Differential Gene Expression Analysis in Melon Roots under Drought Stress Conditions

Margarita M. Rojas[1], Kevin M. Crosby[2] and Eliezer S. Louzada[1]

[1]Texas A&M University- Kingsville Citrus Center, 312 N. International Blvd, Weslaco, TX 78596
[2]Texas Agricultural Experiment Station, 2415 E. Highway 83, Weslaco, TX 78596

ABSTRACT

Plants respond to environmental stresses such as drought through changes in gene expression, which make them more adapted to adverse conditions. The molecular basis for plants drought tolerance is not completely understood. The root system is a critical part of the plant that perceives and responds to signals of water stresses, and therefore, a knowledge of how genes are expressed in roots responding to water stress may contribute to a better understanding of this condition. Differential display RT-PCR was used to study differential gene expression in melon roots under stress conditions. Eighteen differentially expressed partial cDNAs were selected, with fourteen being down regulated in non-irrigated plants. From these, fifteen were able to be reamplified and cloned, eleven of which were down regulated in non-irrigated plants. Reverse northern blot confirmed that five of the clones represented genes that are differentially expressed. The nucleotide sequence of three of the clones were 98% similar within them and the other two were 100% similar to each other. Similarity searches for the amino acid composition at GenBank revealed that one of the clones had 46% identity to a 28 amino acid fragment of a cell wall invertase from maize and the other had 83% identity with a fragment of 287 amino acids of a unknown protein of *Arabidopsis thaliana*. Further studies need to be done to confirm the function of these gene fragments.

RESUMEN

Las plantas responden a las condiciones ambientales adversas, tales como la sequía, por medio de cambios en la expresión de los genes, lo cual las vuelve mejor adaptadas a estas condiciones. La base molecular de la tolerancia a la sequía de las plantas no esta comprendida completamente. El sistema radical es una parte crítica de la planta que percibe y responde a la señal de estrés hídrico, y por lo tanto, el conocimiento de como los genes se expresan en las raíces como respuesta al estrés hídrico puede contribuir a una mejor comprensión de esta condición. Se utilizó la técnica de despliegue diferencial por reacción de polimerización en cadena con transcripción inversa para estudiar la diferencia en la expresión de genes en las raíces de melón bajo condiciones de sequía. Se seleccionaron 18 cDNA parciales expresados diferencialmente, de los cuales 14 fueron regulados negativamente en las plantas no irrigadas. De estas, fue posible reamplificar y clonar 15, de las cuales 11 fueron regulados negativamente en las plantas no irrigadas. Un northern blot inverso confirmó que 5 de los clones representaron genes que fueron diferencialmente expresados. La secuencia de nucleótidos de 3 de los clones presentó 98% de similitud mientras que los restantes 2 tuvieron un 100% de similitud. La revisiones de similaridad para la composición de aminoácidos en GenBank reveló que uno de los clones tuvo un 46% de similitud con el fragmento de 28 aminoácidos de una invertasa de la pared celular del maíz y el otro clon tuvo un 83% de similitud con un fragmento de 287 aminoácidos de una proteína desconocida de *Arabidopsis thaliana.*

Melon (*Cucumis melo* L.) is an important fruit crop cultivated widely in many regions of the world, including the Lower Rio Grande Valley in Texas. The most prevalent environmental stress affecting melon is drought, which considerably impacts plant growth and fruit yield. Usually, melon fruits under drought stress become soft, wrinkled, and turn brown. Additionally, water deficiency hastens premature ripening, reducing fruits size (Shetty et al., 1997). The economic impact of drought in Texas is high with losses of up to 75% for non-irrigated vegetable crops (Miller, 1998). To survive the environmental stresses, plants respond and adapt through physiological, developmental and biochemical changes, including the induction of gene expression and synthesis of a number of proteins (Takahashi et al., 2000). Plants under water stress display changes in foliar enzyme activity, mRNA accumulation, photosynthesis, carbohydrates, and amino acid contents (Foyer et al., 1998).

