Sugarcane genes differentially expressed during water deficit

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Abstract

To identify genes that are up and down-regulated by water deficit in sugarcane we used the macroarray methodology and the expression level of 3 575 independent sugarcane cDNAs was measured by hybridization with RNA extracted from plants submitted to mild, moderate and severe water deficit. We identified approximately 1 670 differentially expressed genes from which 62 % were up-regulated by different stress-conditions, whereas many repressed genes were exclusive for each time-point. Analysis of similarity showed that approximately 24 % of the differentially expressed genes shared homology with proteins involved in different processes such as signal transduction, hormone metabolism, photosynthesis, transcription and stress response. Transcripts with no known function accounted for approximately 39 % and those without similarity represented 36 % of the sequences. Five genes analyzed by RT-PCR confirmed the macroarray results.

Additional key words: drought, induced genes, macroarray, repressed genes, *Saccharum* sp., transcriptome.

Introduction

Drought induces physiological changes that deeply affect the plant metabolism. Plants develop protection mechanisms against immediate dehydration (Trewavas 2000) and modify their metabolism through gene expression alterations that vary according to duration and severity of the stress (Bray *et al*. 2000). Plants that survive or maintain their growth rate in long drought periods are considered tolerant. Mild water deficit resulted in stomatal closure (*e.g*. Brodribb and Holbrook 2003), leaf blade rolling (Munns *et al*. 2000) and changes in root morphology and anatomy (Steudle 2000, Wu and Cosgrove 2000). The increase in drought or exposure time results in moderate water deficit affecting chloroplast biochemistry (Hsiao and Xu 2000, Tardieu *et al*. 2000) or severe water stress leading to leaf abscission (Quirino *et al*. 2000).

The secondary messengers, such as calmodulins, proteins kinases and phosphatases are involved in the transmission of an extracellular signal to trigger biological responses. Calcium is involved in several

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Abbreviations: ABA - abscisic acid; BSA - bovine serum albumin; bZIP - basic-leucine zipper; CDPK - kinase protein calcium-dependent; dATG - deoxyadenosine triphosphate; dCTP - deoxycytidine triphosphate; dGTP - deoxyguanosine triphosphate; DTT - dithiothreitol; dTTP - deoxythymidine triphosphate; EDTA - ethylenediaminetetracetic acid; HSP - heat shock protein; LTP - lipid transfer proteins; RWC - relative water content; SAMDC - s-adenosyl-methionine decarboxylase; SDS - sodium dodecyl sulfate, SSC - saline sodium citrate, Tris-HCl - (hydroxymethyl) aminomethane hydrochloride; ZEP - zeaxanthin epoxidase.
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functions of plant development and it also seems to be necessary to mediate specific responses, such as stomatal closure, protein binding, and activation of a cellular signalling cascade. Calcium-dependent protein kinase (CDPK) depends on calcium to function (Hirschi 2004). Several stress-responsive genes have been described (Seki *et al*. 2003, Riera *et al*. 2005).

Abscisic acid (ABA) is a plant hormone which endogenous concentration is increased under water deficit to protect plants (Seki *et al*. 2007). Since, the expression of certain genes seems to be regulated by ABA during stress, this hormone is important for drought-exposed plants (Rabbani *et al*. 2003). In ABA-dependent pathways, genes have an ABA-responsive element (ABRE) with affinity for MYB and bZIP transcription factors, that signalling for expression of specific genes involved in plant stress response. The transcription factors DREB acts on dehydration-responsive cis-acting element (DRE) to trigger gene expression in an ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki 2007, Agarwal and Jha 2010). In sugarcane, gene expression profile studies detected genes related to sucrose accumulation, such as those expressed in maturing internodal tissues (Carson *et al*. 2002), sugar transporter genes (Casu *et al*. 2003, Rae *et al*. 2005) and genes participating in signal transduction pathways during biotic and abiotic stresses (Papini-Terzi *et al*. 2005, Rocha *et al*. 2007).

The characterization of the gene expression profiles under stress is an important tool for plant breeding (Shinozaki and Yamaguchi-Shinozaki 2007), because the development of drought-tolerant plants is an alternative for areas with restricted water availability (Cushman and Bohnert 2000, Hu *et al.* 2010). Our goal was to identify genes that are differentially expressed under water deficit conditions in sugarcane, in order to understand their roles in the plant defence mechanism so that they may be employed as plant breeding candidate genes. In the present study, a DNA macroarray was used to describe sugarcane genes induced under water deficit conditions.

