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RESEARCH ARTICLE

Identification of drought-responsive transcripts in peanut (*Arachis hypogaea* L.)

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We have used a reverse transcriptase polymerase chain reaction procedure (differential display) to identify cDNAs corresponding to transcripts affected by water stress in peanuts (*Arachis hypogaea* L.). Using this method, we have identified several mRNA transcripts that are up- or down-regulated following water stress. With 21 primer combinations, a total of 1235 differential display products were observed in irrigated samples, compared to 950 differential display products in stressed samples. These products demonstrated qualitative and quantitative differences in the gene expression. The differentially expressed transcripts were collectively named PTRD (Peanut Transcripts Responsive to Drought). We have identified a total of 43 PTRD, which were significantly altered due to water stress. Slot blot analysis of 16 PTRD indicated that 12 were completely suppressed due to prolonged drought, two were down-regulated, and two were up-regulated under drought stress conditions. The 12 completely suppressed transcripts were studied further by RNA dot-blot analysis to compare their expression in drought tolerant and susceptible line , which underwent three weeks of water stress. PTRD-1, -10, and –16 expressed for longer period in tolerant line compared to the susceptible lines and can be used as molecular markers for screening peanut lines for drought tolerance.

Plants are uniquely suited for coping with periods of severe water deficit during certain stages of their life cycle. During the late phase of embryo maturation, embryos undergo severe desiccation resulting in a dry, mature embryo that can survive in a quiescent phase for longer periods. However, exposure of plants to water-limiting environments during the plant's vegetative, reproductive, or early embryo development phases appears to trigger a set of physiological and developmental changes. These are characterized by a number of biochemical changes that ultimately result from a selective increase or decrease in the biosynthesis of a large number of distinct proteins that alter enzyme activity. Changes in the protein profile are due to changes such as transcription rate, RNA stability, post-transcriptional control, and protein turnover, etc. (Smirhoff and Colombe, 1989). Several genes have been described that respond to dehydration at the transcriptional level in

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a variety of plant species (Skriver and Mundy, 1990; Iuchi et al. 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Oliver et al. 1998; Tabaeizadeh, 1998). Although several genes induced by drought have been identified in a wide range of plant species (Bray, 1997; Oliver et al. 1998; Tabaeizadeh, 1998), a molecular basis for plant tolerance to water stress remains unclear (Ingram and Bartels, 1996; Cellier et al. 1998). Depending upon the developmental stage or the external stimuli applied, these genes are classified as dehydrin (dehydration induced), RAB [responsive to abscisic acid (ABA)], or LEA (late embryogenesis abundant) genes, and more than 65 plant dehydrin genes have been sequenced (Close, 1997).

Metabolic changes in response to water stress include reduction in photosynthetic activity (Ritchie et al. 1990), accumulation of organic acids such as malate, citrate and lactate accompanied by accumulation of proline, sugars and betaine (Bohnert et al. 1995; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Tabaeizadeh, 1998), and an overall reduction in protein synthesis (Mason et al. 1988). Exposure of plants to low water potential often leads to loss of cell turgor and plants undergo osmotic adjustments by the rapid accumulation of ABA (Skriver and Mundy, 1990; Bray, 1997) and osmoprotectants (Grumet and Hanson, 1986). In many plant species, the induction of ABA synthesis is rapid (~30 min) which in turn induces changes in gene expression (Guerrero and Mullet, 1986; Gomez et al. 1988; Close et al. 1989; Bray, 1997). Expression of ABA responsive genes is modulated during seed development (Choi et al. 1987; Galau et al. 1987), in response to plant dehydration (Heikkila et al. 1984; Guerrero and Mullet, 1986; Close et al. 1989; Bray, 1997) or low temperature (Shinozaki and Yamaguchi-Shinozaki, 1996; Close, 1997). Peanut (*Arachis hypogaea* L.) is grown throughout the world, especially in tropical and sub-tropical regions. Pre-harvest contamination of peanut with aflatoxin, a carcinogenic fungal secondary metabolite, is a recurrent problem. Proper irrigation of peanuts during drought decreases the severity of aflatoxin contamination (Wilson and Stansell, 1983; Sanders et al. 1985).

