

PATTERNS OF GENE EXPRESSION IN SUGARCANE MONITORED USING cDNA MACROARRAYS

DL CARSON^{1,2}, BI HUCKETT^{1,2} AND FC BOTHA²

¹SASA Experiment Station, Private Bag X02, Mount Edgecombe, 4300

²Institute for Plant Biotechnology, University of Stellenbosch, Private Bag X01, Matieland, 7602

Abstract

A sugarcane Expressed Sequence Tag (EST) database has been established which has led to the identification of genes in different tissues. Gene identity alone, however, does not provide information about patterns of gene expression nor allow elucidation of gene function. As a first step towards functional genome analysis in sugarcane, expression profiles are being developed for a random selection of sugarcane gene sequences with a specific focus on tissue differential expression. In this study, cDNA macroarrays containing 1400 sugarcane clones were screened with total cDNA probes prepared from leaf, leaf roll, immature culm and mature culm tissue. Hybridisation patterns obtained suggest that the majority of genes are constitutively expressed between the different tissue types tested. Work is in progress to identify and characterise the 20% pool of differentially expressed genes. A preliminary functional analysis of gene expression is discussed.

Introduction

An Expressed Sequence Tag (EST) database was initiated at SASEX in 1994 in order to identify sugarcane genes expressed in different parts of the plant (Carson *et al.*, 1998, Carson and Botha, 2000). ESTs are established by random selection of cDNA clones from cDNA libraries followed by DNA sequence analysis and sequence homology comparison with genes of known function. The SASEX sugarcane EST database contains partial gene sequence information for over 500 genes from the leaf roll and culm tissue of varying maturity. Although these data are informative and have contributed towards sugarcane genomics, knowing the identity and general function of a gene frequently does not provide an insight into the specific role in the plant. Expression patterns of individual genes provide clues as to their possible function. Random EST analysis of total cDNA libraries is limited, however, in its capacity to identify differentially expressed genes and can only provide crude indications of transcript abundance. One possible approach towards the identification of differentially expressed genes is through the development of subtracted cDNA libraries. These libraries are enriched for transcripts with differential expression profiles and are therefore potentially more effective for the functional analysis of expressed genes.

Recent advances in the development of macroarray technology have allowed the systematic parallel monitoring of expression of large numbers of genes. This is accomplished through the hybridisation of complex cDNA probes prepared from mRNA to arrays of individual gene sequences immobilised on solid supports. This approach was chosen to assess tissue-specific ex-

pression patterns of randomly selected cDNA clones derived from sugarcane leaf roll and mature culm total cDNA libraries and two subtractive cDNA libraries prepared by reciprocal subtraction between immature and mature culm. As a first step towards functional genome analysis in sugarcane, this paper reports the preliminary analysis of expression patterns of sugarcane cDNA clones in the leaf, leaf roll, immature and mature culm.

Materials and Methods

Field-grown material of commercial cultivar NCo376 and AA40 clone 126 were used for total and subtractive cDNA library construction, respectively. Tissue was harvested from the leaf, leaf roll, immature (internode 2) and mature (internode 7) culm tissue and stored at -80°C until use. Total cDNA libraries were prepared from mRNA isolated from leaf roll and internode 7 tissue according to the protocol described previously (Carson *et al.*, 1998 and Carson and Botha, 2000). Two subtractive cDNA libraries were constructed by reciprocal subtraction between internode 2 and internode 7 using the PCR-based Subtractive cDNA Cloning technique described in Current Protocols in Molecular Biology (Patel and Sive, 1996). All cDNA libraries were cloned into the EcoRI site of the λ ZAPII phage cloning vector (Stratagene).

