PHYSIOLOGICAL AND MOLECULAR RESPONSES DURING WATER DEFICIT IN COTTON (*G.hirsutum*)

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Abstract

Resistance to drought in plants clearly is not a simple trait, but a complex of mechanisms working in combination to avoid or to tolerate water deficits. All physiological, morphological and developmental changes that confer drought tolerance in plants must have a molecular genetic basis. Thus, genotypes which differ in tolerance to water stress should have qualitative or/and quantitative differences in gene expression. To identify and isolate genes which may differ among four cotton genotypes with diverse responses to water deprivation, we used Differential Display (DD). A total of 109 cDNA fragments differentially displayed were identified. These fragments were extracted from the DD gels and reamplified. Sixty five reamplified fragments were cloned in pGEM-T vectors and sequenced. GeneBank searches showed 17 clones with high homology to known genes, and 20 clones with low homology, while 15 clones had no homologous entries. Ribonuclease protection assays (RPA) were used to confirm expression of most of the interesting differentially displayed fragments. These fragments can be used as riboprobes to screen germplasm banks to identify genotypes presenting similar water-deficit tolerance characteristics found in the genotypes used in this study.

Introduction

When plants started to evolve about 1.5 billion years ago (Lehninger et al., 1993) they went through innumerable changes in structures and processes to enable them to survive in relatively dry environments. As a result, single genes that substantially altered plants' capacity to survive dry circumstances during the first steps of land colonization became difficult to find (Boyer, 1996) or even might have disappeared. Over time evolutionary pressure turned plant responses to stress into a complex web of responses beginning with stress perception, which initiates signal transduction pathways, and ending in changes at many metabolic, physiological and developmental levels. Therefore, responses to drought will be conditioned not only by the nature and intensity of the environmental factors involved, but also by the ecological histories of species, ecotypes, cultivars and genotypes. Because several genes are likely to be involved in each trait for drought resistance, molecular biology can aid in identifying and selecting these genes and determining their influence in yield (Turner, 1997). Differentially expressed genes are usually identified by comparing mRNA abundance (Wan et al., 1996). Therefore, a partial understanding of these developmental events may be obtained by analyzing and comparing mRNAs isolated from well-watered and water-stressed plants.

Differential display (Liang and Pardee, 1992) is a technique that uses sub-populations of the total mRNA pool as template for representative cDNA synthesis by reverse transcription. The cDNA sub-populations are then PCR amplified resulting in the generation of a PCR profile representative of the mRNAs contained within each population. PCR products are then displayed side-by-side on polyacrylamide gels to identify treatment-specific expression (Liang and Pardee, 1992). The Differential display has advantage over other techniques (e.g. subtractive hybridization) because it requires only very small amounts of RNA for analysis, is not limited by redundancy of highly expressed mRNAs or under-representation of rare mRNAs, and it has rapid output (Wan et al., 1996). Differentially displayed fragments should be confirmed by Northern blots or Ribonuclease Protection Assay(RPA). Even though, RPA does not give the size of the transcript, it is superior to Northen blots for detection and quantification of low abundance RNAs. With Northern blots, RNA transfer and binding to the membrane may be inefficient, some RNAs may not be accessible for hybridization and low integrity RNA samples produce erroneous bands (Lee and Costlow, 1987).

Water deficit is a major concern in cotton production. Identifying and understanding mechanisms of water stress tolerance is crucial to the development of new tolerant cultivars. The objective of this study was to identify and isolate genes that differ among select four cotton genotypes with diverse responses to water deprivation. The unique expression of genes in stress tolerant genotypes could be used to study drought tolerance mechanisms and to identify genotypes with similar characteristics.

Material and methods

Characterization of Water-Stress Tolerance

Two water-deficit tolerant (Siokra L-23 Australian cultivar, and T-1521 wild type) and two water-deficit sensitive (Stoneville 506 American cultivar, and CS-50 Australian cultivar) genotypes were used (Nepomuceno et al., 1996). These genotypes were submitted to four periods (of four hours each, during the dark period) of water deficit (-0.3MPa) induced in nutrient solution by polyethylene glycol (PEG 6000), and in pots with sand by withholding irrigation. During and after the stress these cultivars were characterized in relation to their osmotic adjustment,

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photosynthetic rate, relative water content, carbon discrimination and other physiological parameters (Nepomuceno et al., 1996).

