



South African Sugarcane Research Institute: Embracing biotechnology for crop improvement research

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Received: 20 September 2007 / Accepted: 7 December 2007

Abstract The South African Sugarcane Research Institute (SASRI) is a leading agricultural research institute and the only one of its kind in Africa conducting research into the development of new varieties, improving crop management and farming systems to enhance sugarcane productivity. In addition, effective delivery of new knowledge and technology make a significant contribution to the sustainability of the sugar industry in southern Africa. Variety Improvement and Crop Protection are key focus areas of research that have embraced modern molecular technologies to advance both conventional breeding and integrated control of pests and diseases.

Keywords Sugarcane, South Africa, SASRI, biotechnology, crop improvement

The South African sugar industry: structure and strategic challenges

The earliest accounts of sugarcane cultivation in South Africa stem from 1635 when Portuguese explorers, shipwrecked near the mouth of the Umzimkulu River, recorded that sugarcane was one of the crops grown by the local inhabitants. Following the establishment of the Agricultural Society in South Africa in 1848, the industry expanded, and

today produces on average 2.5 million tons of sugar per season from 428 000 hectares (Moore and Hudson, 2007).

The southernmost sugarcane industry in the world the South African industry is faced with numerous challenges, not least of which is the topography of the cane growing regions. Extending between the latitudes of 25°S – 31°S, and mostly within the eastern province of KwaZulu-Natal (Fig. 1), conditions are not ideal for sugarcane farming. Of the 428 000 hectares currently under sugarcane about 68% is grown within 30 km of the coast and 17% in the high rainfall area of the KwaZulu-Natal midlands. The balance is grown in the northern irrigated areas. Unlike many other industries, most of South Africa's cane land is sloping, which has resulted in a primarily hand-harvested crop. Further, the relatively low rainfall levels (averaging between 950 mm and 1100 mm per annum) experienced throughout the region contribute to the marginal conditions (O'Reilly, 1998).

There are approximately 45 300 registered growers in South Africa, of which some 43 500 are small-scale farmers that account for approximately 11% of the crop. Their plots of land range in size, but on average are less than 10 ha. Furthermore, these growers farm mostly on land to which they have no title or ownership.

The industry is served by 14 sugar mills. Of these, five are owned by Illovo Sugar Limited, four by Tongaat Hulett Sugar Limited, two by TSB Sugar RSA Limited, one by Umvoti Transport (Pty) Limited, one by Ushukela Milling (Pty) Limited and one is a co-operative - UCL Company Limited. Four of the mills are known as "white end" mills and produce their own refined sugar. Raw sugar produced by TSB Sugar RSA Ltd is exported via the sugar terminal in Maputo. Raw sugar produced at the remaining mills is routed to Durban where it is either refined or stored at the South African Sugar Association Sugar Terminal prior to export. Diversity is the

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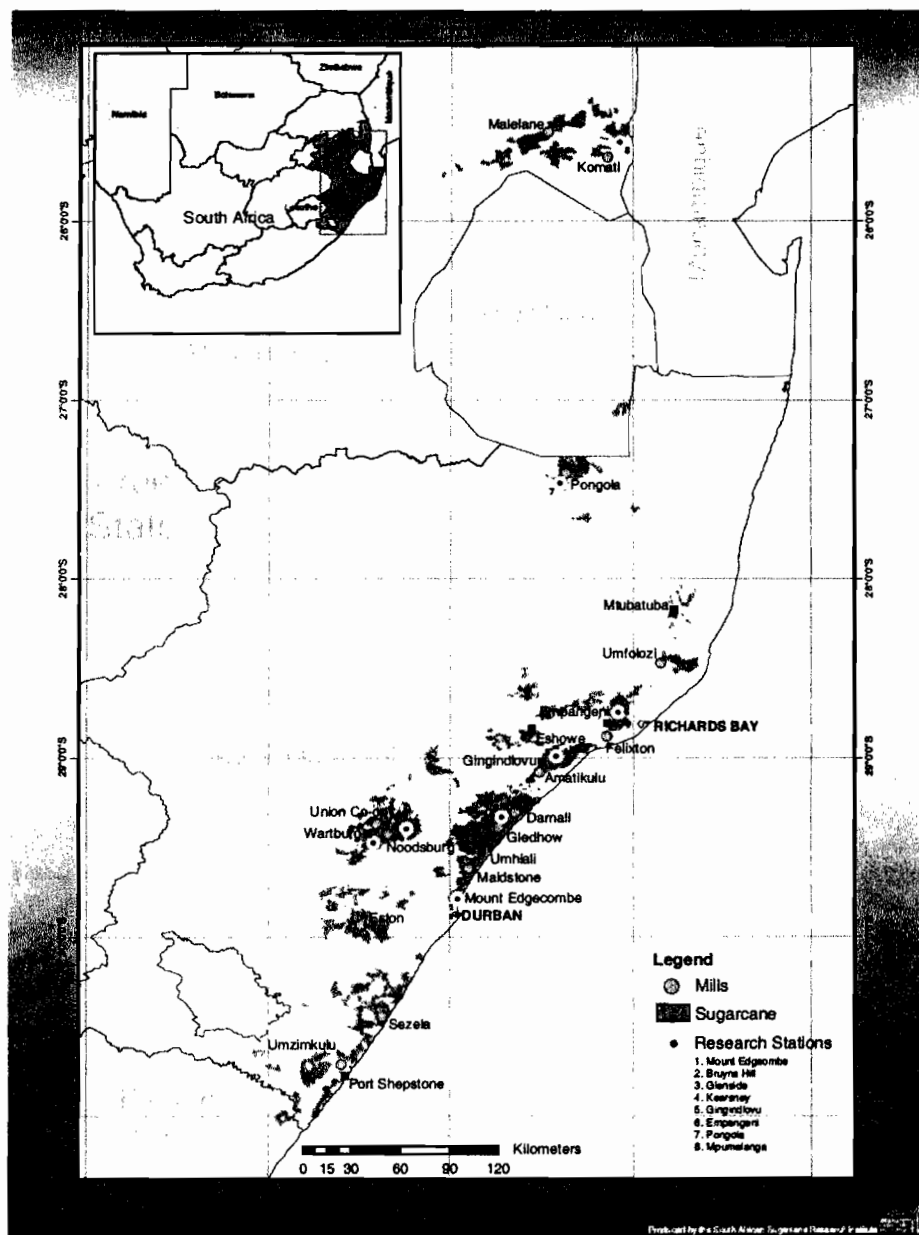


Fig. 1. A map representing the sugarcane industry in South Africa. The Research Stations are managed by SASRI primarily for plant breeding in the Variety Improvement Programme. SASRI is located at Mount Edgecombe, just north of Durban in KwaZulu-Natal.

key factor in today's highly integrated sugar milling operations and the mills produce a range of other products such as ethyl alcohol and furfural and its derivatives.

