

Research Article

Identification of genes encoding drought-induced transcription factors in peanut (*Arachis hypogaea* L.)

Phat M. Dang¹, Charles Y. Chen² and C. Corley Holbrook³

¹USDA-ARS, National Peanut Research Laboratory, PO Box 509, 1011 Forrester Dr. SE, Dawson, GA 39842

²Department of Agronomy and Soils, Auburn University, 201 Funchess Hall, Auburn, AL 36849

³USDA-ARS, Crop Genetics and Breeding Research Unit, 115 Coastal Way, Tifton, GA 31793

Received on June 14, 2012; Accepted on October 7, 2012; Published on October 18, 2012

Correspondence should be addressed to Phat M. Dang; Phone: +1 229 995 7432, Fax: +1 995 7416, Email: Phat.Dang@ars.usda.gov

Abstract

Transcription factors play key roles in the regulation of genes involved in normal development as well as tolerance to biotic and abiotic stresses. Specific transcription factors that are induced in peanut under drought conditions have not been identified. The objectives of this study were to compare gene-expression patterns of various transcription factors of a drought tolerant versus a susceptible peanut genotype under drought conditions and to identify transcripts that were regulated in a drought dependent manner. Twelve putative transcription factors were identified and real-time PCR analysis was performed which resulted in the identification

of three unique transcripts in which ahERF1 was highly induced in the recovery stage; ahERF7 and ahERF8 were also highly induced by drought and returned to nominal levels after recovery. These sequences contain DNA binding domains that are present in the APETALA2/Ethylene Responsive Factors (AP2/ERF) family of transcription factors which have been shown to be induced by stress. Induction levels and patterns of gene-expression of ahERF1, ahERF7 and ahERF8 may be used to select plants that may have higher drought tolerance.

Introduction

Drought is a major environmental stress that adversely affects plant growth and can significantly reduce yield and quality. In order to cope with dynamic environmental changes, plants have evolved a complex network of perception and signal transduction (Huang *et al.* 2012; Hubbard *et al.* 2012; Ishibashi *et al.* 2012). It has been established that signal transduction involves both abscisic acid (ABA) dependent and ABA independent pathways (Zhang *et al.* 2006; Boneh *et al.* 2012). Transcription factors have been identified as key effectors that regulate the expression of downstream genes involved in the acclimation process (Yamaguchi-Shinozaki & Shinozaki 2006; Mizoi *et al.* 2012; Rushton *et al.* 2012). The APETALA 2/ethylene-responsive element binding factor (AP2/ERF) represents a large family of plant-specific transcription factors that are divided into four major subfamilies: AP2, ERF, RAV (related to ABI3/VP1) and DREB (dehydration-responsive element binding protein) (Sakuma *et al.* 2002), which are involved in various

stresses such as low or high temperature and water stress (Mizoi *et al.* 2012). These proteins contain a conserved DNA-binding motif approximately 60 residues in length (Kim *et al.* 2006) and contain 1 or 2 AP2/ERF domains (Sakuma *et al.* 2002). AP2/ERF transcription factors regulate gene expression by binding to the *cis*-acting dehydration-responsive element/C-repeat (DRE/CRT) or GCC-box sequence on the promoters of the affected genes (Yamaguchi-Shinozaki & Shinozaki 2006) and over-expression in transgenic plants can enhance tolerance to various stresses (Kang *et al.* 2011).

Peanut (*Arachis hypogaea* L.) is a warm-season legume that provides an important source of proteins and oil for people living in the U.S. and many parts of the world. Its production is adversely affected by drought depending on duration or severity. Even in peanut under irrigation, the amount and timing of water application can be critical to optimize peanut yield depending on variety and location (Hamidou *et al.* 2012). Plant selection for superior drought tolerance has been difficult due to extreme variability based on

year and location (Araus *et al.* 2002). Low genetic polymorphisms have been observed in cultivated peanut (Kottapalli *et al.* 2007), resulting in the need for development of large number of molecular markers as well as the identification of genes that are regulated under drought stress. Recent advances in the development of genomic resources for variety development are promising (Pandey *et al.* 2012); however, selecting plants with higher tolerance continues to be difficult due to the multigenic nature of the drought response and the strong environmental interactions (Ravi *et al.* 2011). Thus, development of gene-expression profiling of tolerant and susceptible peanut genotypes may provide a better approach for plant selection.

The effect of early season or pre-flowering stage drought does not seem to negatively affect peanut yield and quality, and some reports have even shown yield increases (Puangbut *et al.* 2010; Jongrunklang *et al.* 2011). The recognition of drought can lead to the development a larger and deeper root system in peanut which can result in better drought tolerance (Jongrunklang *et al.* 2011) or a significant rate of growth after the release of drought stress (Awal & Ikeda 2002). Understanding molecular processes that are involved in the recognition and signal transduction of drought stress will enable the selection of plants with better resistance. The goal of this study was to identify transcription factors that respond early to drought stress and return to normal levels upon water availability, by comparing a drought susceptible genotype (AP-3) and a tolerant genotype (C76-16) under a short-term (21 days) drought treatment and recovery.

Materials and Methods

Experimental design and plant treatments

Peanut genotypes C76-16 (drought resistant) and AP3 (susceptible) were selected for evaluation. Plants were grown in a randomized block design in 2 (5.5 m X 12.2m) environmental controlled rainout shelters (Blankenship *et al.* 1989) at the National Peanut Research Laboratory, Dawson, GA, USA. Peanuts were planted at a seeding rate of 20/m with 0.76m spacing and irrigated to provide uniform germination. Three 5.5 m rows were planted for each genotype in each treatment plot: full irrigation and drought treatment. 10-14 days after planting (DAP), germinated seeds were counted for all plots to ensure consistent germination efficiency.