Differential Display

During and after the water stress, leaves of the four cotton cultivars were collected for RNA isolation according to Wan and Wilkins (1995). Before the reverse transcription (RT) reaction 2µg of total RNA was mixed in a 200µL tube with 10µL of 5x MMuLV RT buffer, 1.24µL dNTP (10mM), 2.42µL anchor primer (50µM), 1µL RNAsin (Promega, Madison, WI), and DEPC-treated water to a total volume of 50µL. The reaction mix was heated at 65^0C for 5 min, after which 1.5µL of the MMuLV Reverse Transcriptase (Promega, Madison, WI, 200 U/µL) was added to the reaction mix. After 1 hour at 37 ^{0}C the reaction mix was heated to 99^0C for 5 min to inactivate the reverse transcriptase. Five µL of the reverse transcription reaction cDNA was then mixed in a 200µL tube with 5µL dNTP (100µM), 2.5µL 10x Taq buffer, 2.5µL $MgCl_2$ (25mM), 0.5µL 10-mer primer (50µM), 1µL anchor primer (50µM), 0.5µL ^{35}S-dATP (1250 Ci/mmol), 0.5µL Taq polymerase (Promega, Madison, WI, 5 U/µL) and DEPC-treated water to make a final volume of 25µL. PCR cycling conditions were: 94^0C for 30 sec, 40^0C for 1 min, 72^0C for 30 sec, 40 cycles, followed by 5 min final extension at 72^0C (Koonce and Haigler, personal communication; Song et al., 1995). PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. Bands were analyzed and compared between stressed and non-stressed cultivars in the four cotton cultivars. Bands that appeared differentially displayed were excised from the gel and reamplified. Five anchor primers **(**A2- $^{5'}(T)_9AC^{3'}$; A5- $^{5'}(T)_9GC^{3'}$; A8- $^{5'}(T)_9CG^{3'}$; A11- $^{5'}(T)_9GG^{3'}$; A12- $^{5'}(T)_9CC^{3'}$**)** and eight ten-mers **(**B1- $^{5'}$GAGCTTGAAC$^{3'}$; B3- $^{5'}$CTGATCCATG$^{3'}$; B5- $^{5'}$TAGAGCGATC$^{3'}$; B7- $^{5'}$ATCTCGCTAG$^{3'}$; B9- $^{5'}$GAATTTCCCC$^{3'}$; B11- $^{5'}$AGGGATCTCC$^{3'}$; B13- $^{5'}$AAGCTGCGAG$^{3'}$; B15- $^{5'}$GTGCGTCCTC$^{3'}$**)** were used.

Cloning, Sequencing and Ribonuclease Protection Assay

Reamplified cDNAs fragments were cloned in pGEM-T vectors (Promega, Madison, WI) and sequenced using a universal primer with a AutoRead^{TM} 200 sequencing kit in a ALF^{TM} DNA sequencer (Pharmacia Biotech). Data base search was carried out using the BLASTN2 and BLASTX2 programs provided by Bork Group's Advanced Search Services at EMBL. Anti-sense riboprobes ^{32}P radiolabed were produced using a MAXIscrip^{TM} kit (Ambion Inc.). Ribonuclease protection assay was performed using a HybSpeed RPA^{TM} kit (Ambion Inc.) and visualized in a 5% denaturing polyacrylamide gel.

Results and discussion

Physiological Characterization

Siokra L-23 and T-1521 maintained leaf photosynthetic rate under water-stress whereas the rates significantly decreased in CS-50 and Stoneville 506 (Table 1). Also, Siokra L-23 and T-1521 exhibited a decrease in leaf osmotic potential at full turgor (osmotic adjustment) that did not appear in CS-50 and Stoneville 506 (data not shown). The decrease in the osmotic potential in the tolerant cultivars probably was responsible for the higher relative water content of their leaves (Table 1) which, consequently, maintained photosynthesis near the unstressed control. However, free radical scavenging may also be in effect, whereby water stress disrupts cellular redox homeostasis and, therefore, chloroplast functions; which inevitably leads to the generation of oxygen-radical species. Thus, osmotic adjustment probably performs additional functions in plant tolerance to stress beyond helping to retain water (Bohnert and Jensen, 1996).

Molecular Characterization

Any phenotypic expression in water-stressed plant is related to gene expression. As water is lost from the cell, regulatory processes are initiated that adjust the cellular metabolism to the new cellular conditions. Simultaneously, growth inhibition and alterations of developmental pathways will result in more changes in gene expression. Many genes induced by water-deficit encode gene products predicted to protect cellular function. Genes that function during changes in metabolism, regulation, signaling, and recognition of stress are also expected to be induced. Water-deficit induced genes will promote: cellular tolerance of dehydration, protective functions in the cytoplasm, alterations of cellular osmotic potential to increase water uptake, control of ion accumulation and further regulation of gene expression.

Gene bank searches showed 17 clones with high homology to known genes, and 20 clones with low homology, while 15 clones had no homologous entries. Table 2 shows some of the differentially displayed cDNA clones organized according to homology ranking. Clone A12B15-5 (Table 2) has show a high homology with a NAD(P)H oxidase (rbohA gene) found in *Oryza sativa* (Groom et al., 1996). This enzyme seems to induce a respiratory burst during stress situations. Torres and Jones (personal communication, University of Newcastle, UK, 1997) identified 6 homologs of this gene *in Arabidopsis thaliana* that were up regulated during infection with fungi and bacteria. Clone A12B15-6 (Table 2) showed high homology with a Heat Shock protein that binds to Calmodulin (Lu el al., 1995). Calmodulin is know to be involved in signal transduction. The presence of this Heat Shock protein differentially expressed during drought in both water-stress tolerant genotypes indicates a possible signal transduction pathway related to their tolerance mechanisms. Even though more studies are necessary, the unique expression of these genes, confirmed by RPA, makes it possible to use them as probes to identify other genotypes presenting the same characteristics.