Materials and methods

Plant growth and induction of water stress: Sugarcane (*Saccharum* sp. cv. SP83-2847) plants were cultivated under greenhouse for 60 d in sterilized soil and kept at 26 ºC under 16-h photoperiod with a photon flux density of 70 μmol m^{-2} s^{-1} and 56 % relative humidity. The experiment was arranged in a completely randomized design, with three biological replications for each control and treated group. All plants were irrigated daily with 25 % Hoagland solution (Hoagland and Arnon 1950) as previously described (Broner and Law 1991). After two months, water stress was induced by irrigation suppression and the soil water content was measured by gravimetric method. Daily irrigation was maintained in control plants. Water-stressed plants were collected on the 2^{nd}, 8^{th} and 10^{th} d of the experimental treatment, which were designated as mild, moderate and severe water deficit conditions, respectively. Leaf water content (RWC) was determined gravimetrically as described by Silva *et al.* (2007). Maximum photochemical efficiency (variable to maximum chlorophyll fluorescence ratio, F_v/F_m) was monitored using *Plant Efficiency Analyzer* (*Hansatech Instruments*, Hoddesdon, UK). The net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (c_i), transpiration rate (E), leaf temperature (T_l) were measured by portable photosynthesis system *Li-6400* (*LI-COR*, Logan, USA) under photon flux density of 1 000 μmol m^{-2} s^{-1}. At the same time all leaves from each plant were collected and frozen at -86 ºC prior to use.

High-density membranes confection: For the macroarrays experiments 3 575 non-redundant expressed sequences tags (ESTs) from sugarcane leaf cDNA libraries containing fragments of approximately 1 260 bp were used (Vettore *et al*. 2003). To eliminate the background nonspecific hybridization were also added in nylon membranes 12 spots representing the empty vector performing as negative control. Plasmid DNA diluted in 50 % dimethyl sulfoxide (DMSO) was spotted on 8.5 × 12.5 cm positively charged nylon membranes, (*'Q' Filters*, *Genetix*, New Milton, UK) using *Q-Bot*. The clones were immobilized in a 5 × 5 array and each clone was spotted in duplicate. After spotting, nylon membranes were placed in denaturing (1.5 M NaCl + 0.5 M NaOH) and neutralizing solution (1.5 M NaCl + 1M Tris, pH 7.0) for 10 and 5 min, respectively, and then exposed to UV radiation (*CL-1000 Ultraviolet Crosslinker*, Upland, USA).

Total RNA extraction and cDNA probe synthesis: Total RNA was extracted from the leaves of treated and control plants using the commercial *Trizol* reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. The cDNA probes for a total of 18 samples were individually produced according the methods used by Schummer *et al*. (1999). We used 30 μg of total RNA, 5 mm^3 of 5× *First Strand Buffer*, 2.5 mm^3 of 100 mM DTT, 3 μM oligo-dT_{18} primer, 1 U RNAseOUT (*Invitrogen*) and unlabeled dATP, dTTP and dGTP (10 mM each). After 20 min 1.11×10^{14} Bq $mmol^{-1}$ [α-^{33}P]dCTP and 1.25 mm^3 of *Superscript II Reverse Transcriptase* (*BRL* 200 U mm^{-3}) (*Invitrogen*) were added and the reaction mixture was incubated for 40 min at 42 ºC. The reaction was stopped with 1.4 mm^3 of 5 M NaOH, 1.8 mm^3 of 3.94 M HCl, and 7.0 mm^3 of 1 M Tris-HCl pH 7.5 and sterilized water was added to a final volume of 50 mm^3. The probe was purified using *Sephadex G-50* columns (Sambrook *et al*. 1989), and the radioactive label incorporation was measured with a

Table 1. The primer sequences used in PCR amplifications are described along with the specific annealing temperatures (TA) and the sizes of the fragment expected for each reaction. Macroarray expression under mild, moderate and severe stress.

Clones	Primes sequences	TA [ºC]	Fragment [bp]	Mild	Moderate	Severe
SCBGLR1120F01.g (CA119309) lipid transfer protein	F 5'gACgATggCTgTgCTgAAC3' R 5'gCTgCTCCTCCTgTTACCAC3'	60	466	3.20	2.91	3.52
SCVPLR2027E06.g (CA130078) kinase protein	F 5'CAATgCCACCCgCTTATC3' R 5'TTTgCCCATTTgTgTCAgg3'	55	370	2.27	2.55	2.66
SCBGLR1112D12.g (CA118685) drought-induced hydrophobic protein	F 5'TCTCgCCTTCTTgACCTgg3' R 5'AAgTCCCATCTgCTgCCAC3'	57	472	2.64	-	2.86
SCBGLR1120G10.g (CA119363) 60 ribosomal protein L38	F 5'gATgAAgCAgCAggAAggAC3' R 5'ggAggACgACgAAgAgAATg3'	55	462	2.16	2.09	2.39
SCRLLR1111E05.g (CA125801) DNA binding protein	F 5'TggAggAAATggAACTggC3' R 5'TgCTTTCCACCTTCTgCg3'	56	385	3.43	3.21	3.34
SCMCLV1032E10.g (CA299954) β-actin (*Saccharum* sp.)	5'CgAggCACAgTCCAAgAgAg 3' 5'gCTTCTCCTTgATgTCCCTTAC3'	62	479	-	-	-

scintillation counter (*Beckman LS6500*, Fullerton, USA), to guarantee the use of cDNA probes with the same amounts of radioactive label.