Industry structure

The industry is a proceeds-sharing partnership between the growers and millers. The South African Sugar Association (SASA) administers this partnership on behalf of the South African Cane Growers' Association and the South African Millers' Association Ltd. Equal numbers of representatives from each association sit on the SASA Council, the decision-making body of the organisation. SASA is an autonomous

organisation that operates free of government control, since in terms of the Sugar Act and Sugar Industry Agreement it has been granted statutory powers of self governance.

The association itself comprises a number of divisions that support either core industry functions or the industry partnership. Included amongst the latter is the National Market division that is responsible for managing national marketing of sugar to consumers, administration of industrial rebates and monitoring of sugar supply and demand, while the International Marketing division manages the marketing and logistics associated with bulk raw sugar. Just over half of South African sugar is exported through the Bulk Sugar Terminal located in Durban's Maydon Wharf. The sugar is supplied as VHP (very

high pol) and during loading is coated with high test molasses to meet importer specifications.

Divisions supporting core industry functions are the South African Sugarcane Research Institute (SASRI), the Cane Testing Service (CTS), Autolab and the Shukela Training Centre (STC). SASRI provides for all agricultural research and development for the industry while the CTS is responsible for determination of cane quality at each of the mills. Autolab supports all computerised systems installed at the mills and is responsible for maintaining the systems that track sugarcane through the milling process. Both agricultural and industrial training is conducted at the STC to meet the needs of the industry and beyond.

Challenges

Challenges facing the South African industry are diverse. Land issues are a concern and in recognition of the urgency to promote diverse ownership of agricultural land, the sugar industry has already assisted in the transfer of 16% of freehold land to black growers. An industry target of 30% black ownership of freehold sugarcane land by 2014 has resulted in the development of an independent land reform company (Inkezo), which focuses on achieving this target through the 'willing-buyer willing-seller' principle.

Environmental responsibilities are not peculiar to the South African industry, and it actively promotes sound and sustainable agricultural practices in line with national legislation and international requirements. The industry is engaged in sustainable resource management and provides support to local environmental committees located in the cane producing areas. Codes of Good Practice have been established regarding issues of cane burning, protection of water courses, minimum tillage, responsible herbicide use and so on. Within the milling environment, companies are also engaging better management practices.

In view of the geographic position of the industry and the attendant climatic conditions, development of productive sugarcane varieties is a key focus of activities at SASRI. An extensive plant-breeding programme, that seeks to provide varieties that are able to withstand local pests and diseases and that are adapted to either rainfed or irrigated conditions, successfully produces one or two new varieties on average each year.

Meeting the agricultural needs of the diverse range of growers in the South African industry demands specialised technology transfer systems. Associated with SASRI is an extensive Extension service, whose principal role is to encourage adoption of improved farming practices. Extension specialists are located throughout the industry, many of which work in conjunction with the provincial Departments of Agriculture. Further, to enhance the skill level amongst all industry stakeholders, two divisions of the South African Sugar Association provide agricultural and industrial training that

supports both the farming and milling sectors.

Since cane supply in the industry is limited by land and water availability, efficiencies of scale are significant drivers for efficiencies in the milling and processing activities. The Sugar Milling Research Institute provides technical support to the milling sector through its research, analytical support, advisory services and specialist equipment design and training. However, it is the competitive nature of the industry that has led to South Africa's leading role in the development of cane preparation, diffusion, continuous pan boiling, clarification processes and direct white sugar technology.

The South African sugar industry has responded to its unique combination of challenges to become one of the world's leading cost competitive producers of high quality sugar. Both its structure and organisation have positioned it to embrace the challenges that the future imposes.

Research and development: institutional configuration and strategy

Research and development form the focal centre of all SASRI activities. Research is informed by industry strategy and is designed to produce pertinent outcomes addressing grower problems in the field. Through Knowledge Management and innovative technology transfer techniques, SASRI ensures that these outcomes lead as seamlessly as possible into Extension advisory services, which are also managed from within the institute. In a restructuring of SASRI during 2003–2005, discipline-based departments with their own dedicated research profiles were replaced with programme-based research conducted in virtual networks. The three Programmes at SASRI are Variety Improvement, Crop Protection and Resource Optimisation. Within these, approximately 85 projects in total are running at any one time. New research ideas are vetted through a rigorous central system of project evaluation, and progress is controlled and reported through project management techniques. Each project draws on Resources Centres where scientists, technicians, working space and equipment are housed and coordinated. This new way of conducting research has enhanced multidisciplinary communication and effort significantly and has increased productivity and accountability, among other benefits.

Research, Development & Extension and Knowledge Management are supported by in-house services such as Human Resources and Finance & Administration as well as the technically based Fertiliser Advisory Service.

At present, SASRI employs 475 people (100 of those on contract) across all facilities, including the central institute at Mount Edgecombe and seven research farms situated in varied geoclimatic zones across the industry. Approximately 50 employees are professional scientists, half of whom have doctorates. There is a strong culture of postgraduate training; at any one time there are between 10 and 20 postgraduate students studying for higher degrees at the SASRI centre.

Additional students working on collaborative projects conduct their research at partner institutions.