Pre-harvest aflatoxin contamination is a common occurrence in peanuts that are grown under non-irrigated conditions and exposed to prolonged drought and elevated soil temperatures during seed development. Drought tolerant lines generally display lower rates of pre-harvest aflatoxin contamination (Holbrook et al. 1994) indicating that they may possess some degree of resistance to aflatoxin contamination. However, the unavailability of reliable tools to screen peanut genotypes for drought tolerance are the major hurdles in the genetic improvement of peanut for drought tolerance (Rucker et al. 1995).

The mechanism of drought response has been extensively investigated in the model plant, *Arabidopsis thaliana,* and a resurrection plant, *Craterostigma plantagineum* (Yamaguchi-Shinozaki et al. 1995; Shinozaki and Yamaguchi-Shinozaki, 1996). However, in peanut little is known about the physiological and molecular events regulating gene expression under drought conditions. It is important to analyze drought-responsive gene expression in water-stressed and irrigated peanuts, as it may increase our understanding of the molecular mechanism of water stress and the role of differential gene expression in drought tolerance.

The aim of the present study was to examine the differential expression of transcripts under drought stress and irrigated conditions. Specific transcripts uniquely affected due to water stress can be used as markers for selecting drought tolerant lines. We have focused on isolating and identifying genes suppressed due to drought in leaves from seedlings because it is not possible to grow peanuts to a mature stage without microbial contamination and the microbial RNA may lead to false DDRT profiles. Initially, we have focused on those transcripts that are turned-off or down-regulated during water stress. The expression of these down-regulated transcripts in a drought tolerant and a susceptible genotype was compared to identify putative transcripts that can be used as molecular markers in screening peanut genotypes for drought tolerance.

