INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–1391/2018/20–5–1152–1160
DOI: 10.17957/IJAB/15.0633
http://www.fspublishers.org

Full Length Article

Mild Water Deficit in the Field Down Regulated Drought Responsive Genes in Peanut Leaf Tissues

Liping Wang[1], Diane Rowland[1], Xiping Yang[1], Edzard Van Santen[1], Barry Tillman[1,2] and Jianping Wang[1,3,4*]
[1]*Agronomy Department, University of Florida, Gainesville, Florida, 32611, USA*
[2]*North Florida Research and Education Center, Marianna, FL 32446, USA*
[3]*Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, Florida 32611, USA*
[4]*Genetics Institute, University of Florida, Gainesville, Florida 32610, USA*
[*]For correspondence: wangjp@ufl.edu

Abstract

Drought is a major abiotic stress limiting production of cultivated peanut (*Arachis hypogaea* L.). Exposure of plants to mild water deficit at early developmental stage has potential to maintain pod yield equal to fully irrigated plants in some cropping systems. The molecular mechanism regulating peanut response to mild water deficit at field condition is unknown. To explore possible gene responses under mild water deficit, 11 peanut putative drought responsive genes were examined at 34 and 92 days after planting in the leaf tissues of Florida-07 and Tifguard cultivars treated with either mild water deficit or full irrigation. The results showed that the mild water deficit suppressed the expressions of three transcriptional factor genes, *AhMYB* (P = 0.028), *AhNAC* (P = 0.047) and *AhZFP* (P = 0.038), indicating that plant might use different gene expression profile to cope with mild water deficit and severe drought conditions. Three genes, *AhAREB1*, *AhMYB,* and *AhZFP* expressed differently between the two cultivars indicating the different genetic background could impact the gene expression levels. Expressions of eight of the 11 genes were impacted by crop developmental stages. Though none of the 11 genes were upregulated by the mild water deficit in the peanut field, the results in this study approved the sensitivities of RT-qPCR in detecting gene expression at different field conditions and provided a quick glimpse and incentive for further investigation of molecular mechanisms of crops in response to mild and severe water deficit in the field conditions. © 2018 Friends Science Publishers

Keywords: Mild drought; Abiotic stress; RT-qPCR; *Arachis hypogaea*; Gene expression
Abbreviations: DAP, days after planting; RT-qPCR, reverse transcription quantitative PCR; EST: expressed-sequence tag; ABA: abscisic acid; ABRE1: ABA-responsive element 1; ERF: Ethylene response factor; MYB: MYB DNA-binding protein; ZFP: zinc finger protein; NAM/NAC: no apical meristem protein;LEA3: late embryogenesis abundant protein 3; GOLS2:glycosyltransferase family 2; Hsp70: heat shock protein 70 family gene; Met2:type 2 metallothionein; PLDα1: phospholipase D alpha 1; Sps: Spermidine synthase

Introduction

Drought is a major abiotic stress limiting crop yield and quality (Abreu *et al.*, 2013). The effects of water limitation will likely become worse in the future because of climate change as well as the diminishing supplies of fresh water related to urban expansion (Claeys and Inze, 2013). Understanding of how plants react and acclimate to drought stress has been increasingly the focus of research in agriculture with an effort to reduce crop damage due to water scarcity (Abreu *et al*., 2013). However, many drought studies to date have been conducted under two controlled conditions: 1) utilizing lethal or severe drought levels; and 2) under laboratory or highly artificial conditions. The fact that drought tolerance has been almost exclusively evaluated under severe drought conditions has led to the near lack of identification of genotypes with successful tolerance to drought under field conditions, as evidenced by the lack of yield maintenance under water scarce production-relevant conditions (Skirycz *et al*., 2011; Claeys and Inze, 2013). Therefore, this has led to a generally poor understanding of what occurs under mild drought stress, a condition that more accurately reflects what the crop experiences during most of a typical growing season in the field and is normally the prevailing environmental state prior to severe stress (Harb *et al*., 2010; Clauw *et al*., 2015). In addition, most studies have utilized model plant species, primarily *Arabidopsis*, with comparatively little research conducted using relevant crop species. These approaches can present major stumbling blocks to understanding many of the mechanisms involved

To cite this paper: Wang, L., D. Rowland, X. Yang, E.V. Santen, B. Tillman and J. Wang, 2018. Mild water deficit in the field down regulated drought responsive genes in peanut leaf tissues. *Int. J. Agric. Biol*., 20: 1152–1160

in drought responses because there are meaningful differences between model and crop species at the genetic and physiological levels (Skirycz *et al*., 2011).

The general stress response at a molecular level in plants involves processes related to regulatory signal transduction and metabolism, as well as transcriptional control of functional genes (Bartels and Sunkar, 2005; Harb *et al*., 2010; Verelst *et al*., 2010; Obata and Fernie, 2012). While these general responses may be understood, relatively little is known about the earliest molecular, biochemical, and physiological signaling processes in plants during the initial exposure to stress (Harb *et al*., 2010). An understanding of differential gene expression involved in differing levels of water stress is important. Some major differences in plant transcriptome responding to mild as compared to severe drought were observed. Many of the gene responses at early stress exposure involve the expression of expansin genes that are partially responsible for changes in cell wall expansion, one of the first physiological changes experienced by plants under early, mild water deficit (Harb *et al*., 2010). Others may involve important up-regulation of genes specifically expressed in the roots involved in transport and osmoprotection (Sečenji *et al*., 2010). But there are many instances where gene expression patterns differed significantly between mild and severe stress levels (Watkinson *et al*., 2003; Harb *et al*., 2010; Sečenji *et al*., 2010; Li *et al*., 2012; Clauw *et al*., 2015). For example, Kano-Nakata (Kano-Nakata *et al*., 2014), found that differences among rice genotypes in carbon isotope discrimination (a surrogate for water-use efficiency; WUE) was only manifest under mild, but not severe drought conditions, making it critical that screening for improved WUE be done under moderate water reductions. Similarly, physiological and transcript expression variability among wheat genotypes was only evident under mild water stress (Csiszár *et al*., 2012). Increasing the tolerance to water restriction early in plant development may be a critical breeding target because this could considerably improve the agricultural water-use efficiency of cropping systems by allowing growers to reduce water application during the early season.