Active links with other research organisations have developed strongly over many years and research collaborations are now more strategically important than ever. SASRI has partnership status with various South African universities and ongoing collaborations with individual researchers in a number of academic and research institutions internationally. In some cases SASRI contracts university specialists to conduct specific research or supports international consortia where high-level research is contracted by members. Because of its speed of development and high financial demand, biotechnological research has benefitted greatly from these kinds of alliances and is sure to continue to do so in the future.

Molecular and *in vitro* technologies: progression of integration into research and development

Tissue culture research was initiated at SASRI in the late 1980s but it was only in the early 1990s that molecular biology and tissue culture became the focus of coordinated attention. A dedicated Biotechnology department was put in place with new facilities and additional staff and students in 1992. The main focal areas were: (1) studies in basic sugarcane physiology, (2) genetic modification through transgenesis, (3) the development of molecular markers and (4) provision of genetic and technical resources through tissue culture, genomics and cloning for use in these research thrusts. Biotechnological resources and approaches soon became incorporated into the work of other Departments, Pathology in particular, with both diagnostic and fundamental research benefitting extensively. By the time the restructuring of SASRI took place (see above), biotechnological applications were represented in an even greater range of projects. Currently, biotechnology has a place in all three programme areas and assists in enhancing sucrose accumulation, parent selection, distribution of new varieties, pest and disease research, quarantine control, crop nutrition and crop modelling.

Molecular and *in vitro* technologies in variety improvement

Marker-assisted breeding

Sugarcane has a complex genome that makes improvement through classical breeding consuming and laborious in terms of screening progeny, and makes cross outcomes difficult to predict. The use of molecular markers linked to major genes or quantitative trait loci (QTL) associated with a desirable phenotype is becoming an important tool in breeding for crop improvement. In early marker research at SASRI, several genetic markers associated with pest and disease resistance were characterised reviewed by (Butterfield *et al.*, 2004). In this work, differentially expressed cDNA fragments or

expressed sequence tags (ESTs) were identified during biotic challenge to the smut fungus (*Ustilago scitaminea*), the stalk borer (*Eldana saccharina* Walker) and Sugarcane mosaic virus (SCMV). Using a Restriction Fragment Length Polymorphism (RFLP) approach, fragments with putative association to resistance were used as probes on a population of sugarcane with known resistance ratings to sugarcane smut, *E. saccharina* borer and SCMV. Polymorphic markers were scored and association with phenotype were analysed using statistical methods developed in house (Butterfield *et al.*, 2004). Most probes (76%) yielded at least one RFLP marker associated with smut, eldana or SCMV resistance, illustrating the efficiency of this marker generating strategy.

Validation of the efficiency of molecular markers as opposed to phenotype selection of *E. saccharina* resistance has been demonstrated recently using a mass screening technique across families (Butterfield *et al.*, 2007). SASRI has 125 parent genotypes with known markers for eldana / smut resistance. Further markers are currently being screened across a population of 100 genotypes. Marker-based parent selection has been an active aspect of breeding strategy at SASRI since 2002.

Recent marker research has focused on linkage disequilibrium mapping of cane quality and yield component traits. Some of this work has been conducted at SASRI in collaboration with CIRAD, France, as part of a multi-industry funded project under the auspices of the International Consortium for Sugarcane Biotechnology (ICSB). An extension of this approach currently in progress involves mapping the reference cultivar R570 and a population of commercial sugarcane genotypes using Diversity Arrays Technology (DART), and development of bioinformatics tools to use this information.

The demonstration that complex or polygenic traits can be reduced to their individual genetic components will give sugarcane breeders important tools for studying the individual and combined effects of specific genes in selected genetic backgrounds. Furthermore, application of these techniques will increase the efficiency of sugarcane breeding and selection programmes.

Genetic engineering: introducing novel and improved traits

Although no genetically modified sugarcane is grown commercially in SA, SASRI has been working on genetic engineering technology to: (1) elucidate mechanisms and key enzymes associated with sucrose metabolism and (2) introduce specific traits in to elite cultivars. The main emphasis of the latter area of research has been 'proof of concept' as no commercial contracts have been negotiated with companies that hold intellectual property rights on transformation technologies and genetic constructs.

SASRI began developing transformation technology by establishing *in vitro* culture systems (Snyman *et al.*, 1992).

Subsequent refinement of embryogenic callus production and microprojectile delivery by transient expression of the GUS gene (Snyman *et al.*, 1996) paved the way for optimization of transformation efficiencies. SASRI capitalized on the significant advances made in the production of transgenic plants in Australia (Bower *et al.*, 1996) and USA (Gallo-Meagher and Irvine, 1996) in the early 1990's. Transformation efficiencies are variable depending on genotype, method of gene delivery and length of time spent in culture, so further refinement of the technology is generally conducted by each laboratory, depending on their industry requirements. The use of alternative target material, i.e. leaf discs and explants containing pre-emergent inflorescences, followed by a more rapid route of direct morphogenesis, has significantly decreased production times in our laboratory (Snyman *et al.*, 2000, 2001a, 2006).

Much of the initial evaluation of transgenic lines at SASRI was conducted on plants engineered with herbicide tolerance. Resistance to the compounds glufosinate ammonium and glyphosate were used as model systems to investigate transgene stability over several vegetative ratoons and to evaluate growth and yield parameters under field conditions (Leibbrandt and Snyman, 2001, 2003, 2004; Snyman *et al.*, 2001b). Our results indicated that the transgenes were stably inherited and expressed over several vegetative ratoons.

Due to specific needs of the South African sugarcane industry, other input traits such as pest and disease resistance have been investigated. *E. saccharina* is a highly damaging stalk-boring pyralid moth affecting yields in the South African sugarcane industry. Economic impact of this pest may be in the order of ZAR 60 million per annum. As part of an integrated pest management programme, transgenic sugarcane lines containing the insecticidal cryIAb gene were generated. Lines that produced levels of Δ -endotoxin detectable by commercially available lateral flow strips were subjected to a replicated bioassay. This involved artificial infestation with borer eggs at cane age of 10.5 months and assessment of borer damage and survival 66 days later. Visual inspection of data showed that several lines had substantially less larval survival and stalk damage than non-transformed resistant control plants.