Irrigation treatment

Plants were irrigated based on evapotranspiration (ET) replacement for peanut as described by Stansell *et al.*

(1976). Watermark moisture sensors (Irrometer, Riverside, CA) were placed at 4" and 8" depth and read every 4th day. Irrigation was triggered based on an average reading of -60 kPa for both 4" and 8" depth. For drought treatment, water was completely withheld for 3 wks, and then re-irrigated. Control plots were fully irrigated throughout the experiment.

Sample collection and processing

Samples were collected once a wk for 5 consecutive wks: stage 1 (43 DAP), stage 2 (50 DAP), stage 3 (58 DAP), stage 4 (64 DAP) and stage 5 (71 DAP). Fully expanded leaves from the main stem (second nodal) were randomly selected. Two plants were sampled per row with 3 row replications per treatment. Fresh leaves were collected in the morning (8-9 a.m.), placed into plastic bags and stored on ice until all samples were collected (~30 min). Approximately 0.1 to 0.15 g leaf samples were placed into 2 mL homogenization tubes and immediately frozen in liquid nitrogen for RNA extraction. Third nodal leaves from the same main stem were collected to determine relative water content (RWC), specific leaf area (SLA) and leaf density moisture content (LDMC) on the same collection day.

RWC, SLA, and LDMC measurements

Freshly collected leaves were immediately weighed after collection and were subsequently fully submerged in deionized water and placed under a white light lamp for 2 h to ensure tissues were completely turgid. Leaves were blotted dry and weighed. Leaf area (LA) was measured using the LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA). Leaves were placed into a 65°C oven for 2 days to ensure complete dryness and then weighed.

RWC was determined based on the following formula (Barrs & Weatherley 1962):

$$\text{RWC (\%)} = [(FW - DW)/(TW - DW)] \times 100$$

where FW is fresh weight; DW, dry weight and TW, turgid weight. SLA is the ratio of leaf area to leaf dry mass (LA/DW) and LDMC is the ratio of leaf dry mass to saturated fresh mass (DW/TW).

RNA extraction

Frozen leaves were pulverized with 0.28 mm ceramic beads in 2 mL tubes using an Omni Bead Ruptor 24 (Kennesaw, GA, USA). Total RNA was extracted using the method described by Chomczynski & Sacchi (1987) with the modifications of adding 1% PVPP in the homogenization solution and a LiCl precipitation step to further separate DNA from RNA. RNA concentration was determined using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington,

DE); quality was evaluated by RNA gel electrophoresis (Sambrook *et al.* 1989) and images were captured on the Gel Logic 200 Imaging System (Kodak, Rochester, NY). Prior to reverse-transcription, RNA was DNase-treated with Turbo DNA-free (Ambion, Austin, TX).

Identification of peanut transcription factors

The peanut expressed-sequence tags (EST) project identified twelve putative transcription factors that may have a role in biotic stress (Guo *et al.* 2009). Sequence alignment was performed by Sequencher sequence analysis software v5.0 (Gene Codes, Ann Arbor, MI). Partial sequences were utilized to search against the EST NCBI database to identify other homologous sequences that can align to produce longer sequences and provide a higher number of sequences in overlapping regions for greater confidence. The original NCBI accession numbers with the corresponding consensus and new identifiers are listed in Supplementary Table 1. Open-reading frames (ORFs) were electronically translated to produce putative protein and searched against the NCBI protein database. Protein physical parameters were performed using ProtParam (<http://web.expasy.org/protparam>). This web-based program is a tool which allows the computation of various physical and chemical parameters for a given protein. These parameters include molecular weight, amino acid composition, theoretical pI value and extinction coefficient of a particular protein which can be compared to other proteins to associate similar structure or function.

Primer design and testing

Primers for real-time PCR were designed using Primer Express v3 (Applied Biosystems). Serial dilutions (1:2) of pooled cDNAs from drought treated peanut leaves were utilized to determine primer efficiency. Regression analysis was performed on the Ct values generated from real-time PCR plotted against the log of transformed dilution series.

Reverse transcriptase

For cDNA synthesis, 1 mg total RNA was denatured at 65°C for 5 minutes in the presence of 1 mL oligo dT (50 mM) in a total volume of 13 mL and immediately placed on ice for 2 minutes. To each sample, 4 mL of 5x Reaction Buffer (Invitrogen, Carlsbad, CA) was added and allowed to incubate at 25°C for 10 minutes followed by the addition of 2 mL 10x Reaction Enzyme. The reaction mix was incubated at 42°C 1 hour, heated to 85°C for 5 minutes to terminate the reaction, diluted 1:100 with 10 mM Tris pH 7.5 and stored at 4°C until use.

Real-time PCR conditions

Real-time PCR was performed on an ABI 7500 real-time PCR machine utilizing RT² SYBR Green qPCR Mastermix with ROX (Qiagen, Valencia, CA) as follows: 4 mL of diluted cDNA, 0.4 mM of each primer and 12.5 mL of qPCR mastermix in a 25 mL reaction volume. PCR cycling conditions consisted of 4 stages: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, and a dissociation curve analysis which consisted of 15 sec at 95°C, 20 sec at 58°C and 15 sec at 95°C. Three independent reactions were performed for each sample.

Data analysis

Raw Ct values from the real-time PCR were analyzed according to Livak & Schmittgen (2001). All samples were first normalized to the Actin gene as an internal control, and then treated samples were normalized to controls to determine the relative changes in gene-expression. Levels and standard errors at difference stages for both C76-16 and AP-3 were graphed using SigmaPlot v10 (Systat Software, Chicago, IL). Analysis of variance was performed using Statistix 9 (Analytical Software, Tallahassee, FL). Differences between means were determined using Student's paired t-test method at the $P = 0.05$ level.

