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Research Article

In silico analysis of ESTs from roots of Rangpur lime (*Citrus limonia* Osbeck) under water stress

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Abstract

CitEST project resulted in the construction of cDNA libraries from different *Citrus* sp. tissues under various physiological conditions. Among them, plantlets of Rangpur lime were exposed to hydroponic conditions with and without water stress using PEG6000. RNA from roots was obtained and generated a total of 4,130 valid cDNA reads, with 2,020 from the non-stressed condition and 2,110 from the stressed set. Bioinformatic analyses measured the frequency of each read in the libraries and yielded an *in silico* transcriptional profile for each condition. A total of 40 contigs were differentially expressed and allowed to detect up-regulated homologue sequences to well known genes involved in stress response, such as aquaporins, dehydrin, sucrose synthase, and proline-related synthase. Some sequences could not be classified by using FunCat and remained with an unknown function. A large number of sequences presented high similarities to annotated genes involved with cell energy, protein synthesis and cellular transport, suggesting that Rangpur lime may sustain active cell growth under stressed condition. The presence of membrane transporters and cell signaling components could be an indication of a coordinated morphological adaptation and biochemical response during drought, helping to explain the higher tolerance of this rootstock to water stress.

Key words: expressed sequence tags, transcription, rootstock, drought, molecular response.

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Introduction

Water stress limits crop production worldwide and has major impact on plant growth and development. Responses to abiotic stresses are complex and involve multiple stages of plant responses, according to the event (Chinnusamy *et al.*, 2004). For instance, less water available at the root zone implies additional regulation to balance photosynthesis, water transpiration, and overall metabolism. Secondary consequences may also happen, such as the rise of a nutrient concentration to a toxic level, eventual foliar depletion, and reduction of crop yield.

The advent of new technologies, especially available during the 'post-genomic' era, allowed foresights into the complexity of plant responses (Oono *et al.*, 2003). Even before that, abiotic stresses have received proper attention in different studies, comparing non-stressed to stressed conditions caused by low temperature (Nordin *et al.*, 1993), salinity (Forsthoefel *et al.*, 1995), and water deficit (Vartanian, 1996). Helped by emerging protein and model plant databases, many molecular mechanisms have been envisioned in non-model plants as well, and citrus has been one of them (Sanchez-Ballesta *et al.*, 2004).

Many studies have been conducted to elucidate the mechanisms that underlie plant responses to abiotic stress. The use of model plants like *Arabidopsis thaliana* has permitted some improvement in different studies, and recent efforts have focused on molecular response to water-deficit stress (Bray, 2004). Some drought-inducible genes have been identified: those involved in osmoprotection (*e.g.* sucrose, trehalose, and proline), protective factors of macromolecules (chaperone, heat shock proteins, and late embryogenesis abundant proteins), membrane transporters (aquaporins) and regulatory genes (transcription factors)

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(Yamaguchi-Shinozaki *et al.*, 2002). In addition, it has been proven that other pathway components (including protein kinases) are required in many signal transduction pathways for expression of drought-inducible genes (Mikami *et al.*, 1998; Soderman *et al.*, 1996; Urao *et al.*, 1994). Nevertheless, functions of most of the responsive genes during abiotic stress remain unknown.

Water is normally an agricultural limiting factor for most Brazilian soils. Commercial citrus groves, for instance, rely on grafting a scion of economic interest onto a more drought tolerant rootstock, which may endure from 4 to 6 months of low levels of water in the soil, depending on the region (Carlos *et al.*, 1998). The industry has made efforts to implement irrigation, but the cost of energy and the necessary investment have restrained further development. Thus, achieving drought tolerance in citrus is still extremely important. For half a century, this problem has been partially managed in São Paulo state using Rangpur lime (*Citrus limonia* Osbeck), a drought-tolerant rootstock. This rootstock also shows other attributes, making it possible to grow citrus under different limiting conditions, such as low nutritional and general grove maintenance conditions (Pompeu Jr., 2001). Nationally recognized by growers, Rangpur lime has been widely used after the industry collapsed due to the incidence of Tristeza during the 1940s (Grant, 1961). In the following decades, its reputation grew markedly, reaching preference levels of above 90% in nurseries for new plantings (Pompeu Jr, 2001). Thus, during this period, the orchards became supported almost completely by a single species, which led, as expected, to critical phytosanitary limitations for the Brazilian citrus industry. During the 1970s, citrus blight was reported (Rossetti *et al.*, 1991) and more recently, citrus sudden death, a poorly characterized disease that causes high losses in the citrus groves (Bassanezi *et al.*, 2003). Both problems have been extremely important and have stimulated rootstock changes. Unfortunately, among the alternative rootstocks, drought tolerance has been a limiting characteristic. Therefore, Rangpur lime is still the most preferred option for most growers in spite of the phytosanitary risks.

The development of new drought tolerant rootstocks in citrus, through classical plant breeding and/or innovative strategies that may help plants to survive longer periods under water deficient condition, is essential for the citrus industry to maintain its position in the competitive international market. Consequently, understanding the tolerance of plants to drought and exploiting its potential has been a priority in many recent studies.

Actually, strategies to investigate drought tolerance in citrus should include artificial systems to mimic water stress and the correspondent gene expression. To create such drought condition, a hydroponic system using polyethylene glycol (PEG) has been considered. PEG is a non-penetrating osmotic agent that lowers water potential causing a water stress condition (Piro *et al.*, 2003) and previous studies using this system to induce a stress condition have shown its efficacy (Villela *et al.*, 1991; Furlani, 1997; Piro *et al.*, 2003; Wu *et al.*, 2005).

In addition, an alternative to the study of gene expression is to use a citrus transcriptome database such as the citrus EST project (CitEST) created by the 'Centro APTA Citros Sylvio Moreira'. CitEST bioinformatic analyses allow the study of *in silico* gene expression and represent a powerful tool for understanding mechanisms involved in water stress. The databases permit the study of genes under specific condition or different tissues and the expression patterns may reflect the transcript frequency under such condition. Several water stress responsive transcripts can be identified *in silico*, and hopefully they can provide valuable information for understanding the mechanisms of the drought tolerance in Rangpur lime. The present study aims to evaluate transcriptional responses of Rangpur lime rootstock to osmotic stress in hydroponic systems, identifying genes possibly involved in drought tolerance.