Advances in genetic engineering via post-transcriptional gene silencing or interfering-RNA have resulted in an alternative approach to controlling the viral disease, mosaic (Ingelbrecht *et al.*, 1999; Gilbert *et al.* 2005). The SASRI breeding programme eliminates up to 16% of its superior cultivars at a late stage of selection due to the incidence of mosaic (Butterfield and Thomas, 1996). Transformation vectors containing the maize polyubiquitin and CaMV 35S promoters and viral coat protein gene from a local strain of SCMV were delivered to susceptible cultivars (Sooknandan *et al.*, 2003). Field trials to evaluate resistance are currently underway.

SASRI is also engaged in a promoter isolation programme because: (1) gene silencing is a phenomenon frequently

observed in transgenic sugarcane that may be related to high transgene copy number and/or multiple copies of the genome and (2) intellectual property constraints. Tissue-specific promoters have been isolated from sorghum and maize for expression in mature and immature sugarcane stem and roots. The promoter sequences have been incorporated into a GUS reporter gene vector and expression in sugarcane is being assessed histochemically and fluorometrically.

In an attempt to move away from using the *npII* antibiotic selectable marker gene, a positive selection system using the phosphomannose isomerase (PMI) system was evaluated in our laboratories (Gill *et al.*, 2004). Although expression of the *manA* gene confers a metabolic advantage on transgenic cells, enabling them to grow on mannose as a sole carbon source, results indicated that regeneration could only proceed if some sucrose was added to the growth medium.

The last suite of input traits that SASRI is focusing on, are those that relate to sucrose accumulation. Although some of the genes involved in sucrose metabolism have been identified and used to transform sugarcane (reviewed by Grof and Campbell, 2001; Moore, 2005), manipulation of cell sucrose content involves complex, and sometimes still poorly understood, pathways. Our understanding of these metabolic pathways by perturbation of specific pathways via up or down regulation of key enzymes using transgenesis has been furthered (Botha, 2007).

Germplasm conservation

Active field collections of sugarcane clonal material are necessary for day-to-day operations at SASRI. Approximately 3 ha of land and a glasshouse are dedicated to germplasm maintenance. However, base or back-up collections would be advantageous for museum lines used for plant breeding and for transgenic line preservation. Ideally these lines should not be conserved exclusively in the field or in the glasshouse because maintenance of plants *in situ* is labour-intensive, expensive and may result in loss of material due to environmental or biological hazards. *In vitro* storage techniques offer alternative strategies for germplasm conservation, storage and management (Withers, 1988).

Short-term storage (usually at 10–18 °C on media containing a reduced nutrient concentration) by minimal or slow growth of embryogenic callus and plantlets has been evaluated at SASRI. We have demonstrated that embryos can be stored for 12 weeks at both 18 and 24 °C on medium containing half-strength MS salts and vitamins (Murashige and Skoog, 1962) supplemented with casein (0.5 g/l), sucrose (20 g/l), 2,4-D (3 mg/l) and gelrite (2 g/l), pH 5.8. In addition, plantlets can be stored in Magenta vessels for 8 months at 18 °C on the same medium without growth hormone. The establishment of these techniques gives flexibility in terms of transferring lines to the glasshouse or field should environmental conditions not be suitable at the time, if space

is limiting, or if other constraints exist.

Micropropagation

Conventional propagation of sugarcane in the South African sugar industry is by vegetative means; a process which permits a tenfold propagation rate per annum. In this method, sugarcane stalks, referred to as ‘seedcane’, are planted horizontally in the furrow, with the new crop of plantlets being derived from buds at each node. In addition to the low propagation rates, the potential transmission of pathogens from the seedcane to the subsequent crop limits the efficiency of this method. Hence, the potential for *in vitro* micropropagation approaches to overcome both of these limitations has been explored. Research conducted at SASRI has demonstrated that *in vitro* culture on semi-solid media increases propagation rates 300 fold (Snyman *et al.*, 2007). In addition, further increases in propagation rates, up to 3,000 plants per leaf roll, can be achieved using liquid or temporary immersion culture (Meyer *et al.*, 2007). These plantlets have been successfully hardened off at SASRI and at two commercial seedling nurseries. Although the local sugar industry has not implemented such a scheme for widespread use, there has been interest in the development of propagation via somatic embryogenesis (Snyman *et al.*, 2000, 2001a, 2006), NovaCane®, for use in seedcane schemes.

Directed basic research: sugarcane physiology

For many years, the South African sugar industry has recognised that a thorough knowledge of sugarcane biology will ultimately provide novel strategies for improving the crop. As a result, the industry supports directed basic research projects, particularly those that aim to elucidate physiological processes underlying agronomically important characteristics of the crop. Consequently, considerable research effort has been focused on aspects of sugarcane physiology that permit the accumulation of sucrose in the stalk to concentrations of up to 650 mM (Welbaum and Meizner, 1990). Recent evidence from Australia indicates that sugarcane has the capacity to store sucrose well above this level (Wu and Birch, 2007), which adds credence to SASRI’s attempts to analyse and manipulate sucrose accumulation using molecular technologies. To a lesser extent, modern biotechnological approaches have also been used to characterise the plant-environment interactions, including pathogen infection, and soil aluminium phytotoxicity.

Sucrose accumulation

In the study of sucrose accumulation, SASRI has benefited from strong collaborative links with South African universities, particularly the Institute of Plant Biotechnology (IPB) at the University of Stellenbosch. These alliances have provided a suitable level of intellectual capital and expertise to advance

understanding of the complex biochemical, genetical and physiological processes underpinning sucrose accumulation in sugarcane.

