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Differential gene expression in sugarcane induced by salicylic acid and under water deficit conditions

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ABSTRACT

The objective of this work was to determine the transcript profile of sugarcane plants after foliar application of salicylic acid (SA) as well as to analyse gene expression of a gene involved in the response to water deficit. The SA plays an important regulatory role in multiple physiological processes including plant immune response. The suppression subtractive hybridization (SSH) technique was used to generate a cDNA library enriched for transcripts differentially expressed. RT-PCR and quantitative RT-PCR (qRT-PCR) was used to analyse gene expression of trehalose-6-phosphate in a tolerant genotype under water deficit conditions. A total of 122 clones were identified in the SA library which allowed the identification of four gene groups that play different roles. One such group of genes identified is responsible for the synthesis of sucrose-phosphate, which is involved in response to drought. The SSH technique is effective for identification of tolerant genes activated by salicylic acid. qRT-PCR analysis, showed increased expression of trehalose in plants subjected to 10% soil moisture, confirming that the gene is differentially expressed in water deficit conditions. No studies of this nature have been reported so far for sugarcane. These results reveal the potential of the identified gene for future studies aiming at more water deficit-tolerant varieties based on genetic transformation.

Keywords: *Saccharum sp.*; abiotic stress; drought; cDNA library; suppression subtractive hybridization.

INTRODUCTION

Expression profiling has become an important tool to investigate how an organism responds to environmental changes. Plants have the ability to alter their gene expression patterns in response to environmental changes such as temperature, water availability or the presence of deleterious levels of ions (Rodriguez et al.

2005). In plants, exogenous application of salicylic acid (SA) or its derivatives affects diverse processes which trigger defense response to abiotic stress tolerance. Among abiotic stresses, SA has been reported to counter water stress (Hayat et al. 2009; An and Mou 2011). The production of sugarcane is negatively influenced by a

vast number of environmental factors that affect growth, metabolism and yield. Of these, the strongest and most frequent negative influence on sugarcane yield in Brazil is drought. Plants commonly respond to water deficit by accumulating sugars and other compatible osmoprotecting solutes. One of these components is the non-reducing sugar trehalose, a glucose disaccharide that works as a compatible solute in the cell (Mahajan and Tuteja 2005).

Considering the importance of the sugarcane crop and water deficit as an environmental factor limiting its production, it is necessary to identify and characterize candidate genes involved in the response to water deficit to obtain more tolerant varieties. To understand the molecular regulation of the stress response, the relevant subsets of differentially expressed genes of interest must be identified, cloned, and studied in detail. High throughput detection of differential expression of genes is an efficient means of identifying genes and pathways that may play a role in biological systems under certain experimental conditions. There exist a variety of approaches that could be used to identify groups of genes that change in expression in response to a particular stimulus or environment. Suppression subtractive hybridization (SSH) has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Rodriguez et al. 2005).

To investigate alterations in the transcriptional profiles of sugarcane after foliar application of salicylic acid, suppression subtractive hybridization (SSH) was employed to identify differentially expressed genes. A second objective was to analyse gene expression of a gene involved in the response to water-deficit. Together, these results provide important information for developing of future studies aiming at more water-deficit-tolerant varieties based on genetic transformation.

MATERIAL AND METHODS

SSH experiment

Sugarcane plants, variety RB72910 were germinated aseptically on MS culture medium (0.8% w/v agar), with the pH adjusted to 5.8 ± 0.2 , photoperiod of 16 hours of light and 8 hours of dark, and temperature at $25 \pm 2^\circ\text{C}$. Plants were kept in these conditions for four weeks after germination. Four-week old plants were, then, treated with 5 mM sodium salicylate (Sigma USA) dissolved in autoclaved distilled water. Control plants were sprayed with autoclaved distilled water. Leaves of ten plants were harvested 24 hours after the treatment, mixed and used for total RNA extraction. The leaves of young plants were used to extract the mRNA required for the synthesis of a cDNA library.

For total RNA extraction, the leaves from untreated and SA treated plants were used with Trizol (100 mg mL^{-1}).