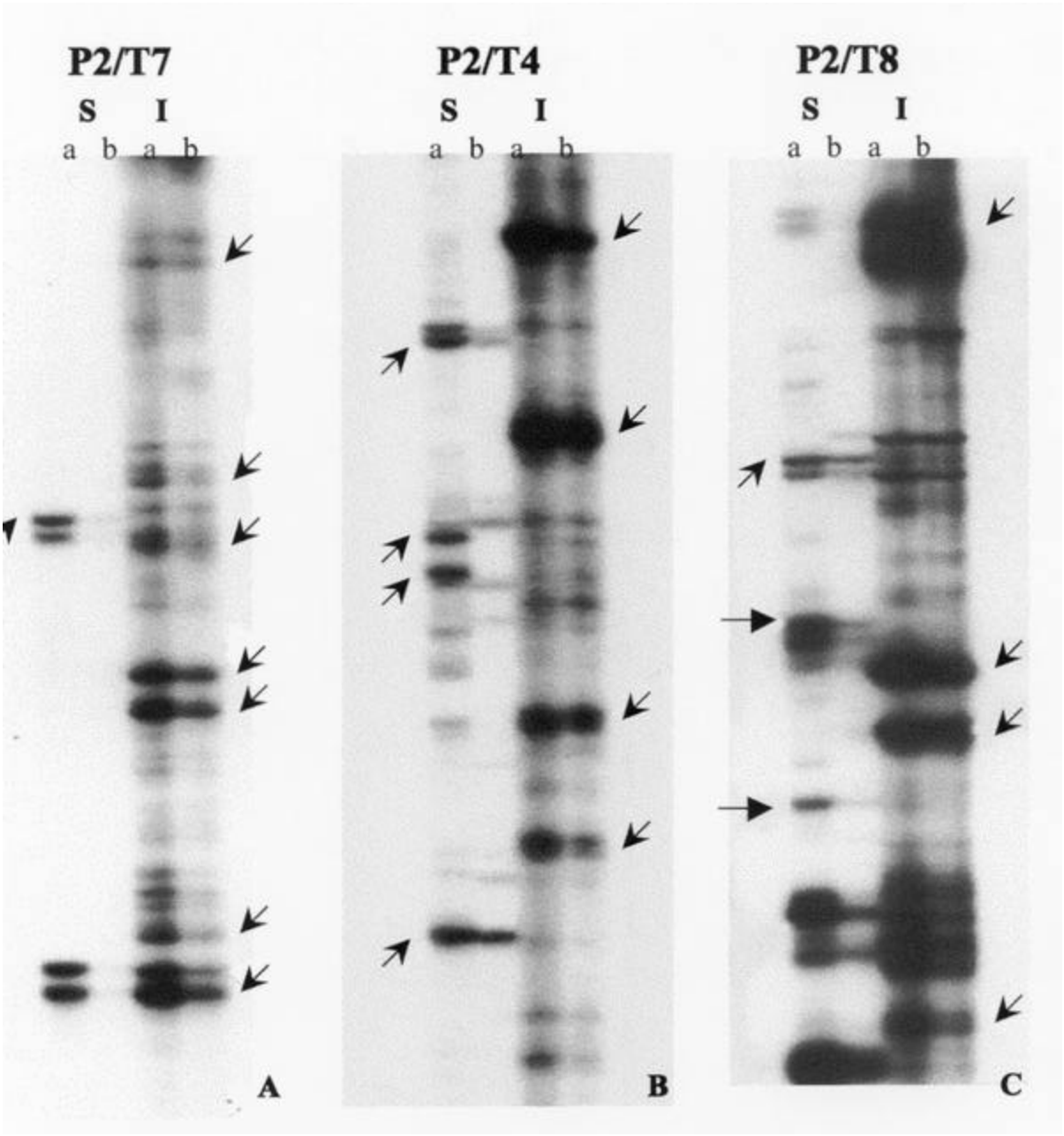
Materials and Methods

Plant material, stress induction and RNA isolation

Peanut plants were grown in one-gallon pots filled with potting soil in a greenhouse and irrigated regularly (pot soil was saturated with tap water every alternate day). It is well known that peanuts are predisposed to aflatoxin contamination when exposed to prolonged drought and drought tolerant lines display lower rates of pre-harvest aflatoxin contamination (Holbrook et al. 1994). Hence, experiments were designed to determine the molecular responses of peanut following prolonged drought stress, and for use in identifying drought tolerant genotypes. Therefore, to monitor the molecular responses to water stress, 30-day-old seedlings were drought stressed for 14 days (when plants exhibited symptoms of wilting). For irrigated control seedlings were watered regularly until 45 days. Fully developed, expanded leaves (5^{th} – 9^{th} leaves from the shoot tip) were collected from stressed and irrigated plants. Total RNA was isolated from 5 g of fresh leaf tissue, which was collected in liquid N, stored at –80°C and homogenized in 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M β-mercaptoethanol, and then extracted with an equal volume of phenol: chloroform (1:1). Total RNA was pelleted and was further purified using LiCl (final concentration 2 M) precipitation and re-dissolved in sterile distilled water.

Differential display

Differential display of cDNA was performed (Liang and Pardee, 1992) using Delta Differential Display Kit following the manufacturer's protocol (Clontech, Palo Alto, CA). First strand synthesis was performed in a total volume



↙ Indicate down-regulated (suppressed) transcripts following water stress.
↗ Indicate up-regulated (induced) transcripts following water stress.
→ Indicate new (activated) transcripts following water stress.
(A) Primer combination P2/T7 was effective to identify down-regulated transcripts.
(B) P2/T4.
(C) P2/T8 were good in identifying both down- and up-regulated transcripts.

Figure 1. DDRT profile of stressed and irrigated peanut plants. Differentially displayed bands in DDRT-PCR of total RNA extracted from water stress (S) and irrigated (I) peanut (*Arachis hypogaea* L. cv. Florunner) plants using different primer combinations (P indicate arbitrary and T indicate anchored primer, for details see Table 1). Two lanes (a, b) under S and I reflect two different dilutions of cDNA used in DDRT reaction.

of 10 µl. The reaction mixture consisted of 2 µg total RNA and 1 µl of 1 µM oligo dT (dT15) primer, 200 units of MMLV reverse transcriptase, 2 µl of 5 mM dNTP and 2 µl of 5X first strand buffer [250 mM Tris (pH 8.3), 30 mM $MgCl_2$, 375 mM KCl]. Differential display PCR was performed in a 20 µl reaction mixture, using two dilutions of the first strand cDNA (dilution 'a' consisted of 4 ng, and dilution 'b' consisted of 1 ng cDNA). Each reaction mixture contained 1 µl of each anchored oligo-dT and arbitrary primers (20 µM), 0.2 µl dNTP (5 mM), 60 nM of

