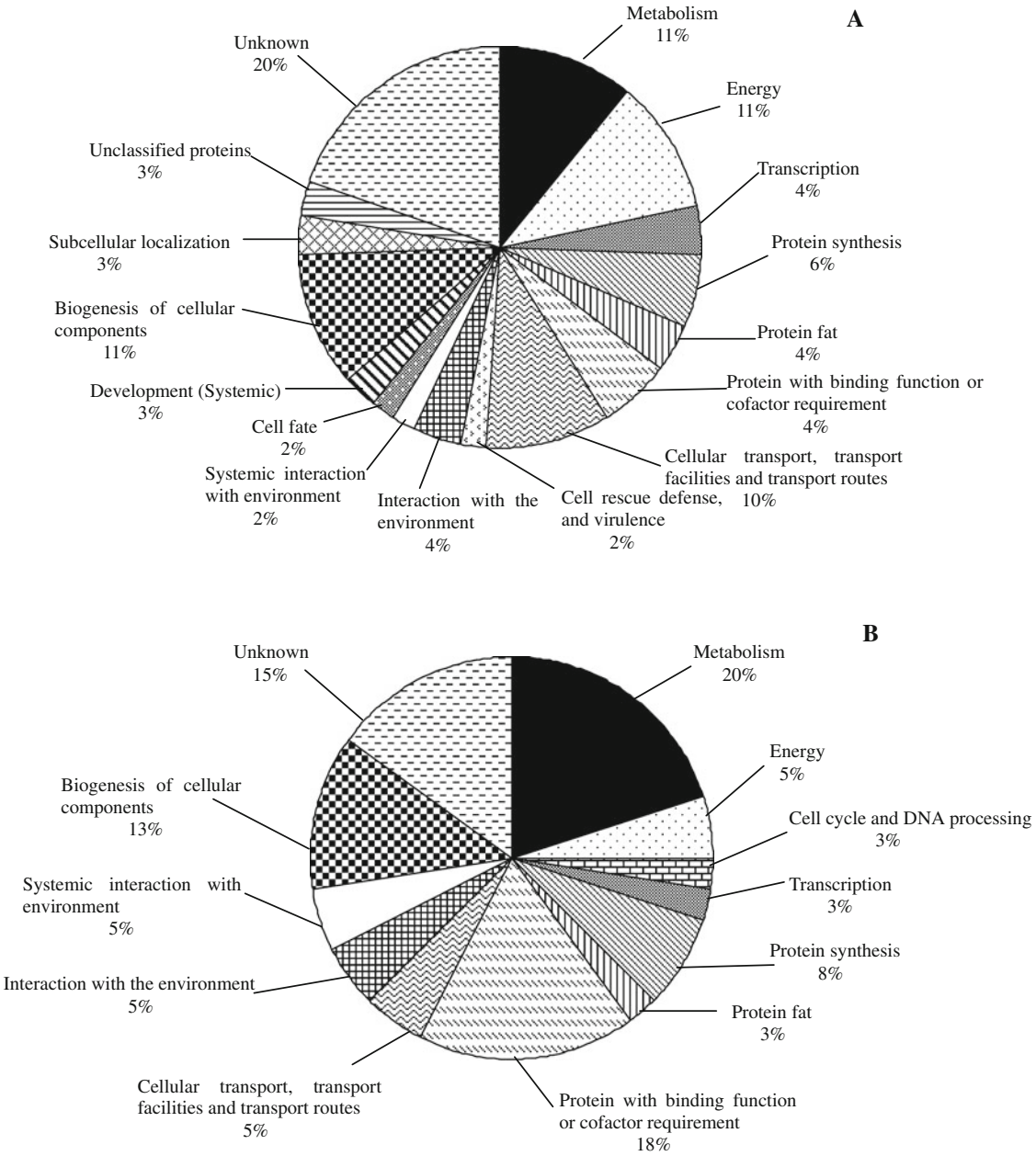

Fig. 2 Functional classification of upregulated (**a**) and downregulated (**b**) clones identified from subtractive library based on MIPS functional categories. The percentage of gene transcripts in each group is listed

