

Genetic engineering of sugarcane for increased sucrose and consumer acceptance

by
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Declaration

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Abstract

Sugarcane is a crop that is farmed commercially due to the high amounts of sucrose that is stored within the mature internodes of the stem. Numerous studies have been done to understand sugar metabolism in this crop as well as to enhance sucrose yields. Until now sugarcane improvement strategies have been implemented through either breeding programs or transgenic manipulation. Public mistrust and regulatory hurdles, however, have made the commercialisation of transgenic crops difficult, expensive and time-consuming.

In this thesis two projects will address issues relating to the above. The first will address an effort to increase sucrose accumulation within the sugarcane culm. This was attempted via the expression of an *Arabidopsis thaliana vacuolar pyrophosphatase (AtV-PPase)* gene, linked to the maize *ubiquitin* promoter, in sugarcane callus. It was anticipated that increased activity of the tonoplast-bound AtV-PPase will result in increased sucrose accumulation in the vacuole. Transgenic sugarcane callus lines were tested for soluble sugar content which suggested no significant increase in sucrose content. However, this may change upon further assessment of sugarcane suspension cultures and glasshouse plants.

The second project was concerned with the development of a novel sugarcane transformation technology that utilises only sugarcane sequences. This 'cisgenic' approach to sugarcane transformation will require a native sugarcane promoter, terminator, vector backbone and selection marker. It was attempted to first isolate a

functional promoter as well as developing a selection system based on an endogenous selection marker.

A promoter was amplified from sugarcane, using primers designed on a sorghum template, and its expression assessed using a *GFP* reporter gene. Unfortunately expression could not be confirmed in transgenic sugarcane callus. Currently, an alternative approach is followed by using short fragments of constitutively expressed genes to screen sugarcane Bacterial Artificial Chromosome (BAC) libraries to isolate their corresponding promoters.

Lastly, it was attempted to develop a selection system for transgenic sugarcane based on resistance to the herbicide chlorosulfuron. A mutant acetolactate synthase (*alsb*) gene from tobacco, which has shown to confer resistance to the tobacco, was transformed into sugarcane callus. It was anticipated that this gene will confer chlorosulfuron resistance to transgenic sugarcane. If resistance is achieved, the corresponding sugarcane gene will be mutated via site-directed mutagenesis and checked if it also confers resistance to sugarcane. Results showed that although transgenic lines were generated, resistance development is still inconclusive.

Samevatting

Suikerriet is 'n kommersiële gewas wat verbou word as gevolg van die hoë hoeveelhede sukrose wat gestoor word in die volwasse tussenknope van die stam. Verskeie studies is al gedoen om suiker metabolisme in die gewas te ondersoek, sowel as om die sukrose opbrengs te verhoog. Huidige strategieë vir suikerriet verbetering word beywer deur middel van teel-programme of transgeniese manipulasie. Die kommersialiseëring van transgeniese gewasse word egter bemoeilik deur publieke wanpersepsies, sowel as regulatoriese uitdagings.

Hierdie tesis beoog om boenoemde kwessies aan te spreek, deur middel van twee projekte. Die eerste projek poog om sukrose akkumulاسie in suikerriet te verhoog. Dit was onderneem om die *Arabidopsis thaliana* vakuolere pirofosfatase (*AtV-PPase*) geen, wat verbind is met die mielie *ubiquitien* promoter, uit te druk in suikerriet kallus. Daar was verwag dat die verhoogde aktiwiteit van die tonoplast-gebonde *AtV-PPase* sal veroorsaak dat meer sukrose in die vakuool akkumuleer. Oplosbare suiker inhoud was getoets in transgeniese suikerriet kallus lyne, maar geen merkbare verhoging in sukrose inhoud was waargeneem nie. Hierdie mag egter verander met verdere ondersoeke in suikerriet suspensie-kulture en glashuis-plante.