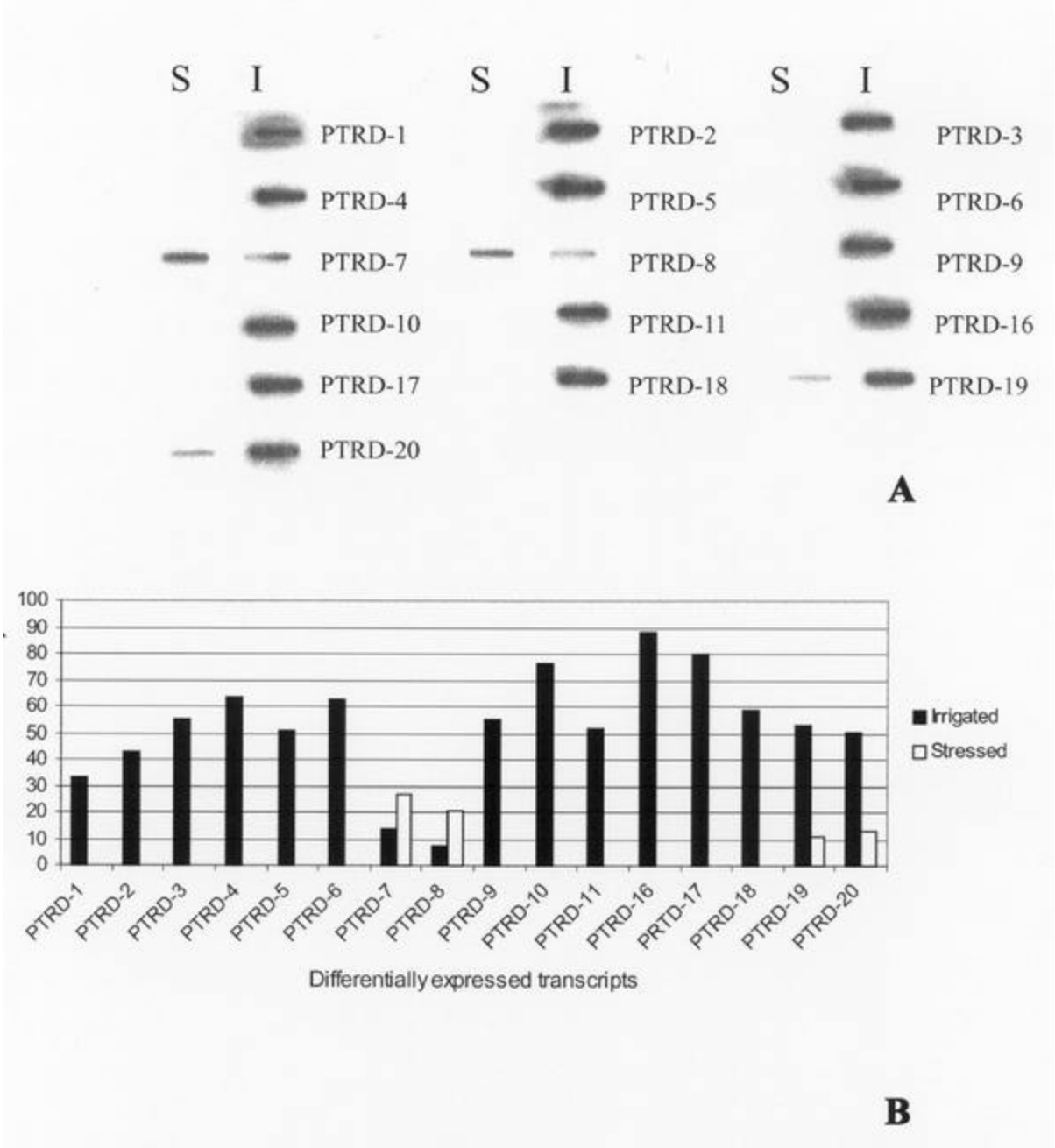


Figure 2. Comparative profile expression of differentially expressed transcripts in stressed and irrigated peanuts.
(A) Total RNA isolated from stressed (S) and irrigated (I) peanut leaf tissue corresponding to the samples used in the differential display experiments were subjected to slot blot analysis using the re-amplified DDRT fragment (named as PTRD-1, PTRD-2 etc) as a probe.
(B) Quantitative detection of differentially expressed transcripts by estimating relative intensity of radioactivity found with each band in slot blot analysis shown in Figure 2A. Similar results were obtained in two independent slot blot experiments.

