SUGARCANE GENETIC ENGINEERING RESEARCH IN SOUTH AFRICA: FROM GENE DISCOVERY TO TRANSGENE EXPRESSION

By

D.A. WATT[1,2], D.L. SWEBY[1], B.A.M. POTIER[1] and S.J. SNYMAN[1,2]

[1]*South African Sugarcane Research Institute,*
Private Bag X02, Mount Edgecombe 4300, South Africa
[2]*School of Biological and Conservation Sciences,*
University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa
derek.watt@sugar.org.za

KEYWORDS: *In vitro* Micro-Propagation, Genetic Transformation, Gene Promoters, Sugarcane.

Abstract

DURING the past 15 years, recombinant DNA and *in vitro* culture technologies have been used in concert at the South African Sugarcane Research Institute (SASRI) to genetically engineer sugarcane. The purpose of such research has been two-fold, *viz*. to establish proof-of-principle regarding the delivery of novel input and resistance traits to sugarcane and to investigate the genetic basis of sucrose accumulation. Underpinning these goals has been the in-house development of essential genetic resources, including the isolation of appropriate transgenes and gene promoter elements, and the optimisation of transformation and tissue culture technologies. With regard to the latter, emphasis has been placed on the development of tissue culture strategies that minimise the potential for somaclonal variation, while maximising the efficiency of germplasm transformation, selection, regeneration and acclimation. The isolation of promoters to drive high-level and targeted transgene expression, as well as the identification of DNA sequences with the potential to deliver the desired phenotype, have been strongly driven by advances in gene discovery and expression technologies. Early approaches relied on the analysis of Expressed Sequence Tag libraries constructed from sugarcane tissues at different developmental stages or challenged with selected abiotic and biotic stress agents. Subsequent improvements in DNA subtractive hybridisation technologies (e.g. suppression subtractive hybridisation) and gene expression analysis (e.g. Affymetrix® Sugarcane Genome GeneChip) provided new and powerful means for gene and promoter discovery. Reviewed here are the advances in *in vitro* culture, gene discovery and transgene expression research, which have formed the foundation of the genetic engineering program at SASRI. Also discussed are the opportunities and challenges in these areas of research that are presented by the availability of ultra-high throughput DNA sequencing (e.g. the 454 Sequencing System) for transcriptome analysis and technologies for rapid and accurate profiling of the sugarcane metabolomes.

Developing capacity for genetic engineering

Since the release of the first 'N' variety, N53/216, in 1964, the South African Sugarcane Research Institute (SASRI) has released 45 varieties that have been bred and selected by conventional means for the five major agro-bioclimatic zones comprising the South African sugar belt.

In the late 1980s, the industry recognised that rapid advances in molecular biology, *in vitro* plant propagation, and recombinant DNA technology offered the potential to serve as powerful adjuncts to conventional breeding; an insight which led to the establishment of a dedicated

biotechnology facility in 1992. The Institute further recognised the value that could be derived from participation in consortia and collaborations to enhance capacities in biotechnology, particularly those pertaining to molecular breeding and genetic engineering.

As a result, SASRI became a founding member of the International Consortium for Sugarcane Biotechnology (ICSB) and entered into agreements with research service providers, such as the Institute for Plant Biotechnology (IPB) at Stellenbosch University, South Africa. The development of in-house capacities at SASRI over the past 15 years, together with outcomes from collaborative and contract research, has led to a thrust in genetic engineering research. Research in this area has focused on establishing Proof-of-Concept for the engineering of resistance and novel input traits, as well as in the investigation of the genetic basis of sucrose accumulation.

Establishing a genetic engineering toolkit

Several technologies and resources are required for effective sugarcane genetic engineering, including: (1) a high throughput plant transformation platform that effectively discriminates for desired transformation events but which also minimises somaclonal variation; (2) sequences of endogenous genes to be targeted for up- or down-regulation and of heterologous genes for the delivery of novel phenotypes; and (3) gene promoters to drive high-level transgene expression that is targeted to the appropriate plant organ or tissue.

Technologies: tissue culture and genetic transformation

In vitro culture is an integral part of plant genetic transformation, with the former technology having been investigated in sugarcane since the late 1960s (e.g. Heinz and Mee, 1969) and which has been recently reviewed by Lakshmanan and co-authors (2005). The availability of such information, together with the protocol for sugarcane transformation described by Bower and Birch (1992), facilitated the development of an effective technology platform for the transformation of South African genotypes (Snyman *et al*., 1996, 2001a; Snyman, 2004).