genes, the metabolism category, energy and biogenesis of cellular components were the higher proportion of the known genes. For the downregulated genes, the largest set was assigned to the metabolism category. Cellular transport, transport facilities and transport routes and protein with binding function or cofactor requirement represented the second largest group in upexpressed and downregulated genes, respectively. The unknown proteins still represented a large group in both forward library and reverse library.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out using specific primers based on the 35 selected ESTs which probably related to the abiotic stress tolerances according to the sequence analysis. Hierarchical cluster and *k* mean cluster analysis of the expression profiles demonstrated differences both AM-colonized and non-colonized seedlings under drought and control treatment. The results indicated that there were at least six patterns (Fig. 3). Six ESTs represented type I which had higher expression in AMC and NAMC than other treatments. However, type IV (6 ESTs) displayed higher expression in AMD and NAMD. Type V (4 ESTs) and type VI (6 ESTs) had similar expression patterns, which generally showed their highest expression in AMC, but the lowest expression in NAMD and NAMC, respectively. The expression level of four other ESTs (type III) peaked in AMD and bottomed in NAMC. Nine ESTs (type II) showed the lowest expression levels in NAMD and highest levels in AMD or NAMC.

Discussion

Abiotic stresses present a major challenge in the quest for sustainable food production as these may bring about 70% reductions in plant yield. Among the abiotic stresses, drought was labeled as the first adverse factors (Gosal et al. 2009). Pigeon pea was proved to be an ideal crop for Karst areas of southwest China. Previously, AM-colonized pigeon pea physiologically demonstrated a further tolerance enhancement to drought stress (Qiao et al. 2011). The illumination of molecular foundation of its responses to water deficit might facilitate the improvement of the drought tolerance. Recently, innovative biotechnological approaches had enhanced our understanding of the processes underlying plant responses to drought stress at molecular level (Gosal et al. 2009). In current investigation, SSH strategy was used to identify drought-responded genes preferentially expressed in AM-colonized pigeon pea seedlings. The 142 upregulated ESTs and the 49 downregulated ESTs were obtained after dot-blotting analysis.

In current investigation, we also detected semi-quantitative RT-PCR data agreed with the SSH data for 29 out of