Differences in drought tolerance are likely related to a genotype's ability or inability to acclimate to stress. Studies of mild stress necessarily include the process of acclimation, defined as physiological and morphological plasticity that may occur on short or seasonal time scales in response to stress (Gaspar *et al*., 2002). Through acclimation, the overall response to stress may even be favorable to plant performance under some conditions (Lichtenthaler, 1988; Rao *et al*., 2012). Acclimation is particularly critical to crop plants because it is a means of maintaining yield under stress. Thus, selecting for genotypes with high acclimation potential has great promise for improving drought tolerance in crops. While exposure to mild water deficit early in development may have little impact to final harvest yield, this is not necessarily a universal response across all genotypes that could be particularly sensitive to water scarcity (Passioura, 2007; Rowland *et al*., 2012). A full understanding of the gene expression differences between mild vs. severe drought of different genotypes will have critical implications for breeding for drought tolerance.

Peanut as a highly important global food crop is grown in more than 100 countries in the world under diversified agro-climates with 80% under semi-arid climates with erratic rainfall and frequent heat (Rao *et al*., 2012). Drought is a major abiotic stress constraining peanut yields and affecting pod grades in those regions (Sharma and Bhatnagar-Mathur, 2006; Daryanto *et al*., 2015). Peanut had the potential for acclimation through possible early mild drought recognition responses that could induce some protection, resulting in the maintenance of growth and reproduction processes later in the season during crucial flowering and pod development stages (Rowland *et al*., 2012). Understanding peanut drought regulated genes and gene networks, particularly those related to mild stress leading to acclimation, could provide information for drought tolerance improvements in breeding in this globally important crop (Sharma and Bhatnagar-Mathur, 2006).

In this study, 11 peanut candidate genes were selected from drought marker gene families and observed their expression profiles by RT-qPCR during vegetative and reproductive developmental stages in the field with two cultivars of commercial importance in the U.S. The analysis provided insights into the possible molecular changes underlying the field-relevant mild water deficits.

Materials and Methods

Peanut Planting, Irrigation and Sample Collection

The field trial was arranged in a randomized complete block design with a split-plot arrangement of three replications. The irrigation treatment was assigned to the main plot and two cultivars, cultivar 'Florida-07' (Gorbet and Tillman, 2009) and 'Tifguard' (Holbrook *et al*., 2008) were provided by Dr. Tillman in Florida peanut breeding program and were assigned to the sub-plot. The two cultivars were planted using conventional tillage in plots consisting of eight rows spaced 0.91 m apart and 19.8 m in length with a two-row Monosem planter on 24 May 2011 at the Plant Science Research and Education Unit in Citra, Florida. Seedling density was six seeds per 0.3 m of row. Irrigation treatments were applied based on daily measurements of potential evapotranspiration (ET) modified by a crop coefficient for peanut (Food and Agriculture Organization of the United Nations) and subtracting any rainfall received from daily accumulated ET. Daily potential ET values were obtained from the Florida Automated Weather Network (FAWN weather system; http://fawn.ifas.ufl.edu/) for the station located at Citra, Florida. Irrigation was triggered based on ET accumulated to 25 mm. Once triggered, irrigation treatments were applied to replace ET losses at

100% (100) and 60% (60). The 100% irrigation treatment was used as the control for assessing gene expression for RT-qPCR analysis. Two developmental stages were targeted for tissue collection: at the early vegetative stage at 34 DAP, and at optimum physiological performance during pod fill at 92 DAP (Fig. 1). At these two time points, leaf samples were randomly collected from 10 plants in each plot at the second nodal position on the main stem as one sample, resulting in 24 total samples collected (2 cultivars x 2 time points x 3 biological replicates x 2 levels of treatment). Fresh leaf samples were individually wrapped with aluminum foil and immediately submerged into liquid nitrogen in the field. Frozen samples were transferred to an 80°C freezer until RNA extraction could be carried out.

Identification of Peanut Drought Response Marker Genes

The peanut genes for gene expression analysis in this study were chosen based on published studies related to molecular signal transduction during plant drought response. A handful of genes (Table 1) representing upstream and downstream of the signal transduction network relevant to drought responses were selected. The gene sequences were identified from NCBI EST database which were further used as query to search their orthologous genes in *Glycine max* (soybean) databases in Phytozome (Phytozome v9.1 www.phytozome.net) and peanut genomes in Peanutbase.org (Bertioli *et al*., 2016).

RNA Sample Isolation and Purification

Frozen leaf samples (about 200 mg) were manually ground into fine powder in liquid nitrogen. Total RNA was extracted from the powder with 1 mL Plant RNA Purification Reagent (Life Technologies) following the product protocol with the following modifications: after the 70% ethanol precipitation step, the RNA sample in ethanol was transferred to RNeasy Mini column in RNeasy Plant Mini kit (QIAGEN). The column was centrifuged and washed with the buffer RW1 included in the kit. To each column, 10 µL of RNase-free DNase I (QIAGEN) in 70 µL of buffer RDD was added and the column was allowed to sit at room temperature for 30 min; then washed once with buffer RW1 and twice with buffer RPE. The RNA sample was eluted from the column with 50 µL of RNase-free water and checked for quality by running a formaldehyde agarose gel or using 2100 Bioanalyzer (Agilent Technologies). The quantity of RNA was measured by using Qubit RNA BR assay kit (Life Technologies).

Quantitative PCR Primer Design and Primer Efficiency Check

Primers for quantitative PCR were designed from peanut EST sequences using Primer 3 Plus program (www.bioinformatics.nl/primer3plus) with a 100–200 bp optimal amplicon size, 60°C optimal annealing temperature, 50–60% GC content, and other default parameters. Candidate primers were further analyzed with Oligo Analyzer tools (http://www.idtdna.com) to exclude potential primer-dimer-forming primers and hairpin-forming primers. Surviving primers were synthesized by Life Technologies and tested for real-time melt curves and validated for qPCR amplification efficiency with cDNA templates generated from Florida-07 100% irrigation sample RNAs and in 3-fold serial dilutions. The threshold cycle (C_T) values obtained from the real-time PCR experiment were plotted against cDNA template dilutions in log3 to generate a linear function y= ax +b where a = slope (1.58) and amplification efficiency $E = 3^{-1/slope}$. Primer sets that produced percentage amplification efficiency (E% = (2-1) x 100%) between 90–110% (Bio-RAD Laboratories, 2006) were chosen for qPCR experiments.

Internal Reference Gene Selection for qPCR

Five reference genes that were previously published for peanut qPCR studies were tested in our systems: *Act2*, *Ubi2* (Morgante *et al*., 2011), and *yls8*, *60sL7* and *adh3* (Brand and Hovav, 2010). Besides tests of primer amplification efficiency, the expression of reference genes was further tested for expression stabilities in our samples. To perform a stability test, one set of eight biological samples were co-run in technical triplicates using one reference gene primer pair in RT-qPCR. The primers that generated the most stable expression, judged by the least variance of C_T across all samples tested were chosen to be used as a reference gene in this study.