The accumulation of some gene products may be an adaptive response. cDNA clone A12B13-1 (Table 2) which

shows homology with trehalose-6-phosphate synthase from *Arabidopsis thaliana*. This enzyme is involved in the production of trehalose, a disacharide know to osmoprotect cell membranes during dehydration (Crowe et al., 1993; Muller et al., 1995; Majara et al., 1996). The presence of trehalose in microorganisms and invertebrate animals is well documented, however, only a few plant species are know to synthesize trehalose although most have trehalase which hydrolyzes trehalose. The presence of trehalase seems to inhibit trehalose protective effects (Muller et al., 1995). Also, the presence of a transporter is necessary to transport trehalose across the cell membrane, since its presence is necessary on both sides to give the protective effects (Crowe et al., 1993). Clone A12B13-1 appears in all four genotypes during the stress. Considering the discussion above, genotypes that lack trehalase activity (Goddijn et al., 1997) might express higher water-deficit tolerance because trehalose will be present. Also, genotypes lacking a transporter to move trehalose to both sides of the membrane will be more sensitive to deficit. The combination of these two possibilities should be considered when analyzing the physiological and genetic characterization of the genotypes studied here. Both Siokra L-23 and T-1521 show a significant osmotic potential decrease during the applied stress (Nepomuceno et al., 1996). Presence of active trehalose might be one of the factors involved in this result.

Some of the differentially displayed cDNA fragments did not show homology with any other known genes. Clone A12B15-13 is expressed only during stress and only in Siokra L-23. Additional work must be done to identify any role these genes might be playing in the water-deficit tolerance of Siokra L-23 and T-1521.

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Table 1. Net photosynthesis and relative water content of four cotton genotypes after four periods of water-deficit induced by PE G6000 (-0.3MPa).

Hours after last stress	Genotype	Photosynthesis (mmol $CO_2.m^{-2}.s^{-1}$)		Relative Water Content-RWC (%)	
		Non Stressed	Stressed	Non Stressed	Stressed
12	*Siokra L-23*	7.04±0.3*	6.80±0.3	91.3±0.3	90.2±3.1
	CS 50	6.48±0.2	5.86±0.2	84.4±2.7	88.3±3.7
	Stoneville 506	9.34±0.4	7.32±0.3	90.4±1.4	86.6±3.7
	T-1521	7.12±0.1	7.02±0.1	95.3±0.5	92.2±0.7
36	*Siokra L-23*	8.01±0.5	7.61±0.1	83.4±0.4	83.2±0.8
	CS 50	8.52±0.3	7.50±0.1	81.8±2.5	79.1±2.2
	Stoneville 506	9.01±0.3	6.82±0.7	90.8±1.4	83.9±3.9
	T-1521	7.38±0.2	7.12±0.1	92.7±1.4	91.4±0.8

*Mean of five observations ± standard error.

Table 2. Clone identification according to homology ranking.

Clone Identification[1]	Homology [2]	Homology P(N)	Identities[3]
A12B15-5	NAD(P)H oxidase - *Oryza sativa*	1.7e-52	44(aa) 88%
A12B13-1	trehalose-6-phosphate synthase	6.6e-33	45(aa) 81%
A12B15-6	heat shock protein, Calmodulin-binding	2.9e-32	57(aa) 69%
A5B1-13	GTP-binding protein, fusA-homolog (yihK)	1.2e-23	45(aa) 93%
A12B13-4	*Avena fatua* and *Petroselinum crispum*DNA-binding protein	1.3e-22	59(aa) 77%
A5B1-18	GTP-binding protein, fusA-homolog (yihK)	9.0e-22	58(aa) 83%
A12B11-2	thioesterase homolog	1.9e-17	48(aa) 67%
A5B1-11	GTP-binding protein, fusA-homolog (yihK)	3.8e-15	42(aa) 93%
A12B3-1	Nuclear receptor co-repressor N-CoR-mouse	1.4e-13	41(aa) 57%
A5B1-8	cf-9 protein precursor - tomato, gene for resistence to *Cladosporium fulvum*	5.4e-11	28(aa) 71%
A5B1-9	*A.thaliana* BAC IG005I10, weak similarly to S.cerevisiae BOB1 protein	3.4e-08	34(aa) 72%
A11B1-α	*Glicine max* TGACG-motif binding protein (STGA1 mRNA	1.1e-05	34(aa) 70%
A12B15-8	translation initiation factor eIF-4 gamma - human	1.4e-05	33(aa) 47%
A12B11-6	hypothetical 31KD protein R107.2 in chromosome III	1.6e-05	43(aa) 55%
A5B1-14	*Cryptosporidium parvum* cDNA 5'	5.3e-05	102(n) 72%

[1] first letter+number represent anchor primer; second letter+number represent 10-mer; third number represents band position in the dried gel; greek letter represent band in non-denaturing gels.

[2] homology searches using the BLASTN2 and BLASTX2 programs provided by Bork Group's Advanced Search Services at EMBL

[3] aa - represents amino acid, n - represents nucleotides