α-^{32}P dATP and 6 units of Taq polymerase. The reaction was performed using a thermal cycler (MJ Research, Inc., Model PTC-100) programmed to 95°C for 5 min, followed by annealing at 45°C for 45 sec, extension at 72°C for 90 sec, and denaturation at 95°C for 45 sec, repeated to annealing temperature for a additional 39 cycles. The DDRT-PCR mixture was denatured with an equal volume of gel loading buffer [95% formamide, 0.1% xylene cyanole FF and 0.1% bromophenol blue] at 90°C for 2 min. The denatured products (2 µl) were separated by electrophoresis at 70 W constant powers on 6% polyacrylamide/7M urea DNA sequencing gel. The gel was dried under vacuum at 80°C on filter paper, and exposed to X-ray film. A total of 21 primer combinations were tested randomly using nine arbitary primers and nine anchored oligo-dT primers (Table 1). Changes in mRNA transcripts

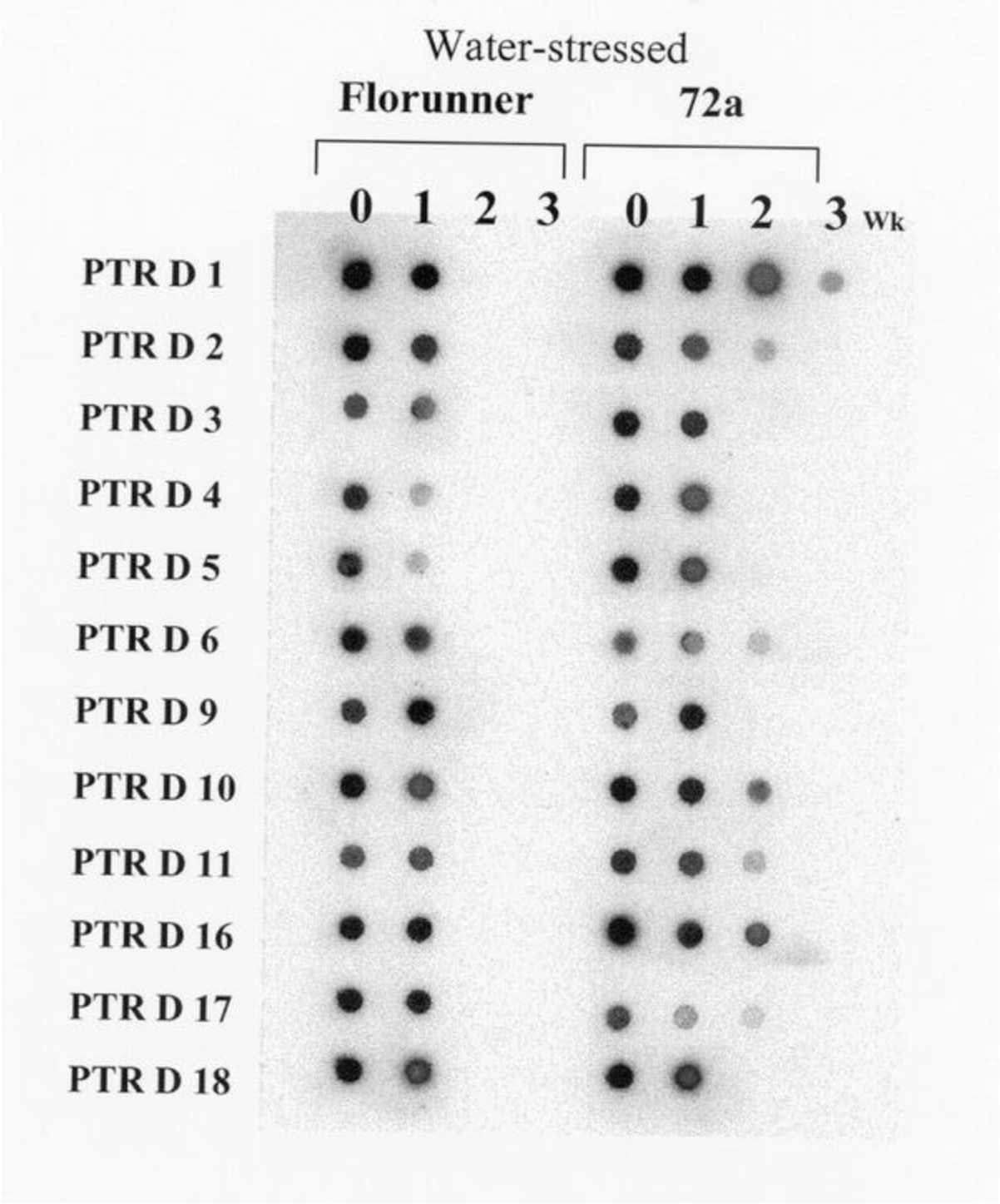


Figure 3. Comparison of down-regulated transcripts in dought-tolerant and -intolerant peanut lines. Dot-blot analysis of down-regulated transcripts in a drought-intolerant (Florunner) and tolerant peanut line (72a, PI 145681) for identification of specific transcripts related to drought tolerance.

between stressed and irrigated samples were recorded for each set of primers.

Isolation of differentially expressed transcripts and slot blot analysis

Twenty differential products were isolated from the gel and selected bands were cut out, DNA was eluted by soaking the gel slice in 50 µl of TE buffer [10 mM Tris – HCl (pH 8.0), 1mM EDTA] followed by heating at 100°C for 5 min. The eluted fragment was precipitated in the

Table 1. Primers used in differential display.

Primers	Sequence
5' Arbitrary primers	
P1	5'-ATTAACCCTCACTAAATGCTGGGGA-3'
P2	5'-ATTAACCCTCACTAAATCGGTCATAG-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
3' Anchored oligo-dT primers (30 mer)	