Results

Physiological indicators of plant water stress

Relative water content (RWC), specific leaf area

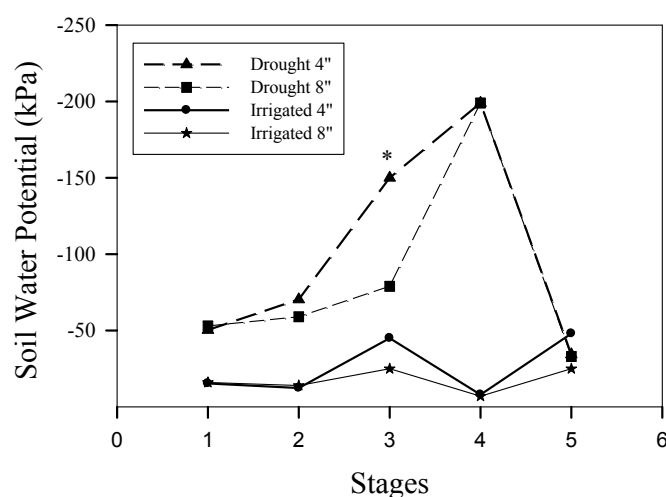


Figure 1. Soil moisture measurements at 4" and 8" depths of drought and irrigated plots. Watermark readings were taken once a week for soil dry-down stages (1-4) and a re-irrigation stage (5). More negative readings (kPa) in drought-treated plot at both 4" and 8" indicated that soil moisture was decreasing over time and returned to standard levels after irrigation. Fully irrigated plot showed minor fluctuation in readings over all stages (1-5).

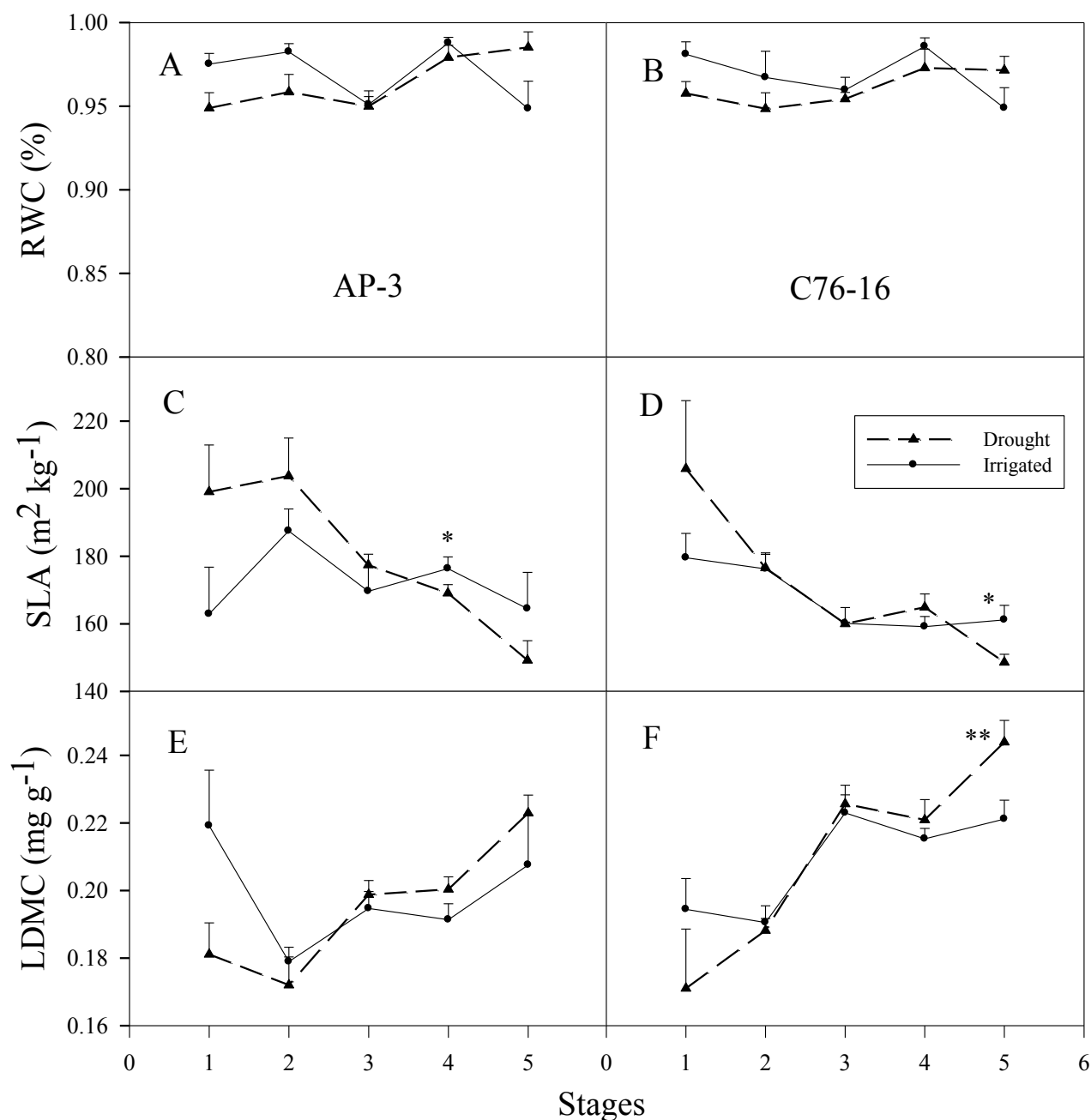


Figure 2. RWC (A, B), SLA (C, D), LDMC (E, F) measurements for AP-3 and C76-16 at drought stages (1-4) and recovery (5). Treated samples were compared to irrigated controls.