RNA yield was 50 mg per 100 mg of plant tissue. Complementary DNA (cDNA) was obtained through SMART PCR cDNA Synthesis Kit. Suppression subtractive hybridization was performed with the PCR-select cDNA subtraction kit. Treated and control samples were processed simultaneously to reduce false positives. Complementary DNA, prepared from the treated samples, was used as “tester”, and that from the control sample as “driver”, for the forward subtraction carried out to isolate fragments corresponding to genes whose expression level was increased following the treatments. Cloned PCR products, in the vector T/A PCR2.1 TOPO were, then, transformed with DH5a competent cells to produce all the subtracted libraries (forward SSH), according to manufacturer's instructions. Cell colonies were transferred to LB liquid medium containing $100 \mu\text{g mL}^{-1}$ kanamycin, and were cultured for 16 hours. Plasmid DNA was extracted by the alkaline lysis miniprep method (Sambrook and Russell, 2001). Recombinant plasmids were identified, by restriction analysis, and subjected to DNA sequencing. Nucleotide sequences of each insert were determined in an automatic DNA Sequencer (ABI PRISM 377), with the Big Dye Terminator kit. Conventional M13- forward and M13-reverse primers were used to determine DNA sequences. Obtained sequences were compared with the ones in the databanks of: the National Center for Biotechnology Information, NCBI (<http://www.ncbi.nlm.nih.gov/blast>), using the program BlastX (Altschul et al., 1997); the Institute for Genomic Research, TIGR (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi>); and the DNA Data Bank of Japan, DDBJ (<http://www.ddbj.nig.ac.jp/search/blast>). Sequences were aligned with the software ClustalW (<http://www.ebi.ac.uk>) (Thompson et al. 1994).

After the cDNA library construction by SSH, genes that play a role in stress tolerance such as synthesis of sucrose-phosphate was observed. In this study the trehalose gene of *Saccharum officinarum* (SoTPS) was gene expression analysed into a tolerant genotype by the quantitative real-time PCR technique under water deficit conditions.

Plant material and treatment

Sugarcane plants, variety RB 72910, were planted and grown in PVC pots containing 30 kg soil for 60 days with no water restriction. After this period, to induce water deficit, plants were subjected to one of five soil water levels, determined by the oven official method: 100% gravimetric soil moisture was considered the control treatment (T0); 50% was no stress (T1); 40% was moderate stress (T2); and 20% (T3) and 10% (T4) were severe stress. The experiment was carried out in a greenhouse (29°C and 70% RH).

Amplification of trehalose gene

RT-PCR analysis

Total RNA was extracted from leaves using the reagent Trizol[®] (Invitrogen) according to the manufacturer's instructions. An aliquot of 1 µg total RNA was used as the template in the semi-quantitative RT-PCR analysis using a One-Step RT-PCR kit (Invitrogen). The specific-primers pair (F: 5'GCAGATATCGAATTCGGG3' and R: 5'TTAAGGTGAGGAAGTGGTGTCTAG3') were designed to anneal in a conserved trehalose region of plants. RT-PCR was performed under the following conditions: 50°C for 30 min and 94°C for 3 min, followed by 30 amplification cycles (94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) and 7 min at 72°C. The plant-specific primer pair that amplifies the translation elongation factor 1α (EF-1αF:5'GACAATCAAGCACTGGAGCA3' and EF-1αR: 5'GATGCTACCACCCCAAGTA3') was used as the control in the RT-PCR experiments. The final amplification product was separated on a 1.5% agarose gel.

Real-time PCR analysis (qRT-PCR)

Complementary DNAs (cDNA) were synthesized from 5 µg of total RNA using Superscript III[®] as described by the manufacturer. A region of the trehalose mRNA was amplified using primers F: 5'GGCGGATGCATGAGAAGTACAAT3' R: 5'TGTCCTCTGGCCACCTCAATTCTT3'. qRT-PCR were performed in a Mastercycler ep realplex (Eppendorf) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The reaction conditions were as follows: initial denaturation at 95°C for 2 min followed by 25 cycles at 95°C for 15 s, annealing at 60°C for 20 s, 68°C for 20 s followed by melting curve analysis. The relative quantification followed the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), normalized to the constitutive gene 14-3-3 using the specific primers 14-3-3F (5'CAACTTGACCCTGTGGACCT3') and 14-3-3R (5'ACCCACCTCGTAGCAATGTC3').