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
T9	5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

presence of D-glycogen (10 mg/ml), purified and resuspended in 25 µl of sterile distilled water. The eluted fragments were reamplified in 50 µl PCR mixture using the same set of arbitrary and anchored primers that generated the differential product. Reamplified PCR fragments (10 µl) were resolved on a 1.2% agarose gel. For slot-blot analysis 15 µg of total RNA from stressed and irrigated samples were blotted onto a nitrocellulose membrane (MSI, Westborough, MA) using a vacuum apparatus, Bio-Dot SF (Bio-Rad Laboratories, CA). RNA was denatured under alkaline conditions (10 mM cold NaOH and 1 mM EDTA) before being applied to the slots. Slots were rinsed with 500 µl of 10 mM NaOH and 1 mM EDTA. The membrane was rinsed in 2 X SSC, 0.1% SDS and RNA was UV cross-linked using the GS Gene Linker (Bio-Rad Laboratories, CA). Membrane pieces were prehybridized at 42°C in 5 X SSC, 50% formamide, 5 X Denhardt's solution [50 X stock solution consisting of 1% Ficoll 400, 1% polyvinylpyrrolidone, 1% BSA (Albumin Bovine Fraction IV)] in distilled water, 0.5% SDS and 100 µg/ml denatured calf thymus DNA for 16 h. The reamplified PCR product (100 ng) was radiolabelled with ^{32}P dATP following the Prime-a-Gene Labeling System (Promega) and used as a probe. Hybridization was performed at 42°C for 8 h. Blots were washed twice at 65°C in a solution containing 1 X SSC and 0.1% SDS for 30 minutes. Hybridized membrane pieces were exposed to Kodak-X-OMAT AR film for 6 h with an intensifying screen. An 18S ribosomal RNA antisense probe was prepared from *Nicotiana tabacum* clone and used as an internal RNA standard. The autoradiogram was analyzed for the relative RNA expression levels by densitometry with the Image Quant software analysis program (Bio Image R, Intelligent Quantifier, Version 2.2.1).