Materials and Methods

Stress induction

Two month-old Rangpur lime (*C. limonia* Osbeck) plantlets were transferred to pots (2 L) containing hydroponic system with Hoagland's nutritive solution (Hoagland and Arnon, 1950) and aeration. The experimental design consisted of four plants per pot and two replications. All plants were maintained in this system during 30 days. After this period, they were subjected to water-stress treatment induced by 372.524 $g.L^{-1}$ of PEG6000 (Synth, São Paulo, Brazil) diluted in water to allow an osmotic potential of -1.5 MPa (Villela *et al.*, 1991). Roots were collected periodically, after 30, 45, 180, and 360 min after stress induction, washed with sterile water, surface-dried and kept at -80 °C until mRNA extraction. Equal weights of roots from each of the four collection times were mixed in order to generate a composite sample, from which the RNA was extracted. Roots from Rangpur lime plantlets, maintained in Hoagland's solution without PEG treatment, were collected and processed as described above and used as a mock (non-stressed) control.

cDNA libraries

RNA was extracted from 1 g of a pool of roots collected in the above-mentioned periods (30, 45, 180, and 360 min after stress induction) by using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA poly A was isolated from 1 mg of total RNA using the polyATract mRNA Isolation System (Promega, Madison, WI). Libraries were constructed using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen). One library of stressed roots (a mix of each collection time) and one library of the control

(non-stressed roots) were constructed and used in the *in silico* analysis.

EST sequencing and data analysis

Sequencing was carried out using the Big Dye Terminator v.3.1 Kit as described by the manufacturer (Applied Biosystems, Foster City, CA). Products were separated by capillary electrophoresis using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA).

An *in silico* 2x2 hybridization experiment was performed for the stressed x non-stressed libraries. The ESTs were grouped in clusters using CAP3, with default parameters. The composing reads for each assembled contig were counted by library, and the significance was given by using the methodology described in Audic and Claverie (1997) for two libraries.

The chosen threshold was a p-value ≤ 0.05. The resulting contigs were compared with public protein databases through BLASTX search (http://www.ncbi.nlm.nih.gov/BLAST/), and an automatic categorization of them was performed using the FunCat Functional Catalogue (Ruepp *et al.*, 2004). In addition, a manual categorization was utilized, especially when the result of automatic categorization had contigs classified into the 98 (classification not yet clear cut) or 99 (unclassified protein) classes.

Results and Discussion

In silico analysis

A total of 4,130 valid reads were sequenced (2,110 and 2,020 reads from the stressed and non-stressed roots libraries, respectively), resulting in 852 contigs. After *in silico* hybridization, 39 significant contigs had different frequencies in each library, of which 28 were up-regulated (higher frequency) and 11 down-regulated (lower frequency) in roots of Rangpur lime under stressed condition. Contigs representing differentially expressed transcripts were classified according to functional category based on similarities to Arabidopsis (FunCat 2.0; Ruepp *et al.*, 2004; Figure 1) and their expression profiles are shown in Figure 2. Most of them belonged to the "unclassified protein" (FunCat category 99). Other up-regulated transcripts were classified in the energy (FunCat 02), protein synthesis (12), protein metabolism (14), cellular transport (20), stress response (32) and cellular sensing and response (34.11) categories. Two contigs, C507 and C429, corresponding respectively to Dehydrin RAB18 and HAT22, after manual annotation, were associated with genes related with the ABA-dependent (abscisic acid) response to water stress. Additional genes related with ABA pathway were not found in this analysis.

Of the 11 down-regulated contigs, only one (contig 313 = C313) was classified as involved in biogenesis of cellular components (category 42.26.03), while all of the others fell in the unclassified proteins or in a classification of not yet clear-cut categories.

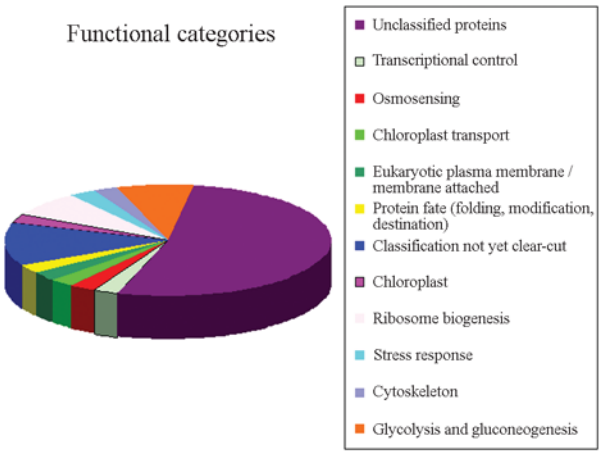

Figure 1 - MIPS functional categories for the 39 putative Rangpur lime water stress-related EST contigs.

Genes potentially associated with Rangpur lime tolerance to water stress

Transcripts identified in the *in silico* analysis of Rangpur lime roots under water stress had high sequence similarity with genes involved in well-known water stress responses in other species, like proline-related synthase (P5CS), sucrose synthase, membrane proteins (aquaporins), and dehydrin (Table 1). Other contigs revealed similarity with genes involved in responses to different abiotic stresses. However, yet other genes found to be differentially transcribed in this study are not normally associated with any kind of stress. The putative up-regulated genes found in the present analysis are discussed below within their potential role during water stress response.

Osmoprotectants and carbohydrate synthesis

According to previous studies, osmoprotectants such as proline, glycine betaine and sugars may help plants to overcome water deficit stress. Osmoprotectants were shown to function as osmolytes which protect cells from dehydration by turgor maintenance of roots and shoots in response to drought (Yamaguchi-Shinozaki *et al.*, 2002).

Free proline (Pro) accumulation is common in many eubacteria, algae and higher plants in response to osmotic stresses (Schobert, 1977; Csonka and Hanson, 1991) and was first observed in wilted rye grass (Kemble and MacPherson, 1954). Under normal conditions, Pro is preferentially synthesized via ornitine (Orn), whereas it is made directly from glutamate (Glu) under stress conditions (Delauney *et al.*, 1993). The first two steps of the proline biosynthesis from glutamate in *Arabidopsis* are catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), a bifunctional enzyme that catalyzes the conversion of Glu to Δ^1-