Changes in primary metabolism are a general response to stress in plants. Increased levels of certain enzymes are related with increased energy demand, such as those involved in sugar metabolism, which are critical in dessication tolerance, and enzymes involved in the synthesis of compatible solutes (Ingram and Bartels, 1996). Transcripts of some enzymes for proline and glycine biosynthesis are up-regulated in leaves during drought conditions. They produce compatible solutes and osmoprotectants as a way of maintaining the water

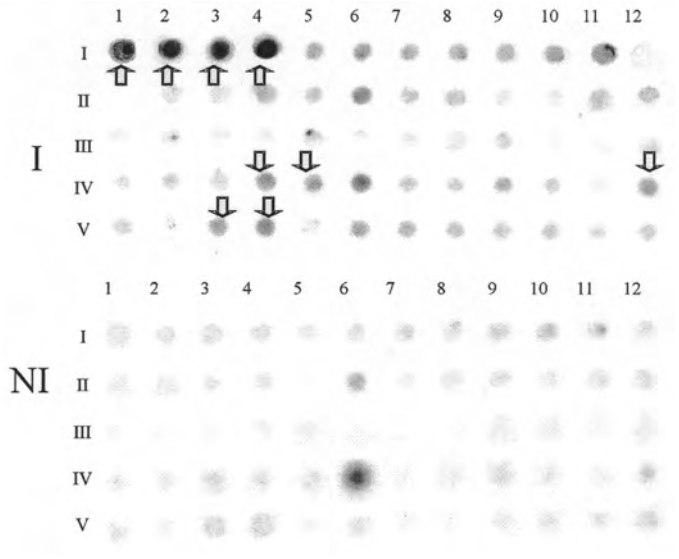

Fig. 1. Reverse Northern dot-blot of fifteen clones containing fragments of differentially expressed genes. I: irrigated. NI: Non-irrigated. Dots a1-4= G10-8, a5-8= G10-10, a 9-12= G10- 11, b1-4= C10- 6, b5-8= C2-1, b9-12= C7-10, c1-4= C7-11, c5-8= C7-12, c9-12= A10-5, d1-4= A10-6, d5-8= A10-7, d9-12= A10-8, e1-4= A10-11, e5-8= A10-12, and e9-12= A2-3. Arrows show the colonies chosen for sequencing.

requirements of the plant by osmotic adjustment (Kawasaki et al., 2000). Ion and water channel proteins are also important in regulating water flux and this is supported by the isolation of channel protein genes expressed in response to water deficit in leaves (Takahashi et al., 2000). Genes encoding proteins with sequence similarity to proteases, that are induced by drought, have been isolated from both pea (*Pisum sativum* L) and *A. thaliana* (Ingram and Bartels, 1996). It is suggested that one of the functions of these enzymes could be to degrade proteins which were irreparably damaged by the effects of drought.

Studies comparing drought-tolerant and drought-sensitive lines of sunflowers showed that the genes HaDhnl and HaDhn2 were up-regulated in leaves of the tolerant line which is characterized by the maintenance of turgor pressure in shoot cells. Accumulation of corresponding transcripts of these genes was correlated with the drought-adaptive response. HaDhn1- and HaDhn2-deduced proteins belong to the dehydrin family. Dehydrins are proteins highly abundant in desiccation-tolerant seed embryos and accumulate during periods of water deficit in vegetative tissues (Cellier et al., 1998). Although the function of dehydrin in plant cells has not yet been elucidated, it has been proposed that it plays a role in the stabilization of macromolecules, and in the maintenance of cellular turgor (Close 1997; Cellier et al., 1998).

Two cDNA clones corresponding to genes that are responsive to dehydration were isolated using differential screening of a cDNA library prepared from dehydrated drought-tolerant cowpea (*Vigna unguiculata*) plants. One of the cDNA clones had a homology to 9-cis-epoxycarotenoid dioxygenase (named VuNCED1), which is involved in abscisic acid (ABA) biosynthesis. Both ABA accumulation and VuNCED1 expression were strongly induced by drought stress (Iuchi et al., 2000). It is widely accepted that abscisic acid (ABA) mediates general adaptative responses to drought. Rapid translocation of ABA in shoots via xylem flux and its increased concentration in plant organs have been correlated with major physiological changes during plant response to drought (Zeevaart and Creenman, 1998). However, analyses of stress inducible genes have revealed the presence of multiple signal transduction pathways between the perception of water stress and changes in gene expression (Gosti et al., 1995). Some genes respond to stress rapidly, whereas others are activated after ABA accumulates. It is suggested that at least four signaling pathways are involved in water stress response; two of them are ABA-dependent and two are ABA-independent (Gosti et al., 1995; Shinozaki and Yamaguchi-Shinozaki, 1997, 1999; Takahashi et al., 2000). Several other genes regulated by water stress and abscisic acid have been identified in the last decade (Cellier et al., 1998; Tabaeizadeh, 1998), some of them encode enzymes that are also induced by other biotic and abiotic stresses (Flowers and Yeo, 1995). The molecular response of commercially important crops, especially melon, to drought stress has not been extensively studied. In this paper we will report the results of a differentially expression analysis of melon root genes induced by drought stress.