(SLA), and leaf density moisture content (LDMC) represent measurable physiological traits that indicate plant stress (Girdthai *et al.* 2010; Vile *et al.* 2005). Treated plants were observed to be healthy with no signs of water stress compared to irrigated controls even when soil moisture was observed to decrease over time (Figure 1). Figure 2 illustrates the RWC, SLA, and LDMC trends of drought treatment compared to the irrigated control. For the first collection date, RWC, SLA and LDMC of treated plants were not different than the irrigated control. At stage 2, soil moisture was slightly reduced in the drought treatment.

However, RWC, SLA, and LDMC showed similar trends under both treatments. At stage 3, RWC, SLA and LDMC for both AP-3 and C76-16 showed no difference compared to the irrigated control while soil water content was significantly reduced ($P < 0.05$) from -70 to -150 kPa at 4" and -59 to -79 kPa at 8" depth. At stage 4, RWC and LDMC showed upward trends similar to the irrigated control for AP-3 and C76-16 even when soil moisture continued to a negative trend above the maximum reading threshold of the moisture sensor of 199. SLA showed a negative trend different ($P < 0.05$) than the control for AP-3 while

C76-16 showed no difference compared to the irrigated control. After tissue collection for stage 4, plants were irrigated to evaluate plant recovery. At stage 5, RWC of drought treatment showed no difference from the irrigated plot for both AP-3 and C76-16 with soil moisture similar to the irrigated plot. SLA and LDMC were significantly different ($P < 0.05$) compared to the control for C76-16 while AP-3 was not different. Observed patterns of RWC, SLA and LDMC for the drought treatment and irrigated control indicated that a mild and short-term water stress was applied.

Identification of putative peanut transcription factors

Twelve putative transcription factors were identified from a reported EST dataset (Guo *et al.* 2009) and were designated as ahERF1 to 12. An NCBI protein database search identified that ahERF1-2 and ahERF5-12 contained a DNA binding domain commonly found

in the AP2/ERF family of transcription factors and ahERF3-4 contained a multiprotein bridging factor 1 (MBF1) with a prokaryotic DNA binding domain helix-turn-helix (HTH) found in the xenobiotic response element (XRE) family of transcription factors. Overexpression of the AP2/ERF transcription factor or of MBF1 in transgenic plants enhanced tolerance to drought and/or heat (Zhang *et al.* 2009; Kim *et al.* 2007). These represented candidate genes that were evaluated in a peanut drought experiment. Primer and amplicon details are listed in Table 2.

Real-time PCR results

The variation in fold change ranged from 0 to 12 for ahERF1, 0 to 5 for ahERF2-6 and ahERF9-12 while ahERF7 and ahERF8 were between 0 and 60. For ahERF1, no difference in the levels of gene-expression was observed at stage 1 between the two genotypes (Figure 3A). C76-16 was 2.0 (1.51/0.760) fold higher ($P < 0.05$) at stage 2, 1.3 (4.19/3.20) and 2.9

Table 2. Primers and amplicons characteristics.

Gene ID	Direction	Primers	Amplicon size (bp)	T _m	Efficiency (%)	Regression coefficient R ²
ahERF1	F	GGCAGAGGCCCTGGGGCAAG	71	70	102.0	0.930
	R	GCCGAGCCAGACACGGACCC		70		
ahERF2	F	TCGCGTTACACGGCGGTTAC	81	66	94.3	0.998
	R	GCCCCTCCTCTTGCCGGA		68		
ahERF3	F	TGTGGGGCCCATATCGCAGGA	70	66	101.9	0.990
	R	CTGGCCGCAGCGTTTGGTG		66		
ahERF4	F	CTGGCGAAGCAGATCAATGAG	64	62	83.8	0.999
	R	GCTGGGCTTTGCCGTTCT		62		
ahERF5	F	GGCAAGAAAGCCAAGGTGAAT	71	61	91.7	0.997
	R	AGATTGGCCTGGCACGTT		61		
ahERF6	F	CCCGTTTTTCGCGGTGTCCGA	111	66	91.4	0.989
	R	CAGCCTCGGCGGTGTCCAAG		68		
ahERF7	F	CCGAACCTCGGCCCCGACCTT	138	66	83.3	1.000
	R	ACGCGGACACGATTTCGGCTGG		68		
ahERF8	F	GTTTCGGCGGCGGAGCTTCA	84	66	87.6	0.957
	R	TGCGTTGGCCGAAGGTGTCC		66		
ahERF9	F	GGGGAAGTGGGTGGCGGAGA	81	68	94.2	0.999
	R	GCGGCCACCGGTGTTGTGTA		66		
ahERF10	F	CTTGTTGCTGCTGTTACGTTGTTC	72	62	86.1	0.999
	R	TTGCCTCCGCCGTTACAC		62		
ahERF11	F	GCCATTGGTGCTGCCCTTGC	76	66	109.8	0.973
	R	TGGGGAAGTTGAGGCGGGCA		66		
ahERF12	F	GGCAAGAAAGCCAAGGTGAAT	71	61	84.2	0.999
	R	AGATTGGCCTGGCACGTT		61		
Actin	F	ACGAGCTTCGTGTGGCTCCTG	68	66	91.8	0.996
	R	GGCCTTTGGGTTGAGGGGTGC		68		