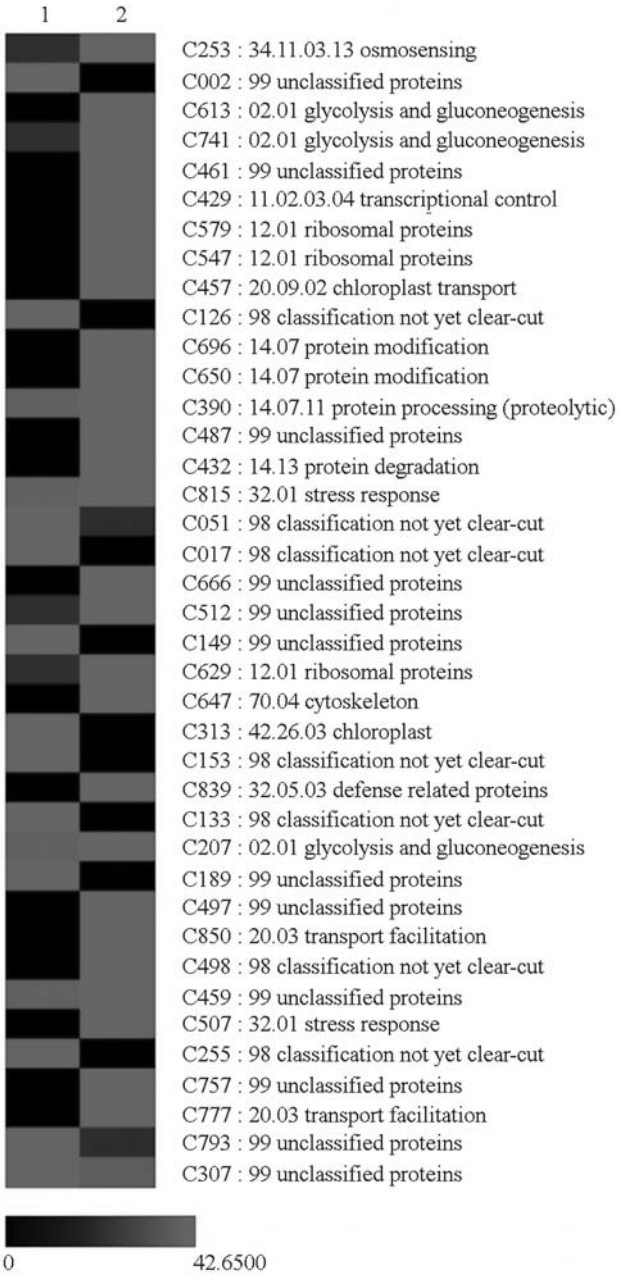

Figure 2 - Expression profiles of putative Rangpur lime water stress-related EST contigs. Scale represents relative abundance of reads in: 1-non-stressed root library and 2- roots under osmotic stress. Each profile is followed by the contig ID, the number of the MIPS functional category and the description of the functional category.

pyrroline-5-carboxylate, which is then reduced to proline (Hu *et al.*, 1992).

There are several studies showing biosynthesis of Pro from Glu under stress conditions (Stewart and Hanson, 1980; Delauney *et al.*, 1993). In our study, P5CS was differentially transcribed (p-value = 0.0099) in Rangpur lime under osmotic stress induced by PEG (see Table 1). These data suggest rapid synthesis and accumulation of Pro as one of the potential mechanisms used from this rootstock for its already known tolerance against drought.

Osmoregulation under water stress can also involve sucrose synthase activation, the most important enzyme of the sucrose biosynthesis pathway. The allocation of sucrose in leaves, stems, roots and nodules of soybean during water stress has been well characterized as well as after plant recovery (Fellows *et al.*, 1987). Concentration of sucrose was significantly greater in leaves, roots and nodules (up to 3 times) during the stress period. Upon rehydration, the elevated concentrations of sucrose in roots and nodules decreased within 1 day to values of control plants. In our experiments, Rangpur lime appears to respond to water stress similarly. A putative sucrose synthase was differentially transcribed (p-value = 0.0499), suggesting adjustments of the carbohydrate biosynthesis as an additional mechanism of response to water stress.

Within the carbohydrate biosynthesis pathway, the cytosolic glyceraldehyde-3-phosphate dehydrogenase (Gap3) was also differentially transcribed (p-value = 0.0382) in Rangpur lime under PEG induced stress. Indeed, as shown by Yang *et al.* (1993), heat shock stress (and some responses are very similar to water stress) increased expression of the GapC in Arabidopsis, which could be expected. Additionally, Yang and collaborators showed similar induction of GapC (up to 10-fold) in response to sucrose supplied for plants *in vitro*. This study has a different orientation, but there is a chance that GapC induction may be a response to both the stress itself and its consequent increase on the cellular sucrose concentration, which is to be expected from higher sucrose synthase expression.

Expression of cysteine proteinase has been studied in different stages of plant development (Beyene *et al.*, 2006), in response to a variety of stresses and also in programmed cell death (Schaffer and Fischer, 1988; Koizumi *et al.*, 1993; Harrak *et al.*, 2001; Solomon *et al.*, 1999). Distinct drought-inducible cysteine proteinases were induced by changes in the osmotic potential of plant cells elsewhere (Koizumi *et al.*, 1993). In the present work, a drought-inducible cysteine (Table 1) which was probably involved in the maintenance of osmotic potential was found to be up-regulated in water deficit conditions.

Membrane transporters

Aquaporins are membrane proteins that are related to water translocation between cells. Members of this family make water-specific channels for ions and solutes (Bray, 1993). The channels facilitate the water flow under osmotic gradient. Aquaporins facilitate the uptake of water from the soil and mediate the regulation of root hydraulic conductivity [Lp(r)] in response to a large variety of environmental stresses (Boursiac *et al.*, 2005). A study using antisense lines of PIP1 and PIP2 aquaporins indicates that PIPs play an important role in the recovery of Arabidopsis from water-deficient conditions (Martre *et al.*, 2002). During water stress, these water-channel proteins accumulate in the tonoplasto (vacuole membrane) and the movement of water and

Table 1 - Up-regulated genes under water stress in Rangpur lime roots.

Gene product	MIPS code	E-value	ID/SIM[1] (%)	p-value[2]	Contig number
Genes typically associated with water stress response					
Delta-1-pyrroline-5-carboxylate synthetase (P5CS)	At3g55610	e-141	83/89	0.0099	C253
Sucrose synthetase	At4g02280	2e-24	89/96	0.0499	C741
Drought-inducible cysteine proteinase	At1g47128	1e-122	74/87	0.0244	C390
Plasma membrane intrinsic protein 2a (Aquaporin)	At3g53420	5e-08	74/96	0.0170	C850
Putative membrane protein	At3g62580	1e-16	90/92	0.0333	C777
Dehydrin RAB18	At5g66400	6e-08	41/59	0.0086	C507
Genes associated with other abiotic/biotic stresses					
Glyceraldehyde-3-phosphate dehydrogenase C subunit (Gap3C)	At3g04120	e-168	92/97	0.0382	C207
Bax inhibitor-1 like	At5g47120	9e-35	83/97	0.0087	C839
Putative Germin-like protein	At1g18980	1e-29	51/68	0.0382	C815
Phosphoenolpyruvate carboxykinase	At5g65690	1e-25	76/85	0.0170	C461
Putative protein kinase	At2g30040	4e-20	53/65	0.0333	C696
Polyubiquitin 4 UBQ4	At5g20620	9e-72	99/99	0.0298	C427
Polyubiquitin (UBQ11)	At4g05050	5e-80	100/100	0.0011	C432
Homeobox protein HAT22	At4g37790	4e-39	75/82	0.0011	C429
40S ribosomal protein S28	At5g64140	2e-26	92/95	0.0297	C629
40S ribosomal protein S9-like	At5g39850	6e-57	88/97	0.0333	C547
Others					
Chloroplast Cpn21 protein	At5g20720	2e-47	79/90	0.0170	C457
MO25 protein family	At5g47540	6e-12	72/88	0.0333	C757
PGPD14 protein	At5g22920	e-101	64/81	0.0382	C459
Glycogenin glucosyltransferase	At3g18660	2e-36	71/79	0.0333	C613
Nodulin-like protein	At5g25940	1e-24	57/66	0.0333	C498
Actin depolymerizing factor2 (ADF2)	At1g01750	2e-31	88/95	0.0333	C647