Die tweede projek het beywer om 'n nuwe suikerriet transformasie tegnologie te ontwikkel, wat slegs van suikerriet genetiese materiaal gebruik maak. Hierdie 'cisgeniese' benadering tot suikerriet transformasie sal 'n inheemse suikerriet promoter, terminator, vektor ruggraat en seleksie-merker, benodig. Dit was eers beoog om 'n funksionele promoter te isoleer, sowel as om 'n seleksie sisteem, gebasseer op 'n inheemse seleksie merker, te ontwikkel.

Deur gebruik te maak van primers wat op 'n sorghum templaar gebaseer is, was 'n promotor geïsoleer vanuit suikerriet; die uitdrukking hiervan is bepaal deur gebruik te maak van 'n *GFP* verklikker geen. Ongelukkig kon uitdrukking nie bevestig word in transgeniese suikerriet kallus nie. Tans word suikerriet Kunsmatige Bakterieële Chromosoom (KBC) biblioteke geskandeer, deur gebruik te maak van geen-fragmente van globaal-uitgedrukte gene, om ooreenstemmende suikerriet promotors te isoleer.

Die tweede deel van die cisgeniese projek het beoog om 'n seleksie sisteem vir transgeniese suikerriet te ontwikkel, wat gebaseer is op weerstand teen die plantdoder chlorosulfuron. Suikerriet kallus was getransformeer met 'n mutante tabak geen – asektolaktat sintase (*ats*) – wat chlorosulfuron weerstand in tabak meebring. Daar was verwag dat die geen chlorosulfuron weerstand aan transgeniese suikerriet sou oordra. Indien weerstand ontwikkel, sal die ooreenstemmende suikerriet geen deur gerigte mutagenese gemuteer word; dan sal dit kan bepaal word of weerstand ook oorgedra word aan suikerriet. Daar is bevind dat alhoewel transgeniese lyne gegenereer is, daar steeds nie 'n konklusiewe bevestiging van weerstand ontwikkeling is nie.

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They don't make them like they used to...

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Chapter 1

General Introduction

Sugarcane (*Saccharum spp.*) is a C₄ grass grown commercially in tropical and sub-tropical regions worldwide (Grof and Campbell, 2001). Its market viability is due to its ability to produce and store high levels of sucrose in the mature internodes of the stem (Rae et al., 2005b). Stalks are harvested and ground to extract this sugar and commercial cultivars have the capacity to store up to 25% of their fresh weight as sucrose (Moore and Maretzki, 1996), corresponding to a concentration of approximately 650 mM in the culm tissues (Rae et al., 2005b). Sugarcane is grown over an estimated area of 22 million hectares in almost 100 countries (FAOSTAT, 2008). Although sucrose is stored by many plant species, only two are grown to a significant extent by farmers for sugar production. Sugarcane contributes about 75% of the world sucrose production with sugar beet producing the rest (Baucum and Rice, 2009). Sucrose has traditionally been used as a food additive; however its use is growing in popularity in the production of bio-ethanol. While other cereal crops, such as rice, wheat and maize are cultivated on a much larger fraction of the world's arable land, sugarcane produces the world's greatest crop tonnage (FAOSTAT, 2008).

In South Africa sugarcane is grown from the Northern Pondoland region in the Eastern Cape to the Mpumalanga Low-veld. According to the South African Sugar Association (SASA) there are in the region of 35 500 registered growers who generate on average 20 million tonnes of sugarcane. South Africa is rated among the top 15 most cost-competitive producers of high quality sugar and it is estimated that the 2011/2012 season will produce

16 537 000 tonnes of crushed sugarcane, leading to production of 1 923 000 tonnes of saleable sugar (www.sasa.org.za, 2011).