MATERIALS AND METHODS

Plant Material. Melon seedlings (*C. melo* L. var.1207) were planted in sand, watered daily, and nutrient solution (Peters 20-20-20, plus micronutrients) was added twice a week. After two weeks of growth in well-watered conditions, plants were kept in a greenhouse, and drought stress was initiated by withholding watering for 8 days. Control plants were watered daily. Roots from irrigated and non-irrigated three-week-old-plants were sampled, frozen in liquid nitrogen, and stored at -80ºC until used for RNA isolation.

RNA Isolation. RNA was extracted from irrigated and non-irrigated root tissue using the Totally RNA isolation Kit (Ambion, Austin, TX) as described. Denaturation solution was added to powdered frozen tissue and the RNA was purified by phenol:chloroform: Isoamyl alcohol and acid phenol: chloroform extractions, followed by one chloroform extraction. An equal volume of isopropanol was added and the preparations were stored at -20ºC for 2 h. The RNA was precipitated by centrifugation, and dissolved in DEPC (diethyl pyrocarbonate) treated H_2O. A solution of 8 M LiCl was added, and the RNA precipitated after overnight storage at 20ºC. The RNA was resuspended in RNase free dH_2O / 0.1 mM EDTA. The RNA samples were treated with RNase free DNase treatment (Ambion) according to manufacturer instructions prior to use.

Differential display. DNA-free total RNA (0.2 µg) from irrigated and non-irrigated melon plants were reverse transcribed using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA); with three $HT_{11}M$ anchor oligo-dT primers (5'-AAGCTTTTTTTTTTTM-3', where M represents A, C, or G). The same oligo-dT primers and eight arbitrary 15-mer primers (Table 1) were used to perform 24 different PCR reaction for

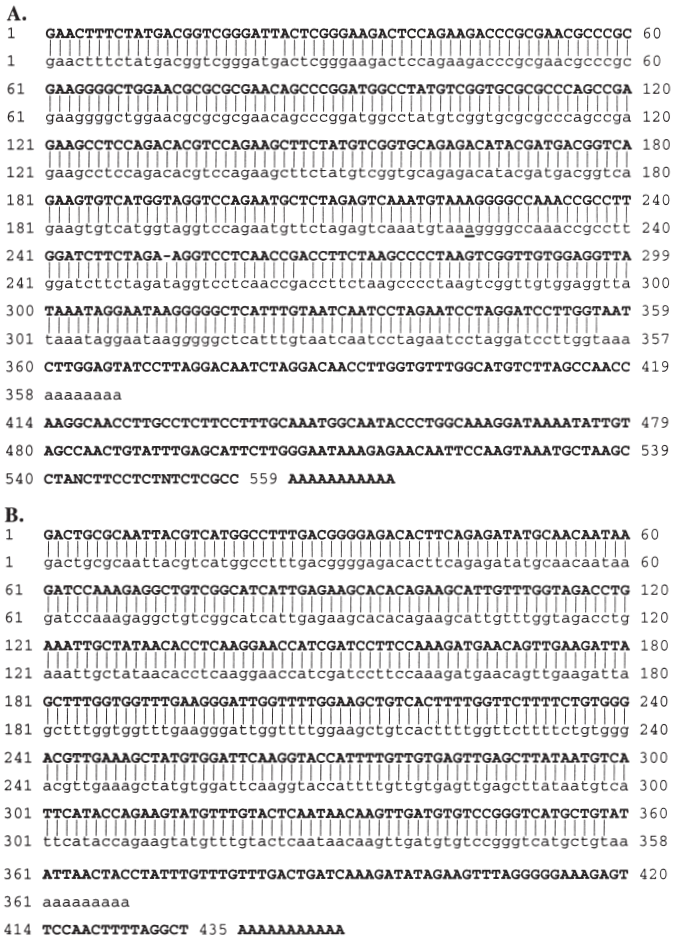

Fig. 2. Nucleotide sequences of partial cDNA clones, and alignment of (A) G10-8 (uppercase) with A10-6 and A10-7 (lower case), and (B) A10-8 (uppercase), with clone A10-11 (lower case).