Comparison of down-regulated transcripts in drought-tolerant and -intolerant peanut line

The twelve transcripts showing complete suppression were selected for further screening to compare their expression in the -tolerant and -intolerant genotypes. Seedlings of a drought-tolerant breeding line namely 72a [plant inventory (PI) number 145681 from the USDA peanut core germplasm] and drought-intolerant Florunner were grown in the greenhouse and stressed up to three weeks as described above. Leaf samples were collected at 0, 1, 2, or 3 weeks post water-stress and total RNA was isolated as described earlier. Total RNA (10 µg) from tolerant and intolerant lines was dot-blotted onto a nitrocellulose membrane (MSI, Westborough, MA) using the Bio-Dot system (Bio-Rad Laboratories, CA), probed with reamplified PCR product (PTRD-1 to -6, PTRD-9 to –11 and PTRD-16 to -18) and radiolabelled with ^{32}P-dATP. An 18S ribosomal RNA antisense probe was used as an internal RNA standard. The conditions for pre-hybridization, hybridization and washing were the same as described earlier. After washing, the membrane was exposed to Kodak-X-OMAT AR film and the autoradiogram was

Table 2. Differentially expressed transcripts affected severely following water-stress in peanut[1].

Primer Combination	Suppressed transcript (DDRT product from irrigated sample)	Induced transcript (DDRT product from stressed sample)
P1/T1	2	-
P1/T6	6(PTRD 1-6)	2(PTRD 7-8)
P1/T7	2	-
P1/T9	2	3
P2/T1	6	-
P2/T4	3	4(PTRD 19-20)
P2/T7	3(PTRD 9-11)	-
P2/T8	3	1
P3/T1	1(PTRD 16)	-
P3/T5	2(PTRD 17-18)	1
P4/T5	2	-
Total	32	11

[1] These DDRT products were selected for slot-blot and dot-blot hybridization, numbers in parentheses represent corresponding PTRD used in slot-blot and dot-blot hybridization as shown in Figure 2 and Figure 3.

analyzed for the comparative expression of PTRD.

Results and Discussion

Differential cDNA display between water-stressed and irrigated peanut

The differential display reverse transcriptase (DDRT) technique resulted in equal detection of most cDNA bands in RNA samples from both water-stressed and irrigated plants. Some bands, however, were unique to either water-stressed or irrigated samples (Figure 1). The total number of cDNA bands resolved on the autoradiograph with each primer combination was recorded in stressed and irrigated samples. Although differences in the number of bands were observed between different primer combinations, the number ranged from 28 to 83 cDNA bands for irrigated samples and 2 to 69 cDNA bands for water-stressed samples. A total of 1235 cDNA bands were found with 21 primer combinations for leaf samples from irrigated peanut. Based on the comparative intensity, the cDNA bands expressed following water stress were grouped into three classes: induced (up-regulated), suppressed (down-regulated), and newly expressed (activated following water stress). Out of 1235 cDNA bands from irrigated peanut, the intensity of 63 cDNA bands was lower in water-stressed samples (Figure 1, arrowhead facing downward) indicating that these cDNA species were down-regulated upon stress.

On the other hand, a total of 950 cDNA bands were recorded in water-stressed samples, of which 46 cDNA bands appeared to represent up-regulated transcripts. Comparison of band intensity between irrigated and stressed samples on the autoradiogram revealed that 498 bands were of lighter intensity in stressed samples as compared to their levels in irrigated samples. This indicates that water stress suppressed mRNA synthesis both qualitatively and quantitatively, reducing overall protein synthesis, as reported in soybean (Mason et al. 1988). Water stress also activated several new transcripts, and a total of 212 cDNA bands were recorded in stressed samples with light to medium intensity, which were absent in irrigated samples (Figure 1C, horizontal arrowhead). Activated transcripts have been recorded in other plant species (Bray, 1997; Tabaeizadeh, 1998) and analysis of these genes has indicated that their products might function cooperatively to protect cells from dehydration, and hence, may play an important role in the plant adaptive mechanism during water stress (Bohnert et al. 1995; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Oliver et al. 1998; Tabaeizadeh, 1998). Primer combinations P1/T1, P1/T6, P2/T1, P2/T2, P2/T7 and P4/T4 used for DDRT were very effective in amplifying the suppressed transcripts (genes turned-off following water stress).