Reverse Transcription Quantitative PCR

Three micrograms of total RNA sample was first reverse-transcribed into first-strand cDNA with 1 µL of Random Primer Mix (New England Biolabs) and 10 µL SuperScript III First-Strand Synthesis SuperMix (Life Technologies) in a 20 µL reaction using SuperScript III protocol. Completed cDNA reactions were then diluted 1:100 with DNase-free H_20. For a 20 µL real-time PCR reaction, 5 µL of the diluted template, 1 µL primer mix (0.25 µM each) and 10 µL SYBR Green Master Mix (Life Technologies) were used with the following PCR reaction profile: 95°C for 10 min for one cycle, 95°C for 10 sec, and 60°C for 30 sec for 40 cycles using MyiQ™ real-time PCR instrument (Bio-Rad Laboratories). Every sample was co-run with a control sample using one set of target gene primers and adh3 reference gene primers in technical triplicates. The control sample was a pooled cDNA sample of three biological samples of Florida-07 under 100% irrigation treatments and collected at 34 DAP. The C_T values of target genes were adjusted by subtracting C_T values of the reference gene and then that of the control sample to generate $\Delta\Delta C_T$, from which $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) values were produced as measurements of relative gene expression levels.

Table 1: The 11 *Arachis hypogaea* genes for RT-qPCR analysis

Gene	[1]GenBank ID	[2]Gene model	[3]*A. thaliana ortholog*	[4]*G. max*ortholog	Reference of peanut EST
AhAREB1	JF766570.1	Araip.M1IPR	AT3G19290.3 [5](2e-104)	Glyma.07G213100.1 (1e-172)	Xu*et al*., direct submission, 2011
AhERF	ES721792	Araip.F3QBW	AT3g14230.3 (2e-37))	Glyma.07G044300.1 (1e-84)	Guo*et al*., 2008
AhMYB	GO264242.1	Araip.76FEY	AT3G06490.1 (1e-65)	Glyma.17G037500.1 (6e-67)	Beilinson*et al*., direct submission, 2009
AhNAC	JK206678.1	Araip.DL86S	AT3G15500.1 (3e-111)	Glyma.12G221500.1 (1e-115)	Koilkonda*et al*., 2012
AhZFP	EG030307.1	Aradu.42P16	AT2G28200.1 (2e-30)	Glyma.13G333400.1 (7e-44)	Fu*et. al*., direct submission, 2006
AhLEA3	ES721105.1	Araip.18621	AT2G36640.1 (3e-55)	Glyma.10G064400.1 (7e-58)	Guo*et al*., 2008
AhGOLS2	GO339374.1	Aradu.ZK8VV	AT1G56600.1 (1e-137)	Glyma.19G227800.1 (1e-153)	Beilinson*et al*. ,direct submission, 2009
AhHsp70	CX128230.1	Araip.48K15	AT5G02490.1 (1e-157)	Glyma.18G289600.1 (2e-167)	Yan*et al*., direct submission, 2004
AhMet2	JK183252.1	Araip.91WSG	AT3G09390.1 (8e-21)	Glyma.07G132000.1 (1e-26)	Huang *et. al*., direct submission, 2012
AhPLDα1	AB232322.1	Araip.YJB9F	AT3G15730.1 (0.0)	Glyma.13G364900.1 (0.0)	Nakazawa*et al*., 2006
AhSps	GO325684.1	Araip.L7Y2B	AT5G53120.6 (3e-83)	Glyma.06G126700.1 (6e-93)	Beilinson*et al*., direct submission, 2009

1: A. hypogaea EST in GenBank; 2: PeanutBase; 3, 4: Phytozome database; 5: E values for CDS alignments with peanut genes

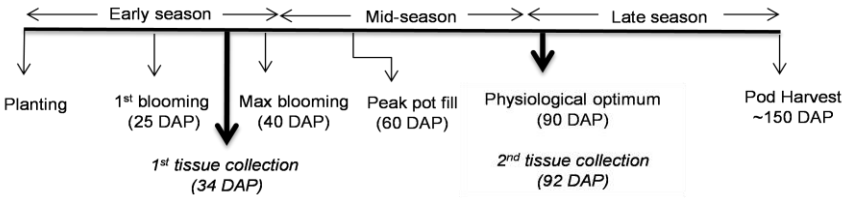

Fig. 1: Peanut growth season, developmental stages, experimental planting and sample collection time

Statistical Analysis

Statistical analyses were accomplished using R program (R Development Core Team, 2013, https://www.r-project.org/). The mean values of $2^{-\Delta\Delta CT}$ of qPCR analysis for each target gene were subjected to ANOVA analysis with respect to irrigation treatments, cultivar, and collection dates and their interactions using a linear mixed model and an α level of 0.10 (if P-values exceed 0.05, they are noted in the text specifically). The initial analysis was based on the underlying field design of a randomized complete block design (r=4) with a split-block restriction on randomization plus a repeated measures component, where water treatment and cultivar where the two main-plot effects, treatment x cultivar the subplot effect, and DAP the repeated measures component. Water treatment, cultivar, and DAP and their respective interactions were treated as fixed effects. Block, Block * Water Treatment, Block * Cultivar, and Block * Water Treatment * Cultivar were the design components, where the latter three are the appropriate experimental errors associated with main-plots and sub-plot. We first fit a complete model as indicated by the field design. Noticing that both main-plot error terms were extremely small and close to the subplot error term we then fit a model with all fixed effects but pooled MP 1 error and MP 2 error with SP error and compared the AICC (penalized fit statistic) values. The AICC values for the two models were very close; in most cases either the simple RCB model or CRD models had a better fit, except for *AhNAC*, which was analyzed based on the underlying RCB-SB design. We next fitted the residual covariance structure for the repeated measures portion. There were only two time points, thus the unstructured model, allowing a separate variance for each time point and a covariance between two, was a logical choice. Again the decision was based on AICC. For four out of 11 genes, the simpler model either RCB-SB or RCB was chosen as the best model. For the remaining cases, the CRD-UN model was deemed the best one. In the final step, all interaction effects with $P > 0.10$ were dropped from the model. Only for *AhZFP* was the full interaction model warranted. For all other response, a main effects model with terms Water Treatment, Cultivar, and DAP was deemed to be sufficient. Estimation of means and standard errors was based on these final models. The correlation matrix of the expression of the target genes was generated by running the entire data set ($2^{-\Delta\Delta CT}$) using Pearson correlation coefficient.

