PROGRAM 2

New Product Development from Sugarcane (Bioproducts)

This program will produce high-value biomaterials from sugarcane either as alternative products to sugar or as co-products

PROGRAM 2 OVERVIEW

OVERVIEW

Recent research into producing high-value biomaterials from plants has confirmed sugarcane as an excellent plant option. The need to find alternatives other than oil to produce bioplastics and biofuels also offers Australia's sugarcane industry new and potentially profitable business opportunities.

These biomaterials can be produced either in sugarcane as alternative products or co-products with sucrose (through R&D from the Biofactory subprogram) or by downstream processing of pre-existing compounds harvested from sugarcane (from the Biorefinery subprogram). To develop these new materials, the industry will rely on this CRC's underlying research knowledge, scientific platforms, enabling technologies and strategic alliances.

PRODUCTION OF HIGH-VALUE MATERIALS

The Biofactory subprogram aims to establish sugarcane as the preferred host for plant-based production of specific high-value materials and ensure Australia leads the field with associated IP-protected technologies. It brings together different technologies for producing high-value alternative sugars and biopolymers in sugarcane. Its strategic research will inform selection or engineering of high-value sugarcane varieties. High-value varieties potentially produce higher yields or improved compositions of materials that assist downstream biological or chemical conversions into specific value-added products.

Sugarcane's high photosynthetic efficiency and ability to generate high quantities of stored sucrose, cellulosic fibres, lignins and surface waxes make it an ideal vehicle for generating these downstream products. Its capacity to store soluble products and the composition of its lignins and epicuticular waxes also make it an ideal industrial crop for synthesising several types of high-value products. Because it is vegetatively propagated, sugarcane's genetic make-up does not change, making bio-product development extremely reliable. Opportunities also exist for the co-generation of electricity needed to extract and process from bagasse (the fibre or biomass left over after liquid sugar has been extracted from the cane).

There is a growing market for plant-derived food additives and nutraceuticals. Some high-value waxes and flavonoids are already biosynthesised by sugarcane and are likely to be produced by the industry soon. The opportunity exists for the industry to establish as a leader in supplying this growing market if economic co-production levels and separation processes can be established.

The CRC's approach to this challenge combines:

- application of gene technologies to understand the biosynthesis of these materials, enhance production levels and shift production to economically preferred forms; and
- development of growing, harvesting and extraction technologies for optimal recovery of these materials, as well as sucrose.

TAKING ADVANTAGE OF RENEWABLES

While production of high-value materials in plant biofactories is a strategy for the future, production of materials from available renewable resources by chemical and/or biological conversion is well established. Ethanol, organic acids (lactic acid, citric acid), amino acids (lysine, glutamate, phenylalanine), sugar alcohols (e.g. sorbitol) and many fine

PROGRAM 2 OVERVIEW CONTINUED

chemicals (e.g. enzymes, penicillins) are examples of products derived from renewable resources.

The Biorefinery subprogram seeks to show the potential of taking greater advantage of sugarcane streams for large-scale production of such materials and identify similar new high-value products for the industry.

Fermentation facilities are usually sited next to sugarcane mills. This offers the advantages of an efficient supply of biomass from the sugar milling stream and electricity co-generated from bagasse, as well as use of dunder, the dregs of cane juice, to fertilise surrounding cane fields. Substantial scope exists to generate IP related to increased extraction efficiencies and conversion to high-value downstream products from the chemical and process engineering and molecular biotechnology expertise in this CRC.

In partnership with the sugarcane industry, the CRC is developing the base for producing value-added materials from sugarcane-derived feedstocks or harvesting, milling and refining by-products and wastes. Examples include alcohols, biofuels, fibre products, biopolymers, biosurfactants, industrial enzymes and renewable biomaterials to replace industrial petrochemicals used in plastics manufacture.

This CRC is developing the base for producing value-added materials

2008>09 HIGHLIGHTS

- Two Program 2 projects moved into the commercialisation phase. IP related to lignin based barrier coats is being commercialised in-house, while IP for production of high molecular weight hyaluronic acid has been licensed to UniQuest.
- The CRC was partner in a successful grant application – Korea-Australia Bio-Product Alliance – with Korea Advanced Institute of Science and Technology (KAIST) and The University of Queensland. The project will see nearly $7.7m invested in Korea and Australia for continued development of technologies underpinning sucrose based biorefineries.
- Two provisional patents have been filed for PHA production in sugarcane.
- Field trials confirmed that three different transgenic technologies can generate commercially useful sugarcane lines.
- The CRC lodged a provisional patent application in April 2009 for a potentially useful pharmaceutical molecule showing inhibitory activity against enzymes involved in digestion of complex carbohydrates.
- Researchers produced a natural supplement with strong potential to modify glycemic index (GI).