Initial protocols for gene delivery involved the use of embryogenic Type 3 callus with morphogenesis proceeding via indirect embryogenesis (Snyman *et al*., 1996; Snyman, 2004). With these protocols, the time required for the production of a transgenic plant for acclimation ranged between 24 and 36 weeks (Snyman *et al*., 2000, 2001a). A period of 8 to12 weeks in culture is required for each of three stages, *viz.*: callus initiation and proliferation; secondary embryogenesis on a selection agent; and germination of embryos which give rise to transgenic plantlets.

Although this timeframe was considered acceptable in a research and development context, SASRI researchers recognised that a reduction in the culture period would be essential to meet commercial imperatives. It was further recognised that an important additional advantage would result from such a reduction, *viz.* reduced duration of exposure to growth hormones would minimise the risk of somaclonal variation.

Consequently, a novel approach for gene delivery was developed at SASRI, based on the use of leaf discs containing floral initials as recipient material, with subsequent plant regeneration via direct embryogenesis (Snyman *et al*., 2006). Application of this method has resulted in the production of transgenic plants within 14 weeks and also increased the range of germplasm that may be targeted for effective genetic transformation. This high throughput and benign transformation protocol has proven to be an essential element of SASRI's genetic engineering toolkit.

Genetic resources: accessing transgenes

Over the last 20 years many tools have been developed to study gene structure and function. One of the most powerful of these has been the construction of large public DNA databases, readily accessible via the Internet. When it was demonstrated in the 1980s that partial nucleotide sequences of random clones from cDNA libraries could be aligned to sequence data of known genes contained

in such databases, the capacity for the assignment of putative identities to genes of relatively uncharacterised organisms became a reality. These partial gene sequences, termed Expressed Sequence Tags (ESTs), formed an invaluable resource for research on organisms with complex and non-sequenced genomes, such as sugarcane.

SASRI pioneered EST–based genomic research for sugarcane in the mid 1990s, which was closely followed by similar projects in Australia, USA and Brazil. The SASRI effort was modest in comparison to these later initiatives (Carson and Botha, 2000). For example, Brazilian researchers constructed 26 cDNA libraries from several sugarcane tissues at various stages of development, which yielded a collection of several hundred thousand ESTs (Vettore *et al*., 2001). SASRI, in contrast, elected to pursue a more targeted strategy, whereby a limited number of cDNA libraries were constructed with a focus on specific targets. As an important SASRI research goal was to determine the genetic basis of sucrose accumulation, considerable effort was devoted to the development of EST libraries that reflected key shifts in gene expression associated with sucrose accumulation.

Two tissue-types were targeted for cDNA library construction, *viz.* the apical meristem (leaf roll) and maturing culm (internode 7). It was hypothesised that ESTs derived from the meristem would provide access to a wide range of genes associated with growth and development, while those from the maturing internode would provide insights into the identity of genes associated with sucrose accumulation (Carson and Botha, 2000; Carson and Botha, 2002). As expected, meristem ESTs reflected genes encoding products associated with active growth, *viz.* cell wall synthesis, protein synthesis and protein modification.

Surprisingly, however, the maturing culm yielded very few ESTs depicting enzymes directly associated with sucrose metabolism. Hence, to increase resolution of the EST-based approach, reciprocal subtractive cDNA hybridisation was deployed to enrich for transcripts differentially expressed between internode two (immature) and internode 7 (maturing) (Carson *et al.*, 2002a). Despite this more focused approach, only 10% of ESTs from the subtracted libraries matched genes associated with carbohydrate metabolism; none of which were direct participants in sucrose metabolism. Those results revealed that growth and maturation of the culm is associated with the expression of genes encompassing a wide variety of processes, suggesting that the mechanisms regulating sucrose accumulation were comprised of a complex interplay between various aspects of primary metabolism.

While the SASRI EST collections provided direct access to genes for use in transgenesis, additional value was extracted from the libraries through comparison of EST expression patterns among various target tissues. Central to this research was the in-house development of gene expression profiling strategies based on array screening. Comparative gene expression analyses between tissue-types and developmental stages using membrane-based macroarrays identified a significant number of differentially regulated genes (Carson *et al.*, 2002 a, b). These included genes associated with cell wall metabolism, various regulatory and signal transduction processes, as well as a suite of stress-induced genes.