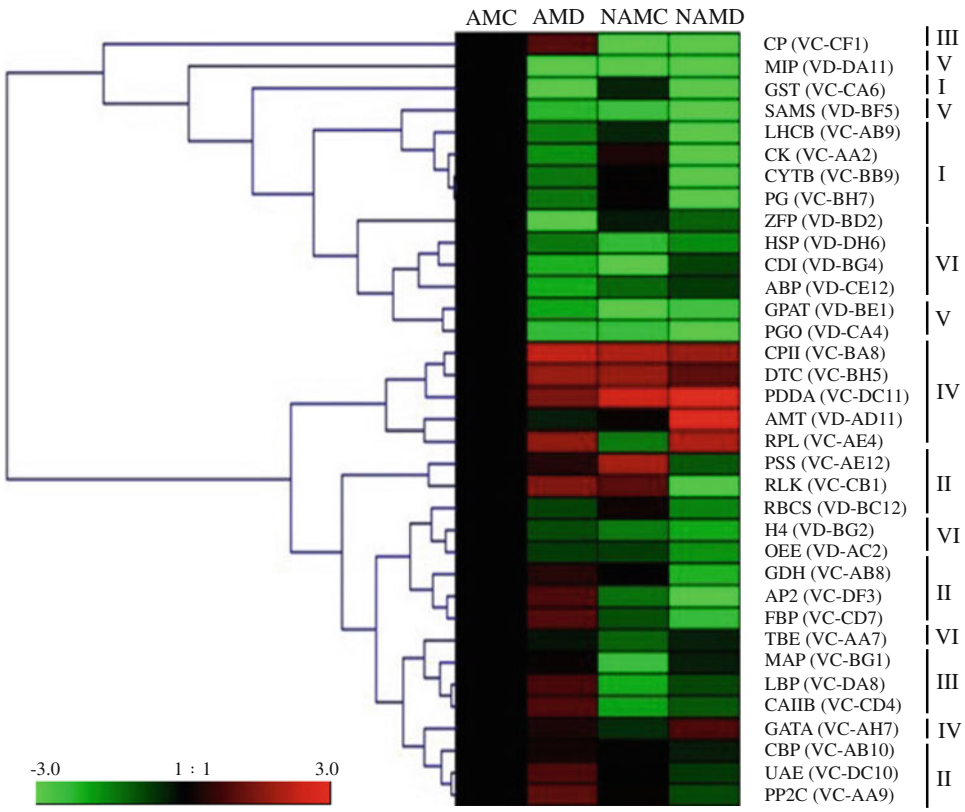

Fig. 3 Hierarchical cluster and *k* mean cluster analysis of selected ESTs derived from AM-colonized and non-AM pigeon pea under drought and control treatment. I, II, III, IV, V, and VI represent different types of expression patterns. The *rows* represent individual genes. The up- and downregulated proteins are indicated in *red and green* respectively. *Color brightness* represents the degree of the difference, as shown in the *bar* at the top (color figure online)

35 data points, which indicated that 83% of clones of SSH were authentic positive. Seventeen differentially expressed genes, which upregulated based on the SSH analysis were more abundant in AMD in comparison with those of the AMC, such as cationic peroxidase 2 (VC-CF1), lipid binding protein (VC-DA8), ribosomal protein L14 (VC-AE4). Conversely, 12 differentially expressed genes were downregulated, e.g., peroxisomal glycolate oxidase (VD-CA4), myo-inositol 1-phosphate synthase (VD-DA11), cyclin-dependent kinase inhibitor (VD-BG4). The rests (6 differentially expressed genes) demonstrated contrary tendency compared with SSH data. The semi-quantitative RT-PCR data proved that SSH technology was also reliable to identify the differentially expressed genes involved in drought stress of pigeon pea inoculated by AMF.

Water stress tolerance might be ascribed to a complex cascade of molecular events including genes activation (Ramanjulu and Bartels 2002), and/or expression inhibition (Shinozaki et al. 2003). In the current case, most of the genes, irrespectively up or downregulated, demonstrated significant homology to the known genes from other plants (Table 2). Functional categories showed that the known genes represented by those cDNAs had a wide range of roles in different cell activities, e.g., metabolism, biogenesis of cellular components and energy. Although most of differentially expressed genes belonged to different categories, they presumably functioned not only in protecting cells from water deficit by the production of important metabolic proteins, but also in the regulation of genes for signal transduction in the water-stress response based on the sequence analysis. The former included proteins that probably function in stress tolerance, e.g., glutathione S-transferase (VC-CA6), ubiquitin-activating enzymes (VC-DC10) and s-adenosylmethionine synthetases (VD-BF5). Ubiquitin-activating enzymes played vital role in the degradation of many cellular proteins. Together with transcription control and protein phosphorylation, it regulated many basic cellular processes including cell cycle and antigen presentation (Glickman and Ciechanover 2002). The expression of s-adenosylmethionine synthetases during drought and re-watering after serious drought indicated that it was involved in the response to drought and re-watering and might be one of the key genes for drought tolerance and water use efficiency (Lin et al. 2008). The latter contained proteins involved in further regulation of signal transduction and gene expression that probably function in stress response, e.g., myo-inositol 1-phosphate synthase (VD-DA11), calmodulin-binding proteins (VC-AB10), and phosphatidylserine synthase (VC-AE12). Myo-inositol 1-phosphate synthase was a precursor of inositol phospholipids and played a crucial role in signal transduction, actin remodeling, phytic acid biosynthesis, membrane, and cell wall biosynthesis (Downes et al. 2005; Suzuki et al. 2007). Ca^{2+} and IP_3 were the most probable candidates as second messengers in water-stress responses in plant cells, thus calmodulin-binding proteins and phosphatidylserine synthase were regarded to have important roles in various signal transduction cascades in plant as well as in yeasts and animals (Song and Yang 2006).