Results

Mild Drought Levels

Based on relatively ample precipitation received during 2011, irrigation was not triggered frequently. By the 34 DAP tissue collection, two irrigation treatments had been applied for a resulting difference between the 100 and 60% treatments of 10 mm of irrigation applied. By the 92 DAP collection, six irrigation treatments had been applied for a total differential of 27 mm. This represents a 5% difference in total amount water received between the 100% and 60% irrigation treatments, confirming that a mild water deficit condition was achieved in this study.

Gene Expression Variation Detected by RT-qPCR

A total of 11 putative peanut target genes were selected and used for this study (Table 1), among which, *AhAREB1*,

AhERF, *AhMYB*, *AhNAC* and *AhZFP* are transcription factors in the upstream of the signal transduction pathway during drought response and *AhHsp70*, *AhSps*, *AhLEA3*, *AhGOLS2*, *AhMet2* and *AhPLDα1* are functional genes in the downstream of the pathway. To choose a suitable reference gene for the RT-qPCR experiment, five reference genes used in different studies were test for the primer efficiency and stability (Fig. 2). Results showed that *Adh3* gene (Table 2) had the optimal primer efficiency and expression stability, thus was chosen as a reference gene in this study (Fig. 2). The primer amplification efficiency of the 11 tested genes were in the range of 0.90 to 1.09 (Table 2), thus was used for the RT-qPCR experiment in this study.

To characterize the differential gene expression under mild water deficit, ANOVA analysis was carried out for the 11 gene expressions across the 24 samples (Table 3). Of the 11 examined genes, the single factors of irrigation treatment, cultivar type and sampling day or the interactive effects of the factors had varying impacts on expressions of nine gene, with two exceptions being the gene *AhLEA3* and *AhGOLS2*, which showed no significant response for single factors or multi-factors ($P<0.1$). Mild water deficit treatment had significant effects on expression of three genes, *AhMYB*, *AhNAC* and *AhZFP* with only *AhZFP* 3-way interactions being significant (Table 3). Compared to full irrigation, the expression of these three transcription factor genes were down regulated by reduced water irrigation with less than 2-fold change in gene expression level (Fig. 3).

Besides water deficit treatment effect, two cultivars also showed significant difference in gene expression for three genes (Table 3). AhAREB1 and *AhMYB* genes had higher expression in FL-07 than Tifguard without any interaction being significant. However, *AhZFP* expression was lower in FL-07 than in Tifguard in general (Fig. 4) with significant ($P<0.1$) multi-factor interactions (Table 3). Specifically, the gene expression of *AhZFP* was affected by interaction of sampling day and cultivar type as well as three-way interactions among irrigation treatment, cultivar types and sampling day (Table 3). AhZFP gene expressed significantly higher in fully irrigated than water deficit treated Tifguard at early growing season (34 DAP). But there was no change in FL-07 at both early and mid-late (92 DAP) growing seasons, which was the same for Tifguard at the late growing season (Fig. 5).

The early and mid-late growth seasons had significant impact on gene expression of eight of the11 genes studied (Table 3). Compared to 34 DAP, *AhAREB1*, *AhERF*, *AhHsp70*, *AhNAC*, *AhPLDα* and *AhSps* were upregualted, and *AhMet2* and *AhZFP* were down regulated at 92 DAP. Among the upregulated genes, *AhNAC* gene expression displayed more than 4-fold change and the rest of the genes showed less than 2-fold change in expression level (Fig. 5). The results reflected effects of developmental changes on gene expression level.

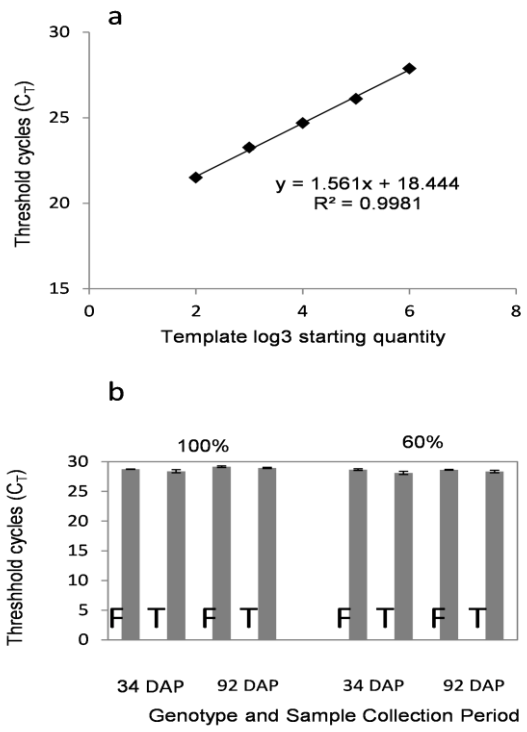

Fig. 2: The validation of adh3 reference gene amplification efficiency test (a); stability test (b).F: Florida-103; T: Tifguard

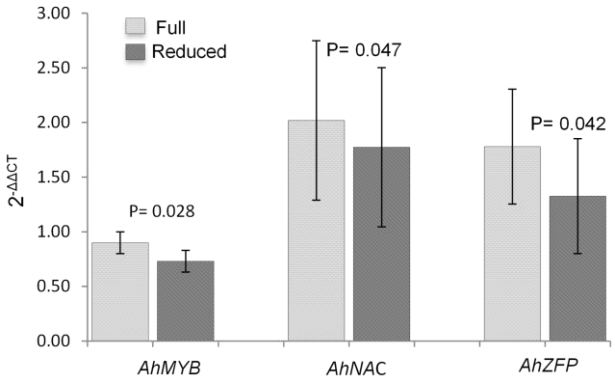

Fig. 3: Gene expression affected by water deficit treatment. Relative gene expression levels of three genes under 100% full irrigation and reduced 60% irrigation were significant. Error bars are 95% CI (μ) and contrast P values between the two water treatments are provided

Gene Expression Correlation

Significant correlations in gene expression were observed among seven of the 11 genes tested (Table 4). Correlation coefficients (CC) of the seven genes were larger than 0.5 and their corresponding P values were smaller than 0.01, which indicates the likelihood of co-expression of one gene