[1]ID/ SIM = identity/ similarity; [2]Audic and Claverie (1997) statistics.

solutes from vacuole to the cytoplasm is affected, changing the amount of water and the cytoplasmic osmotic potential (Maurel *et al.*, 1993). Aquaporins are found mainly in cells involved in hydraulic flow, such as root epidermis, root tip tissues and tissues near the xylem cells in roots (Bohnert *et al.*, 1995). The expression of aquaporins of the tonoplast and cell membrane has been correlated also with cell elongation (Yamaguchi-Shinozaki *et al.*, 1992; Daniels *et al.*, 1994), supporting the idea that cells of Rangpur lime sustain continuous growth of the root system. Under stress conditions, trees with longer and deeper roots are favored to reach underground water. The gene expression analysis of aquaporins in response to abiotic stress in *Arabidopsis thaliana* revealed the presence of 35 genes of aquaporins, and from those, 13 had identity with intrinsic proteins of the plasmatic membrane (PIP) (Jang *et al.*, 2004). The subfamily PIP is divided into 2 subgroups: PIP1 and PIP2, according to the aminoacid sequence at the amino- and the carboxy-terminal. Jang *et al.* (2004) observed using Real Time PCR approach, that different genes of PIP are predominantly expressed either in roots or in aerial tissues (PIP1;3, PIP1;4, PIP2;1 and PIP2;5) under water stressed condition. The same work also reported that the expression of aquaporin can be either dependent or independent of ABA. Here, we found membrane proteins that showed homology with PIP2.1 class, but we did not evaluate whether or not it is dependent on ABA.

Protective factors of macromolecules

Cellular protectors refer to the group of genes that code for certain late embryogenesis abundant (LEA) proteins, more commonly called dehydrins (Close, 1996). The exact function of these proteins is still unclear but numerous studies have demonstrated the induction of dehydrins by drought stress (Maitra and Cushman, 1994; Giordani *et al.*, 1999; Thompson and Corlett, 1995). Dehydrins are normally synthesized in maturating seeds during their desiccation, and in vegetative tissues of plants treated with ABA or exposed to environmental stress factors that result in cellular dehydration. Allagulova *et al.* (2003) described that they are considered as stress proteins involved in the formation of plant protective reactions against dehydration. The

generally accepted classification of dehydrins is based on their structural features, such as the presence of conserved sequences, designated as Y-, S-, and K-segments; and the K-segment representing a highly conserved 15 amino acid motif (EKKGIMDKIKE KLPG), forming amphiphilic alpha-helix, has been found in all dehydrins.

Therefore, finding a putative dehydrin in citrus root tissues, taken from a water deficient condition, was not a surprise. In field conditions, Rangpur lime rootstock has relatively good drought resistance, resulting in reasonable harvests even without irrigation during the 3 to 6 months dry winter period in São Paulo state. This resistance mechanism remains unclear, but it may have higher levels of protective factors than other rootstocks, such as dehydrin molecules. This contribution to drought resistance remains quantitatively unconfirmed.

Although not included in the present analysis, multiple putative heat shock proteins that mainly belonging to the HSP70 family were found very close to statistical significance, showing a possible role in the response to water stress in Rangpur lime. Perhaps under different experimental conditions, these proteins would present a more significative expression. Most of the HSPs work as chaperones that help to correct folding and/or to prevent protein denaturation. Since stress promotes denaturation and protein aggregation, the higher rate of HSP synthesis in Rangpur lime under stressed condition would help protect the osmotic stress proteins that occur after dehydration of the cell (Zhu *et al.*, 1997). Some of the HSPs induced in Rangpur lime are named binding protein (BiP). Plant BiP expression has also been shown to respond to a variety of abiotic and biotic stress conditions (Jelitto-Van Dooren *et al.*, 1999; Kumar *et al.*, 2004). Transgenic tobacco (*Nicotiana tabacum* L. cv. Havana) plants constitutively expressing BiP genes were more resistant to water stress than control plants (Alvim *et al.*, 2001). The expression of these genes in Rangpur lime is probably involved in drought resistance.

Differential expression of an EST corresponding to the chloroplast Cpn21 protein was also identified in stress-induced Rangpur lime roots (p-value = 0.0170). Cpn21 was characterized as a 21-kDa protein chaperonin 21 (Cpn21), which is a functional homolog of the chaperonin 10 (Cpn10) and consists of two Cpn 10-like domains fused together in tandem (Hirohashi *et al.*, 1999). Also named Cpn20 (TAIR, The Arabidopsis Information Resource), Cpn21 seems to work independently of a chloroplast Cpn10 (both are homologues of the *E. coli* GroEs) on the regulation of specific Cpn60 subunit homologues (*E. coli* GroEl homologues) in plastids (Koumoto *et al.*, 2001). In Arabidopsis, the expression of the chloroplast Cpn10 in mature plants is detected only in leaves and stems, while Cpn20 is expressed in leaves, stems and roots (Koumoto *et al.*, 1999 and 2001). These findings suggest Cpn20 (or Cpn21) as the specific chaperonin involved in regulation of Cpn60 subunits in roots, and so, the assembly of proteins inside chloroplasts. Moreover, differential expression of the Cpn21 in stressed Rangpur lime suggests possible changes in the regulation of protein assembly or needs for increased protein synthesis.

Signaling

Signal transduction pathways for expression of drought-inducible genes require different transcription factors and other components like protein kinases. Phosphorylations promoted by diverse families of kinases are normally switches of important biochemical processes. Kinases that can be turned on and off under processes related to water stress are known (Zhang *et al.*, 2004), and therefore, a putative kinase was expected to be seen in our results. However, a valuable question considering its potential insertion and contribution in different signaling pathways and different rootstock.

Another kinase found in the present analysis was phosphoenolpyruvate carboxykinase, which is an enzyme used in gluconeogenesis to convert oxaloacetate and guanosine triphosphate into phosphoenolpyruvate, guanosine diphosphate and carbon dioxide. It seems to be somehow involved in the stress response in many plants (Saez-Vasquez *et al.*, 1995). In alfalfa, a cDNA library from inoculated and non-inoculated roots identified a homolog of phosphoenolpyruvate carboxykinase expressed in those plants subjected to nematode infection (Potenza *et al.*, 2001). In sorghum, phosphoenolpyruvate carboxylase-kinase (PEPCase-k) activity is highly enhanced by salt stress in leaves (García-Mauriño *et al.*, 2003). Here, we observed a higher expression of this enzyme in roots under water stress, but the exact role during this process needs to be further investigated.

