FUNCTIONAL GENOMICS APPROACHES FOR THE STUDY OF WATER STRESS IN SUGARCANE

By

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Abstract

THE AIM OF this study was to evaluate gene expression in leaf roll tissues of sugarcane plantlets subjected to water restriction under greenhouse controlled conditions using two functional genomics approaches: EST library analysis and differential gene expression by a cDNA macroarray analysis. Sugarcane plantlets of variety SP80-3280 were submitted to water restriction for 17 days and leaf rolls were collected on days 1, 5, 9, 13 and 17 after initiation of the water restriction treatment. Two cDNA libraries were constructed using mRNA isolated from tissues collected at day 9 (LR9 library) and day 17 (LR17 library) and, after sequencing, the generated ESTs were analysed using bioinformatics tools. The results from the EST library analysis showed that transcripts for all the enzymes of the octadecanoid pathway leading to jasmonic acid (JA) biosynthesis were present during both periods, indicating that water-deficit-stress-induced expression of genes was involved in JA production. Transcripts for the antioxidant enzymes catalase, ascorbate peroxidase, polyphenol oxidase and superoxide dismutase were present. A macroarray containing 1202 spotted genes in duplicate were hybridised with cDNAs prepared from mRNA isolated from leaf rolls. An analysis of differentially expressed genes showed that only 147 genes presented differential expression, being induced or repressed. As observed in the cDNA libraries studies, genes coding enzymes of the octadecanoid pathway were induced. Similarly, genes coding antioxidant enzymes (two catalase isoforms, one monohydroascorbate reductase, two ascorbate peroxidase isoforms), indicating the tissue was under oxidative stress.

Introduction

Water deficit is one of the most important environmental stress factors limiting growth and productivity of agronomically important crops (Neill and Burnett, 1999). The changes in metabolism and development induced by stress can frequently be attributed to alterations in the level of gene expression (Taiz and Zeiger, 2004).

A relatively rapid way to obtain information about gene expression is the partial sequencing of cDNAs. Digital analysis of gene expression can be achieved by producing tags, e.g. ESTs, to expressed genes and then inferring transcript abundance from the frequency of these tags (Lee *et al*., 1995).

The DNA macroarrays enable the identification of activated or deactivated metabolic routes, in addition to the depiction of the hundreds of interactions that occur, at the transcriptional level, in response to several physiological events (Nepomuceno *et al.*, 2001).

Material and methods

Leaf rolls were collected from 48-day-old sugarcane plants (var. SP80-3280) after 9 and 17 days of water restriction. Two leaf roll cDNA libraries, LR3 (9-day water restriction) and LR4 (17-day water restriction), were constructed from mRNA.

Total RNA was isolated using the acidphenol-guanidine-thiocyanate protocol (Chomczynski and Sacchi, 1987). Extraction of mRNA was performed with Oligotex mRNA Mini kit (Qiagen, CA, USA).

Sequencing of cDNA inserts was performed from the 5' end using the BigDyeTM Terminator Cycle Sequencing v2.0 (PE Biosystems, CA, USA) and the reactions were subjected to automated sequencing in a 3700 DNA Analyser (PE Applied Biosystems).

Macroarray experiments were based on ESTs from the LR9 and LR17 libraries, in which 12 ESTs most highly expressed were arrayed on the membrane together with 34 ESTs classified as no hits and 250 ESTs associated with stress.

The remaining 905 ESTs selected for the array belonged to stress-related metabolic pathways that were identified in the Sugarcane Expressed Sequence Tag (SUCEST) project (Vettore *et al*., 2003) resulting in a membrane with a total of 1202 ESTs. The membranes were created at Centro Brasileiro de Estocagem de Clones (HTTP://www.bcccenter.fcav.unesp.br).

Results and discussion

A total of 4252 ESTs of good quality (minimum of 140 bases with Phred quality ≥ 20) were obtained for the two libraries under water stress conditions, with 1934 from the LR9 library and 2318 from the LR17 library.

All had the 5' extremity sequenced and 387 clones also had the 3' extremity sequenced, with 237 from the LR9 library and 150 from the LR17 library. The mean size of the inserts (ESTs) was 835 bp for the LR9 library and 502 bp for the LR17 library (data not shown).

In the LR9 library, 8 ESTs (0.414%) coding for lypoxygenase (LOX) were observed while, in the LR17 library, 35 ESTs (1.510%) showed significant similarity to this gene. An analysis of the 43 ESTs for LOX present in the LR9 and LR17 libraries showed that they were distributed in 6 clusters (Table 1).

Table 1—Clusters of ESTs that contain LOX sequences and result of BLASTX against the bank nr of NCBI.