PROGRAM 2 CONTINUED

PROGRAM 2 PROJECT SUMMARIES

TESTING SUCROSE ACCUMULATION

Understanding how sucrose accumulates in sugarcane will underpin future strategies for increasing sucrose or other high-value products in sugarcane.

The aim of the research is to develop improved transformation methods especially for smut-resistant varieties, identify and test key genes in the sucrose accumulation process by manipulation in transgenic sugarcane, test promoters believed to target expression most strongly to the storage parenchyma and complete the PhD training of three PhD students. This project is focused on intellectual property (IP) capture and the provision of a suite of tools to improve the production of transgenic sugarcane plants.

This year produced several significant achievements

The CRC SIIB promoter has been found to drive tissue-enriched expression in storage parenchyma of the maturing stem when compared to vascular bundles and rind. A detailed report was submitted on the promoter studies to the CEO and Commercialisation Manager.

Cohorts of transgenic plants have been developed to down-regulate various sugar transporters and a bZip transcription factor to identify genes that could have a major effect on sugar accumulation. Transcript levels have been assessed in the T0 cohorts for all chosen transcripts, with down-regulated plants identified for all of them. Tissue samples have recently been taken from the two cohorts for transcript, sugars and fibre analysis. In this work, the CRC has successfully demonstrated the possibility of using RNAi-mediated gene silencing to alter the expression of genes involved in core metabolism in sugarcane, which was previously unknown.

Transformation of at least one smut-resistant variety of sugarcane has been achieved, however, transformation efficiencies would still require optimisation which is beyond the scope of this project. The successful testing of the PAR001 promoter fulfils a major objective of this project and contributes significantly to Outcome 1 as listed in the Project Schedule. The use of RNAi-mediated gene silencing of the sucrose metabolism-related genes has been successful but it remains to be seen whether the reductions achieved in the transcript levels correlate with a change in phenotype.

The use of the PAR001 promoter to drive transcript expression in the storage parenchyma has been submitted to our intellectual property assessment panel for their deliberations.

PHA production in sugarcane

Polyhydroxyalkanoates (PHAs) is a family of natural polymers that can replace plastic produced from non-renewable resources. The commercial partner on this project, Metabolix, is already establishing large-scale microbial production from corn starch. Production of PHAs in sugarcane has the potential of significantly reducing cost of production.

This project focuses on determining where and how to best produce PHA in sugarcane. Sugarcane lines that produce PHA only in the leaves have been generated. Lines that produce PHA in plastids (subcellular compartments where light is converted into sugar) at commercially promising levels have been successfully grown under glasshouse conditions. Extensive characterisation revealed that these lines are substrate limited.

In order to guide further engineering, a genome scale metabolic model has been developed for sorghum, a close relative to sugarcane for which the entire genome has been sequenced. Use of a chemical ripener to inhibit competition for a key substrate has been found in tissue culture to increase the level of PHA threefold in the treated lines over non-treated controls. This strategy is currently being tested in mature plants.

In an alternative strategy, PHA production has been targeted to peroxisomes (the subcellular compartment where lipids are recycled) resulting in PHA accumulation greater than 2% of leaf dry weight. This high level of accumulation exceeds the space available in peroxisomes, which is possible because PHA is diverted to the storage vacuole, which greatly increases the space for PHA accumulation inside plant cells. A provisional patent has been submitted covering this strategy and dual targeting to plastids and peroxisomes is currently being evaluated.

PLASTID TRANSFORMATION

This project aims to transform sugarcane plastids to allow them to grow valuable bio-products such as bioplastics.

Plastids are the organelles of the cell where photosynthesis normally occurs. Because of the large number of plastids present in every plant cell, transformation of the plastids results in production of high amounts of transgenic protein. Also, as plastids are not transferred to the progeny via pollen, the risk associated with cross-pollination of transgenic with non-transgenic crops is removed.

However, to transform plastids, special vectors (vehicles which carry DNA of interest into the plant cell) have to be made. The design of the plastid transformation vector is based on the principle that there will be an exchange of similar DNA sequences during plastid division. Thus, the vector used for plastid transformation contains the gene of interest, which is flanked by sequences that are the same as a region of the plastid genome.

Researchers are assessing two types of plastid vectors using the principle outlined above.

1) Regulatory regions of the sugarcane plastid genome have been evaluated for gene expression using the green fluorescent protein (gfp). The sequences resulting in highest expression have been identified and used in producing transformation vectors. Some putative transgenic sugarcane plants have been produced using these transformation vectors and are being characterised at the molecular level.

2) Gene expression of the plastid-encoded genes of sugarcane has been studied using a technique called macroarray analysis. Novel plastid genes expressed constitutively and in a tissue specific manner have been successfully identified using this technology. Regulatory sequences of these genes

PROGRAM 2 CONTINUED

have been determined and transformation vectors are being prepared.