each RNA sample. The PCR reaction contained 2 µL of each cDNA, 1.0 µM of oligo dT primer, 1.0 µM of arbitrary primer; 2 µM dNTPs (-A), 1 unit of HotStartTaq DNA polymerase (Qiagen, Valencia, CA), 2 µL of the correspondent 10 x buffer; and 2 µCi [^{33}-P] dATP (3000Ci/mmol; NEN, MA) in a final volume of 20 µL. PCR conditions were modified from Kammer et al. (2000) as follows: One cycle at 95ºC for 15 min; 94ºC for 30 s; 40ºC for 4 min; 72ºC for 1 min. This first cycle was followed by 35 extra cycles at 94ºC for 45 s; 55ºC for 2 min; 72ºC for 1 min; and one cycle at 72ºC for 5 min. The amplified cDNA was resolved on a 6% polyacrylamide sequencing gel, transferred to a Whatman 3MM paper and dried without fixing. The dried gel was exposed for 12-24 h on Kodak BioMax MR film (Eastman Kodak, Rochester, NY) and developed.

Reamplification and Cloning of cDNA Fragments. Differentially expressed cDNAs were excised from the gel, recovered and reamplified using the same primer set that produced it. The annealing temperature was dropped from 55ºC to 45ºC and the dNTP final concentration increased to 0.2 µM. The PCR reaction consisted of 8 µL of 5 x PCR buffer (300 mM Tris-HCl, 75 mM ammonium sulfate, 12.5 mM $MgCl_2$, 0.01% Triton X-100); 0.2 mM of dNTPs; 0.5 µM of anchor primer; 0.5 µM of random primer; 4 µL of cDNA solution and 1 µL of Thermozyme DNA polymerase (Invitrogen, Carlsbad, CA) in 40 µL volume. The cDNA fragments were cloned into TOPO TA Cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer instructions.

Colony-PCR. The colony-PCR was performed using anchor and random primers or the primers that flank the cloning site (M13 forward and reverse). PCR reactions were carried out for 30 cycles at 94ºC for 30 s; 45ºC for 30 s; 72ºC for 1 min, and 10 min at 72ºC in a 40 µl reaction containing 4 µL of 10 x PCR buffer (50 mM KCl, 10 mM Tris-HCl, 15 mM $MgCl_2$ and 0.1 % Triton X 100), 0.5 µM of each primer, 0.2 mM of dNTPs, 4 µL of colony lysate, and 1 unit of Taq DNA polymerase (Promega, Madison, WI). The insert size was verified by comparing the molecular size of the PCR products before and after cloning in a 1.5 % agarose gel.

Reverse Northern Dot Blot. Blotting was performed by mixing 30 µL of each colony-PCR product with sodium hydroxide and EDTA to a final concentration of 0.4 M sodium hydroxide and 10 mM EDTA. The mixture was boiled for 10 min to completely denature the cDNA. An equal volume of cold 2 M ammonium acetate was added to the target solution. Each sample was blotted onto duplicate Zeta-Probe membranes (Bio-Rad, Hercules, CA) using the Bio-Dot microfiltration system (Bio-Rad, Hercules, CA). After the samples were filtered, the wells were washed with 500 µL of 0.4 M NaOH. The blotted membranes were rinsed in 2 x SSC, air-dried, and crosslinked. Prehybridization and hybridization were carried out in a solution of 1 mM EDTA; 7% SDS; and 0.5 M Na_2HPO_4 at 65ºC. Radio-labeled cDNA probe was generated by reverse transcription of total RNA using an oligo dT20 primer and [∝ -^{32}P]dCTP. After overnight hybridization, the membranes were washed for 30 min twice with wash solution I (1 mM EDTA; 40 mM $NaHPO_4$, and 5% SDS), and twice with wash solution II (1 mM EDTA: 40 mM $NaHPO_4$, and 1% SDS) at 65ºC with gentle agitation. The positive clones were sequenced using an ABI Prism Automatic Sequencing System Model 377. Sequences were compared to the non-redundant database of the National Center for Biotechnology Information (NCBI) using BLASTN and BLASTX.

RESULTS AND DISCUSSION

The differential display method was used to study genes differentially expressed as a result of drought stress in melon roots. Only cDNA bands that were reproducible in six different

Table 1. Arbitrary primers used for differential display.