This information, when analysed in concert to data from biochemical and physiological studies, allowed the construction and analysis of custom cDNA macroarrays. Such arrays, sometimes referred to as boutique arrays, were designed using specific ESTs known to encode gene products associated with sucrose metabolism and transport (Watt *et al.*, 2005).

Expression profiling of these ESTs revealed information about key genes and pathways regulating sucrose accumulation. Such array-based gene expression profiling approaches gained considerable momentum after the development of the Affymetrix® Sugarcane Genome GeneChip (Affymetrix chip); an important innovation driven by Casu *et al.* (2006) in Australia. Using this resource, researchers at SASRI have examined the role of sugar sensing and signalling in mediating

the source-sink relationship in sugarcane (McCormick *et al.*, 2006). That work revealed a potentially central role of trehalose metabolism in the sink-mediated modulation of photosynthetic activity (McCormick *et al*., 2008). Work is currently in progress to unravel further the relationship that may exist between the capacity of the sugarcane plant to modulate sucrose production in the leaf according to the demand for the sugar by the stalk. In this regard, a combination of Suppression Subtractive Hybridisation (SSH) and ultra-high throughput DNA sequencing, based on 454-technology, is proving pivotal.

Genetic resources: isolating gene promoters

Gene promoters are an essential component of any genetic engineering toolkit, as they are required to regulate transgene expression to an appropriate level and to target expression to the desired site within the plant. The unavailability in the public domain of suitable promoters to drive high-level tissue- or organ-specific transgene expression in South African sugarcane germplasm prompted SASRI to establish a promoter isolation research programme. The overall strategy used in this endeavour has three components, *viz.*: (1) identification of transcripts displaying a suitable tissue-specific expression pattern; (2) identification and isolation of the corresponding promoter region from genomic DNA; and (3) functional assessment of the promoter *in planta*. In this regard, SASRI has sought to isolate functional promoters for targeting transgene expression to the leaf, culm or root.

Identification of tissue-specific transcripts has relied on the macroarray and the SSH technologies that were developed and deployed in SASRI's early EST research. More recently, the Affymetrix chip technology has also been used to good effect in detecting differentially expressed genes to serve as a basis for promoter discovery. However, further validation of the differential gene expression patterns, detected by these technologies, is a vital step prior to promoter isolation. To this end, SASRI researchers have used two validation protocols, *viz.* (1) virtual confirmation of laboratory observations using the numerous online tools and data available at the National Centre for Biotechnology Information; and (2) experimental confirmation using either northern hybridisation analysis or quantitative PCR (qPCR), with the latter being the current method-of-choice.

Initial promoter isolation approaches at SASRI used the nucleotide sequences of well-characterised differentially expressed genes to retrieve the corresponding promoter region from the sugarcane genome. Such retrieval was based on the screening of sugarcane genomic libraries, which were either in the form of Bacterial Artificial Chromosome (BAC) or bacteriophage libraries.

Numerous conceptual and practical challenges arose during the screening of such libraries, primarily as a result of the complex, polyploid nature of the sugarcane genome. These challenges included accounting for the potential presence in the genome of multiple pseudogenes with associated non-functional promoter sequences, in addition to allelic promoter variants with different levels of activity.

Of additional concern was the propensity for gene silencing in sugarcane observed at SASRI and also reported in the literature (Hansom *et al.*, 1999, Mudge *et al.*, 2009). Hence, to-date, the maize *Ubi-1* promoter (Christensen and Quail, 1996) remains the most widely used plant-derived promoter for sugarcane transformation, primarily due to the difficulties encountered by many research groups in isolating promoters that are capable of stable and high-level transgene expression (Brumbley *et al*., 2008). To reduce the risk of promoter silencing by transcriptional gene silencing (TGS) events, a strategy has been implemented at SASRI to isolate tissue-specific promoters from close *Saccharum* relatives, *viz. Sorghum bicolor* and *Zea mays*, for use in sugarcane. Availability of the sorghum genome sequence (Paterson *et al.*, 2009) has made this approach particularly efficient, in that promoter regions are easily targeted for PCR-mediated amplification by means of sequence-specific oligonucleotide primers.