Sequence and phylogenetic analysis

The trehalose gene *SoTPS* was sequenced on both strands in an ABI Prism 3100 (Applied Biosystems) sequencer. Sequences were compared to those in Genbank (<http://www.ncbi.nlm.nih.gov>). Sequence analyses of amino acids deduced from gene *SoT6P* and TPSs of other organisms were aligned using Clustal W, and a phylogenetic tree was constructed by the neighbor-joining method with MEGA 3.1 (Kumar et al. 2004). The reliability of the tree was measured by bootstrap analysis with 1500 simulations and edited with MEGA 3.1.

RESULTS AND DISCUSSION

As described above, aiming to isolate cDNA clones corresponding defense genes related abiotic stress in sugarcane, cDNA library was constructed through SSH technique, with genes induced by salicylic acid (SA library). One of the main advantages of SSH is that it normalizes the cDNA abundance, so that cDNAs encoded by genes which are expressed infrequently, but nonetheless differentially, can be identified readily (Kürkcüoğlu, et al. 2006). After SSH, 122 cDNA clones were obtained with Inserted length ranged from 200 to 1.600 bp. Clone similarity was considered at E-values lower than $10e-05$ in the different data banks analyzed, being, thus, significant (Fernández et al, 2003). In the SA library were identified four gene groups presenting different functions (Figure 1). Of these, 40 clones showed no similarity with data bank sequences, 37 represented genes involved in defense mechanisms, 24 were similar to putative proteins with unknown roles and 21 clones were associated to cell maintenance and plant development. Plant genes with known roles, involved in biotic and abiotic stress response and associated to cell maintenance and plant development, were identified in SA library. Some of these genes (Table 1) as those encoding sucrose-phosphate synthase are related to cell metabolism. Sucrose occupies a central position in the metabolism of all plants and has many roles: transport sugar, storage reserve, compatible solute, and signal compound. (Castleden et al. 2004).

A better understanding of the genes that are expressed in response to abiotic stress in grasses, such as water deficit are needed to characterize fully the mechanisms that permit adaptation to limiting water conditions (Nakashima et al. 2009). SSH combines suppression PCR with subtraction and normalization steps in a single reaction, increasing, therefore, the possibility of identifying low expressed genes (Moody 2001). Genes with unknown roles were identified in SA library which indicates the possibility of identifying new genes which have not yet been reported in previous studies of stress/tolerance response. Similar results were observed in SSH libraries from *Arabidopsis* in abiotic stress conditions (Mahalingam et al. 2003) and Tomato (Amaral et al. 2008).

After the identifying genes we investigated the differential gene expression of a trehalose-6-phosphate in sugarcane plants under water-deficit conditions.

One of the best-documented physiological/molecular responses to water deficit in plants is the ability of some species to their osmotic adjustment performance (Nepomuceno et al. 2002). Non reducing disaccharides, e.g., trehalose, have been reported as one of the most effective osmoprotectants in terms of minimal required concentration (Avonce et al. 2004; Iordachescu and Imai 2008).

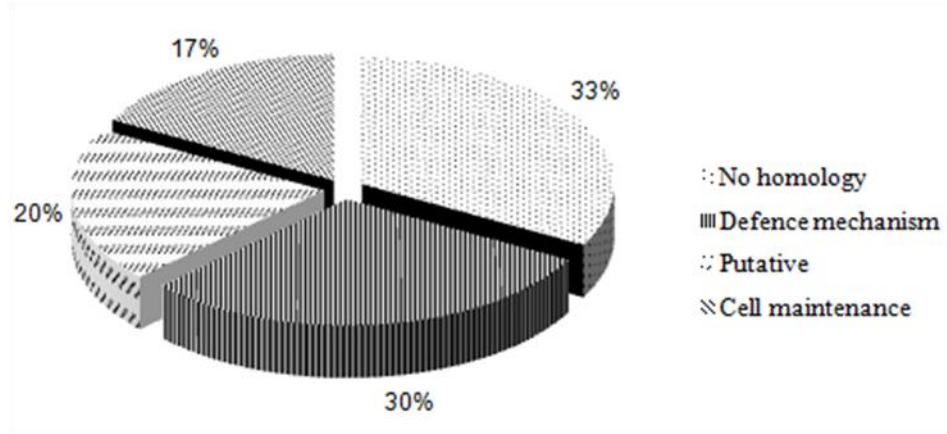


Figure 1. Genes activated in the sugarcane plants treated with salicylic acid (SA). Genes were isolated by suppression subtractive hybridization, from sugarcane mRNAs, 24 hours after SA treatment