Macroarray hybridization and data analysis: The membranes were hybridized for 18 h at 58 ºC, in a solution containing 1 % (m/v) BSA (*Sigma*, St. Louis, USA), 1 mM EDTA, 7 % SDS (99.9 %) and 0.5 M Na_2HPO_4. After the hybridization the membranes were washed twice in 2× SSC with 0.1 % SDS for 15 min at 65 ºC, once in 1× SSC with 0.1 % SDS for 15 min at 65 ºC, twice in 0.1× SSC with 0.1 % SDS for 15 min at 65 ºC and once in 0.1× SSC for 5 min at room temperature. The filters were sealed with plastic film and exposed to *Imaging Plates* (*Fujifilm*, Tokyo, Japan) for 96 h. The membrane images were scanned using *Phosphorimager FLA3000-G* (*Fujifilm*), which produces a digital image of the radioactive signals. The images were analyzed using the *ArrayVision* software (*Imaging Research*, St. Catherines, ON, Canada, www.imageresearch.com) to identify the spots and quantify the respective foreground and background intensities.

The data were stored in text files and analyzed by the statistical program *R* (*R Development Core Team 2005*; Ihaka and Gentleman 1996). We verified the raw data quality through the visualization of scatter plot, MA plot and box plot graphics (data not shown). The obtained raw values were background corrected and normalized by the variance stabilization and normalization method (VSN) in *R* (Huber *et al*. 2002). No signal was observed for the negative controls used in membranes showing the absence of nonspecific hybridization (data not shown). Subsequently, a linear model was used to compute and identify the differentially expressed genes in the limma package (Smyth *et al*. 2003, Smyth 2004). The genes were classified as being differentially expressed in decreasing order of the B-statistic, considering the false discovery rate (FDR) proposed by Benjamini and Hochberg (1995) and implemented on limma. The analyses were conducted with using *Bioconductor* project software (Gentleman *et al*. 2004).

The differentially expressed genes were obtained setting a FDR equal to 5 %, B-statistic (log-odds of differential expression higher than zero and $P < 0.0001$). The relative gene expression is shown by M ($\log_2$ fold change) whose unit represents two-fold change. Sequences were compared using the *BlastX* tool (Altschul *et al*. 1997), from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nhi.gov/) against sequences from *Arabidopsis*, rice, wheat, maize and others. The determined similarities allowed classification according to the biological role played by the protein, as proposed in the *SwissProt* (http://ca.expasy.org/sprot/) and *TrEMBL* (http://us.expasy.org) databases. Genes showing similarity with unknown proteins as well as genes with no similarity were submitted to the *InterPro* database (http://www.ebi.ac.uk/interpro/) for motif and domain searches (*e*-value $\leq 10^{-5}$ cut off).

Semi-quantitative RT-PCR Analysis: Some genes were arbitrarily selected to analyze the differential expression *via* the RT-PCR semi-quantitative method (Meadus 2003). Primers were designed using *Gene Runner* software (*Hastings Software*, New York, USA). The sequences and the annealing temperature (TA) of each primer are shown in the Table 1. Based on literature, the sugarcane β-actin gene (SCMCLV1032E10.g) was used as a positive control (Kim *et al*. 2007, Lee *et al*. 2007, Yu *et al*. 2007, Ahmad *et al*. 2008, Sperotto *et al*. 2008). A set of amplifications were performed to verify the influence of water stress on β-actin gene expression and determine the appropriate cycle number within of the linear range. Total RNA (5 μg) from each selected gene was treated with DNAse I (*Invitrogen*) and translated into first strand cDNA using 0.5 μg oligo-dT_{18} primer, 1 mm^3 10 mM dNTPs mix (dATG, dCTP, dTTP, dGTP), 4 mm^3 5× RT buffer, 2 mm^3 0.1 M DTT, 40 U RNAseOUT and 200 U Superscript II RT (*Invitrogen*) in a final volume of 20 mm^3 at 42 ºC for 50 min. From the resulting cDNA, 0.5 μg was amplified with 10 pmol of each specific

primer, 4 mm^3 of 50 mM $MgCl_2$, 2 mm^3 of 10 mM dNTP mix, 5 mm^3 of 5× RT buffer, and 0.4 mm^3 of *Taq* DNA polymerase (5 U mm^{-3}) in a total volume of 50 mm^3. An identical reverse transcription reaction was carried out without *Superscript II* RT and was used as negative control. Amplifications were performed using *PT-100*™ (*MJ Research*, Waltham, USA) at 95 ºC for 2 min and 27 cycles at 95 ºC for 45 s, TA for 45 s, 72 ºC for 45 s and finally an extension at 72 ºC for 10 min. The PCR products (15 mm^3) were electrophoresed on a 1.6 % (m/v) agarose gel under 88 V for 90 min and photographed using the *Kodak* electrophoresis documentation system (*Eastman Kodak Company*, Rochester, USA)