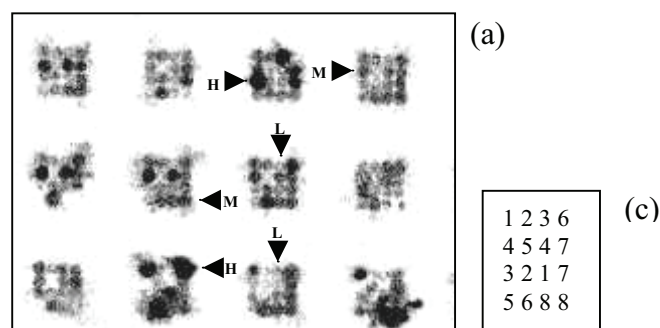
Individual recombinant phages were randomly selected from plated out aliquots of the libraries and stored in SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 20 mM Tris-HCl pH 7.5, 0.01% gelatin) at 4°C. Individual phagemids containing the cloned cDNA inserts were obtained by *in vivo* excision of the phagemid from the λ ZAP II vector according to the manufacturer's instructions (Stratagene). Phagemid clones were stored as -80°C bacterial glycerol stocks in 96-well microtitre dishes. A total of 1400 cDNA clones were sent to Clemson University Genomics Institute (SC, USA) for preparation of cDNA macroarrays. Bacterial clones were arrayed on 22 X 7 cm Hybond N⁺ nylon membranes (Amersham) in a 4 X 4 duplication pattern using a robot (Qbot, Genetix). Membranes were hybridised with ³³P-labeled first-strand cDNA probes. The probes were prepared by reverse transcription of 1 μ g mRNA according to the method of Sambrook *et al.* (1990) using ExpandTM Reverse Transcriptase (Roche Molecular Biochemicals) and with the addition of 200 μ M ddCTP (Decraene *et al.*, 1999). Prehybridisation, hybridisation and washing of the membranes were performed according to the recommended protocol supplied with the membranes. Hybridised membranes were exposed to a Super Resolution Cyclone Phosphor Screen (Packard) for 4-16 hours and data captured and analysed with OptiQuantTM software (Packard).

Individual phagemid cDNA inserts were sequenced, analysed and assigned putative identities by GenBank database searches as described previously (Carson *et al.*, 1998 and Carson and Botha, 2000).

Results and Discussion

The objectives of this study were to examine the expression patterns of individual sugarcane gene sequences in a variety of tissue types and to identify differentially expressed genes. In addition, the quality and effectiveness of two subtractive cDNA libraries were assessed by examining the expression patterns of subtracted cDNA clones. Macroarray membranes were prepared with 1400 sugarcane cDNA clones comprising 500 clones each from a leaf roll and internode 7 total cDNA library and 200 clones each from an internode 2 and internode 7 subtractive cDNA library. Radioactively-labeled total cDNA probes were prepared from leaf, leaf roll, internode 2 and internode 7 and hybridisation profiles of the cDNA clones compared. Initial data analysis was performed manually whereby the hybridisation signals for individual clones were designated as “low”, “medium” or “high”, according to the intensity of the signal (Figure 1). Quantification of a random selection of representative signals using the OptiQuant software (Packard) was performed to verify the results. The hybridisation signals designated “medium” were shown to be consistently 2-fold higher than “low”, while “high” signals were 2-3 fold higher than “medium”.

The tissue-dependent expression pattern of leaf roll and mature culm cDNA clones is summarised in Table 1. Results indicate that the majority of genes are constitutively expressed



Signal Intensity	Density Light Units (mean of 16 samples)
Low	7338.94
Medium	16802.61
High	42368.68

Figure 1: Representative portion of a cDNA macroarray hybridised with a leaf roll total cDNA probe. Individual clones corresponding to low (L), medium (M) and high (H) signal intensity are annotated in (a) while signal quantification using the OptiQuant™ software (Packard), expressed as Density Light Units, for 16 randomly selected samples is indicated in (b). The 4 X 4 duplication pattern of clone placement on the macroarray is represented diagrammatically in (c), where each number corresponds to an individual clone.

between the different tissue types tested. These include typically “housekeeping” genes that are associated with core metabolic processes. A small group (3%) of genes was found to exhibit significantly high levels of constitutive expression. These included genes involved with protein synthesis, DNA methylation and stress (DNAJ protein). A reduction in expression level from the leaf to the maturing culm was observed for 9% of the clones. Many of these clones have been identified as coding for genes involved in protein synthesis and protein modification. Conversely, an increase in expression from the leaf to the maturing culm was detected for 4.4% of clones. Preliminary analyses suggest that several of these clones code for stress-response associated genes as well as genes involved in various signal transduction pathways. Northern blot analysis of the putative differentially expressed genes is in progress to validate the results obtained from the cDNA macroarray screening.