(1.88/0.66) fold lower ($P < 0.05$) for stages 3 and 4, and 4.9 (12.5/2.56) fold higher ($P < 0.05$) at stage 5, compared to AP-3. C76-16 was 1.2 (2.26/1.86) fold higher ($P < 0.05$) at stage 1 for ahERF2, 1.4 (1.6/0.816) fold lower ($P < 0.05$) at stage 3, and 1.2 (1.30/1.13) fold higher ($P < 0.05$) at stage 5, compared to AP-3 (Figure 3B). No differences were observed at stages 2 and 4. For ahERF3, no differences were observed for stages 1 to 3 between the two genotypes (Figure 3C). C76-16 was 1.2 (0.579/0.496) and 1.4 (1.22/0.876) fold lower ($P < 0.05$) at stages 4 and 5 than AP-3. No difference was observed at stage 1 for ahERF4 between genotypes (Figure 3D). C76-16 was 2.1 (2.59/1.21) fold higher ($P < 0.05$) at stage 2, 1.77 (0.380/0.214) and 1.85 (2.02/1.09) fold lower ($P < 0.05$) at stages 3 and 4, and 4.1 (1.11/0.496) fold lower ($P < 0.05$) at stage 5, compared to AP-3. For ahERF5, no difference was observed at stage 1 between genotypes (Figure 3E). C76-16 was 1.4 (1.47/1.09) fold higher ($P < 0.05$) at stage 2 but 2.0 (2.15/1.05) and 2.2 (1.44/0.659) fold lower ($P < 0.05$) for stages 3 and 4, respectively. No difference was observed at stage 5. The level of gene-expression for ahERF6 in C76-16 at stage 1 was 1.8 (3.16/1.75) fold higher but showed no difference at stages 2 and 3, when compared to AP-3 (Figure 3F). At stage 4, C76-16 was 1.9 (1.44/0.741) fold higher and no difference was observed at stage 5. For ahERF7, C76-16 was 13.2 (6.29/0.476) fold lower ($P < 0.05$) at stage 1 and no difference was observed at stage 2 (Figure 3G). At stage 3, C76-16 was 447 (47.83/0.107) fold higher ($P < 0.05$) compared to AP-3 and 100 (47.83/0.476) fold higher, ($P < 0.05$) compared to stage 1 of C76-16. No difference was observed for stage 4 and C76-16 was 18.6 (3.85/0.207) fold lower ($P < 0.05$) than AP-3. No difference was observed for stage 1 of ahERF8 between genotypes (Figure 3H). C76-16 was 2.7 (11.2/4.16) fold higher ($P < 0.05$) at stage 2, 7.2 (14.9/2.06) higher ($P < 0.05$) at stage 3, and 4.5 (34.9/7.82) fold higher ($P < 0.05$) than AP-3. However, C76-16 was 11 (9.47/0.864) fold lower than AP-3 at stage 5. For ahERF9, C76-16 was 2.0 (2.32/1.18) fold higher ($P < 0.05$) at stage 1 while no difference was observed at stage 2, compared to AP-3 (Figure 3I). C76-16 was 1.5 (2.36/1.59) fold higher ($P < 0.05$) at stage 3 and 2.5 (1.01/0.406) fold lower ($P < 0.05$), compared to AP-3. At stage 5, C76-16 was 1.3 (2.45/1.84) fold lower ($P < 0.05$) than AP-3. No differences were observed for stages 1 to 3 for ahERF10 (Figure 3J). C76-16 was 1.9 (1.25/0.659) fold lower ($P < 0.05$) than AP-3 at stage 4 and no difference was observed at stage 5. For ahERF11, no differences were observed for stages 1 to 4 with a decreasing trend with the lowest point at stage 4 and increasing trend at stage 5 with C76-16 at 1.9 (2.90/1.52)

fold lower ($P < 0.05$), compared to AP-3 (Figure 3K). No difference was observed at stage 1 for ahERF12 between the two genotypes (Figure 3L). C76-16 was 3.5 (3.19/0.903) fold higher ($P < 0.05$) at stage 2, and 2.2 (2.17/0.990), 1.5 (1.86/1.22) and 2.0 (2.84/1.43) fold lower ($P < 0.05$) at stages 3 to 5, respectively.

Protein elements

The DNA binding element found in APETALA2 and ethylene responsive factors were identified in ahERF1-2 and ahERF5-12, while MBF1 was present in ahERF3-4 (Table 2). ahERF1 was of interest since it showed a strong gene-induction in the recovery stage for C76-16, compared to AP-3; and ahERF7 and ahERF8 were of interest since they showed coordinated and strong levels of gene induction and returned to almost comparable levels as in the controls after the release of drought. ahERF1 was composed of 90 residues (270 aa) with a single AP2/ERF binding domain located between aa 105 and 135 (YRGIRQRPWGKWAAEIRDPHKGVRVWLGTF). It was predicted to be 29.9 kDa with a pI value of 5.12 and an extinction coefficient of 44,460, indicating instability. Asparagine (10.0%), Aspartic acid (9.3%), Glycine (8.1%) and Leucine (7.8%) were highly represented. ahERF7 was composed of 76 residues (228 aa) with a single AP2/ERF binding site. It was predicted to be 24.8 kDa with a pI value of 6.22 and an extinction coefficient of 34,950, also indicating instability. Alanine (13.2%), Proline (10.5%) and Serine (14.0%) amino acids were highly represented but devoid of Cysteine. A conserved DNA binding domain was located between aa 45 and 75 (YRGVRRMRQWGKWVSEIREPKKRNRIWLGTF) that matched a cd00018 sequence cluster in the NCBI protein database. This binding domain is found in APETALA2 and EREBP (ethylene responsive element binding protein) transcription factors that specifically bind to an 11-bp GCC-box on the promoters of ethylene responsive genes (Allen *et al.* 1998). ahERF8 was a partial sequence with 280 aa in length. Protein database search revealed that it contained two AP2 binding domains (YRGVTRHRWTGRYEAHLWDNSCRREGQSRKGRQVYLGG) and (YRGVTRHHQHGRWQARIGRVAGNKDLYLGT), representative of AP2 belonging to the AP2/ERF family of transcription factors (Allen *et al.* 1998) and also matched the cd00018 sequence cluster.