Results

Identification of differentially expressed genes: Physiological parameters were measured during the experimental treatment to verify the effects of water deficit on plants. The soil water content (SWC) corresponded to approximately 83, 46 and 22 % under mild, moderate and severe water stress, respectively. The leaf relative water content (RWC) decreased from 87 % during mild stress to 64 % under severe stress. Maximum photochemical efficiency (F_v/F_m) and net photosynthetic rate (P_N) were reduced with the water deficit increase. Also stomatal conductance (g_s) and transpiration rate (E) decreased progressively during water deficit period. Leaf temperature (T_l) was higher in treated plants compared to control plants, mainly under moderate and severe drought (Table 2).

Table 2. Physiological parameters of the plants during water deficit. The plants were collected when the soil water content was approximately 83 % (mild stress), 42 % (moderate stress), and 22 % (severe stress). Means ± SD of three replicates.

Parameters	Plants	Mild	Moderate	Severe
Soil WC [%]	control	97.86 ± 1.72	99.49 ± 1.99	98.41 ± 2.30
	stressed	83.94 ± 1.97	46.37 ± 2.82	22.07 ± 4.11
Leaf RWC [%]	control	98.33 ± 0.92	99.49 ± 0.99	96.86 ± 1.30
	stressed	87.94 ± 2.31	71.37 ± 1.52	64.07 ± 0.67
F_v/F_m	control	0.95 ± 0.02	0.90 ± 0.00	0.89 ± 0.00
	stressed	0.88 ± 0.01	0.78 ± 0.05	0.69 ± 0.04
P_N [µmol m^{-2} s^{-1}]	control	34.90 ± 1.97	30.87 ± 1.48	31.73 ± 1.71
	stressed	32.99 ± 0.98	8.03 ± 0.90	5.01 ± 0.68
c_i [µmol mol^{-1}]	control	198.70 ± 17.7	125.80 ± 26.9	145.30 ± 20.8
	stressed	277.30 ± 21.0	395.80 ± 24.8	667.90 ± 6.7
T_l [° C]	control	30.40 ± 0.39	29.77 ± 1.09	29.41 ± 1.76
	stressed	31.00 ± 0.82	32.30 ± 1.12	33.37 ± 0.68
g_s [mol m^{-2} s^{-1}]	control	0.35 ± 0.01	0.32 ± 0.03	0.30 ± 0.00
	stressed	0.27 ± 0.02	0.10 ± 0.03	0.06 ± 0.02
E [mmol m^{-2} s^{-1}]	control	7.32 ± 0.56	7.26 ± 0.59	8.27 ± 0.23
	stressed	6.56 ± 0.13	2.36 ± 0.67	1.22 ± 0.22

This gene expression study evaluated 3 575 sugarcane cDNA clones from which 1 670 (46 %) were differentially expressed under the water stress. We observed an up-regulation of expression for 1 038 genes and down-regulation was noted for 632 transcripts. The profile of genes induced and repressed overtime, the number of exclusive genes and the number of repeated genes for the different days under water deficit conditions are summarized in Fig. 1. Genes whose expression levels changed exclusively under mild, moderate or severe stress conditions were more frequently repressed than induced. In contrast, genes common to multiple conductions were more frequently induced (*e.g.*, of the

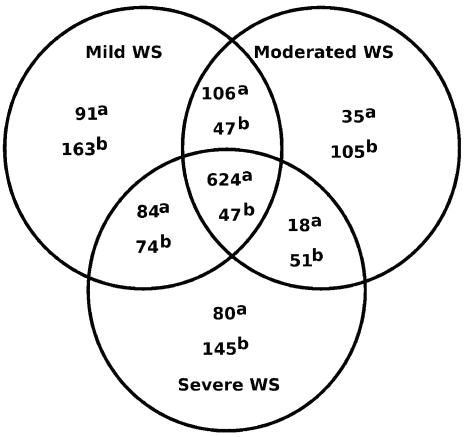

Fig. 1. Venn diagram showing the shared expression of genes under mild, moderate and severe water deficit. Some genes were identified in exclusive periods whereas others were expressed in different conditions. [a] - up-regulated genes, [b] - down-regulated genes under water stress.