A third transcription factor, a homeobox-leucine zipper homolog to the Arabidopsis HAT22 gene, was differentially expressed in the Rangpur lime stressed roots. A type of homeobox-leucine zipper gene has been identified in *Arabidopsis thaliana* (Schena and Davis, 1994). The authors suggested that these genes mediate specific regulatory events. In another study, Deng *et al.* (2002) reported five novel dehydration-responsive homeodomain leucine zipper genes (HDZips) from the resurrection plant *Craterostigma plantagineum*. All families of these genes were modulated in their expression in response to dehydration in leaves and roots, supporting the role of HDZips in related regulatory pathways in *C. plantagineum*, which lead to desiccation tolerance. In a recent work, Dezar *et al.* (2005) transformed Arabidopsis plants with a Hahb-4 homeobox-leucine zipper transcription factor from *Helianthus annuus* (sunflower). Transgenic plants were more tolerant to water stress conditions, showing improved plant development, healthier appearance and higher survival rates than wild-type plants. Plants under either normal or drought conditions produce approximately the same seed weight per plant as wild-type plants under normal

growth conditions. Since Hahb-4 is a transcription factor, it was proposed that it may participate in the regulation of the expression of genes involved in developmental responses of plants to desiccation. Thus, the expression of a HAT22 homologue in our experiment probably indicates a regulatory role in stress response in citrus.

Other proteins activated during stress response

Several other deduced proteins could also be related to stress response and among them, an orthologue of Bax-inhibitor was found to be significantly transcribed in stressed, but not in non-stressed, Rangpur lime root cDNA libraries. The Bax-inhibitor 1 (BI-1) is a membrane protein that plays an important role in preventing PCD (programmed cell death) related to Bax in mammals (Hückelhoven *et al.*, 2003). In plants, it has been shown that expression of a Bax-inhibitor homolog is rapidly up-regulated during wounding or pathogen challenge, suggesting its role in responses to biotic and abiotic stresses (Sanchez *et al.*, 2000; Hückelhoven *et al.*, 2003; Matsumura *et al.*, 2003; Kamauchi *et al.*, 2005). No Bax-inhibitor like sequences had been reported in *Citrus* sp. prior to this work. Interestingly, all of the six putative Bax-inhibitor sequences tags found in our citrus EST libraries came from the drought stressed Rangpur lime root library. Even though no clear correlation has not been shown between Bax-inhibitor like protein and drought stress in the literature so far, it is reasonable to presume that the Bax protein might play a role in such a situation as well, and could possibly be overexpressed in order to avoid cell collapse and death.

Earlier reports have shown that water deficit causes significant decreases in protein synthesis (Hsaio, 1970; Dhindsa and Cleland, 1975; Rhodes and Matsuda, 1976; Mason *et al.*, 1988; Bensen *et al.*, 1988; Valluri *et al.*, 1989). Interestingly, in our experiment we observed an increase in the levels of two proteins that constitute the 40S subunit of the ribosomes, S28 e S9. *De novo* protein synthesis is necessary for cold response. Three genes encoding different ribosomal proteins were isolated from soybean and are induced by low-temperature stress (Kim *et al.*, 2004). This could have happened to enhance the translational process or help the proper ribosome assembly and functioning under this condition. We can also speculate that under water stress, Rangpur lime shows a higher level of ribosomal proteins in order to maintain the physiological processes that lead to tolerance to drought. On the other hand, higher expression of the ubiquitination related genes, encoding ubiquitin-conjugating enzymes, was also observed, suggesting that stress responses were taking place, leading to increased degradation of proteins. The ubiquitin-dependent proteolytic pathway regulates many essential processes in eukaryotes by selective protein breakdown (Ciechanover and Schwartz, 1998) and the ubiquitin conjugating enzyme is an E2 protein that synthesizes multi-ubiquitin chains from free ubiquitin (Chen and Pickart, 1990; van Nocker and Vierstra, 1991; van Nocker *et al.*, 1996).

Another protein, a germin-like protein (C815), and classified in the stress response categories – as well as in the DNA processing/ DNA synthesis and replication – was found to be significantly up-regulated in stressed Rangpur lime root library. Interestingly, these proteins have been involved in an array of functions, most of them related to plant development and cell wall biogenesis, but also to C-compound and carbohydrate catabolism, osmotic regulation, photoperiodic oscillation, and defense (Çaliskan, 2000; Patnaik and Khurana, 2001).

The importance of germin-like proteins to salt stress tolerance in both monocot and dicotyledoneous plants has been known for years (Hurkman *et al.*, 1991; Michalowski and Bohnert, 1992). However, their main and most-studied role is in early plant development (Lane *et al.*, 1993). Wissel *et al.* (2003) demonstrated that regulation of a germin-like gene of Aspen is strongly related to the tree developmental stage, with overexpression only in young tissues and during the growth phase of the tree, regardless of the environmental conditions.

Up-regulated proteins not directly related to stress response

A putative protein found only in the stressed Rangpur lime library presented similarity with members of the MO25 (early mouse development) protein family, involved in cell differentiation. Even though there is no information about the role of such proteins in plants, hypothetical MO25-like genes have been found in *Arabidopsis thaliana* (www. arabidopsis.org).

In mammals, it has been shown that the MO25a/b protein interacts with STRADa/b and increases their ability to bind, activate and localize LKB1 (a 50 kDa serine/threonine kinase involved in suppression of tumor) in the cytoplasm (Boudeau *et al.*, 2003). The complexes between the LKB1, STRADa/b and MO25 a/b are upstream kinases in the AMP-activated protein kinase (AMPK) cascade. AMPK is a sensor of cellular energy charge that acts as a 'metabolic master switch' inhibiting cell proliferation and protecting cells against nutritional or environmental stresses (Hardie and Hawley, 2001; Hardie *et al.*, 2003; Hawley *et al.*, 2003; Baas *et al.*, 2004).

In plants, kinases have been shown to be activated by various abiotic stresses (Ikeda *et al.*, 1999; Kelner *et al.*, 2004; Thelander *et al.*, 2004). It remains unknown and to be addressed in further studies whether the MO25 homologue has any implication in such activation or in root cell differentiation in outgrowing the stressful condition.

Another putative up-regulated protein in water stressed roots was a nodulin. Nodulins modulate metabolizing carbon and nitrogen, and transport compounds within the nodule. Kuster *et al.* (1996) studied different modular structures and compared the timing of gene expression. Cit-

rus does not produce nodules, such as beans, but it does have a root system populated by diverse organisms. Therefore, it is possible that water stress affects root-microorganism interactions in the citrus ryzosphere, and thus, affects gene expression of related mediators.