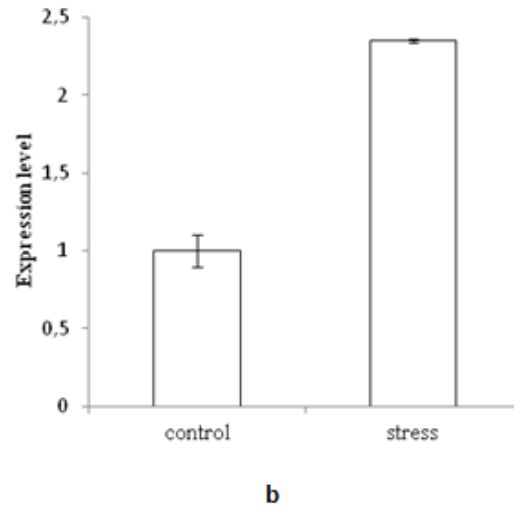
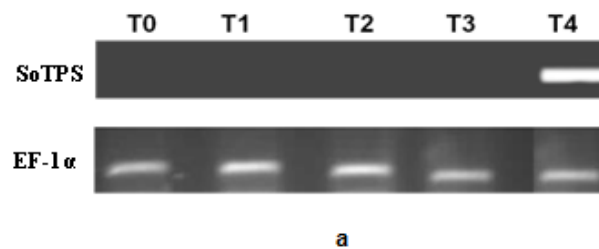


Figure 2. RT-PCR and Real-time PCR analysis. (A) Gene expression analysis of SoTPS. Pattern of RT-PCR expression of gene SoT6P analyzed in leaves of sugarcane plants under water stress. T0 - Control, T1 - 50% gravimetric soil moisture, T2 - 40% gravimetric soil moisture, T3 - 20% gravimetric soil moisture and T4 - 10% gravimetric soil moisture. EF-1 α , expression control. (B) Relative gene expression. Level of trehalose gene expression in RB 72910 sugarcane variety estimated by qPCR. Control (no stress), 100% gravimetric soil moisture; Stress (severe stress), 10% gravimetric soil moisture