Cluster	No. ESTs in the libraries		Results of BLASTX against NCBI (first hit)			
	LR9	LR17	GeneID	Gi	Locus in maize	Maize chromosome
JFLR17001D07.g	0	3	542495	162459823	NP_001105515	??
JFLR17011G01.g	0	5	542495	162459823	NP_001105515	??
JFLR9010F11.g	3	17	541856	162461114	NP_001105003	3
JFLR9016D09.g	1	0	541856	162461114	NP_001105003	3
JFLR9042F11.g	2	2	100037802	162462799	NP_001105973	3
JFLR9011B01.g	2	8	100037802	162462799	NP_001105973	3
Total	8	35				

Clone analysis revealed the presence of four ESTs for catalase (CAT; three in LR9 and one in LR17), three for ascorbate peroxidase (APX; one in LR9 and two in LR17), five for polyphenol oxidase (PPO; all in LR9) and two for superoxide dismutase (SOD; all in LR17) (Table 2).

To protect cells and subcellular compartments from the harmful effects of ROS, plants synthesise a suite of antioxidant enzymes, among which CAT, SOD, APX and PPO are the most important (Mittler, 2002; Soares and Machado, 2007).

Table 2—ESTs present in the LR9 and LR17 libraries and that codify antioxidant enzymes.

Enzyme	Cluster	Nº ESTs in the libraries		Total ESTs
		LR9	LR17	
Catalase (CAT)	JFLR9081A10.g	3	1	4
Ascorbate peroxidase (APX)	JFLR17007F02.g	0	2	3
	JFLR9043F11.g	1	0	
Polyphenol oxidase (PPO)	JFLR9075F12.g	3	0	5
	JFLR9084F11.g	2	0	
Superoxide dismutase (SOD)	JFLR17025D09.g	0	2	2

The data generated by the macroarrays analysis indicated that 147 ESTs exhibited differential expression during the water stress period evaluated. As observed in the cDNA libraries, genes coding enzymes of the octadecanoid pathway were induced. Similarly, genes coding antioxidant enzymes (two catalase isoforms, and two ascorbate peroxidase isoforms) indicated a state of oxidative stress. According to Kumari *et al*. (2006) the possibility that H_2O_2 oxidative stress produced in response to water deficit, with the direct participation of JA itself, and the stress induced on account of the functioning of the octadecanoid pathway (β-oxidation stage in the peroxisomes/glyoxysomes) can contribute to the activation of genes coding Bowman-Birk (BBI) protease inhibitors.

These two mechanisms could be acting concomitantly during the induction of defensive mechanisms to counter the negative effects of water-deficit stress (Ferro, 2008).

The profiling of the EST clones from the LR9 and LR17 libraries, and the analysis of their expression with macroarrays indicated that the octadecanoid pathway or oxylipin pathway was activated by water stress, appearing more active as the severity of stress increased. During activation of this pathway, JA and H_2O_2 are produced which then lead to induction of antioxidant enzymes, signaling pathway genes and defence gene expression (Figure 1).

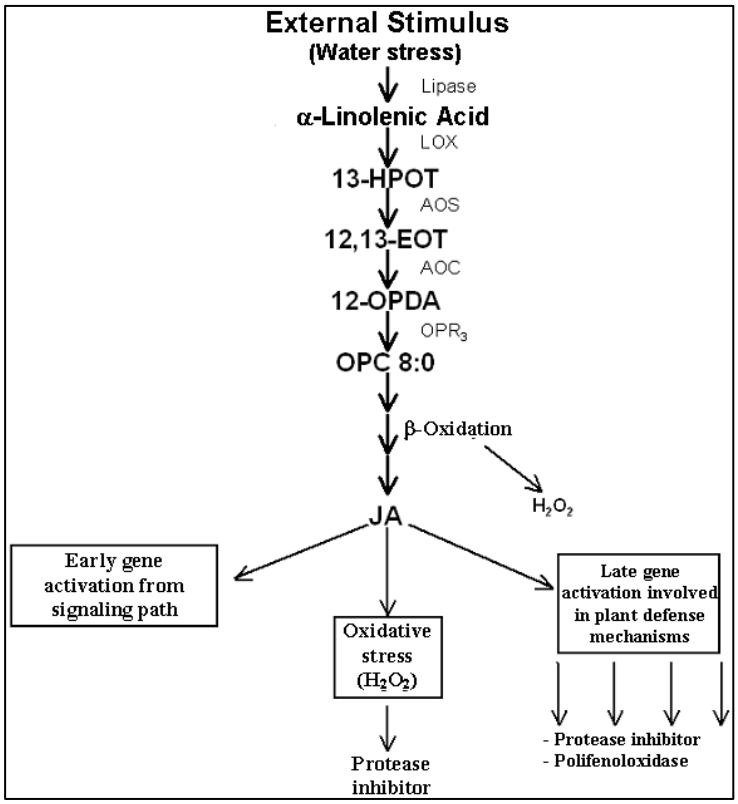

Fig. 1—Schematic representation of jasmonic acid (JA) production by the octadecanoid pathway and cellular processes and responses that can be triggered by JA (Ferro, 2008). **AOC**: allene oxide cyclase; **AOS**: allene oxide synthase; **JA**: jasmonic acid; **LOX**: Lipoxygenase; **OPR_3**: 12-oxo-PDA reductase (isoenzyme 3).