Primer combinations P1/T3, P1/T9 and P5/T5 were more effective in amplifying the newly expressed (activated) transcripts (genes turned-on following water stress). Primer combination P2/T4 was good in amplifying both suppressed (down-regulated) and induced (up-regulated) transcripts (Figure 1B). The differentially expressed transcripts were collectively named PTRD (Peanut Transcripts Responsive to Drought). As a first step towards understanding the molecular mechanism of water stress in peanut, a total of 43 PTRD, which were severely affected following water stress, were identified for further characterization and were classified as 32 suppressed transcripts and 11 induced transcripts (Table 2).

Slot blot analysis

Sixteen of the PTRD, out of 43 PTRD identified above, were radiolabelled and tested individually by hybridization on total RNA slot blots from irrigated and water stressed samples. All the 16 PTRDs were hybridized to different levels with RNA from irrigated samples, indicating variation in gene expression at transcription levels as observed earlier in DDRT results. Twelve PTRDs did not show hybridization signals with RNA from stressed samples (Figure 2A). This suggests that prolonged drought completely suppressed these twelve transcripts indicating corresponding genes were turned-off following water stress. Of the four PTRDs hybridized with mRNA from stressed samples, two PTRDs (PTRD-19 and PTRD-20) expressed at lower level while the other two PTRDs (PTRD-7 and PTRD-8) expressed at higher level compared to mRNA from irrigated samples. This would suggest down-regulation and up-regulation of these genes. The relative mRNA expression levels of the 16 PTRD fragments were quantified (Figure 2B) by densitometry. The highest levels were recorded for PTRD-16 (88%) followed by PTRD-17 (80%), PTRD-10 (76%) and PTRD-4 (63%) (Figure 2B).

The differential display technique enabled the detection of transcripts that are expressed differentially between irrigated and water-stressed plants by using different combinations of primer sets. In the present study, we employed 21 primer sets, and were nevertheless able to detect a number of cDNA bands that were specific to either irrigated or water-stressed samples. The results clearly demonstrated transcript differences between irrigated and stressed peanut samples.

Dot-blot analysis

Slot-blot results showed that twelve of the PTRDs were completely suppressed within 2 weeks of water stress in the intolerant peanut line 'Florunner'. These down-regulated PTRDs were further targeted for their differential expression in a drought-tolerant line 72A (PI 145681) and drought-intolerant (Florunner) genotype up to 3 weeks following water stress. The comparative expression of down-regulated PTRDs in a dot-blot analysis (Figure 3) showed quantitative variation in levels and duration of expression of these PTRDs in the tolerant and intolerant genotypes. Two weeks of water stress to the intolerant line completely suppressed these transcripts. On the other hand, six of these transcripts (PTRD-2, -6, -10, -11, -16 and -17) showed expression up to two weeks and one (PTRD-1) up to three weeks of water stress in the tolerant line. These results indicate that the tolerant line is capable of expressing certain genes for a longer period during drought. Out of these six PTRDs, PTRD-1, -10 and -16 showed higher levels of expression up to two weeks of water stress in the tolerant line (72a) indicating these transcripts may be used as markers for screening genotypes with drought tolerant characteristics. These transcripts may be helpful as additional tools along with other morphological characters such as large root system and visual stress ratings (Rucker et al. 1995; Holbrook et al. 2000) in identifying and selecting a drought tolerant peanut line. Currently, Restriction Fragment Length Polymorphism (RFLP), Restriction Landmark Genome Scanning (RLGS), micro satellite, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) or Florescent *in situ* Hybridization (FISH) techniques are widely used as molecular markers to analyze complex traits and identifying a Quantitative Trait Loci (QTLs) (Burrow and Blake, 1998). However, these techniques have shown limited polymorphism in peanut and have proved inadequate and incapable of identifying unique bands for use as molecular markers (Kochert et al. 1991). As a result, the transcripts (PTRD-1, -10 and –16) identified in this study have great potential for selecting peanut lines with drought tolerant characteristic.

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