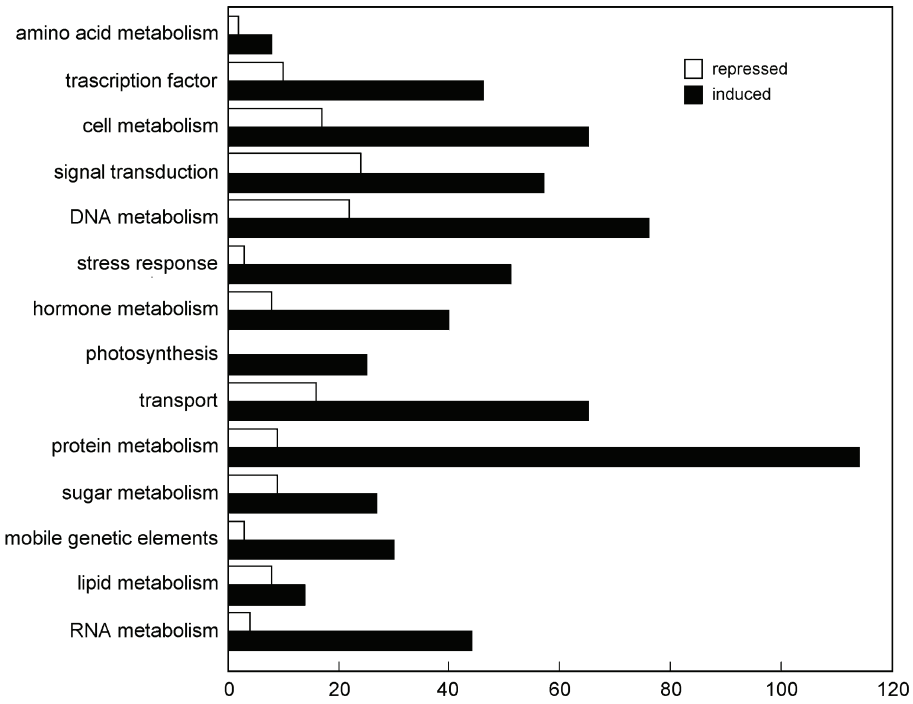

Fig. 2. Functional classification of differentially expressed genes under mild, moderate, and severe water deficit. Genes were categorized according their putative roles in the biological process. Each category shows the number of up- and down-regulated genes.

671 genes differentially expressed in all water stress conditions, 624 were up-regulated) (Fig. 1). Some genes induced during the whole period of water deficit showed similarity with proteins regulated by plant hormones like ABA (SCACLV1021E03.g; SCRLLV1024G07.g), auxin (SCJFLR1035F05.g; SCEZLR1009G12.g; SCBGLR1114G12.g), or those involved direct or indirectly in their synthesis, such as zeaxanthin epoxidase (ZEP) EC 1.14.13.90 (SCBFLR1039E10.g), s-adenosyl-methionine decarboxylase (SAMDC) EC4.1.1.50 (SCCCLR2C02A12.g; SCACLR2014E11.g) and ACC oxidase EC 1.14.17.4 (SCEZLR1009E06.g). The exhibited pattern shows that various genes are highly expressed in mild, moderate and severe stress. The highest induction level was observed for an ABA-inducible gene (SCACLV1021E03.g), which was induced under mild (M-value = 4.62) and severe stress (M-value = 5.49). This gene was not the most highly induced under moderate stress although it did show a high expression value (M-value = 4.13).

Our database searches revealed that sequences without known function accounted for approximately 76 % of the 1 670 genes differentially expressed in mild, moderate and severe conditions (37 % of the transcripts with no similarity in databases and 39 % were matched with putative, hypothetical, and unknown proteins) (data not shown). The others genes that showed similarity (approximately 24 % of the 1 670 genes) were placed in 14 functional categories according to their putative roles on biological processes (Fig. 2). Genes included in protein metabolism category represent ribosomal proteins and the protease ubiquitin. In the transport category we observed genes related to lipid transfer proteins, sugar and sorbitol transporters as well as several aquaporin and ABC transporter proteins. Genes classified in the DNA

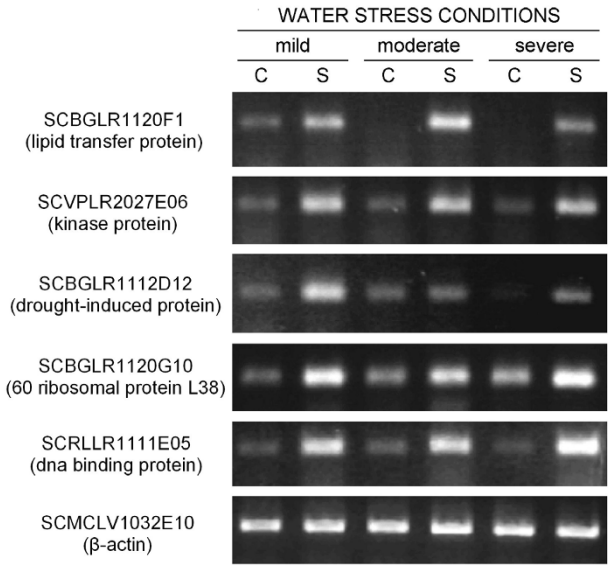

Fig. 3. Differential gene expression analyzed by RT-PCR. Five genes were reverse transcribed into cDNA first strand and were amplified with 27 cycles to show gene expression induction under water deficit. PCR products were electrophoresed on a 1.6 % (m/v) agarose gel and analyzed using *Kodak* 1D image analysis software. The sugarcane β-actin gene was used as an internal control. C - control plants, S - drought-stressed plants.