Discussion

Plants can respond to drought by deploying an escape mechanism in which their life cycle is completed before a severe drought occurs or an avoidance/tolerance mechanism in which physiological, biochemical and

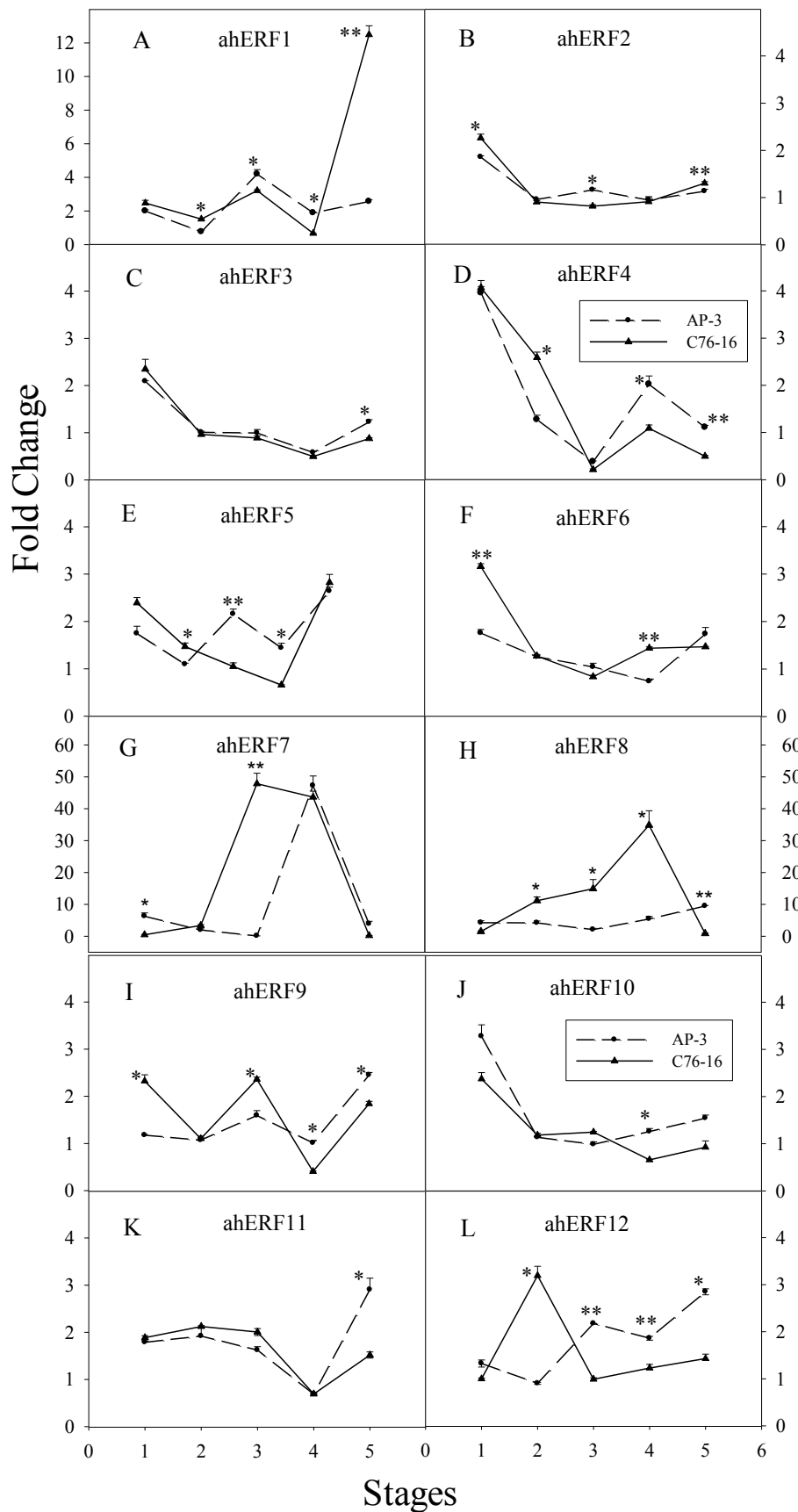


Figure 3. Real-time PCR results of twelve putative peanut transcription factors comparing AP-3 and C76-16. Relative fold changes ranged from 0 to 12 for ahERF1 (A), 0 to 5 for ahERF2-6 (C-F) and ahERF9-12 (I-L) and 0 to 60 for ahERF7-8 (G-H) were plotted in drought stages (1-4) and recovery (5).

physical parameters are modulated to maintain growth and development (Harb *et al.* 2010). In nature, variations in duration and severity of different droughts occur which likely result in the evolution of plants with different combinations of molecular processes. Peanut seems to exhibit an avoidance/tolerance response with changes in root architecture (Puangbut *et al.* 2009; Jongrunklang *et al.* 2011) or a physiological and biochemical modulation in the recognition of stress and recovery (Awal & Ikeda 2002; Puangbut *et al.* 2010). Exogenous expression of *Arabidopsis* DREB1A transcription factor in transgenic peanut resulted in stronger root growth under drought stress, compared to wild type controls (Vadez *et al.* 2012). Peanut transcription factors may be involved in the recognition and response leading to drought acclimation or tolerance. Patterns of gene-expression were evaluated comparing a drought tolerant (C76-16) and a susceptible (AP-3) genotype to identify differences in drought recognition and response.