Table 2: Primer sequence, amplicon size and efficiency for RT-qPCR analysis

Gene	Forward primer	Reverse primer	Amplicon (bp)	Efficiency (%)
AhAREB1	5' TTGACGCTTCCAAGGACTCT 3'	5' TGAAACCCCATTGCTACCTC 3'	126	0.95
AhERF	5' AGAACCAGTTTCGGGGAATC 3'	5' GTTCACCTTGGCTTTCTTGC 3'	173	1.01
AhMYB	5' GGGAAACAGATGGTCGAAAA 3'	5' CGCATGGTGTCTTTGAATTG 3'	144	1.09
AhNAC	5' AAGGCACCAAAACAAACTGG 3'	5' GAGACGATCCATTGCTCCAT 3'	183	0.96
AhZFP	5' TGGTGCCATGACCAAATCTA 3'	5' ATCATTCTCAGAGGGCGTGA 3'	94	0.90
AhLEA3	5' AGAAGGCAAAAGAGGGGAAG 3'	5' AGTCTTCTGCATGGCTGCTT 3'	161	1.00
AhGOLS2	5' TAAAGGAGTGGTGGGTCTGG 3'	5' GAGGATGTTGCGGTGTTCTT 3'	106	1.02
AhHsp70	5' CACTTCTCCACTGGCTCCAT 3'	5' TGAGGACAGCATGTGAGAGG 3'	172	0.93
AhMet2	5' TTGCTGTGGAGGAAACTGTG 3'	5' AGCTGGAACACCCATTTCAG 3'	178	1.09
AhPLDα1	5' GAGGTCAAGAAGCCAGGAGA-3'	5' GGTTGATGTTTGCAGATCCA 3'	160	1.05
AhSps	5' GGCAAGTCAAGGAAAGTCCA 3'	5' TCAACTGAACAATCCCGTCA 3'	172	1.03
**AhAdh3*	5' GACGCTTGGC GAGATCAACA 3'	5' AACCGGACAA CCACCACATG 3'	140	0.95

*Adh3: alcohol dehydrogenase class III. Brand and Hovav 2010

Table 3: P-values of ANOVA analysis

Effect	*AhERF*	*AhMYB*	*AhNAC*	*AhHsp70*	*AhSps*	*AhZFP*	*AhLEA3*	*AhGOLS2*	*AhPLDα1*	*AhMet2*	*AhAREB1*
Treatment (WT)	0.75	**0.03**	0.05	0.12	0.84	**0.04**	0.57	0.21	0.11	0.84	0.83
Cultivar (CV)	0.54	**0.04**	0.16	0.82	0.75	**0.00**	0.47	0.89	0.37	0.60	**0.02**
Sampling Period (SP)	**0.02**	0.80	**0.01**	**0.03**	**0.09**	**0.01**	0.12	0.18	**0.03**	**0.00**	**0.05**
CV×WT	ns	ns	ns	ns	ns	0.27	ns	ns	ns	ns	ns
SP×WT	ns	ns	ns	ns	ns	0.22	ns	ns	ns	ns	ns
SP×CV	ns	ns	ns	ns	ns	**0.00**	ns	ns	ns	ns	ns
SP×WT×CV	ns	ns	ns	ns	ns	**0.09**	ns	ns	ns	ns	ns
Covariance Model†	CRD-UN	RCB-SPT	CRD-UN	CRD-UN	CRD-UN	RCB-SPT	CRD-UN	CRD-UN	CRD-UN	CRD-UN	CRD-UN

† The covariance models were a CRD-UN, where Block, Block x WT and Block x C effects were pooled with experimental error and the residual variance was modeled with an unstructured matrix. In the RCB-SPT model, Block x WT and Block x C effects were subsumed into the experimental error and residuals were found to be uncorrelated. Ns: not significant at P=0.1

Mixed models analysis of real-time PCR data of gene expressions with respect to 100% and 60% irrigation treatments, DAPs (sampling points) and cultivar types and interactions between two or three factors. P< 0.1 are bolded

Table 4: Gene expression correlation matrix with correlation coefficients and P-values

Genes	*AhHsp70*	*AhLEA3*	*AhPLDα1*	*AhMet2*	*AhSps*	*AhGOLS2*	*AhERF*	*AhAREB1*	*AhNAC*	*AhMYB*	*AhZFP*
AhHsp70		0.186	**0.000**	0.397	0.111	0.474	**0.000**	**0.006**	0.023	0.102	0.299
AhLEA3	0.290		**0.000**	0.273	0.326	**0.003**	**0.001**	**0.000**	**0.000**	0.349	0.788
AhPLDα1	**0.720**	**0.750**		0.917	0.247	**0.006**	**0.000**	**0.000**	**0.000**	0.682	0.385
AhMet2	-0.180	-0.240	-0.020		0.545	0.652	0.491	0.426	0.128	0.656	0.276
AhSps	0.330	-0.210	0.250	0.130		0.680	0.705	0.663	0.580	0.830	0.818
AhGOLS2	0.150	**0.600**	**0.540**	-0.100	0.090		0.022	**0.003**	**0.000**	0.587	0.606
AhERF	**0.790**	**0.630**	**0.900**	-0.150	-0.080	0.480		**0.000**	**0.000**	0.106	0.585
AhAREB1	**0.550**	**0.760**	**0.900**	-0.170	0.090	**0.580**	**0.800**		**0.000**	0.629	0.708
AhNAC	0.460	**0.820**	**0.800**	-0.320	-0.120	**0.690**	**0.780**	**0.850**		0.955	0.895
AhMYB	0.340	-0.200	0.090	-0.100	-0.050	-0.120	0.350	0.100	0.010		0.419
AhZFP	0.220	0.060	0.190	0.230	-0.050	0.110	0.120	0.080	0.030	-0.170	

Correlation coefficient matrix is generated by online tool http://www.vassarstats.net/rsig.html for N=23 and r> 0.53 will be significant at P=0.01. Bottom diagonal are correlation coeffcients; top diagonal are P-values associated with a correlation. Significant (P=0.01) numbers are bolded

with one or more other genes among them under the experimental conditions. Based on their correlation coefficients, the seven genes were connected to generate a hypothetical gene co-expression network (Fig. 7). The network was composed of three transcription factors genes *AhAREB1*, *AhNAC* and *AhERF* and four functional genes *AhLEA3*, *AhPLDα*, *AhHsp70* and *AhGOLS2*.

In the network, *AhAREB1* and *AhPLDα* were connected to five other genes and also to each other. AhNAC gene with *AhERF* and *AhLEA3* were connected to other four genes and to each other as well. Based on CC values from high (<0.9) to low (>0.7) with P<0.001, the likelihood of gene co-expression between two genes were very high for *AhAREB1* and *AhPLDα* = *AhAREB1* and *AhERF* (CC = 0.9) (Table 4 and Fig. 7).