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LA GÉNOMIQUE FONCTIONNELLE UTILISER POUR ETUDIER LE STRESS HYDRIQUE CHEZ LA CANNE À SUCRE

Par

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MOTS CLÉS: Stress Hydrique, Expression Différentielle, Réponse au Stress, Tolérance à la Sécheresse.

Résumé

L'OBJECTIF de cette étude consistait à évaluer l'expression génique dans des tissus de feuilles enroulées issues des plantules de canne à sucre sous des conditions contrôlées de serre. Deux approches de génomique fonctionnelle furent utilisées: l'analyse des banques des marqueurs de séquence exprimée (ESTs) et l'expression différentielle de gènes par l'analyse de macroarray d'ADNc. Des plantules de canne à sucre de la variété SP80-3280 furent soumises à une restriction hydrique pendant 17 jours et les feuilles enroulées furent prélevées au bout de 1, 5, 9, 13, et 17 jours après l'initiation du stress hydrique. Deux banques d'ADNc ont été construites en utilisant l'ARNm isolé des tissus prélevés au 9ème (banque LR9) et 17ème jour (banque LR17) et, après le séquençage, les ESTs générés furent analysés en utilisant des outils bioinformatiques. Les résultats des analyses de banques d'EST ont montré que les transcripts pour toutes les enzymes de la voie octadécanoïde de la biosynthèse de l'acide jasmonique étaient présents durant les deux périodes, indiquant que l'expression de gènes induits par un stress hydrique était impliquée dans la production de l'acide jasmonique. Des transcripts pour les enzymes antioxydants suivants: la catalase, l'ascorbate peroxidase, la polyphénol oxidase et la superoxyde dismutase étaient présents. Un microaaray contenant 1202 gènes spottés en double ont été hybridés avec les ADNc préparés avec de l'ARNm isolé des feuilles enroulées. Une analyse des gènes exprimés différentiellement a montré que seulement 147 gènes présentaient des expressions différentielles, étant induits ou réprimés. Comme observé dans des études des banques d'ADNc, des gènes codant pour des enzymes de la voie octadecanoide furent induits. Parallèlement, des gènes codant pour les enzymes antioxydants (deux isoformes de la catalase, une réductase monohydroascorbate, deux isoformes de l'ascorbate peroxydase), furent induits indiquant que le tissu était sous le stress oxydatif.

ENFOQUES EN GENÓMICA FUNCIONAL PARA EL ESTUDIO DEL ESTRÉS HÍDRICO EN LA CAÑA DE AZÚCAR

Por

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PALABRAS CLAVE: Restricción de Agua, Expresión Diferencial, Respuesta al Estrés, Tolerancia a la Sequía.

Resumen

EL OBJETIVO de este estudio fue evaluar la expresión de genes en tejidos foliares de plántulas sometidas a restricción hídrica bajo condiciones controladas en invernadero usando dos enfoques de genómica funcional: el análisis de librerías EST y el análisis de la expresión génica diferencial en una macrocolección de cADN. Las plántulas de caña de azúcar de la variedad SP80-3280 fueron sometidas a restricción hídrica por 17 días y rollos de hojas fueron colectados 1, 5, 9, 13 y 17 días posteriores al tratamiento de restricción hídrica. Las dos librerías de cADN fueron construídas usando mARN aislado de tejidos colectados en los días 9 (librería LR9) y 17 (librería LR17) y, luego de su secuenciación, los ESTs generados fueron analizados usando herramientas de la bioinformática. Los resultados del análisis de la librería de EST mostró que los transcritos para todos los enzimos de la ruta octadecanoide conducente a la biosíntesis de ácido jasmónico (JA) se encontraban presentes durante ambos períodos, indicando que la expresión génica inducida por el estrés hídrico está involucrada en la producción de JA. Entre los transcritos presentes se encontraron aquellos para los enzimos antioxidantes de la catalasa, la peroxidasa del ascorbato, la oxidasa polifenólica y la dismutasa superóxida. Una macrocolección conteniendo 1202 genes contados en duplicado fueron sujetos a una hibridación con los cADN preparados a partir de mARN aislados de los rollos de hojas. Un análisis de los genes expresados diferencialmente mostró que sólo 147 genes presentaron expresión diferencial, ya inducida ya reprimida. Tal y como se observó en los estudios de librerías de cADN, se indujeron los genes que codifican para los enzimos de la ruta octadecanoide. De manera similar, se hallaron los genes que codifican a los enzimos antioxidantes (dos isoformas de la catalasa, una reductasa del monohidroascorbato, dos isoformas del peróxido ascorbato), indicando que el tejido estaba bajo estrés oxidativo.