C76-16 may recognize drought stress earlier than AP-3. ahERF7 gene-expression was significantly ($P < 0.05$) induced up to 100 fold, compared to the initial stage for C76-16 and one wk earlier than AP-3. This result suggests that ahERF7 gene-expression is critical for drought response since it is significantly induced under drought and is reduced to a very low level after drought release. Earlier drought recognition and response maybe provide a selective advantage of C76-16 compared to AP-3. ahERF8 gene-induction was positively correlated with relative soil moisture during drought treatment and was significantly higher ($P < 0.05$) for C76-16 compared to AP-3. Transcription of ahERF8 was highly regulated with only 4 ESTs being identified from a total of

253,274 peanut ESTs in the NCBI database. ahERF8 was induced by drought and returned to a low level after the release of drought stress. This temporal induction indicated that ahERF8 is important in the drought response which may lead to plant acclimation or tolerance.

A strong recovery after the release of drought was observed in peanut with significant increases in relative growth rate, net N₂ assimilation rate, root to shoot (R:S) ratio and a flush of flowers upon the release of drought (Awal & Ikeda 2002). ahERF1 in C76-16 was 4.9 (12.5/2.56) fold induced ($P < 0.05$) following recovery, compared to AP-3 (Figure 3A). In *Arabidopsis*, Expansins that are involved in cell expansion were induced in the acclimation process (Harb *et al.* 2010) and may lead to rapid growth after the release of drought.

The expression of ahERF1 can induce the expression of downstream genes important in the acclimation process. The ethylene responsive element binding proteins interact with *cis*-acting GCC box in promoter regions of defense-related genes and regulate gene-expression (Hao *et al.* 1988). Overexpression of LeERF in tomato enhanced drought tolerance and up-regulated stress related genes such as proline synthetase (P5CS), late embryogenesis protein (LEA), lipid transfer protein (Ltpg2) and cysteine protease (tdi-65) (Lu *et al.* 2010). *Arabidopsis* plants transformed with ERF5 and ERF6 resulted in significant increased resistance against *Botrytis cinerea* and induced a set of 46 genes, a majority of which were associated with the defense response (Moffat *et al.* 2012). In general, specific downstream genes can be activated based on the timing and duration of stress that can result in tolerance or resistance.

Conclusion

Comparison of contrasting drought responsive genotypes emphasized the importance of plant selection in utilizing acclimation capacity in early-season drought. The correlation of plant drought response to specific patterns of gene-expression can identify important genes in the acclimation process. Variation in the patterns of gene-expression of the same transcript between two very similar peanut genotypes suggested differences in the promoter regions. Induction levels and patterns of gene-expression of drought-inducible genes may be used to select plants that may have higher drought tolerance.

Acknowledgements

The authors would like to thank Larry Powell and

Kathy Gray for technical assistance in the field and in the laboratory. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

Conflicts of interest

The authors do not have any conflicts of interest.

References

- Allen MD, Yamasaki K, Ohme-Takagi M, Tatenos M & Suzuki M 1998 A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *Embo J* **17** 5484-5496.
- Araus JL, Slafer GA, Reynolds MP & Royo C 2002 Plant breeding and drought in C3 cereals: what should we breed for? *Ann Bot* **89** 925-940.
- Awal MA & Ikeda T 2002 Recovery strategy following the imposition of episodic soil moisture deficit in stands of peanut (*Arachis hypogaea* L.). *J Agron Crop Sci* **188** 185-192.
- Barrs HD & Weatherley PE 1962 A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Aust J Biol Sci* **15** 413-428.
- Boneh U, Biton I, Schwartz A & Ben-Ari G 2012 Characterization of the ABA signal transduction pathway in *Vitis vinifera*. *Plant Sci* **187** 89-96.
- Blankenship PD, Mitchell BW, Layton RC, Cole RJ & Sanders TH 1989 A low-cost microcomputer system to monitor and control an environmental control plot facility. *Comput Electron Agric* **4** 149-155.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* **162** 156-159.
- Girdthai T, Jogloy S, Vorasoot N, Akkasaeng C, Wongkaew S, Holbrook CC & Patanothai A 2010 Associations between physiological traits for drought tolerance and aflatoxin contamination in peanut genotypes under terminal drought. *Plant Breed* **129** 693-699.
- Guo BZ, Chen X, Hong Y, Liang X, Dang PM, Breneman T, Holbrook CC & Culbreath AK 2009 Analysis of gene expression profiles in leaf tissues of cultivated peanuts and development of EST-SSR markers and gene discovery. *Int J Plant Genomics* doi: 10.1155/2009/715605
- Hamidou F, Ratnakumar P, Halilou O, Mponda O, Kapewa T, Monyo E, Faye I, Ntare BR, Nigam SN, Upadhyaya HD & Vadez V 2012 Selection of intermittent drought tolerant lines across years and locations in

the reference collection of groundnut (*Arachis hypogaea* L.). *Field Crops Res* **126** 189-199.

Hao DY, Ohme-Takagi M & Sarai A 1998 Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J Biol Chem* **273** 26857-26861.

Harb A, Krishnan A, Ambavaram MMR & Pereira A 2010 Molecular and Physiological Analysis of Drought Stress in *Arabidopsis* Reveals Early Responses Leading to Acclimation in Plant Growth *Plant Physiol* **154** 1254-1271.

Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, Liu J, Zhong M & Guo ZF 2012 Signal transduction during cold, salt, and drought stresses in plants. *Mol Biol Rep* **39** 969-987.

Hubbard KE, Siegel RS, Valerio G, Brandt B & Schroeder JI 2012 Absciscic acid and CO₂ signalling via calcium sensitivity priming in guard cells, new CDPK mutant phenotypes and a method for improved resolution of stomatal stimulus-response analyses. *Ann Bot* **109** 5-17.