There are different forms of stress conditions, but for a regular cell expansion and root hair tip growth there is a highly dynamic demand for polar actin cytoskeleton formation. Ketelaar *et al.* (2004) studied such conditions. Under drought conditions, root development can be impaired to avoid dehydration, or initially stimulated to explore yet untouchable soil areas. Since gene families encode actin, in higher eukaryotes, it is possible that this putative actin gene is responsive to drought conditions.

In addition, other genes had a much higher expression induced by water stress, such as glycogenin glucosyltransferase (C613) and PGPD14 protein (C459). In rice, the accumulation of a glycogenin glucosyltransferase (OsGGT) seems to be involved in response to stress, including environmental stresses, such as drought and salt treatment (Qi *et al.*, 2005). PGPD14 protein (pollen germination related protein) does not appear to be involved directly with stress because of lack of further reports.

Other unclassified proteins that also displayed differentially transcribed patterns under stressed condition can be targets for future investigation and perhaps they can help to find new answers about the mechanism involved in Rangpur lime drought resistance mechanism not yet described.

Concluding Remarks

Rangpur lime is a rootstock considered highly tolerant to drought stress. However, its mechanism of tolerance is not fully understood. There is little biological evidence suggesting that this tolerance is mostly related to a capacity to physically grow more and longer roots rather than only a biochemical ability to withstand water stress. Our EST data analysis showed that several of the proteins often associated with drought stress in other plant species, such as proline-related synthase, aquaporins, dehydrins, some sugars and others associated with different types of abiotic stresses, had transcripts that were significantly induced in the Rangpur lime library constructed under such condition. In addition, other proteins not normally related to abiotic stresses were also induced, but their roles remain unclear.

The Rangpur lime tolerance mechanism could be based on the activation of proteins involved in osmoprotection together with other responses that adjust cell turgor and an increase of expression of genes related to sustaining tissue development, which might keep root growth active. The responses of genes activated by abscisic acid (ABA) were infrequently observed. Only one transcription factor, a homologue of the Arabidopsis HAT22 gene known to be activated by ABA, was detected in our analysis. This gene might have a potential role in controlling the response to stress, similar to that occurring in other plants. Additionally, most of the up-regulated transcripts found in this study are related to protein synthesis and some to other functions. Future studies should test this hypothesis *in vivo* and validate the specificity and expression levels of the genes found here, considering related conditions.

Thus, it is possible that the genetic response of Rangpur lime to drought leads to an efficient capacity of the root system to absorb water, potentially in a better way than normally happens to other citrus species when used as rootstocks. Those related genes are potentially interesting subjects for further studies, including development of gene specific markers for selection within hybrid populations generated by Rangpur lime interspecies crossings and genetic engineering of other citrus rootstocks in order to increase their resistance to water stress.

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References

Allagulova CHR, Gimalov FR, Shakirova FM and Vakhitov VA (2003) The plant dehydrins: Structure and putative functions. Biochem 68:945-951.

Alvim FC, Carolino SMB, Cascardo JCM, Nunes CC, Martinez CA, Otoni WC and Fontes EPB (2001) Enhanced accumulation of bip in transgenic plants confers tolerance to water stress. Plant Physiol 126:1042-1054.

Audic S and Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7:986-995.

Baas AF, Kuipers J, van der Wel NN, Batlle E, Koerten HK, Peters PJ and Clevers HC (2004) Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. Cell 116:457-466.

Bassanezi RB, Bergamin A, Amorim L, Gimenes-Fernandes N, Gottwald TR and Bove JM (2003) Spatial and temporal analyses of citrus sudden death as a tool to generate hypotheses concerning its etiology. Phytopathol 93:502-512.

Bensen RJ, Boyer JS and Mullet JE (1988) Water-deficit-induced changes in abscisic acid, growth, polysomes, and translatable RNA in soybean hypocotyls. Plant Physiol 88:289-294.

Beyene G, Foyer CH and Kunert KJ (2006) Two new cysteine proteinases with specific expression patterns in mature and senescent tobacco (*Nicotiana tabacum* L.) leaves. J Exp Bot 57:1431-1443.

Bohnert HJ, Nelson DE and Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7:1099-1111.

Boursiac Y, Chen S, Luu DT, Sorieul M, van den Dries N and Maurel C (2005) Early effects of salinity on water transport in Arabidopsis roots: Molecular and cellular features of aquaporin expression. Plant Physiol 139:790-805.

Boudeau J, Baas AF, Deak M, Morrice NA, Kieloch A, Schutkowski M, Prescott AR, Clevers C and Alessi DR (2003) MO25a/b interact with STRADa/b enhancing their ability to

bind, activate and localize LKB1 in the cytoplasm. EMBO J 22:5102-5114.

Bray EA (1993) Molecular responses to water deficit. Plant Physiol 103:1035-1040.

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot 55:2331-2341.

Carlos EF, Stuchi ES and Donadio LC (1998) Porta Enxertos para a Citricultura Paulista. FUNESP, Jaboticabal, 49 pp.

Çaliskan M (2000) Germin, an oxalate oxidase, has a function in many aspects of plant life. Turk J Biol 24:717-724.

Chen Z and Pickart CM (1990) A 25 kilodalton ubiquitin carrier protein (E2) catalyzes multi-ubiquitin chain synthesis via lysine 48 of ubiquitin. J Biol Chem 265:21835-21842.

Chinnusamy V, Schumaker K and Zhu J-K (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. J Exp Bot 55:225-236.

Ciechanover A and Schwartz AL (1998) The ubiquitin-proteasome pathway: The complexity and myriad functions of proteins death. Proc Natl Acad Sci USA 95:2727-2730.

Close TJ (1996) Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plantarum 97:795-803.

Csonka LN and Hanson AD (1991) Prokaryotic osmoregulation: Genetics and physiology. Annu Rev Microbiol 45:569-606.

Daniels MJ, Mirkov TE and Chrispeels MJ (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol 106:1325-1333.

Delauney AJ, Hu C-AA, Kavi Kishor PB and Verma DPS (1993) Cloning of ornithine 8-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. J Biol Chem 268:18673-18678.

Deng X, Phillips J, Meijer AH, Salamini F and Bartels D (2002) Characterization of five novel dehydration-responsive homeodomain leucine zipper genes from the resurrection plant *Craterostigma plantagineum*. Plant Mol Biol 49:601-610.

Dezar CA, Gago GM, Gonzalez DH and Chan RL (2005) Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants. Transgenic Res 14:429-440.

Dhindsa RS and Cleland RE (1975) Water stress and protein synthesis. I. Differential inhibition of protein synthesis. Plant Physiol 55:778-781.

Fellows RJ, Patterson RP, Raper Jr CD and Harris D (1987) Nodule activity and allocation of photosynthate of soybean during recovery from water stress. Plant Physiol 84:456-460.

Forsthoefel NR, Vernon DM and Cushman JC (1995) A salinity-induced gene from the halophyte m-crystallinum encodes a glycolytic enzyme, cofactor-independent phosphoglycero mutase. Plant Mol Biol 29:213-226.