As transient expression assays do not necessarily depict *in planta* promoter expression characteristics, the functionality of promoters isolated at SASRI was assessed in numerous plants regenerated from multiple transgenic events. The rapid *in vitro* propagation and genetic transformation protocols developed greatly assisted in the generation of the large numbers of transgenic plants required for promoter functional testing. Results obtained to-date indicate that overcoming the phenomenon of TGS remains a challenge. As a result, SASRI researchers are currently exploring ways to alleviate promoter methylation, which is a recognised cause of transgene silencing. A construct, pKOMet, is being used to alter the expression of the endogenous sugarcane DNA methyltransferase, while the efficacy of a construct bearing a viral suppressor of gene silencing, pUbi P1/HcPro is also under evaluation. The availability of tissue-specific promoters to drive high-level transgene expression will be an extremely valuable tool in SASRI's genetic engineering research.

Establishing Proof-of-Concept: examples from SASRI's research portfolio

Proof-of-Concept for transgenesis was first generated at SASRI using herbicide resistance conferred by the *pat* gene from *Streptomyces viridochromogenes* against the herbicidal compound glufosinate ammonium (Leibbrandt and Snyman, 2003) (Table 1). Gene constructs were delivered by microprojectile bombardment and selection was facilitated by the *npt*II gene using G418 or paromomycin as selection agents (Snyman, 2004). Gene expression of both the gene of interest and the selectable marker was driven by the maize ubiquitin promoter. Subsequent field trials with selected herbicide resistant lines established that the *pat* transgene was stably inherited and expressed over multiple ratoons (Leibbrandt and Snyman, 2003). Since that initial study, two additional Proof-of-Concept projects, focusing on the delivery to elite South African sugarcane germplasm of input and resistance traits, have been successfully completed, *viz.* (1) increased resistance to the sugarcane stem borer, *Eldana saccharina*, through expression of the *Cry1A(c)* δ-endotoxin gene from *Bacillus thuringiensis* and (2) tolerance of the herbicide, glyphosate, through expression of a transgene derived from *Agrobacterium* sp. strain CP4 encoding the enzyme 5-*enol*pyruvyl-shikimate-3-phosphate synthase (Table 1).

Technologies to modify the expression of genes encoding key enzymes of carbohydrate metabolism in sugarcane have generated insights into the complex biochemical, metabolic and physiological processes underlying sucrose accumulation. This research area at SASRI has benefited from a strong collaboration with the IPB at Stellenbosch University, as well as from data generated from the early EST and gene expression experiments. For example, the gene encoding UDP-glucose dehydrogenase (UDPG-DH) was shown to be preferentially expressed in the immature culm (Carson *et al*, 2002a). Using this information as a starting point, research has been conducted to down-regulate UDPG-DH activity using antisense and RNAi technologies (Bekker, 2007) (Table 1). It was hypothesised that assimilated carbon would be directed away from cell wall synthesis and towards sucrosesynthesis. Internodes from transgenic lines grown under glasshouse conditions demonstrated an increase in sucrose content that was correlated with a decrease in UDPG-DH activity. Currently, several other lines with modified levels of key enzymes of sucrose metabolism, which demonstrated desirable phenotypes in cell suspension culture and under glasshouse conditions, are under analysis in field trials (Table 1).

Future directions

The application of genetic engineering technology at SASRI has yielded several invaluable outcomes, including Proof-of-Concept for the delivery of beneficial input and resistance traits, as well as insights into the regulation of sucrose accumulation.

As the prospect of commercial production of transgenic sugarcane becomes increasingly less distant, SASRI management and researchers have begun to focus their efforts on issues that will ultimately facilitate the realisation of that goal. Such issues include refinement of

transformation technologies and addressing, as far as is possible, the socio-political, freedom-to-operate and regulatory issues surrounding genetic engineering with a commercial intent.

Refinements to the genetic engineering toolkit currently underway at SASRI encompass the development of: (1) transformation protocols that offer an alternative to biolistics; (2) benign selection methodologies for transformation events; and (3) novel approaches to isolate promoters which are not susceptible to TGS.

Table 1—Novel input traits, resistance traits and modified sucrose metabolism conferred via transgenesis to South African sugarcane germplasm. Also reflected are the nature and source of transgenes and the extent to which transgenic lines have been characterised to-date.