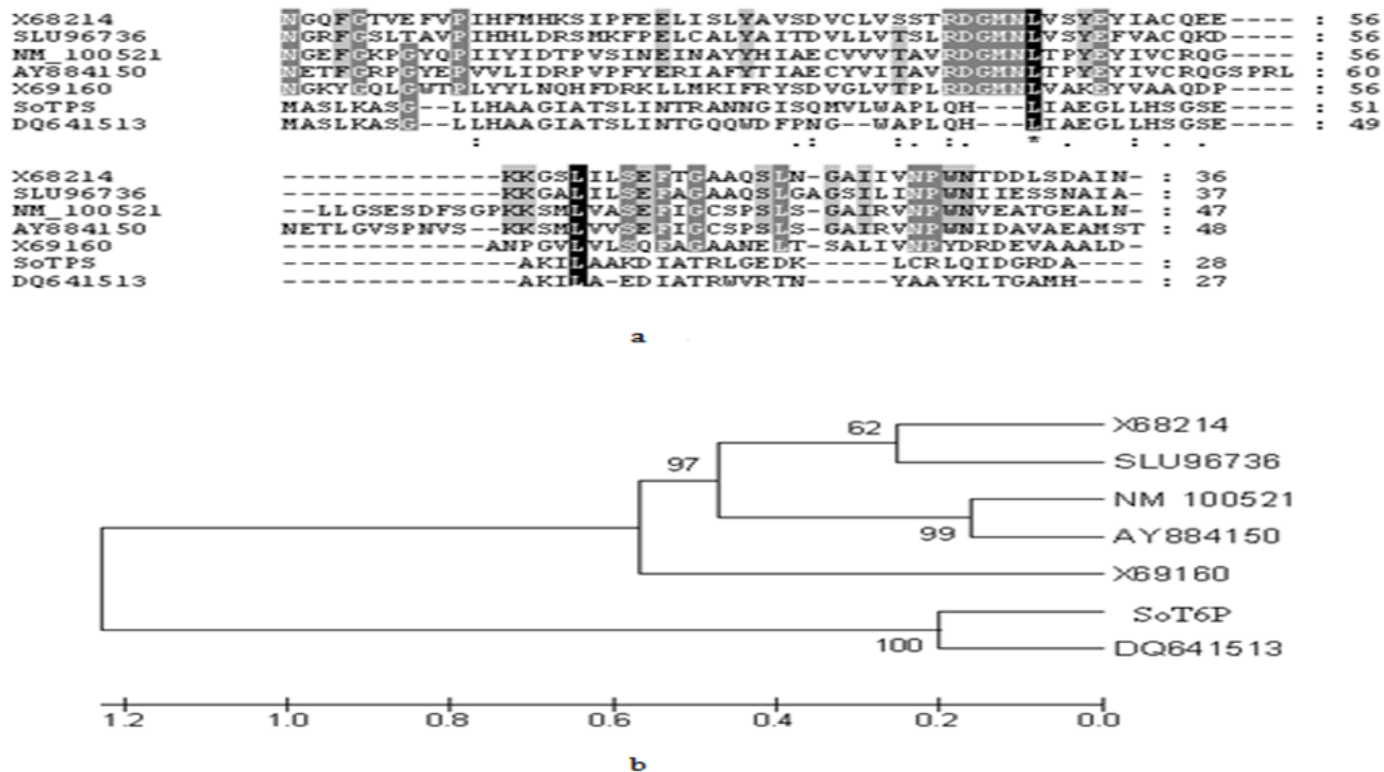


Fig. 3: Alignment of SoTPS sequences with trehalose of different organisms. A- Numbers on the right indicate the number of amino acids of the predicted protein. The sequences marked black dark gray and clear grey represent identical amino acids, 80% and 60% identity, respectively. *S. officinarum* (GenBank, accession no. DQ641513); *S. cerevisiae* (GenBank, accession no. X68214); *A. thaliana* (GenBank, accession no. NM100521); *G. biloba* (GenBank, accession no. AY884150) and *E. coli* (GenBank

Table 1. Identification of salicylic acid-induced genes in sugarcane RB 72910, with similarity to known genes from *Sacharum officinarum* species.

Clone ¹	Gene description (Access no.) ²	E-value ³
SA-SSH-18	Glutamine Synthase [<i>Sacharum officinarum</i>] gb AY 835457.1	0.0
SA -SSH-30	Acil - CoA synthase [<i>Sacharum officinarum</i>] TC 50035	3e-33
SA -SSH-35	Endopeptidase NP1 (precursor) [<i>Sacharum officinarum</i>] TC 4835.1	1.6e-62
SA -SSH-53	Peptidyl-polyl cistrans isomerase [<i>Sacharum officinarum</i>] TC 49521	1.6e-67
SA -SSH-141	Sucrose-Phosphate Synthase [<i>Sacharum officinarum</i>] TC 49521	2.2e-52

¹Genes isolated by suppression subtractive hybridization, from salicylic acid treated sugarcane mRNAs.

²Gene roles were identified with homologue sequences from NCBI, TIRG and DDBJ, using the program BlastX. 3E-value was used to indicate the significance of the similarity for each gene. 0.0 correspond to 100% similarity.

Diverse techniques are used in gene expression analysis, e.g., Northern blotting, RT-PCR and quantitative real-time PCR, of which Northern blotting is most commonly used. However, this technique is slow and requires a relatively large quantity of mRNA. RT-PCR, on the other hand, is quicker, more sensitive and can be more specific than Northern blotting (Dean et al. 2002). Additionally, qPCR provides higher sensitivity for the detection of DNA or RNA due to the combination of amplification performed by PCR and the detection system (Bustin 2000).