Furlani PR (1997) Instruções para o Cultivo de Hortaliças de Folhas pela Técnica de Hidroponia NFT. Boletim Técnico n. 168. Instituto Agronômico de Campinas, Campinas, 30 pp.

García-Mauriño SJM, Alvarez R, Vidal J and Echevarría C (2003) Characterization of salt stress-enhanced phosphoenolpyruvate carboxylase kinase activity in leaves of *Sorghum vulgare*: Independence from osmotic stress, involvement of ion toxicity and significance of dark phosphorylation. Planta 216:648-55.

Giordani T, Natali L, D'Ercole A, Pugliesi C, Fambrini M, Vernieri P, Vitagliano C and Cavallini A (1999) Expression of a dehydrin gene during embryo development and drought stress in ABA-deficient mutants of sunflower (*Helianthus annuus* L.). Plant Mol Biol 39:739-748.

Grant TJ (1961) Citrus variety reaction to tristeza virus in Brazil when used in various rootstocks and scion combinations. Plant Dis Rep 45:416-421.

Hardie DG and Hawley SA (2001) AMP-activated protein kinase: The energy charge hypothesis revisited. Bioessays 23:1112-1119.

Hardie DG, Scott JW, Pan DA and Hudson ER (2003) Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett 546:113-120.

Harrak H, Azelmat S, Baker EN and Tabaeizadech Z (2001) Isolation and characterization of gene encoding a drought-induced cysteine protease in tomato (*Lycopersicum esculentum*). Genome 44:368-374.

Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR and Hardie DG (2003) Complexes between the LKB1 tumor suppressor, STRADa/b and MO25a/b are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2:1-16.

Hirohashi T, Nishio K and Nakai M (1999) cDNA sequence and overexpression of chloroplast chaperonin 21 from *Arabidopsis thaliana*. Biochim Biophys Acta 1429:512-515.

Hoagland DR and Arnon DL (1950) The Water Culture Methods for Growing Plants without Soil. University of California, Berkeley, 32 pp.

Hsaio TC (1970) Rapid changes in levels of polyribosomes in *Zea mays* in response to water stress. Plant Physiol 46:281-285.

Hu C-AA, Delauney AJ and Verma DPS (1992) A bifunctional enzyme (A'-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA 89:9354-9358.

Hückelhoven R, Dechert C and Kogel K-H (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis.* Proc Natl Acad Sci USA 100:5555-5560.

Hurkman WJ, Tao HP and Tanaka CK (1991) Germin-like polypeptides increase in barley roots during salt stress. Plant Physiol 97:366-374.

Ikeda Y, Koizumi N, Kusano T and Sano H (1999) Sucrose and cytokinin modulation of WPK4, a gene encoding a SNF1-related protein kinase from wheat. Plant Physiol 121:813-820.

Jang JY, Kim DG, Kim YO, Kim JS and Kang H (2004) An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. Plant Mol Biol 54:713-725.

Jelitto-Van Dooren EP, Vidal S and Denecke J (1999) Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. Plant Cell 11:1935-1944.

Kamauchi S, Nakatani H, Nakano C and Urade R (2005) Gene expression in response to endoplasmic reticulum stress in *Arabidopsis thaliana*. FEBS J 272:3461-76.

Kelner A, Pekala I. Kaczanowski S, Muszynska G, Hardie DG and Dobrowolska G (2004) Biochemical characterization of

the tobacco 42-kD protein kinase activated by osmotic stress. Plant Physiol 136:3255-3265.

Kemble AR and MacPherson HT (1954) Liberation of amino acids in perennial rye grass during wilting. Biochem J 58:46-59.

Ketelaar T, Anthony RG and Hussey PJ (2004) Green fluorescent protein-mTalin causes defects in actin organization and cell expansion in Arabidopsis and inhibits actin depolymerizing factor's actin depolymerizing activity *in vitro*. Plant Physiol 136:3990-3998.

Kim K-Y, Park S-W, Chung Y-S, Chung C-H, Kim J-I and Lee J-H (2004) Molecular cloning of low-temperature-inducible ribosomal proteins from soybean. J Exp Bot 55:1153-1155.

Koizumi M, Yamaguchi-Shinozaki K, Tsuji H and Shinozaki K (1993) Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene 29:175-182.

Koumoto Y, Shimada T, Kondo M, Hara-Nishimura I and Nishimura M (2001) Chloroplasts have a novel Cpn10 in addition to Cpn20 as co-chaperonins in *Arabidopsis thaliana*. J Biol Chem 276:29688-29694.

Koumoto Y, Shimada T, Kondo M, Takao T, Shimonishi Y, Hara-Nishimura I and Nishimura M (1999) Chloroplast Cpn20 forms a tetrameric structure in *Arabidopsis thaliana*. Plant J 17:467-477.

Kumar V, Mills DJ, Anderson JD and Mattoo AK (2004) An alternative agriculture system is defined by a distinct expression profile of select gene transcripts and proteins. Proc Natl Acad Sci USA 101:10535-10540.

Kuster H, Fruhling M, Puhler A and Perlick AM (1996) The modular nodulins Nvf-28/32 of broad bean (*Vicia faba* L.): Alternative exon combinations account for different modular structures. Mol Gen Genet 252:648-657.

Lane BG, Dunwell JM, Rag JA, Schmitt MR and Cumin AC (1993) Germin, a protein marker of early plant development, is an oxalate oxidase. J Biol Chem 268:12239-12242.

Maitra N and Cushman JC (1994) Isolation and characterization of a drought-induced soybean cDNA encoding a D95 family late-embryogenesis-abundant protein. Plant Physiol 106:805-806.

Martre P, Morillon R, Barrieu F, North GB, Nobel PS and Chrispeels MJ (2002) Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol 130:2101-2110.

Mason HS, Mullet JE and Boyer JS (1988) Polysomes, messenger RNA, and growth in soybean stems during development and water-deficit. Plant Physiol 86:725-733.

Matsumura H, Nirasawa S, Kiba A, Urasaki N, Saitoh H, Ito M, Kawai-Yamada M, Uchimiya H and Terauchi R (2003) Overexpression of bax inhibitor suppresses the fungal elicitor-induced cell death in rice (*Oryza sativa* L.) cells. Plant J 33:425-434.

Maurel C, Reizer J, Schroeder JI and Chrispeels, MJ (1993). The vacuolar membrane protein gamma-TIP creates water specific channels in *Xenopus oocytes*. EMBO J 12:2241-2247.

Michalowski CB and Bohnert HJ (1992) Nucleotide sequence of a root-specific transcript encoding a germin-like protein from the halophyte *Messembryanthemum crystallinum.* Plant Physiol 100:537-538.