Trait	Gene and nature of modification	Source of gene	Stage of assessment	Reference
Novel input trait				
Herbicide resistance: glufosinate ammonium glyphosate	*pat* gene: heterologous expression *CP4* gene: heterologous expression	*Streptomyces viridochromo-genes* A*grobacterium* sp. strain CP4	Field trial* (five ratoons) Field trial* (two ratoons)	Leibbrandt and Snyman (2003) Snyman *et al*. (2001b)
Resistance traits Insect resistance: *Eldana saccharina* (Lepidoptera: Pyralidae))	*Cry1A(c)*: heterologous expression	*Bacillus thuringiensis*	Pot bioassay: inoculation with *Eldana saccharina* (Lepidoptera: Pyralidae) larvae.	Unpublished
Virus resistance: (Sugarcane Mosaic Potyvirus (SCMV))	SCMV Coat Protein: heterologous expression in antisense and untranslatable forms	SCMV Strain D (predominant strain in South African Midlands region)	Natural infestation field trial* (two ratoons)	Sooknandan (2002); Sooknandan *et al*. (2003)
Modification of sucrose metabolism Enzyme and transporter targets				
Pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP)	PFP gene: down-regulation of endogenous gene by anti-sense expression	*Saccharum* spp hybrid var NCo310	Performance evaluated in field trials* (one ratoon)	Groenewald and Botha (2007)
Neutral invertase (NI)	NI gene: down-regulation of endogenous gene by anti-sense expression	*Saccharum* spp hybrid var NCo310	Performance evaluated under glasshouse conditions and in cell suspension culture.	Roussow *et al*. (2007)
UDP-glucose dehydrogenase (UDPG-DH)	UDPG-DH gene: down-regulation of endogenous gene by RNAi and anti-sense expression	*Saccharum* spp hybrid var NCo310	Performance evaluated under glasshouse conditions	Bekker (2007); Patent number 2006/07743
H^+-translocating vacuolar pyrophosphatase (VPPase)	VPPase gene: overexpression of endogenous gene	*Saccharum* spp hybrid var NCo310	Performance evaluated under glasshouse conditions	Swart (2007); Patent number 2007/02680

* Field trials conducted under permit from the South African National Department of Agriculture, Directorate: Biosafety, in accordance with the GMO Act 15 of 1997.

In parallel to these practical issues, researchers are beginning to draw on the potential offered by ultra-high throughput technologies, such as those for nucleotide sequencing and metabolite profiling, for potential application to gene discovery and demonstration of substantive equivalence of transgenic lines to wild-type. SASRI's genetic engineering research is conducted in

a favourable environment, in that the South African sugar industry is supportive and the national regulatory framework is well-established. Given this context, the expertise held by SASRI and the availability of an established repertoire of technologies, the future for sugarcane genetic engineering research in South Africa holds considerable promise.

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LA RECHERCHE EN GÉNIE GÉNÉTIQUE EN AFRIQUE DU SUD : DE LA DÉCOUVERTE DU GÈNE À L'EXPRESSION DU TRANSGÈNE

Par

D.A. WATT[1, 2], D.L. SWEBY[1], B.A.M. POTIER[1] et S.J. SNYMAN[1,2]

[1]South African Sugarcane Research Institute,
Private Bag X02, Mount Edgecombe 4300, Afrique du Sud
[2]School of Biological and Conservation Sciences,
University of KwaZulu-Natal, Private Bag X54001, Durban 4000, Afrique du Sud
derek.watt@sugar.org.za

MOTS CLÉS: Micro-Propagation *In Vitro*,
Transformation Génétique, Promoteurs des Gènes, Canne à Sucre.