As well as the vectors, researchers have studied sugarcane plastid division and tested factors that could affect transformation efficiency (for example type of particles, shooting pressures as well as timing and intensity of selection pressure applied). Putative transgenic plants produced with these different variables are being characterised.

FIELD EVALUATION OF GM SUGARCANE

This project aims to assess the field performance of GM sugarcane produced by three different transgenic technologies: agrobacterium-mediated transformation, conventional plasmid-based and the new minimal DNA-based biolistic methods.

Trials harvested this year confirm that these technologies can generate commercially useful lines, but because of high structural variation rigorous evaluation is needed to identify the best lines.

In 2007, 82 transgenic lines with appropriate controls were planted in the field at BSES Woodford Pathology Station. This partially replicated trial produced a very good crop. A sample of this population, a total of 32 lines representing all treatments selected based on growth and yield in the propagation stage, was planted in BSES Woodford in August 2008. This trial was harvested in the end of July 2009. In general, the negative effect of transgenesis observed in the propagation stage was reduced in the current trial. Cane and sugar yield (TCH and TSH) and morphological measurements taken at harvest indicate that a significant proportion, about 20%, of transgenic lines was comparable to wild type, suggesting that practically useful clones can be generated with the current technologies. It is, however, important to note that nearly 50% of the transgenic lines showed morphological variation with reduced stalk thickness being the most affected. Tissue samples collected from this trial are being anlaysed for transgene expression. The results obtained so far emphasise the need for rigorous clonal evaluation to identify useful lines.

BIOACTIVE NATURAL PRODUCTS FROM SUGARCANE

In 2008>09, two commercial opportunities were identified in the discovery of bioactive molecules from the sugarcane leaf.

The first was IP in the form of a provisional patent application (lodged in April 2009) that is of interest in terms of the structure/function of molecules displaying potent inhibitory activity against enzymes involved in the initial digestion of complex carbohydrates.

The second was production of a natural supplement for use as a glycemic index (GI) modifier that could be sold as an ingredient for functional foods or sold independently to be consumed with food. The production of a supplement would come from the sugarcane processing waste stream (sugarcane leaf and bagasse), which potentially adds significant value to the sugarcane industry.

A laboratory-scale process has been developed to produce the natural supplement as extracts from the sugarcane leaf and bagasse that has an enhanced GI lowering activity. The process has been scaled up and identification of biomarkers for the active extract is ongoing.

Researchers isolated and identified inhibitory compounds and submitted a provisional patent application. A simple process for producing ethanol extracts from sugarcane leaf (SCL) and bagasse with enhanced enzyme inhibitory activity has been developed.

Bagasse extract showed higher activity than SCL extract, and was deemed a better source of the potent extract. Compared to acarbose, bagasse extract is a 4.5 times greater inhibitor of α-amylase and more than 125 times greater against α-

glucosidase. The identification of biomarkers for the active extracts is ongoing and the process has been scaled up.

An Australian provisional patent application was lodged in April 2009 entitled 'Compounds affecting glycemic index' in the name of Sugar Industry Innovation Pty Ltd, the CRC SIIB's intellectual property holding company.

ALTERNATIVE SUGARS

This project aims to identify technologies to produce alternative sugars for food applications or as industrial chemicals. In the past year researchers have cloned and expressed enzymes with the potential to modify sucrose and create new products. The applications of these novel sugars in the food or chemical industries will depend on their physical and sensory properties. Sweeteners ideally need to have similar taste and properties to sucrose but with improved health benefits. CRC researchers have developed laboratory methods to test industry-relevant properties of sugars, including sweetness, crystallisation, digestibility and cariogenicity (ability to form dental caries). The results highlight structure–function relationships among sugars.

Assays for sweetness, digestibility and cariogenicity have been used to test two of the candidate sugars as well as a panel of commercially available sugars. Sweetness relative to sucrose has been determined by a two-way preference ingestion assay with *Drosophila melanogaster* (fruit flies). Digestion by oral and intestinal enzymes *(invertase* and *alpha-glucosidase*) was assayed to determine whether the sugars would be calorigenic. Growth of an oral isolate of *Streptococcus mutans* was used to measure the potential for the sugars to promote tooth decay. Both candidate sugars tested so far appear to have the properties required for an alternative sugar product. These properties match the activities of commercial sweeteners. The remaining two sugars are still being produced enzymatically and will be tested as soon as possible.

The first output from this project was a scoping study to assess candidate sugars for their technical feasibility, IP and market opportunities. The project is on track to deliver the second output – IP for production of sugars. The project will also deliver information on the structure–property relationships among sugars and know-how/methods for analysing sugar properties on a laboratory scale.

The candidate sugars and processes underlying their production represent IP of potential value to the CRC. However, the commercial value of this IP can only be assessed once the properties of the sugars and their potential applications have been determined. These properties are still being assessed by methods developed in the project.