Initial work focused on unravelling the processes in sugarcane that facilitate the dramatic increases in dry matter accumulation and sucrose concentration in the internodes just below the stalk apical meristem (Moore, 1995). This transition from a growing to a storage function, which is characteristic of internode maturation, has provided SASRI researchers with an ideal system for the identification of potential regulatory points in sucrose accumulation. The work of Whittaker and Botha (1997) demonstrated that internode maturation coincides with a partitioning of carbon towards storage, at the expense of allocation to insoluble matter and respiration. That seminal research provided first evidence of the identity of enzymes that may play crucial roles in allocating carbon towards sucrose storage. Since that time, these enzymes and the genes encoding them have been the subject of intensive study at SASRI and the IPB (Table 1), in whole plants (Whittaker and Botha, 1997; Rose and Botha, 2000, Botha and Black, 2000), tissue slices (Bindon and Botha, 2001; 2002) and, more recently, in cell suspension cultures (Roussouw *et al.*, 2007).

To complement the biochemical and physiological analysis of sucrose accumulation during internode maturation, SASRI established one of the world’s first sugarcane EST profiling programmes (Carson and Botha, 2000). That technology was later refined by the concomitant use of DNA subtractive hybridisation (Carson and Botha, 2002a,b; Carson *et al.*, 2002). The complexity of processes involved in stalk development and maturation was clearly revealed by that work, which informed the direction and scope of SASRI’s subsequent strategies. To provide a desired focus, later profiling research deployed ‘boutique’ arrays, which bore defined sets of ESTs

Table 1. Selected enzymes identified as playing a key role in sucrose accumulation in the sugarcane stalk. Enzymes listed have been subjected to extensive molecular and kinetic characterisation (Characterisation) and assessment of tissue- or organ-specific expression and activity (Localisation)

Characterisation	Enzyme	Reference
	Pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP)	Groenewald and Botha (2007b); Whittaker and Botha (1999)
	Sucrose synthase	Schäfer <i>et al.</i> (2005; 2004a)
	Fructokinase	Hoepfner and Botha (2004)
	UPD-glucose dehydrogenase	Turner and Botha (2002)
	Neutral invertase	Vorster and Botha (1998)
Localisation	Sucrose synthase	Schäfer <i>et al.</i> (2004b)
	Neutral invertase	Bosch <i>et al.</i> (2004); Rose and Botha (2000); Vorster and Botha (1999)
	Fructokinase	Hoepfner and Botha (2003)
	Sucrose phosphate synthase	Botha and Black (2000)
	Sucrose synthase	Botha and Black (2000)

with known involvement in sucrose metabolism and transport (Watt *et al.*, 2005). That approach revealed that, during internode maturation, a decrease occurred in transcript abundance of genes participating in cell wall biosynthesis (including UDP-glucose dehydrogenase), triose phosphate metabolism and sugar-mediated signalling. In contrast, significant increases were revealed in the expression of sucrose synthase and sucrose phosphatase. Those EST profiling studies were invaluable as they provided: (1) further evidence of potential regulatory points in sucrose accumulation, confirming results obtained from biochemical and physiological studies (Table 1); (2) sequence information for accessing gene promoters with desirable expression patterns; and (3) a first indication of the potential importance of signalling and transport in the control of sucrose accumulation.

Information on the biochemical, physiological and gene expression changes associated with internode development revealed the identity of a small set of enzymes playing a potentially key role in sucrose accumulation. For example, pronounced sucrose cycling was revealed as an important component of the accumulation process, mediated in part by the presence of three invertase isoforms (Vorster and Botha, 1998; Rose and Botha, 2000; Bosch *et al.*, 2004), as well as other enzymes, including sucrose synthase (Schäfer *et al.*, 2004b). To examine the role of selected enzymes, transgenic lines in which activity has been reduced were produced by means of RNA interference technology (Table 2). The downregulation of neutral invertase in sugarcane cell suspension cultures resulted in a decreased rate of sucrose hydrolysis, suggesting a reduction of carbon flux through the 'futile' cycle (Rossouw *et al.*, 2007). The impaired growth of those cultures suggested reduced availability of hexoses to support respiration. In contrast, the inhibition of expression of pyrophosphate:fructose 6-phosphate 1-transferase (PFP) resulted in no visible phenotypic abnormalities (Groenewald and Botha, 2007a). However, sucrose concentrations were significantly increased in the immature internodes, although levels of the sugar remained unaltered in mature region of the stalk. The results suggest a role of the enzyme in regulating the flow of carbon into glycolysis, a potentially important step in allocation of sucrose to storage. The outcomes from these two studies clearly reflect the power of SASRI's transgenic

approach in the investigation of sucrose accumulation.

Analysis of the compartmentation of the enzymes, metabolites and transporters of sucrose metabolism in sugarcane is challenging due to the physical nature of the stalk, which is the site of storage. The hardness and high fibre content of the tissue make it extremely difficult to isolate intact intercellular components, including vacuoles. To circumvent this limitation, SASRI, in collaboration with the University of Stellenbosch, developed a kinetic model to assess the intercellular compartmentation and allocation of sucrose (Rohwer and Botha, 2001). Recent refinement of that initial model suggests that sucrose does not accumulate in the cytoplasm of the storage parenchyma, but in another intracellular compartment (Uys *et al.*, 2007). The revised model further indicates that the flux between sucrose storage and hydrolysis may not change markedly during internode maturation. This kinetic model represents a powerful tool at SASRI's disposal for the analysis of the mechanisms underpinning sucrose accumulation.

Initial gene expression profiling research at SASRI indicated that sucrose transport and sugar-mediated sensing and signaling might be an important component of the sucrose accumulation process in sugarcane (Watt *et al.*, 2005). As a result, a study was initiated at SASRI in 2003 to assess the role played by source-sink feedback and determine the molecular mechanism mediating the relationship. That novel work was based on the analysis of physiological and gene expression responses of the plant to source-sink perturbations, which were induced by partial defoliation, leaf shading and cold-girdling. For the first time in sugarcane, communication of stalk sucrose status to the leaf was revealed, a relationship that results in the depression of photosynthetic activity with increasing sucrose accumulation in the stalk (McCormick *et al.*, 2006). Investigation of sugar sensing and signaling pathways mediating source-sink relationship (McCormick *et al.*, 2007) has provided information that will be used ultimately to formulate strategies to uncouple this potentially yield-limiting interaction.