In this study, the *SoTPS* gene trehalose biosynthesis was expressed differentially in variety RB 72910 in the different tissues and treatments. Plant leaves (leaf +1) and roots subjected to diverse stress levels were collected, and total RNA was extracted efficiently. Reverse transcription reactions followed by PCR (RT-PCR) and quantitative PCR (qPCR) were performed to study trehalose expression. For the analysis of quantitative real-time PCR, we designed a new pair of primers. These primers amplify smaller fragments that are only 200 base pairs (bp), which provides more consistent results due to greater efficiency and tolerance of PCR reaction conditions (Wong and Medrano 2005). The constitutive *14-3-3* gene was chosen as a known suitable reference gene for expression normalization in a wide range of sugarcane tissue samples (Papini-Terzi et al. 2005). During the experiments, no expression of the trehalose gene was observed in the root tissue. In the leaves, expression of the trehalose gene was demonstrated in plants subjected to severe stress (Treatment 4: 18 days without watering and 10% soil moisture) (Figure 2A). Plants grown under this treatment were also analyzed by real time PCR, which revealed an expression of the gene for trehalose of approximately two times that of the control treatment. Detection of the trehalose gene in the control treatment was not observed on a semi quantitative gel but was detected by real-time PCR due to the high sensitivity of this method (Figure 2B).

The finding of increasing amounts of trehalose agrees with many studies that describe the importance of osmotic adjustment of species, genotypes or cultivars to drought in grasses (El-Bashiti et al. 2005; Hongbo et al. 2006). These authors assert that this adjustment is related to the accumulation of solutes in tissues in response to drought, which maintains cell turgor and facilitates the adjustment of physiological and biochemical processes to such conditions. Thus, plants that are more tolerant to drought generally show higher amounts of trehalose in plant tissue (El-Bashiti et al. 2005).

Recent studies suggest that trehalose in superior plants may be related to carbohydrate accumulation and the protection of cells against damage caused by abiotic stress. It is further believed that trehalose acts as a molecular signal in the regulation of plant metabolism and development (Bae et al. 2005). The identification of

trehalose as a potential osmoprotectant has raised interest in its metabolism as a means of introducing traits of tolerance to abiotic stresses, using genetic transformation, into crops of economic interest. To date, there are no reports on the participation of the trehalose in the expression of water-deficit tolerance in large crops such as sugarcane.

In this study, the set of primers designed to clone trehalose gene *SoT6P* of the sugarcane variety RB 72910 amplified a fragment of cDNA of approximately 540 bp. After sequencing, the identity with other trehalose sequences was verified. The amino acid-deduced sequence showed 95% identity with trehalose of *Saccharum officinarum*, 70% with trehalose of *Saccharomyces cerevisiae*, 68% with trehalose of *Selaginella lepidophylla*, 65% with trehalose of *Arabidopsis thaliana*, 62% with trehalose of *Ginkgo biloba* and 59% with trehalose of *Escherichia coli* (Figure 3A). A phylogenetic tree was constructed using amino acid sequences deduced from the trehalose gene *SoTPS* and other trehalose types deposited in GenBank. The following two groups were formed: Group I with trehalose of two distinct microorganism types (*S. cerevisiae* and *E. coli*) and three plants (*S. lepidophylla*, *Ginkgo biloba* and *A. thaliana*) and group II with trehalose of sugarcane only (Figure 3B).

In the sugarcane genotype studied here, the expression of the trehalose gene was only verified in plants under more severe stress. A similar result was obtained recently by Wu et al. (2006), who subjected *Ginkgo biloba* plants to different stress types. Furthermore, in cotton plant leaves under water deficit, Kosmas et al. (2006) observed a considerable increase in the expression level of the trehalose gene compared to unstressed plants based on RT-PCR analysis. Studies focusing on the metabolism of trehalose in plants have been conducted particularly with *A. thaliana* (Avonce et al. 2004; Bae et al. 2005). No studies of this nature have been reported so far for sugarcane. The results obtained here reveal the potential of the identified gene for future studies aiming to develop more water deficit-tolerant varieties based on genetic transformation.

CONCLUSION

The techniques of suppression subtractive hybridization (SSH) and qRT-PCR was practicable and efficient to study the transcriptional profiles after foliar application of salicylic acid and to analyse gene expression in the response to water-deficit in sugarcane. While SSH could be used for initial isolation of differentially expressed transcripts, a confirmation of gene expression of a gene involved in water stress can be accomplished efficiently by qRT-PCR analysis.

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