To assess the efficiency of the subtraction procedure, a random selection of clones from the two subtraction libraries were screened with total cDNA probes prepared from immature and mature culm tissue. Results indicated that 37% of clones from the immature culm and 31% of mature culm clones tested exhibited tissue-preferential expression. The remaining 43% and 49%, respectively, showed similar levels of expression in the two tissue types. This indicates that the process of subtraction resulted in an increase in the identification of preferentially expressed genes by approximately 18%, as compared with screening results of the total cDNA libraries. The combination of enrichment of gene sequences through subtraction with the efficiency of macroarray screening may therefore be a valuable way of identifying differentially expressed genes. Tissue-preferential clones from the subtraction libraries have been sequenced and putatively identified by sequence homology search of GenBank database. Some of the genes identified in the immature culm library included UDP-glucose dehydrogenase and xyloglucan endotransglycosylase which are associated primarily with cell wall synthesis. Other immature culm clones exhibited sequence similarity to an environmental stress-induced protein, a drought-induced protein and a TMV resistance protein homolog. Several clones preferentially expressed in the mature culm were also putatively identified as stress-responsive exhibiting sequence similarity to genes such as an abscisic acid and salt stress responsive clone and jacalin, a lectin. It is not yet known, however, what the exact function of these stress-responsive genes might be in sugarcane and whether the different genes preferentially expressed in the two tissues may serve different roles. Other genes identified in the mature culm subtractive library included cellulase and genes involved in glycerol metabolism. Northern blot analysis of the preferentially expressed genes identified in both subtraction libraries is in progress to provide further information about expression patterns in other tissues such as the leaf and leaf roll.

Further investigations will focus towards developing an understanding of the specific function of the various differentially expressed genes during sugarcane growth and maturation. These genes may then be targeted for promoter isolation, marker development or use as transgenes.

Table 1: Variations in expression patterns of sugarcane ESTs in leaf, leaf roll, immature culm and mature culm tissue.

Expression Pattern	% Clones	Selected Examples	Source of cDNA Clone	Putative Function
Constitutive	80	Cellulose synthase Pyruvate dehydrogenase Calcium-dependent protein kinase	Leaf roll Leaf roll Leaf roll	Cell wall synthesis Cellular respiration Protein modification
High constitutive	3	Triose phosphate isomerase Calmodulin S-adenosyl-methionine synthetase	Leaf roll Internode 7 Internode 7	Glycolytic enzyme Signal transduction DNA methylation
Reduction from leaf to maturing culm	9	Vacuolar H ⁺ -ATPase 40S ribosomal protein S13 Protein phosphatase 2A	Leaf roll Internode 7 Internode 7	Proton pump Protein synthesis Protein modification
Increase from leaf to maturing culm	4.4	ADP-ribosylation factor Heat shock protein 82 Adenosine kinase	Internode 7 Internode 7 Leaf roll	Protein trafficking Stress response Nucleotide metabolism
Variable (no fixed pattern)	3.6	60S ribosomal protein L13	Internode 7	Protein synthesis

REFERENCES

- Carson, DL, Hockett, BI and Botha, FC (1998). The identification of sugarcane genes by random sequencing of cDNA clones. *Proc S Afr Sug Technol Ass* 72: 143-145.
- Carson, DL and Botha, FC (2000). Preliminary analysis of Expressed Sequence Tags for sugarcane. *Crop Science*: 40 (6): 1769-1779.
- Decraene, C, Reguigne-Arnould, I, Auffray, C and Piétu, G (1999). Reverse transcription in the presence of dideoxynucleotides to increase the sensitivity of expression monitoring with cDNA arrays. *BioTechniques* 27: 962-966.
- Patel, M and Sive, H (1996). PCR-based subtractive cDNA cloning. *In: Current Protocols in Molecular Biology*. Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA and Struhl, K (Eds.). Vol 1, Greene and Wiley-InterScience, New York.
- Sambrook, J, Fritsch, EF and Maniatis, T (1990). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.