Plant-environment interactions

SASRI has employed modern biotechnological approaches to examine sugarcane interactions with biotic and abiotic factors in the environment that are known to affect crop productivity. Research of this nature has included analysis of interactions with the fungal pathogen causing sugarcane smut (*U. scitaminae*) (Thokoane and Rutherford, 2001) and phytotoxic levels of aluminium (Watt, 2003). Examination of the response of sugarcane to these environmental cues has embraced a variety of technologies, including cDNA-AFLP differential display (Thokoane and Rutherford, 2001) and suppression subtractive hybridisation (Heinze *et al.*, 2001; Watt, 2003). Although that work yielded useful information on sugarcane biology, the primary goal was the isolation

Table 2. Selected enzymes of sucrose metabolism that have been manipulated through transgenesis

Enzyme	Nature of modification	Reference
Pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP)	Down-regulation	Groenewald and Botha (2007a)
Neutral invertase	Down-regulation in cell suspension cultures	Roussow <i>et al.</i> (2007)
UDP-glucose dehydrogenase	Down-regulation	Bekker (2007)

'perfect' genic markers linked to tolerance or resistance to the abiotic or biotic stressors (Heinze *et al.*, 2001). It is of note that markers depicting resistance to sugarcane smut have been used for parent selection in SASRI's breeding activities.

Molecular and *in vitro* technologies in crop protection

Pathogen epidemiology and the use of molecular methods as an aid to disease management

A large range of pathogens is able to infect sugarcane with more than 40 being identified in South Africa. Many are considered to be of minor importance but diseases such as mosaic, smut (*U. scitaminea*), ratoon stunt (*Leifsonia xyli* subsp. *xyli*), and brown rust (*Puccinia melanocephala*) result in serious losses when outbreaks occur. Yellow leaf (*Sugarcane yellow leaf virus* (ScYLV)) is widespread in the northern irrigated parts of the industry (Rutherford *et al.*, 2004) and recently, maize streak (*Maize streak virus*) was identified in a newly released cultivar, the first report of this disease in sugarcane in South Africa.

General disease management strategies include cultivar resistance, seedcane health and effective crop eradication. Early detection and accurate identification of pathogens is key to successful disease management and molecular techniques are playing an increasingly important role in this regard. A suite of molecular techniques, including Polymerase Chain Reaction (PCR)-RFLPs, reverse-transcription (RT)-PCR, inter-simple sequence repeats (ISSRs) and DNA sequencing, is now available for the detection and characterization of pathogenic viruses, bacteria, fungi and phytoplasmas that occur in sugarcane. Conserved genes are generally used to identify the genus or species involved and less conserved genes are used for investigating genetic diversity within species.

Brown rust was, for many years controlled through cultivar resistance. In 2000, severe infections were observed in a number of varieties that were considered to have acceptable resistance, suggesting the development of a new race of *P. melanocephala*. This was investigated by amplifying the large ribosomal sub-unit of the genome and sequencing of the cloned products. Successfully aligned sequences confirmed that genetic diversity existed in the different samples of *P. melanocephala* collected (Pillay *et al.*, 2005).

Properly managed seedcane schemes to ensure a reliable supply of healthy seed for replanting are once again gaining favour with sugarcane growers. Disease diagnosis in nurseries based on visual symptoms alone can be unreliable. For example, ratoon stunt produces no obvious external symptoms and is routinely diagnosed using serological techniques or phase contrast microscopy. Similarly, leaf scald, yellow leaf and mosaic infections can be latent. Molecular methods such as PCR or RT-PCR using specific primers are now being used to complement or replace the more traditional methods of diagnosis. When unusual disease symptoms are observed, as

was the case with maize streak, universal primers for the different groups of pathogens are used to amplify RNA or DNA within the plant. Any organisms present can then be identified by sequencing the amplified products, facilitating the process of identifying the causal organism.

Plant parasitic nematodes are a serious constraint to sugarcane productivity in South Africa, particularly on the sandy soils (Spaull, 1995). Compared to most other cultivated crops, the community of plant parasitic nematodes associated with sugarcane is diverse. Surveys in South Africa have shown that the number of genera present in the soil ranges from three to nine, with an average of six genera per sample. The most commonly occurring genera are *Helicotylenchus*, *Meloidogyne*, *Paratrichodorus*, *Pratylenchus*, *Scutellonema* and *Xiphinema* (Cadet and Spaull, 2005).

Traditional methods of nematode diagnosis have involved the use of morphological characteristics. This works well, provided that staff are trained and experienced in such diagnoses. However, newer molecular biological methods exist that promise to increase sample throughput, are more sensitive and can theoretically be performed by 'non-Nematologists'. This also has potential promise for nematodes with highly conserved morphological features (such as species of *Helicotylenchus*). Due to the diversity of nematodes associated with sugarcane in South Africa, development of molecular techniques has been more of a challenge than for other crop-nematode combinations elsewhere in the world.

PCR-based methods for identifying plant parasitic nematodes of sugarcane have been developed (Berry *et al.*, 2007). These methods, which target the ITS1 region, enable discrimination of selected genera and species by discernible differences in fragment sizes. In addition to utilizing variation in size of the ITS1 region, which can be limiting between certain genera, specific diagnostic primers have been developed from analysis of ITS1 sequence data (Berry, 2007). Sequence diversity within most species was low except for *M. javanica* and *P. zaeae*. Variation was usually greater between species than within species. Attempts were made to develop primers to seven species: *H. dihystra*, *M. javanica*, *P. lobatus*, *P. sacchari*, *P. zaeae*, *S. brachyurus* and *X. elongatum*. However, of these, only three were specific enough for continued use. The *M. javanica* primer detected not only this species but also other species of *Meloidogyne*. This increased detection can be considered an advantage as two species of *Meloidogyne* (*M. javanica* and *M. incognita*) have been found associated with sugarcane worldwide. The *P. zaeae* and *X. elongatum* primers were specific only to the species-of-interest. These are the two main species of *Pratylenchus* and *Xiphinema* respectively associated with sugarcane in South Africa.