metabolism were matched with histone and DNA binding proteins. The transcription factor class included some stress-induced transcription factor like NAC1, DREB1, bZIP, MYB and MYC proteins, *etc.* In addition to the signal transduction class, there were phospatases, CDPKs and others kinases. Analyzing the expression patterns among the functional classes, we verified that approximately 76 % of genes expressed in each water stress condition were induced. No repressed gene involved in photosynthesis metabolism was observed (Fig. 2). Several transcripts identified in this sugarcane study encoded metallo-thionein, HSP, SAMDC, thioredoxin, LTP, ribosomal protein, calmodulins, chlorophyll *a/b* binding protein, histone proteins, photosystem 2, ABA-inducible gene, ubiquitin, DNA-binding protein, and disease resistance protein; these transcripts were also observed in studies of drought-stressed rice and barley (Gorantla *et al*. 2007, Talame *et al*. 2007). Genes for receptor-like protein kinase, protein phosphatase 2C, ZEP, protein RPM1 and wound-induced proteins were differentially expressed in *Arabidopsis* (Bray 2004) and rice (Rabbani *et al*. 2003, Walia *et al*. 2005) under cold, ABA application, drought and high-salinity. The expression of bZIP transcription factor and glyceraldehyde-3-phosphato-dehydrogenase was changed in water deficit-tolerant bean (Micheletto *et al*. 2007) and sunflower plants (Roche *et al*. 2007) as well as in maize exposed to high-salinity, cold and ABA (Zheng *et al*. 2006). Aquaporins (Luu and Maurel 2005), kinase protein (Shou *et al*. 2004), WRKY transcription factor (Marè *et al*. 2004), and auxin-regulated protein (Wang and Bughrara 2007) were also described as responsive to water stress. This is consistent with our results and shows that the cDNA macroarray was an efficient method for identifying stress-regulated genes.

Validation of results by RT-PCR: To confirm the macroarray results five differentially expressed genes related to signal transduction, DNA metabolism, stress response, transport and protein metabolism were amplified by RT-PCR. RNA samples were primarily treated with DNAse I and translated into first strand cDNA, which was used as template for PCR amplifications. These reactions were performed to measure the expression level of genes that encode kinase protein (SCVPLR2027E06.g), DNA binding protein (SCRLLR1111E05.g), drought-induced hydrophobic protein (SCBGLR1112D12.g), lipid transfer protein (SCBGLR1120F01.g) and 60S ribosomal protein L38 (SCBGLR1120G10.g). The differential gene expression obtained for mild, moderate and severe stress conditions was normalized using the expression of the sugarcane β-actin gene (SCMCLV1032E10.g), which showed no significant differences between control and treated samples according to *ANOVA* analysis (data not showed). No amplification was observed in the negative controls. The PCR product images were analyzed with *Kodak 1D* image analysis software to estimate the intensity of bands as well as the relative expression of these genes (Fig. 3). Our results showed that the sugarcane genes assayed via RT-PCR were differentially expressed under water deficit conditions. Their expression profiles were very similar to those observed in the first method, confirming the information already provided by the macroarray experiments.

Discussion

Gene expression alterations can promote cellular adaptation to water stress. The gene expression profile characterized in a molecular study suggests the complexity of defence mechanisms (Zheng *et al*. 2004). Maize and rice have been investigated at the gene expression level under water stress conditions (Gorantla *et al*. 2007, Zheng *et al*. 2006, Andjelkovic and Thompson 2006).

Techniques used in gene expression studies such as Northern Blot, Dot Blot or Differential Display can be used to monitor the expression pattern under different stress situations. However, the DNA macroarrays is a powerful tool for the identification of differentially expressed genes because this methodology permits the simultaneous analysis of many genes. In our sugarcane study, we observed some stress-related genes, others genes with uncharacterized action in plants under drought conditions and new genes not yet described in the water stress tolerance process.

Physiological modifications are the first responses of the plants to water deficit. The plant response begins with stress recognition at the cellular level *via* activation of signal transduction pathways. The roots signal to leaves through messengers, promoting stomatal closure, leaf rolling and leaf abscission. Our results show that the expression of genes encoding calcium binding proteins (SCRLLV1026E05; SCCCLR1C04H11; SCMCLR1122G02), protein kinase (SCVPLR2027E06) and phosphatases (SCCCLR1072F03; SCJLLR1105C01) is altered under stress. Studies with ABA mutants indicate that the phosphorylation/desphosphorilation of phosphatase proteins type 2C (PP2C) can be involved in ABA signalling during water stress (Leung *et al*. 1997, Yoshida *et al*. 2006). A protein kinase (SCVPLR2027E06.g) and a DNA-binding protein (SCRLLR1111E05.g) were amplified by semi-quantitative RT-PCR. These genes were differentially expressed and showed approximately the same gene expression level at all times under water stress conditions, corroborating macroarray results (Fig. 3).