Discussion

The drought effect is likely caused by a combination of the water deficit and the length of the water stress exposure to the experimental plants because how plants respond to drought depends on drought intensity, duration and progressiveness (Passioura, 2007). Interestingly and unexpectedly, the mild drought did not stimulate the expression of any potentially drought responsive genes analyzed in this study rather it suppressed the expressions of

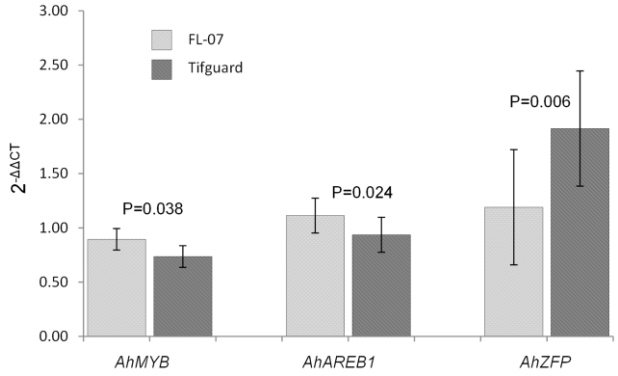

Fig. 4: Differential gene expression by cultivars. Relative gene expressions of three genes are significant between two cultivars. Error bars are displayed as 95% CI(μ) and contrast P-values between the two cultivars are provided

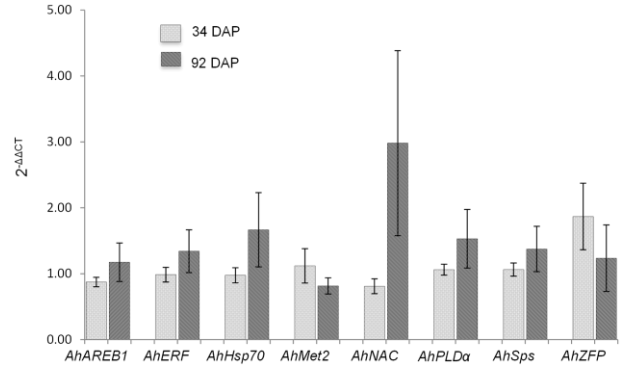

Fig. 5: Gene expression affected by growth stage. Relative gene expression levels of eight genes are significantly different on two sampling days. The contrast P-values between 34 DAP and 92 DAP expression are 0.052, 0.021, 0.030, 0.003, 0.005, 0.025, 0.087 and 0.079 for *AhAREB1*, *AhERF*, *AhHsp70*, *AhMet2*, *AhNAC*, *AhPLDα*, *AhSps* and *AhZFP* genes, respectively. Error bars represent 95% CI (μ)

three transcription factor genes *AhMYB*, *AhNAC* and *AhZFP*. The observation indicated that 1) the mild water deficit in peanut field did impact gene expression; and 2) these three transcription factors may not actively engage in mild water deficit acclimation though they have been reported to play -significant roles in stress response caused by severe drought in cassava (Manihot esculenta) (Ruan *et al.*, 2017), Populus euphratica (Lu *et al.*, 2018) and Arabidopsis thaliana (Yin *et al*., 2017). For example, not all genes from the same family had the same response level to the same drought treatments but with a wide range (0.02 to 78 fold) of changes in gene expression. The gene expression level usually changed more dramatically as drought conditions became more severe (Ruan *et al.*, 2017; Lu *et al*., 2018).

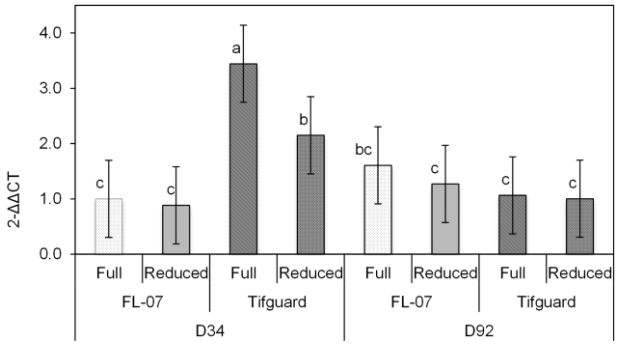

Fig. 6: Multiple factor Interactions of *AhZFP* gene expression. Column boxes are the mean estimates and lines are 95%CI (μ). Treatment combinations sharing the same letter are not significantly different at *P*= 0.05. D34: DAP 34; D92: DAP 92

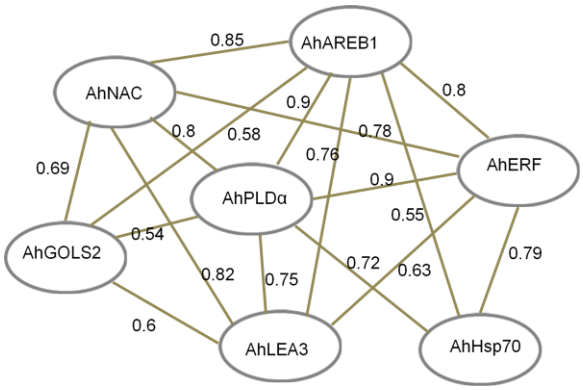

Fig. 7: Predicted gene expression network. Genes with expression correlation coefficients equal to 0.54 or larger (*P*<0.01) were connected to generate the above predicted co-expression network. Correlation coefficients are shown on connection lines, the length of the lines are not proportional to the values

As mentioned previously, gene expression can differ significantly under mild vs. severe drought stress. For example, a microarray study of 15,593 genes in sugarcane found that different number of genes, 77, 361 and 532 were differentially up- or down-regulated by mild, moderate or severe drought, respectively though with some overlapping (Li *et al*., 2016), suggesting that plants might respond to different drought intensity by using different gene expression profiles to achieve physiological changes matching the stress levels. Why the three genes were suppressed instead of promoted by the mild drought treatment is not clear. These genes might be involved in regulatory process of peanut growth and/or development. They are from MYB, NAC and ZFP large transcription factor gene families respectively that play many key regulatory roles in plant development, metabolism and stress response (Nakashima *et al*., 2012; Zhou *et al*., 2015; Park *et al*., 2017). It has been found that down-regulated

genes by mild drought are largely associated with plant growth (Chaves *et al*., 2009; Moumeni *et al*., 2011; Li *et al*., 2016). Because gene network regulation is very complex, a transcription factor gene activity can often lead to multiple downstream cellular/phenotypic effects. Careful studies are necessary to elucidate the functions of the three genes in peanut, especially their functions in gene network regulation of plant response to mild drought, and plant growth and development under sustained mild water deficit.