However, conventional PCR does not enable the quantification of nematode genera and species in soil and root samples. At best, conventional PCR is semi-quantitative. With increased emphasis on ecological management of plant parasitic populations it is essential not only to know which

genera/species are present in a sample but also their relative proportions. Real-time (quantitative) PCR using SYBR Green I dye can provide such a method to not only accurately and sensitively detect nematode species or genera but also to quantify them.

To increase efficiency and reduce cost, multiplex reactions (i.e. the detection of multiple nematode species within a single tube) were tested. However it was found that detection of individuals in mixtures of two or more species in the same tube resulted in competition between species. Thus simplex reactions (i.e. the detection of single nematode species in single tubes) were adopted for further use. Calibration curves for each of the three species, *M. javanica*, *P. zeae* and *X. elongatum*, were constructed and these then used for the quantification of these species in nematode samples extracted from the field. These were strongly correlated with counts carried out with microscopy.

Future work will involve investigating factors that will improve the accuracy of detection. These include: measuring potential inhibiting factors that could lead to false negative results, investigating the upper and lower limits of detection and incorporating these molecular diagnostic techniques into the current laboratory structure.

Quarantine and phytosanitation

SASRI exchanges sugarcane cultivars with many different countries, including Australia, USA, Colombia, Brazil, Barbados and Zimbabwe, mainly to increase the genetic pool for breeding new varieties. Cultivars are subsequently evaluated for their release into commercial production. However, the movement of sugarcane cultivars between countries can cause serious disease risks and therefore needs stringent testing and monitoring. The current Quarantine facility was set up at SASRI, Mount Edgecombe in 1984 to replace the original glasshouse that was located in the Botanic Gardens, Durban which had been operational since 1924. The current facility can be described as a world-class laboratory where molecular techniques are used for the accurate detection of the most important sugarcane pathogens.

The release of imported varieties from Quarantine over the past two years has been seriously hampered by the frequent presence of ScYLV (van Antwerpen *et al.*, 2005). A new tissue culture facility for the 'cleaning' of varieties from diseases such as ScYLV, SCMV and unknown viral diseases was added to the Quarantine building. Meristem tip culture is an *in vitro* culture technique used for pathogen elimination. This enables SASRI to clean imported sugarcane varieties from most pathogens so that disease-free plants can be used in breeding programmes. It also ensures that SASRI is in a position to export healthy, tissue culture-derived plants of SASA 'N' cultivars instead of conventional setts in the future. All imported varieties are processed through this laboratory to eliminate most pathogens from plants. Micropropagated plants

are indexed for diseases before they are exported or moved to the post-quarantine area.

Since 1998, great improvements were made in the detection of disease causal agents in the Quarantine glasshouse with the introduction of molecular diagnostic tests. Current diagnostic tests include PCR for the detection of ratoon stunt (van Antwerpen and Botha, 1999) and *Sugarcane bacilliform virus* (Braithwaite *et al.*, 1995); RT-PCT for the detection of SCMV, *Fiji disease virus* (FDV) (Smith *et al.*, 1994) and ScYLV; and RT-PCR and RFLP for the detection of sugarcane yellows phytoplasma (SCYP), sugarcane white leaf and grassy shoot. PCR and selective plating are used for the detection of pathogens causing leaf scald (*Xanthomonas albilineans*). PCR can also be used for the detection of *U. scitaminea*. In addition, plants are inspected weekly for visible disease symptoms or nutritional problems.

Plant and pest interactions

The pyralid moth *E. saccharina* Walker is an indigenous pest of sugarcane and maize widely distributed throughout sub-Saharan Africa. One of the first steps in research towards the successful implementation of any Integrated Pest Management system is to accurately identify the pest and its origin.

A phylogeographic study on *E. saccharina* from eleven countries of Africa has clearly shown its population structure within the continent (Assefa *et al.*, 2006). A portion of mitochondrial DNA, corresponding to the Cytochrome Oxidase subunit I region, was sequenced to clarify phylogenetic relationships between geographic populations. Mitochondrial DNA data of this type can reveal cryptic lineages representing distinct species or subspecies within apparently morphologically homogeneous organisms. *E. saccharina* separated into four major populations i.e. West African, Rift Valley and two southern African populations. Sequence divergence between the four populations ranged from 1% to 4.98%, enough to suggest that there are cryptic species. The molecular data are congruent with 'isolation by distance' except for some of the specimens from eastern and southern Africa where geographically close populations are nevertheless genetically distant.

Cytoplasmic incompatibility, one of several phenotypes induced by the maternally inherited intracellular bacterium *Wolbachia*, is a possible mechanism for initiation of speciation where populations overlap geographically (Telschow *et al.*, 2002). A study conducted by Jeyaparakash and Hoy (2000) suggested that up to 70% of all insect species might be infected. To investigate further the phenomenon of cryptic speciation within *E. saccharina*, PCR of a *Wolbachia* specific surface protein gene (*wsp*) has revealed the presence of *Wolbachia* in *E. saccharina*.

Recent evidence suggests that isolates of the fungus *Fusarium* colonising sugarcane borings can affect the

development and fecundity of *E. saccharina*, most likely via the production of secondary metabolites such as insecticidal toxins (e.g. fusaproliferin and beauvericin). A project designed to investigate the diversity of *Fusarium* associated with sugarcane resulted in a collection of 223 isolates. Of these, 117 were isolated directly from borings, while 65 isolated from surface sterilised undamaged cane were considered to be endophytic. The remaining 41 were isolated from cane showing symptoms of pokkah boeng, a disease reputedly caused by *F. verticillioides* and *F. subglutinans*. Attenuated isolates incorporated into *E. saccharina* diet, distinguished between those that were beneficial or antagonistic to the development of *E. saccharina*. The proportion of the different secondary metabolites produced could be critical to the effect that various *Fusarium* isolates have on *E. saccharina* development (McFarlane and Rutherford, 2005).

Identification of isolates was performed by PCR using two different primer sets to amplify fragments from genes coding for the translation elongation factor-1 and β -tubulin proteins according to the method described by O'Donnell *et al.* (1998). The isolates were grouped and assigned a preliminary identification by RFLPs of the resulting PCR products. Four groups of fusaria were obtained using RFLP analysis and could be tentatively identified based on their banding patterns. Most common was *F. sacchari*, but *F. proliferatum*, *F. verticillioides*, *F. napiforme* and *F. subglutinans* were also identified.