Mikami K, Katagiri T, Luchi S, Yamaguchi-Shinozaki K and Shinozaki K (1998) A gene encoding phosphatidylinositol 4-phosphate 5-kinase is induced by water stress and abscisic acid in *Arabidopsis thaliana.* Plant J 15:563-568.

Nordin K, Vahala T and Palva ET (1993) Differential expression of 2 related, low-temperature-induced genes in *Arabidopsis thaliana* (L) Heynh. Plant Mol Biol 21:641-653.

Oono Y, Seki M, Nanjo T, Narusaka M, Fujita M, Satoh R, Satou M, Sakurai T, Ishida J, Akiyama K, *et al.* (2003) Monitoring expression profiles of Arabidopsis gene expression during rehydration process after dehydration using ca 7000 full-length cDNA microarray. Plant J 34:868-887.

Patnaik D and Khurana P (2001) Germins and germin like proteins: An overview. Indian J Exp Biol 39:191-200.

Piro G, Leucci MR, Waldron K and Dalessandro G (2003) Exposure to water stress causes changes in biosynthesis of cell wall polysaccharides in roots of wheat cultivars varying in drought tolerance. Plant Sci 165:559-569.

Pompeu Jr. J (2001) Rootstocks and scions in the citriculture of the São Paulo state. In: Proceedings 6th International Congress of Citrus Nurserymen. Ribeirão Preto, pp 75-82.

Potenza C, Thomas SH and Sengupta-Gopalan C (2001) Genes induced during early response to *Meloidogyne incognita* in roots of resistant and susceptible alfalfa cultivars. Plant Sci 161:289-299.

Qi Y, Kawano N, Yamauchi Y, Ling J, Li D and Tanaka K (2005) Identification and cloning of a submergence-induced gene OsGGT (glycogenin glucosyltransferase) from rice (*Oryza sativa* L.) by suppression subtractive hybridization. Planta 221:437-445.

Rhodes PS and Matsuda K (1976) Water stress, rapid polyribosome reductions and growth. Plant Physiol 58:631-635.

Rossetti VV, Beretta MJG and Teixeira ARR (1991) Experimental transmission of declinio by approach-root-grafting in São Paulo State, Brazil. In: International Organization of Citrus Virologists. University of California, 11:250-255.

Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Güldener U, Mannhaupt G, Münsterkötter M, *et al.* (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res 32:5539-5545.

Saez-Vasquez J, Raynal M and Delseny M (1995) A rapeseed cold-inducible transcript encodes a phosphoenolpyruvate carboxykinase. Plant Physiol 109:611-618.

Sanchez P, de Torres Zabala M and Grant M (2000) AtBI-1, a plant homologue of Bax inhibitor-1, suppresses Bax-induced cell death in yeast and is rapidly upregulated during wounding and pathogen challenge. Plant J 21:393-9.

Sanchez-Ballesta MT, Rodrigo MJ, Lafuente MT, Granell A and Zacarias L (2004) Dehydrin from citrus, which confers *in vitro* dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. J Agric Food Chem 52:1950-1957.

Schaffer MA and Fischer RL (1988) Analysis of mRNAs that accumulates in response to low temperature identifies a thiol protease gene in tomato. Plant Physiol 87:431-436.

Schena M and Davis RW (1994) Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. Proc Natl Acad Sci USA 91:8393-8397.

Schobert B (1977) Is there an osmotic regulatory mechanism in algae and higher plants? J Theor Biol 68:17-26.

Stewart CR and Hanson AD (1980) Proline accumulation as a metabolic response to water stress. In: NC Turner and PJ Kramer (eds) Adaptation of Plants to Water and High Temperature Stress. John Wiley & Sons, New York, pp 173-189.

Soderman E, Mattsson J and Engstrom P (1996) The Arabidopsis homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. Plant J 10:375-381.

Solomon M, Belenghi B, Delledonne M, Menachem E and Levine A (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11:431-444.

Thelander M, Olsson T and Ronne H (2004) Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle. EMBO J 23:1900-1910.

Thompson AJ and Corlett JE (1995) mRNA levels of four tomato (*Lycopersicon esculentum* Mill. L.) genes related to fluctuating plant and soil water status. Plant Cell Env 18:773-780.

Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N and Shinozaki K (1994) Two genes that encode Ca^{2+} - dependent protein kinases are induced by drought and high salt stresses in *Arabidopsis thaliana.* Mol Gen Genet 224:331-340.

Valluri JV, Castillon J, Newton RJ and Soltes EJ (1989) Water stress-induced changes in protein synthesis of slash pine hypocotyls. J Plant Physiol 135:355-360.

van Nocker S and Vierstra RD (1991) Cloning and characterization of a 20-kDa ubiquitin carrier protein from wheat that catalyzes multiubiquitin chain formation *in vitro*. Proc Natl Acad Sci USA 88:10297-10301.

van Nocker S, Walker JM and Vierstra RD (1996) The *Arabidopsis thaliana* UBC7/13/14 genes encode a family of multiubiquitin chain-forming E2 enzymes. J Biol Chem 271:12150-12158.

Vartanian N (1996) Mutants as tools to understand cellular and molecular drought tolerance mechanisms. Plant Growth Regul 20:125-134.

Villela FA, Doni Filho L and Siqueira EL (1991) Tabela de potencial osmótico em função da concentração de polietilenoglicol 6000 e da temperatura. Pesq Agropec Brasil 26:1957-1968.

Wissel K, Pettersson F, Berglund A and Jansson S (2003) What affects mRNA levels in leaves of field-grown aspen? A study of developmental and environmental influences. Plant Physiol 133:1190-1197.

Wu L, Fan Z, Guo L, Li Y, Chen Z-L and Qu L-J (2005) Over-expression of the bacterial *nhaA* gene in rice enhances salt and drought tolerance. Plant Sci 168:297-302.

Yamaguchi-Shinozaki K, Koizumi M, Urao S and Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs genes that are responsive to desiccation in *Arabidopsis thaliana*: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 33:217-224.

Yamaguchi-Shinozaki K, Kasuga M, Liu Q, Nakashima K, Sakuma Y, Abe H, Shinwari ZK, Seki M and Shinozaki K (2002) Biological mechanisms of drought stress response. JIRCAS Work Rep 23:1-8.

Yang Y, Kwon H-B, Peng H-P and Shih M-C (1993) Stress responses and metabolic regulation of glyceraldehyde-3-phosphate dehydrogenase genes in *Arabidopsis*. Plant Physiol 101:209-216.

Zhang ZG, Zhou HL, Chen T, Gong Y, Cao WH, Wang YJ, Zhang JS and Chen SY (2004) Evidence for serine/threonine and histidine kinase activity in the tobacco ethylene receptor protein NTHK2. Plant Physiol 136:2971-2981.

Zhu J, Hasegawa PM and Bressan RA (1997) Molecular aspects of osmotic stress in plants. Crit Rev Plant Sci 16:253-277.

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