Gene expression variations according to cultivars are most likely due to the different genetic backgrounds of the cultivars though both cultivars are runner-type peanuts and generated in the Southern United States. Their pod quality and seeds oil content vary in addition to disease resistance. However, the gene expression variations of these genes were not related to mild drought response. In addition, plant developmental changes also have effects of on gene expression level, such as *AhNAC*. The ortholog gene of *AhNAC* in *Arabiposis* was known to associate with processes of water deprivation and jasmonic acid mediated signaling pathway which lead to growth inhibition, senescence and leaf abscission (http://www.uniprot.org/uniprot/Q9LDY8). AhNAC gene thus might play some roles in jasmonic acid pathway which also cross-talk with drought signaling pathway (Shinozaki and Yamaguchi-Shinozaki, 2007). AhZFP gene is the only one among the 11 genes studied which showed gene expression affected by single factor of irrigation treatment, cultivar type or sampling time, and also by two-way interactions between cultivar and sampling time and three-way interactions of all three factors (Fig. 6). The gene could play multiple roles in growth and biotic signaling under mild water deficits and its expression could be affected by different environmental conditions.

The high likely hood co-expression of *AhAREB1* and *AhPLDα* in this study was also discovered in an *Arabidopsis* study demonstrating that AREB1 and PLDα1 were co-upregulated and co-suppressed under the regulation of acyl-CoA-binding proteins which promoted ABA signaling during seed germination and seedling development (Du *et al*., 2013). Although our study did show the upregulation of *AhAREB1* and *AhPLDα* during mild water deficit treatment, the genes might be important players in ABA mediated signaling pathways in peanut and their activation might be detectable by an increased water stress level. Functional gene *AhPLDα* was at the center of the network connecting to all other genes in the network with high likelihood to be co-expressed with transcription factor *AhAREB1*, *AhERF* and *AhNAC* followed by functional genes *AhLEA3*, *AhHsp70* and *AhGOLS2*. The results indicate that *AhPLDα* could be a key gene involved in multiple physiological and developmental processes.

Conclusion

This was the first time that these 11 peanut genes reported to be involved in plant drought tolerance were studied for their expression pattern under mild drought in the field condition with the RT-qPCR method. With the sensitiveness of RT-qPCR, we were able to detect gene expression responses, specifically down-regulation of *AhMYB*, *AhNAC* and *AhZFP* under a mild (5% irrigation deficit) water deficit in field settings using two peanut cultivars and two sampling dates. Gene expression impacted by different crop cultivars and different developmental stages was detected as well. Under a mild water deficit, expressions of several genes, such as *AhAREB1* and *AhPLDα* were co-related and appeared to be aligned with previous findings, which indicated that no matter the various conditions, gene expression are networked to response to the environmental changes. None of 11 genes were up-regulated by mild water deficit across different experimental conditions, thus none of them may positively involve in mild water deficit acclimation, indicating that mild water deficit in the field may induce very different gene expression changes from the manipulated drought condition in model plants at laboratory conditions. Further experiments should be conducted to investigate the mild and several water deficit conditions in the field environment to understand the real world drought acclimation and tolerance in crops.

Acknowledgements

This research is supported by the National Peanut Board and the National Institute of Food and Agriculture, University of Florida, USDA National Institute of Food and Agriculture, Hatch Projects, FLA-AGR-005122, FLA-AGR-005569, and FLA-AGR-005478 under award number 2013-67013-21108.

References

Abreu, I.A., A.P. Farinha, S. Negrao, N. Goncalves, C. Fonseca, M. Rodrigues, R. Batista, N.J. Saibo and M.M. Oliveira, 2013. Coping with abiotic stress: Proteome changes for crop improvement. *J Proteomics*, 93: 145–168

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

Bertioli, D.J., S.B. Cannon, L. Froenicke, G. Huang, A.D. Farmer, E.K. Cannon, X. Liu, *et al.*, 2016. The genome sequences of *Arachis duranensis* and *Arachis ipaensis*, the diploid ancestors of cultivated peanut. *Nat. Genet.*, 48: 438–446

Bio-RAD Laboratories, Inc. 2006. Real-time PCR applications guide

Brand, Y. and R. Hovav, 2010. Identification of suitable internal control genes for quantitative real-time PCR expression analyses in peanut (*Arachis hypogaea*). *Peanut Sci.*, 37: 12–19

Chaves, M.M., J. Flexas and C. Pinheiro, 2009. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Ann. Bot.*, 103: 551–60

Claeys, H. and D. Inze, 2013. The agony of choice: How plants balance growth and survival under water-limiting conditions. *Plant Physiol.*, 162: 1768–1779

Clauw, P., F. Coppens, K. De Beuf, S. Dhondt, T. Van Daele, K. Maleux, V. Storme, L. Clement, N. Gonzalez and D. Inze, 2015. Leaf responses to mild drought stress in natural variants of Arabidopsis. *Plant Physiol.*, 167: 800–816

Csiszár, J., A. Gallé, E. Horváth, P. Dancsó, M. Gombos, Z. Váry, L. Erdei, J. Györgyey and I. Tari, 2012. Different peroxidase activities and expression of abiotic stress-related peroxidases in apical root segments of wheat genotypes with different drought stress tolerance under osmotic stress. *Plant Physiol. Biochem.*, 52: 119–129

Daryanto, S., L. Wang and P.A. Jacinthe, 2015. Global synthesis of drought effects on food legume production. *PloS One*, 10: e012740110

Du, Z.Y., M.X. Chen, Q.F. Chen, S. Xiao and M.L. Chye, 2013. Arabidopsis acyl-CoA-binding protein ACBP1 participates in the regulation of seed germination and seedling development. *Plant J.*, 74: 294–309

Gaspar, T., T. Franck, B. Bisbis, C. Kevers, L. Jouve, J.F. Hausman and J. Dommes, 2002. Concepts in plant stress physiology. Application to plant tissue cultures. *Plant Growth Regul.*, 37: 263–285

Gorbet, D.W. and B.L. Tillman, 2009. Registration of 'florida-07' peanut. *J. Plant Regist.*, 3: 14–18

Harb, A., A. Krishnan, M.M. Ambavaram and A. Pereira, 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant Physiol.*, 154: 1254–1271

Holbrook, C.C., P. Timper, A.K. Culbreath and C.K. Kvien, 2008. Registration of 'tifguard' peanut. *J. Plant Regist.*, 2: 92–94