Kinases are key enzymes in the regulation of both metabolic pathways involved in plant growth and development, as well as stress events. These enzymes are activated by extracellular stimuli, which lead to gene

expression changes (Xu *et al*. 2006). Once activated, transcription factors act as DNA-binding proteins that are capable of mediating the transcription of key proteins in the stress response mechanism. DNA-binding activity has been investigated in plants under water stress. These proteins seem be accumulated in seeds during the late stages of embryogenesis and conditions with low water content (Maskin *et al*. 2007), and they are over-expressed in transgenic plants with an increased salt tolerance (Rom *et al*. 2006). Histone proteins seem to possess multiple functions on plant metabolism and their gene expression is reported in several water stress studies. The action of these proteins on stressed tissues was studied using antisense transgenic plants, but only morphological differences in leaf anatomy were observed. These findings highlighted the complexity of the plant response to water stress (Scippa *et al*. 2004). Methods to increase environmental stimulus perception or intensify cellular communication may facilitate the anticipation of defence mechanisms (Chinnusamy *et al*. 2004).

Genes homologous to ribosomal proteins were up-regulated in plants under salinity, cold, water stress and ABA-treatment (Zheng *et al*. 2004, 2006). They were also up-regulated during stress-associated events suggesting a function in restoring the protein synthesis processes (Rizhsky *et al*. 2004). In our study, a lot of ribosomal proteins were also found. One ribosomal protein (SCBGLR1120G10.g) was used in semi-quantitative RT-PCR validation, and it showed a gene expression profile consistent with that observed in the macroarray experiment (Fig. 3). All genes validated showed an expression pattern very similar to that in the macroarray. Genes were selected randomly for this process, and the consistency of our results highlights the quality of the data and statistical analysis.

Plants have a cuticle on the surface of their leaves that provides a protective barrier against the environmental adversities, such as water stress. Genes encoding lipid transfer proteins (LTPs) were prevalent in libraries prepared from dehydration-stressed tissues of cassava (Lokko *et al*. 2007). Although their functions are not known yet, we believe that these proteins act by transferring wax and cutin monomers from membranes to the cell surface. This idea is supported by the protein's location in the cell wall space of epidermal cells (Locy *et al*. 2002). Cameron *et al*. (2006) studied the relationship between wax and dehydration stress in tobacco leaves. This work verified that the total wax load increased roughly 2.5-fold and the LTP gene expression showed a 6-fold increase, suggesting that the LTPs can be involved in these processes. Our results showed that three transcripts (SCEZLR1031G08.g, SCCCLR1080B06.g SCBGLR1120F01.g) coding for LTPs (gi|75994602|, gb|AAB47967.1|, gi|16904376|) were classified in functional category of transports. The SCBGLR1120F01.g cDNA clone was used in RT-PCR experiments to confirm the macroarray results. These experiments confirmed our macroarray findings that LTP gene expression was increased during all time under water stress. Talame *et al*. (2007) analyzed drought stress in barley plants submitted to 7 and 11 d of slow drying treatment, and this work verified the roughly 2.5-fold increase in the transcript levels of two LTPs.

The endogenous ABA concentration increases under water stress conditions (Bray 2002). The ZEP enzyme EC.1.14.13.90 (SCBFLR1039E10.g) participates in ABA biosynthesis by converting zeaxanthin into violaxanthin, and its role in this pathway during drought conditions has been shown in *Arabidopsis* root tissues (Audran *et al*. 2001). Moreover, ZEP gene expression was enhanced under osmotic stress in a study with *Arabidopsis* mutants, and positive feedback regulation by ABA on ZEP gene expression was observed (Xiong *et al*. 2002). Our results also described two ABA-inducible proteins (SCACLV1021E03.g; SCRLLV1024G07.g), which were induced during all of the stress exposure periods. Although they were classified into different clusters, the corresponding genes show high similarity to gi|22313|. One of them (SCACLV1021E03.g) showed the highest level of expression rate under mild and severe stress conditions.

Water stress can severely reduce the productivity of sugarcane (Silva *et al*. 2007) because the photosynthetic rate reduces progressively as stress becomes more severe (Bloch *et al*. 2006, Dulai *et al*. 2006, Bhatt *et al*. 2009, Santos *et al*. 2009). In this tolerant sugarcane cultivar, several genes related to photosynthesis were induced under stress (Fig. 2). The decrease in the photosynthetic rate during the mild water deficit was not strong enough to produce photoinhibition (Table 2). However, P_N was significantly reduced under moderate and severe stress. According to our data, we hypothesized that the water stress was enough to trigger plants physiological responses to protect them against the stress, but it was not so severe as to repress the expression of the photosynthesis genes. Better adapted plants are also more efficient in water use and show greater tolerance to drought-stress (Munns 2002, Xu and Hsiao 2004).