Soil and plant health monitoring

Farms in the South African sugarcane industry cover a diverse range of soil types, ranging from the deep, sandy Namib form to the shrink-swell, waterlogged Rensburg form. Most, if not all of these soils, have been under long-term monoculture. Associated agricultural practices have adverse effects on the health of the soil (Dominy *et al.*, 2001). Hence, there is a need to identify soils that are poor in health and provide recommendations to growers to improve the soil health status.

Soil health provides an overall picture of the soil functions. Soil health may be considered as the state of soil at a particular time and is influenced by the dynamic properties such as the number and diversity of microorganisms that can show rapid changes in the short term. Although soil health cannot be measured directly, soil chemical, physical and biological indicators can infer the health of a soil. At SASRI, assessments of soil physical indicators are made on texture, depth of topsoil, rooting depth, bulk density, air filled porosity, intake rate, water retention and drainage. The chemical indicators include total organic carbon, pH, electrical conductivity, N_2 mineralization potential, and macro or micro nutrient analysis (Barnard *et al.*, 1990). Biological indicators of soil health that are measured are microbial biomass, microbial enzymes and microbial diversity. Microbial biomass is measured using chloroform fumigation (Graham *et al.*, 2001). Phospholipid fatty acid analysis is used to study the changes occurring in the entire

microbial community structure (Graham *et al.*, 2001), while dehydrogenase activity is measured to determine the metabolic state of the soil microbial population (Beyer *et al.*, 1992).

The study of microbial diversity is not easy because only 1% of the soil microbial population can be cultured by standard laboratory practices (Torsvik *et al.*, 1998). Therefore SASRI's approaches to study soil microbial diversity include, for culturable microorganisms, direct counts, selective isolation plating (Vogel *et al.*, 2002, Guyon *et al.*, 2003; van Antwerpen *et al.*, 2002, 2007), and carbon utilisation patterns using the BIOLOG™ assay. Beneficial microorganisms such as *Trichoderma*, *Burkholderia*, *Actinomyces* etc. are enumerated and it is considered that the higher the numbers of beneficial microorganisms are, per gram of soil, the healthier the soil.

Genetic diversity of culturable bacteria is mostly studied by the diversity of the 16S rDNA genes and 16-23S spacer region, which occur in all bacteria and which show variation in base composition among species. Studying the genetic variation of culturable bacteria provides an indication of the profile of the culturable microbial community. Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Vogel *et al.*, 2002) is one technique that employs PCR with a primer set that targets the 16S rRNA gene of isolated bacteria. Amplified bands are then restricted with a combination of two or more endonucleases and the resulting restriction patterns obtained are used to evaluate the genetic diversity of the cultured soil bacteria. The 18S rDNA gene and 18S- 5.8S- 23S spacer regions are used for detection of fungi and protozoa.

Microbial diversity of non-culturable microorganisms is studied using Denaturing Gradient Gel Electrophoresis (DGGE) (van Antwerpen *et al.*, 2007). The benefit of this approach is that a molecular fingerprint of the bacterial or fungal community structure is generated for each soil. In fact, each band in each lane of the gel theoretically represents a different bacterial or fungal species. In addition, this technique enables the excision and subsequent sequencing of bands, allowing species identification using existing databases. With DGGE, SASRI scientists can compare the microbial community profiles of poor yielding soils to good yielding soils. Monitoring the microbial communities of soil enables us to rate the health of soil in order to advise sugarcane growers on suitable agricultural practices to maintain or improve the health of their soils and thereby to reverse the impact of long-term sugarcane yield decline.

SASRI's approach to plant health is by protecting plants from pathogens and pests. Plant-associated microorganisms are used in the biological control of plant parasitic nematodes, bacterial and fungal pathogens (Rutherford *et al.*, 2002; van Antwerpen *et al.*, 2002; Guyon *et al.*, 2003; Omarjee *et al.*, 2006). Beneficial bacteria and fungi intimately associated with sugarcane are screened *in vitro* against these pests and pathogens (van Antwerpen *et al.*, 2002; Omarjee *et al.*, 2004). Those microorganisms with potential for biological control are inoculated on sugarcane under field conditions. The

colonisation and persistence of potential biocontrol microorganisms, post inoculation, is monitored using Capillary Electrophoresis Single Stranded Conformation Polymorphism (CE-SSCP) (Omarjee *et al.*, 2006). Here, the rRNA genes in total DNA extracted from rhizosphere soil or plant tissue is subjected to PCR amplification with fluorescent primers of the respective genes and analysed using an automated DNA sequencer. A single nucleotide difference in the amplified region is sufficient to obtain different patterns with CE-SSCP allowing easier detection of the inoculated strain.

Endophytic bacteria that play key roles in promoting sugarcane health and growth (Govindarajan *et al.*, 2006) are also studied. Local sugarcane is screened for the colonisation of *Burkholderia tropica* and *B. unamae*, two N₂ fixing bacteria that are known to colonise the stalks and roots of some varieties of sugarcane. Species-specific PCR is conducted to detect these microorganisms in plant tissue. Knowledge on the colonisation of these endophytes provides an indication of which cultivars are more dependent on N₂ fertilizer than others (Omarjee *et al.*, 2006).

With the use of molecular and analytical techniques SASRI specialists are able to monitor changes in soil health. With that in mind, we are able to develop appropriate economically viable management practices through research that can restore soil and plant health and enhance sugarcane profitability.

Conclusions

SASRI strives to be the recognized global leader in innovative sugarcane research at the forefront of a thriving industry. This vision has been furthered by the rapid advances in molecular biotechnology which have revolutionized the approach to sugarcane improvement over the last decade. The future impact of biotechnology on sugarcane offers the potential to further our understanding of the physiology of sucrose accumulation, to deliver alternative high value products, to produce renewable transportation fuel and green energy, and to breed for cultivars to encompass novel industry directions.

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