Kano-Nakata, M., J. Tatsumi, Y. Inukai, S. Asanuma and A. Yamauchi, 2014. Effect of various intensities of drought stress on δ13C variation among plant organs in rice: Comparison of two cultivars. *Amer. J. Plant Sci.*, 5: 1686–1693

Li, F., H.J. Lei, X.J. Zhao, R.R. Tian and T.H. Li, 2012. Characterization of three sorbitol transporter genes in micropropagated apple plants grown under drought stress. *Plant Mol. Biol. Report*, 30: 123–130

Li, C., Q. Nong, M.K. Solanki, Q. Liang, J. Xie, X. Liu, Y. Li, W. Wang, L. Yang and Y. Li, 2016. Differential expression profiles and pathways of genes in sugarcane leaf at elongation stage in response to drought stress. *Sci. Rep.*, 6: 25698–25709

Lichtenthaler, H.K., 1988. In vivo chlorophyll fluorescence as a tool for stress detection in plants. *In: Applications of chlorophyll fluorescene*1st edition, pp: 129–142. Lichtenthaler H.K. (ed.). Kluwer Academic Publishers, Dordrecht, Netherlands

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta deltaC(T)) method. *Methods*, 25: 402–408

Lu, X., X. Zhang, H. Duan, C. Lian, W. Yin and X. Xia, 2018. Three stress-responsive NAC transcription factors from *Populous euphratica* differentially regulate salt and drought tolerance in transgenic plants. *Physiol. Plant.*, 162: 73–97

Morgante, C.V., P.M. Guimaraes, A.C. Martins, A.C. Araujo, S.C. Leal-Bertioli, D.J. Bertioli and A.C. Brasileiro, 2011. Reference genes for quantitative reverse transcription-polymerase chain reaction expression studies in wild and cultivated peanut. *BMC Res. Notes.*, 4: 339–349

Moumeni, A., K. Satoh, H. Kondoh, T. Asano, A. Hosaka, R. Venuprasad, R. Serraj, A. Kumar, H. Leung and S. Kikuchi, 2011. Comparative analysis of root transcriptome profiles of two pairs of drought-tolerant and susceptible rice near-isogenic lines under different drought stress. *BMC Plant Biol.*, 11: 174–190

Nakashima, K., H. Takasaki, J. Mizoi, K. Shinozaki and K. Yamaguchi-Shinozaki, 2012. NAC transcription factors in plant abiotic stress responses. *Biochim. Biol. Physiol. Acta*, 1819: 97–103

Obata, T. and A.R. Fernie, 2012. The use of metabolomics to dissect plant responses to abiotic stresses. *Cell. Mol. Life Sci.*, 69: 3225–3243

Park, Y.R., M.J. Choi, S.J. Park and H. Kang, 2017. Three zinc-finger RNA-binding proteins in cabbage (*Brassica rapa*) play diverse roles in seed germination and plant growth under normal and abiotic stress conditions. *Physiol. Plant.*, 159: 93–106

Passioura, J., 2007. The drought environment: Physical, biological and agricultural perspectives. *J. Exp. Bot.*, 58: 113–117

Rao, R., M. Sheshshayee, N. Karaba, R. Sreevathsa, N. Rama, S. Kumaraswamy, T. Prasad and M. Udayakumar, 2012. Groundnut: Genetic approaches to enhance adaptation of groundnut (*Arachis hypogaea* L.) to drought. *In: Improving Crop Productivity in Sustainable Agriculture*. Tuteja, N., S. Gill and R. Tuteja (eds.), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

Rowland, D., W. Faircloth, P. Payton, D. Tissue, J. Ferrel, R. Sorensen and C. Butts, 2012. Primed acclimation of cultivated peanut (*Arachish ypogaea* L.) through the use of deficit irrigation timed to crop developmental periods. *Agric. Water Manage.*, 113: 85–89

Ruan, M.B., X. Guo, B. Wang, Y.L. Yang, W.Q. Li, X.L. Yu, P. Zhang and M. Peng, 2017. Genome-wide characterization and expression analysis enables identification of abiotic stress-responsive MYB transcription factors in cassava (*Manihot esculenta*). *J. Exp. Bot.*, 68: 3657–3672

Sečenji, M., Á. Lendvai, P. Miskolczi, G. Kocsy, Á. Gallé, A. Szucs, B. Hoffmann, É. Sárvári, P. Schweizer, N. Stein, D. Dudits and J. Györgyey, 2010. Differences in root functions during long-term drought adaptation: Comparison of active gene sets of two wheat genotypes. *Plant Biol.*, 12: 871–882

Sharma, K.K. and P. Bhatnagar-Mathur, 2006. Peanut (*Arachish ypogaea* L.). *Methods Mol. Biol.*, 343: 347–358

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

Skirycz, A., K. Vandenbroucke, P. Clauw, K. Maleux, B. De Meyer, S. Dhondt, A. Pucci, N. Gonzalez, F. Hoeberichts, V.B. Tognetti, M. Galbiati, C. Tonelli, F. Van Breusegem, M. Vuylsteke, and D. Inzé. 2011. Survival and growth of Arabidopsis plants given limited water are not equal. *Nat. Biotechnol.*, 29: 212–214

Verelst, W., A. Skirycz and D. Inze, 2010. Abscisic acid, ethylene and gibberellic acid act at different developmental stages to instruct the adaptation of young leaves to stress. *Plant Signal Behav.*, 5: 473–475

Watkinson, J.I., A.A. Sioson, C. Vasquez-Robinet, M. Shukla, D. Kumar, M. Ellis, L.S. Heath, N. Ramakrishnan, B. Chevone, L.T. Watson, L. van Zyl, U. Egertsdotter, R.R. Sederoff and R. Grene, 2003. Photosynthetic acclimation is reflected in specific patterns of gene expression in drought-stressed loblolly pine. *Plant Physiol.*, 133: 1702–1716

Yin, M., Y. Wang, L. Zhang, J. Li, W. Quan, L. Yang, Q. Wang and Z. Chan, 2017. The Arabidopsis Cys2/His2 zinc finger transcription factor ZAT18 is a positive regulator of plant tolerance to drought stress. *J. Exp. Bot.*, 68: 2991–3005

Zhou, C., Y. Chen, Z. Wu, W. Lu, J. Han, P. Wu, Y. Chen, M. Li, H. Jiang and G. Wu, 2015. Genome-wide analysis of the MYB gene family in physic nut (*Jatropha curcas* L.). *Gene*, 572: 63–71

(Received 08 December 2017; Accepted 13 January 2018)