The integral membrane protein ZmTIP4-2 (gi|13447831|), encoded by a sugarcane gene SCQGLR2017D04.g induced under mild stress, is an aquaporin present in tonoplasts that plays an important role in water transport. These proteins are, therefore, important in adjusting the water status in response to environmental changes (Luu and Maurel 2005). The protection against stress also induces changes in the cellular cycle as well as modifications in the vacuolar system and cell wall architecture. These changes usually affect plant growth and development. Some genes encoding auxin response factors (SCEQLR1050F05.g; SCRULR1042E09.g) and auxin-regulated proteins (SCJFLR1035F05) were observed in our experiments. These proteins have a key role in plant growth and development, and they may be related to the vascular differentiation (Murphy 2002).

A putative gene homologous to a metallothionein-like protein (SCRLLV1026G03.g) was induced by water stress. The metallothioneins are small proteins rich in

cysteine residues, and they work as cell metal chelators efficient in metal detoxification (Schor-Fumbarov *et al*. 2005). The metallothioneins are classified in four groups according to the distribution of the cysteine residues in their domains. All types of metallothioneins were identified in sugarcane, but the metallothioneins-type 1 was the most abundant and it showed a significant expression level in leaves (Figueira *et al*. 2001). The identification of metallothioneins acting in the stress response in sugarcane is an indication that the control mechanisms of uptake and accumulation of essential and non-essential metals is important to plant stress tolerance.

The expression of some genes in response to water stress can be regulated by ABA signalling (Schwartz *et al*. 2003, Riera *et al*. 2005). Some genes coding to MYB (SCEQLR1094D08.g; SCEPLR1051B08.g; SCBGLR1099F12.g), bZIP (SCCCLR1072B02.g) and DREB1 (SCBGLR1115C04.g) proteins were induced in sugarcane leaves exposed to water stress. The actions of the bZIP ABA-regulated transcription factor on gene expression have been reported to enhance drought tolerance in *Arabidopsis* (Fujita *et al*. 2005) and in rice plants, because the salt-stress tolerance of these plants was affected by down-regulation of the gene expression (Zhou *et al*. 2008). In *Arabidopsis*, a specific guard cell MYB protein was involved regulating stomatal movements (Cominelli *et al*. 2005). Transgenic *Arabidopsis* plants overexpressing a MYB gene were more tolerant to drought, cold, and salt stresses (Dai *et al*. 2007). The DREB1 protein responds to cold stimuli and regulates the DNA binding motif DRE, activating the gene expression in response to stress (Shinozaki and Yamaguchi-Shinozaki 2007). Transgenic *Arabidopsis* plants overexpressing the DREB1 gene showed an increased tolerance to drought, high-salt concentration and low-temperature stresses (Ito *et al*. 2006). The gene expression results obtained here are consistent with data described in the literature.

The genes similar to unknown proteins and those without similarity represent approximately 76 % of the genes described in this study. Gorantla *et al*. (2007) identified genes differentially expressed in rice using ESTs libraries produced from plants grown under drought. These results showed that approximately 43 % of the 1 677 unigenes represents functionally unknown genes, such as hypothetical, putative, predicted and unknown proteins. Ozturk *et al*. (2002) monitored 1 463 transcripts from drought-stressed libraries in barley, and this work verified that at least 38 % of the ESTs were functionally unknown. These genes included those similar to unknown protein as well as genes without homology. These uncharacterized genes with unknown functions are important for the study of water stress tolerance because they may represent new variability sources and preserve information thus far unknown about the complex interactions between gene expression and physiological processes involved in plants adaptation to drought.

This study describes genes induced in sugarcane exposed to prolonged drought conditions as well as their possible roles in stress situations. Genes induced by water stress included the signalling molecules, genes responsive to stress, regulated by plant hormones, and genes important for protection against metal toxicity and oxidative stress (Schützendübel and Polle 2002, Jain *et al*. 2006). Based on our results and previously described information, we pointed out a possible metabolic modification that may occur in sugarcane leaves under such adverse conditions. The genetic control of water stress is complex and involves a network of genes acting together. Studies of gene expression are important to elucidate how these mechanisms work, and they should assist plant breeding. Information on this subject is still limited and thus restricts sugarcane breeding. Overall, plants undergo metabolic alterations to maintain growth and development during drought conditions. Identifying the pathways involved in this process and how events are regulated is crucial to understanding stress tolerance. The study of gene expression patterns in plants with different responses to water stress is an alternative method for identifying tolerance genes.

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