Currently, a total of 35 differentially expressed genes of pigeon pea were homologous to the known genes that putatively involved in the abiotic stress tolerances. The relative differences in mRNA levels between AM-colonized and non-colonized pigeon pea subjected to drought stress and stress free was determined by semi-quantitative RT-PCR. We found that the expression level of these selected genes were different in all treatment (Fig. 3). For instance, the protein phosphatase-2c (VC-AA9) and receptor-like protein kinase (VC-CB1) reached the highest expression in AMD and the lowest in NAMD. Biochemical and molecular genetic studies had identified that protein phosphatase-2c enzymes (Rodriguez 1998) and plant receptor-like protein kinases (Joshi et al. 2010) played a fundamental role in sensing external environmental signals to regulate gene expression. Therefore, the present evidences indicated the enhanced tolerance of AMF pigeon pea to drought stress might at least partially be ascribed to the changes in expression level of stress-related genes triggered by the colonization of AMF.

It is worthy to note that two transcription factors among water stress-modulated genes were identified, zinc finger proteins (VD-BD2) and AP2 domain-containing protein (VC-DF3). Zinc finger proteins played a key regulatory role in ABA signaling under drought stress (Golldack et al. 2011). In rice and tomato transgenic modification of zinc finger proteins transcription factor modified the tolerance of plants to water deficit and to salt stress (Amir Hossain et al. 2009; Hsieh et al. 2010). AP2/EREBP genes formed a large multigene family, and they played a variety of roles throughout the plant life cycle (Riechmann and Meyerowitz 1998). The expression of some members in AP2 domain-containing protein family was water stress inducible (Gorantla et al. 2005; Pandey et al. 2005). There had been many instances where overexpression of some AP2 domain-containing genes enhanced stress tolerance, including water stress tolerance (Fu et al. 2007; Dai et al. 2009).

Interestingly, we found that several ESTs had been reported to be induced by other forms of abiotic stress, not only by water stress. Myo-inositol 1-phosphate synthase (VD-DA11) and ribulose-1, 5-bisphosphate carboxylase small subunit (VD-BC12) had been identified in the salt-stressed halophyte smooth cordgrass (Baisakh et al. 2008) and alfalfa (Jin et al. 2010). Light-harvesting chlorophyll-binding proteins (VC-AB9) had been isolated from *Lepidium latifolium* inhibited by cold stress (Aslam et al. 2010). It was suggested that the different environmental stresses

might result in similar non-specific responses at the cellular and molecular level, in addition to stress-specific responses, e.g., the elevation in the endogenous level of free proline under salinity condition (Gomathi et al. 2010), and in the malondialdehyde (MDA) accumulation by heat stress (Ding et al. 2010), or by oxidative stress (Guo et al. 2007). Similarly, our previous investigation demonstrated that the proline and MDA content of the drought-stressed seedlings were higher than that of the stress free (Qiao et al. 2011). It maybe due to the fact that multiple stressors trigger similar downstream signal transduction chains (Jin et al. 2010).

The current investigation focused on the differentially expressed genes that were isolated from young pigeon pea seedlings inoculated by AMF in exposure to drought stress, which may probably inflected that AMF colonization further enhanced drought tolerance. The following work will aim to further investigate whether the expressions of these differentially expressed genes are strictly triggered by AMF or not. Additionally, a large number of response genes regulated by water deficit stress in this study encode unknown proteins, and the ongoing work will also extend to verify how they involved in the drought tolerance, which are essential to fully elucidate the molecular mechanism of the enhanced tolerance to drought stress of pigeon pea caused by AMF, as well as to further improve its stress tolerance via genetic manipulation.

Author contribution Xiaopeng Wen designed the research and instructed the experiment. Guang Qiao and Lifei Yu performed the experiment and analyzed the data. Xiangbiao Ji gave materials support. Guang Qiao and Xiaopeng Wen prepared the manuscript.

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References

Amir Hossain M, Lee Y, Cho JI, Ahn CH, Lee SK, Jeon JS, Kang H, Lee CH, An G, Park PB (2009) The bZIP transcription factor OsABF1 is an ABA responsive element binding factor that enhances abiotic stress signaling in rice. Plant Mol Biol 72:557–566

Aslam M, Sinha VB, Singh RK, Anandhan S, Pande V, Ahmed Z (2010) Isolation of cold stress-responsive genes from *Lepidium latifolium* by suppressive subtraction hybridization. Acta Physiol Plant 32:205–210

Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Augé RM (2004) Arbuscular mycorrhizae and soil/plant water relations. Can J Soil Sci 84:373–381