Résumé

DURANT LES 15 dernières années, les technologies de l'ADN recombiné et de la culture *in vitro* ont été utilisées au South African Sugar Research Institute (SASRI) pour la transformation génétique de la canne à sucre. Cette recherche avait un double but : établir la preuve de principe concernant le transfert de nouveaux traits de production et de résistance à la canne à sucre, et examiner le mécanisme génétique responsable de l'accumulation de saccharose. Pour atteindre ces objectifs, il a été nécessaire de développer en laboratoire les techniques essentielles, y compris, l'isolation des transgènes et promoteurs appropriés, l'optimisation des technologies du génie génétique et de la culture de tissus. Concernant la culture de tissus, l'accent a été mis sur les stratégies qui minimisent le potentiel de variation somaclonale, tout en maximisant l'efficience de la transformation du germoplasme, la sélection, la régénération et l'acclimatation. L'isolation des promoteurs pour une forte expression du transgène ciblée, et l'identification des séquences d'ADN qui ont le potentiel de développer le phénotype recherché, ont fortement évoluées de par les avancées dans la découverte des gènes et leur expression. Les approches initiales dépendaient de l'analyse des banques d'étiquettes de séquences exprimées (EST) produites à partir des tissus de canne à sucre de différents stades de développement ou qui ont été soumis à des stress biotiques et abiotiques. Des améliorations ultérieures des technologies d'hybridation soustractive d'ADN (e.g. hybridation soustractive et suppressive) et de l'analyse de l'expression génique (e.g. Affymetrix® Sugarcane Genome Genechip) ont apporté des moyens nouveaux et puissants pour la découverte de gènes et de promoteurs. Les avancées dans la culture *in vitro*, la découverte de gènes et l'expression de transgènes, qui ont formé la base du programme de génie génétique au SASRI, sont revues ici. Les opportunités et les défis dans ce domaine de recherche, grâce à la disponibilité de séquençage d'ADN à ultra haut débit (e.g. le Système 454 de Séquençage) pour l'analyse du transcriptome, et les technologies pour établir un profil rapide et précis des métabolomes de la canne à sucre sont aussi discutés.

INVESTIGACIÓN EN ÁFRICA DEL SUR SOBRE INGENIERÍA GENÉTICA DE LA CAÑA DE AZÚCAR: DEL DESCUBRIMIENTO GENÉTICO A LA EXPRESIÓN TRANSGÉNICA

Por

D.A. WATT[1,2], D.L. SWEBY[1], B.A.M. POTIER[1] y S.J. SNYMAN[1,2]
[1]*Instituto de Investigación en Caña de Azúcar de África del Sur, Private Bag X02, Mount Edgecombe 4300, África del Sur*
[2]*Escuela de Ciencias Biológicas y de Conservación, Universidad de KwaZulu-Natal, Private Bag X54001, Durbán 4000, África del Sur*
derek.watt@sugar.org.za

PALABRAS CLAVE: Micro-Propagación *In Vitro*, Transformación Genética, Promotores Genéticos, Caña de Azúcar.

Resumen

DURANTE los últimos 15 años, las tecnologías de ADN recombinante y de cultivo *in vitro* han sido utilizadas en combinación en el Instituto de Investigación en Caña de Azúcar de África del Sur (SASRI, por sus siglas en Inglés) para innovar genéticamente a la caña de azúcar. El propósito de tal investigación es doble, *viz.* establecer pruebas contundentes de la incorporación de nuevos caracteres y de aquellos de resistencia, e investigar la base genética de la acumulación de sacarosa. Tales objetivos han sido sustentados por el desarrollo interno de recursos genéticos cruciales, incluídos el aislamiento de transgenes y elementos de promoción genética adecuados, y la optimización de las tecnologías de transformación y en el cultivo de tejidos. Con respecto de lo último, se ha enfatizado en el desarrollo de estrategias en el cultivo de tejido para minimizar el potencial de variación somaclonal, a la par de maximizar la eficiencia en la transformación de germoplasma, la selección, la regeneración y la aclimatación. El aislamiento de promotores para impulsar un alto nivel y el destino de la expresión transgénica, así como la identificación de secuencias de ADN para asegurar la obtención del genotipo deseado, han sido fuertemente impulsados por avances tecnológicos en el descubrimiento y expresión genéticos. Los esfuerzos iniciales dependían del análisis de las bibliotecas de Etiquetas de Secuencias Expresadas (EST, por sus siglas en Inglés) derivadas de tejidos de caña de azúcar en distintos estados de desarrollo o sometidas a agentes selectos de estrés abiótico y biótico. Mejoras subsecuentes en tecnologías de hibridación substractiva (p.e., supresión de hibridación substractiva) y en el análisis de la expresión genética (p.e., *Chip* Affymetrix® de Genes de la Caña de Azúcar) han provisto de nuevos y poderosos medios para el descubrimiento de genes y promotores. Aquí se revisan los avances en el cultivo de tejidos, el descubrimiento de genes y la investigación en la expresión transgénica, los cuales han significado el fundamento del programa de ingeniería genética en el SASRI. Asimismo, se debaten las oportunidades y retos en las áreas de investigación aquí expuestas debido a la disponibilidad en la secuenciación de alto procesamiento del ADN (p.e., el sistema de secuenciación 454) o el análisis en el transcriptomo y en tecnologías para la definición rápida y precisa del perfil de los metabolomas de la caña de azúcar.