Name	Sequence
HPC -2	5'-TGCCGAAGCTTCAGC-3'
HPC -3	5'-TGCCGAAGCTTACAG-3'
HPC -4	5'-TGCCGAAGCTTTCGC-3'
HPC -5	5'-TGCCGAAGCTTGCTA-3'
HPC -6	5'-TGCCGAAGCTTTCTG-3'
HPC -7	5'-TGCCGAAGCTTAGAC-3'
HPC -8	5'-TGCCGAAGCTTCGAT-3'
HPC- 10	5'-TGCCGAAGCTTCTAG-3'

replications were considered differentials. Eighteen cDNA bands were found to be differentially expressed, with fourteen being down regulated in non-irrigated plants. The cDNAs were eluted from the gel slices and reamplified using the same primer combination that generated it, but using a annealing temperature of 45ºC instead of 55ºC. From the 18 cDNAs, two could not be reamplified, one consisted of two poorly defined bands, and 15 produced single, well defined bands. From the 15 reamplified cDNAs, 11 were down regulated in non-irrigated plants. These 15 cDNA fragments were cloned into the pCR®4-TOPO vector and four colonies per plate were picked randomly and submitted to colony PCR to verify the presence of the correct cDNA fragment. The 15 clones were named A2-3, A10-5, A10-6, A10-7, A10-8, A10-11, A10-12, C2-1, C7-10, C7-11, C7-12, C10-6, G10-8, G10-10, G10-11. These clones (four colonies per clone) were submitted to reverse northern blot to identify the truly positive ones as shown in Fig.1. For the clone G10-8 (Fig.1, I-a1-4) all four colonies were positive and were down regulated in non-irrigated plants as expected. For clones A10-6, A10-7, and A10-8 (Figs. 1; I-d1-4, I-d5-8, I-d9-12, respectively), only one colony was positive. The second colony on clone A10-7 was considered a false positive because it was also present in the non-irrigated sample. In clone A10-11 (Fig.1; I-e1-4), two colonies were positives. In total, five clones were considered truly positives and chosen to be sequenced. The nucleotide sequence for three of the partial clones; G10-8, A10-6, and A10-7 were 98% similar within them (Fig. 2A). A10-6 (357 bp) and A10-7 (357 bp) were identical and were 198 nucleotides shorter than G10-8 (559 bp). The clone G10-8 was obtained with anchor primer $HT_{11}G$, while the other two clones were obtained using $HT_{11}A$, however they seem to be a fragment of the same gene. There was no difference among the clones G10-8 obtained from the four colonies, as expected. Partial clones A10-8 (435 bp), and clone A10-11 (358 bp) were 100% similar, however, A10-11 was 76 bp shorter than A10-8 (Fig. 2B). There was no difference between the two clones A10-11 from the two different colonies. The sequence of the clones were compared to existing data deposited at the GenBank database to identify possible homologies. The partial clones G10-8, A10-6, and A10-7 had no similarity at the nucleotide level, however, it showed some similarity at the amino acid level to a fragment of 28 amino acids of a cell wall invertase, a beta-fructosidase from *Zea mays*. The identity was 46%, with 53% similarity and an E value of 6.5. Even though this E value indicate that such similarity could be obtained just by chance, it is interesting that cell wall invertase from wheat anther is down regulated during water stress, which causes a lack of starch deposition, resulting in poorly developed pollen (Minhas and Saini, 1998). Plant invertases seem to be involved in plant response to abiotic and biotic stresses such as wounding, heat, and water stress (Tymowska-Lalanne and Kreis, 1998).

The nucleotide sequence of the partial clone A10-8 showed 83% similarity ($E=2x10^{-8}$) with a 104 bp fragment of the acp5 gene from *A. thaliana* that codes for an acid phosphatase type 5. It also showed exactly the same similarity with a fragment of chromosome 3 of *A. thaliana* that may be a similar gene. The deduced amino acid sequence of this partial clone showed 83% identity, and 95% similarity (E value of $2x\ 10^{-43}$) with a 287 amino acid fragment of two *A. thaliana* proteins with unknown functions (Accessions #BAB02703.1, and #AAF79715.1).

For more conclusive information about these genes and their role in melon water stress, their full length cDNAs need to be obtained, which would allow for a better correlation with existing genes deposited in the GenBank database.

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