Baisakh N, Subudhi PK, Varadwaj P (2008) Primary responses to salt stress in a halophyte, smooth cordgrass (Spartina alterniflora Loisel.). Funct Integr Genomic 8:287–300

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23–58

Dai YL, Qin QL, Dai DL, Kong LS, Li W, Zha XJ, Jin YJ, Tang KX (2009) Isolation and characterization of a novel cDNA encoding methyl jasmonate-responsive transcription factor *TcAP2* from *Taxus cuspidate*. Biotechnol Lett 31:1801–1809

Ding W, Song L, Wang X, Bi Y (2010) Effect of abscisic acid on heat stress tolerance in the calli from two ecotypes of *Phragmites communis*. Biol Plantarum 54:607–613

Domoguen RL, Saxena KB, Mula MG, Sugui F, Dar WD (2010) The multiple uses of pigeonpea. http://www.sunstar.com.ph/baguio/multiple-uses-pigeon pea. Accessed 27 January 2010

Downes CP, Gray A, Lucocq JM (2005) Probing phosphoinositide functions in signaling and membrane trafficking. Trends Cell Biol 15:259–268

Fu XY, Zhang Z, Peng RH, Xiong AS, Liu JG, Wu LJ, Gao F, Zhu H, Guo ZK, Yao QH (2007) Isolation and characterization of a novel cDNA encoding ERF/AP2-type transcription factor OsAP25 from *Oryza Sativa* L. Biotechnol Lett 29:1293–1299

Glickman MH, Ciechanover A (2002) The ubiquitin-protcasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428

Golldack D, Lüking I, Yang O (2011) Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep 30:1383–1391

Gomathi R, Vasantha S, Thandapani V (2010) Mechanism of osmo regulation in response to salinity stress in sugarcane. Sugar Tech 12(3–4):305–311

Gong DY, Zuo DC, Liu QG, Ban XW (2005) The application of pigeon pea in the subtropical regions of Guizhou Province. Subtrop Agric Res 1:24–26

Gorantla M, Babu PR, Lachagari VBR, Feltus FA, Paterson AH, Reddy AR (2005) Functional genomics of drought stress response in rice: transcript mapping of annotated unigenes of an *indica* rice (*Oryza sativa* L. cv. Nagina 22). Curr Sci India 89:496–514

Gosal SS, Wani SH, Kang MS (2009) Biotechnology and drought tolerance. J Crop Improv 23:19–54

Guo Z, Huang MY, Lu SY, Zhao YQ, Qiao Z (2007) Differential response to paraquat induced oxidative stress in two rice cultivars on antioxidants and chlorophyll a fluorescence. Acta Physiol Plant 29:39–46

Hsieh TH, Li CW, Su RC, Cheng CP, Sanjaya, Tsai YC, Chan MT (2010) A tomato bZIP transcription factor, SlAREB, is involved in water deficit and salt stress response. Planta 231:1459–1473

ICRISAT (International Crop Resreach Institute for the Semi-Arid Tropics) (1998) Improvement of pigeon pea in eastern and southern Africa: Project completion report, PO Box 39063. Nairobi, Kenya

Jin HC, Sun Y, Yang QC, Chao YH, Kang JM, Jin H, Li Y, Margaret G (2010) Screening of genes induced by salt stress from Alfalfa. Mol Biol Rep 37:745–753

Joshi A, Dang HQ, Vaid N, Tuteja N (2010) Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress *in planta*. Glycoconjugate J 27:133–150

Li ZH, Zhou CH, Gu Y, Zhang JY (2001) The present status of study and utilization of pigeon pea in China and its prospects. For Res 14:674–681

Lin FY, Wang SQ, Hu YG, He BR (2008) Cloning of a s-adenosyl-methionine synthetase gene from broomcorn millet (*Panicum*

miliaceum L.) and its expression during drought and re-watering. Acta Agron Sin 34:777–782

Liu Q, Zhu AD, Chai LJ, Zhou WJ, Yu KQ, Ding J, Xu J, Deng XX (2009) Transcriptome analysis of a spontaneous mutant in sweet orange [Citrus sinensis (L.) Osbeck] during fruit development. J Exp Bot 60:801–813