Ishibashi Y, Tawaratsumida T, Kondo K, Kasa S, Sakamoto M, Aoki N, Zheng SH, Yuasa T & Iwaya-Inoue M 2012 Reactive Oxygen Species Are Involved in Gibberellin/Abscisic Acid Signaling in Barley Aleurone Cells. *Plant Physiol* **158** 1705-1714.

Jongrunklang N, Toomsan B, Vorasoot N, Jogloy S, Boote KJ, Hoogenboom G & Patanothai A 2011 Rooting traits of peanut genotypes with different yield responses to pre-flowering drought stress. *Field Crop Res* **120** 262-270.

Kang HG, Kim J, Kim B, Jeong H, Choi SH, Kim EK, Lee HY & Lim PO 2011 Overexpression of FTL1/DDF1, an AP2 transcription factor, enhances tolerance to cold, drought, and heat stresses in *Arabidopsis thaliana*. *Plant Sci* **180** 634-641.

Kim S, Soltis PS, Wall K & Soltis DE 2006 Phylogeny and domain evolution in the APETALA2-like gene family. *Mol Biol Evol* **23** 107-120.

Kim MJ, Lim GH, Kim ES, Ko CB, Yang KY, Jeong JA, Lee MC & Kim CS 2007 Abiotic and biotic stress tolerance in *Arabidopsis* overexpressing the Multiprotein bridging factor 1a (MBF1a) transcriptional coactivator gene. *Biochem Biophys Res Commun* **354** 440-446.

Kottapalli KR, Burow MD, Burow G, Burke J & Pupala N 2007 Molecular characterization of the US peanut mini core collection using microsatellite markers. *Crop Sci* **47** 1718-1727.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25** 402-408.

Lu C, Li Y, Chen A, Li L, Zuo J, Tian H, Luo Y &

Zhu B 2010 LeERF1 improves tolerance to drought stress in tomato (*Lycopersicon esculentum*) and activates downstream stress-responsive genes. *African J Biotechnol* **9** 6294-6300.

Mizoi J, Shinozaki K & Yamaguchi-Shinozaki K 2012 AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim Biophys Acta* **1819** 86-96. doi: 10.1016/j.bbagr.2011.08.004

Moffat CS, Ingle RA, Wathugala DL, Saunders NJ, Knight H & Knight MR 2012 ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against *Botrytis cinerea* in *Arabidopsis*. *PLoS ONE* **7** e35995.

Nautiyal PC, Ravindra V, Zala PV & Joshi YC 1999 Enhancement of yield in groundnut following the imposition of transient soil-moisture-deficit stress during the vegetative phase. *Exp Agric* **35** 371-385.

Pandey MK, Monyo E, Ozias-Akins P, Liang X, Guimarães P, Nigam SN, Upadhyaya HD, Janila P, Zhang X, Guo B, Cook DR, Bertoli DJ, Michelmore R & Varshney RK 2012 Advances in *Arachis* genomics for peanut improvement. *Biotechnol Adv* **30** 639-651.

Puangbut D, Jogloy S, Vorasoot N, Akkasaeng C, Kesmala T, Rachaputi RCN, Wright GC & Patanothai A 2009 Association of root dry weight and transpiration efficiency of peanut genotypes under early season drought. *Agric Wat Manag* **96** 1460-1466.

Puangbut D, Jogloy S, Toomsan B, Vorasoot N, Akkasaeng C, Kesmala T, Rachaputi RCN, Wright GC & Patanothai A 2010 Physiological Basis for Genotypic Variation in Tolerance to and Recovery from Pre-flowering Drought in Peanut. *J Agron Crop Sci* **196** 358-367.

Ravi K, Vadez V, Isobe S, Mir RR, Guo Y, Nigam SN, Gowda MV, Radhakrishnan T, Bertoli DJ, Knapp SJ & Varshney RK 2011 Identification of several small effect main QTLs and large number of epistatic QTLs for drought tolerance in groundnut (*Arachis hypogaea* L.). *Theor Appl Genet* **122** 1119-1132.

Rushton DL, Tripathi P, Rabara RC, Lin J, Ringler P, Boken AK, Langum TJ, Smidt L, Boomsma DD, Emme NJ, Chen X, Finer JJ, Shen QJ & Rushton PJ 2012 WRKY transcription factors: key components in abscisic acid signalling. *Plant Biotechnol J* **10** 2-11.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K & Yamaguchi-Shinozaki K 2002 DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Biophys Res Commun* **290** 998-1009.

Sambrook J, Fritsch EF & Maniatis T 1989 Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.

Stansell JR, Shepherd JL, Pallas JE, Bruce RR, Minton

- NA, Bell DK & Morgan LW 1976 Peanut response to soil water variable in the southeast. *Peanut Sci* **3** 44-48
- Vadez V, Rao JS, Bhatnagar-Mathur P & Sharma KK 2012 DREB1A promotes root development in deep soil layers and increases water extraction under water stress in groundnut. *Plant Biol (Stuttg)*. doi: 10.1111/j.1438-8677.2012.00588.x
- Vile D, Garnier E, Shipley B, Laurent G, Navas ML, Roumet C, Lavorel S, Diaz S, Hodgson JG, Lloret F, Midgley GF, Poorter H, Rutherford MC, Wilson PJ & Wright IJ 2005 Specific leaf area and dry matter content estimate thickness in laminar leaves. *Ann Bot* **96** 1129-1136.
- Yamaguchi-Shinozaki K & Shinozaki K 2006 Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol* **57** 781-803.
- Zhang JH, Jia WS, Yang JC & Ismail AM 2006 Role of ABA in integrating plant responses to drought and salt stresses. *Field Crop Res* **97** 111-119.
- Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J & Ma Y 2009 Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought and diseases in transgenic tobacco. *J Exp Bot* **60** 3781-3796.