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–502

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol 149:88–95

Pandey GK, Gran JJ, Cheong YH, Kim BG, Li L, Luan S (2005) ABR_1, an $APETALA_2$-domain transcription factor that functions as a repressor of ABA response in Arabidopsis. Plant Physiol 139:1185–1193

Porcel R, Aroca R, Azcón R, Ruiz-Lozano MJ (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. Plant Mol Biol 60:389–404

Qiao G, Wen XP (2010) Cloning and sequence analysis of actin gene fragment from *cajanus cajan*. Guizhou Agric Sci 38:5–7

Qiao G, Wen XP, Yu LF, Ji XB (2011) The enhancement of drought tolerance for pigeon pea inoculated by arbuscular mycorrhizae fungi. Plant Soil Environ 57:541–546

Rahaie M, Xue GP, Naghavi MR, Alizadeh H, Schenk PM (2010) A MYB gene from wheat (*Triticum aestivum* L.) is upregulated during salt and drought stresses and differentially regulated between salt-tolerant and sensitive genotypes. Plant Cell Rep 29:835–844

Ramanjulu S, Bartels D (2002) Drought- and desiccation-induced modulation of gene expression in plants. Plant, Cell Environ 25:141–151

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol 161:1189–1202

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem 379:633–646

Ritchie JM, Polaszek A, Abeyasekera S, Minja E, Mviha P (2000) Pod pests and yield losses in smallholder pigeon pea in Blantyre/Shire Highlands. In: Ritchie JM (ed) Integrated crop management research in Malawi: developing technologies with farmers. Proceedings of the Final Project Workshop, Club Makokola, Mangochi, Malawi, 29 Nov–3 Dec 1999. Natural Resources Institute, Chatham UK

Rodriguez PL (1998) Protein phosphatase 2C (PP2C) function in higher plants. Plant Mol Biol 38:919–927

Saibo NJM, Lourenc T, Oliveira MM (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann Bot-London 103:609–623

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417

Song YQ, Yang Q (2006) Phospholipase in plant defence signaling transduction and development of high plants. J Harbin Inst Technol 38:243–246

Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. Bioinformatics 18:207–208

Suzuki M, Tanaka K, Kuwano M, Yoshida KT (2007) Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): implications for the phytic acid biosynthetic pathway. Gene 405:55–64

Varshney RK, Chen WB, Li YP, Bharti AK, Saxena RK, Schlueter JA, Donoghue MTA, Azam S, Fan GY, Whaley AM, Farmer AD, Sheridan J, Iwata A, Tuteja R, Penmetsa RV, Wu W, Upadhyaya HD, Yang SP, Shah T, Saxena KB, Michael T, McCombie WR, Yang BC, Zhang GY, Yang HM, Wang J, Spillane C, Cook DR, May GD, Xu X, Jackson SA (2011) Draft genome sequence of pigeonpea (Cajanus cajan), an orphan legume crop of resource-poor farmers. Nat Biotechnol 30:83–89

Wang HG, Zhang HL, Gao FH, Li JX, Li ZC (2007) Comparison of gene expression between upland and lowland rice cultivars under water stress using cDNA microarray. Theor Appl Genet 115:1109–1126

Wu QS, Xia RX (2006) Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. J Plant Physiol 163:417–425

Wu N, Fu K, Fu YJ, Zu YG, Chang FR, Chen YH, Liu XL, Kong Y, Liu W, Gu CB (2009) Antioxidant activities of extracts and main components of pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves. Molecules 14:1032–1043

Xiao BZ, Huang YM, Tang N, Xiong LZ (2007) Over-expression of a LEA gene in rice improves drought resistance under the field conditions. Theor Appl Genet 115:35–46

Xiong LM, Schumaker KS, Zhu JK (2002) Cell signal during cold, drought, and salt stress. Plant Cell 14:S165–S183

Zheng J, Zhao JF, Tao YZ, Wang JH, Liu YJ, Fu JJ, Jin Y, Gao P, Zhang JP, Bai YF, Wang GY (2004) Isolation and analysis of water stress-induced genes in maize seedlings by subtractive PCR and cDNA macroarray. Plant Mol Biol 55:807–823