Although the industry enjoys well-developed export infrastructure, excellent agriculture, industrial research platforms and effective organization, it has difficulties exporting to the world market profitably. One of the major concerns is subsidy-induced overproduction of sugar from major sugar-producing countries like Brazil. This distorts the world market price, which threatens the continued profitability of sugarcane farming and sugar production. Access to major world markets is also constrained by high tariffs and preferential trade agreements in the form of tariff rate quotas. The South African government acknowledges these problems and supports millers and growers via tariff protection against low world prices, equitable export obligations and the Sugar Cooperation Agreement between members of the Southern African Development Community (SADC) (www.sasa.org.za, 2011). To improve efficiency within the industry new varieties have to be developed, and these are currently produced using conventional breeding methods.

In the late nineteenth-century attempts were made through extensive breeding programs to fight diseases like sereh and to increase the sucrose yield in sugarcane (Bremer, 1961). One of the first breakthroughs was with the crossing of a domesticated “noble” cultivar *S. officinarum* to a wild relative *S. spontaneum* “glagah” and then backcrossing the hybrids to *S. officinarum* (Bremer, 1961, Berding and Roach, 1987). The backcrossing was termed “noblilisation”, which occurred three times, with the first noblilisation resulting in the Kassoer cultivar. Originally it was thought that Kassoer was a wild species, but after morphological (Jeswiet, 1925) and cytological (Bremer, 1921) studies, it was determined

that it was a spontaneous cross between *S. officinarum* and *S. spontaneum*. The subsequent third crossing resulted in a cultivar 2878 P.O.J (Proefstation Oost Java), which had high levels of resistance to sereh disease and mosaic leaf roll virus, as well as storing much higher concentrations of sugar (Bremer, 1961).

Sugarcane breeding programmes have led to increases in yields, but not to the same extent as in other major food crops, such as maize, rice and wheat. This is most probably due to the narrow gene pool used (Grof and Campbell, 2001), as most current cultivars are derived from those first crossings of *S. officinarum* and *S. spontaneum*, with a few cycles of intercrossing and selection in addition (Grivet and Arruda, 2001). It has been suggested that the natural genetic potential of commercial cultivars has reached its limit in regards to increasing sucrose yield via breeding and selection (Grof and Campbell, 2001).

Recently, attempts have been made to broaden the germplasm of sugarcane via crossings with other sugarcane sub-species. Reviews by Zhou et al. (2008) and Waclawovsky et al. (2010) respectively discuss efforts to breed cultivars with lower starch content (due to starch being an adverse impurity), as well as greater biomass (for bio-ethanol production). *S. spontaneum*, which is adapted to harsh conditions, is also used as a genetic source for stress resistance (Roach and Daniels, 1987; Ming et al., 2006) and increasing fibre content for higher biomass yields (ISSCT, 2009).

According to the breeding methodologies of the Louisiana State University Agricultural Centre, it takes up to 12 years from the initial cross to release of one new variety to commercial farmers. Unforeseen environmental setbacks can take the form of a range of diseases, insects and even climatic occurrences (Bischoff and Gravois, 2004). Breeding is

thus a time-consuming and labour-intensive method, meaning that transgenic approaches to increasing sucrose yield in sugarcane are of great value, as they bypass the intrinsic difficulties of breeding in this crop (Grof and Campbell, 2001).

During the 1980s the South African sugarcane industry recognised the potential in the advances in molecular biology, *in vitro* plant propagation and recombinant DNA technology, which led to the establishment of the South African Sugarcane Research Institute (SASRI). SASRI is one of the founding members of the International Consortium for Sugarcane Biotechnology (ICSB) and has signed agreements with research providers such as the Institute for Plant Biotechnology, which is located at Stellenbosch University (Watt et al., 2010).

Effective genetic engineering of sugarcane requires the development of technologies that include (1) a high throughput transformation system that minimises somaclonal variation and the effective identification of desired transformed plants; (2) endogenous gene sequences that can be targeted to be used for up- or down-regulation; (3) heterologous genes for the production of novel phenotypes and (4) strong promoters driving high-level gene expression either constitutively or in targeted tissue organs (Watt et al., 2010).

A method publication by Bower and Birch (1992) set the stage for the development of better sugarcane transformation methodologies (Snyman et al., 1996, 2000, 2001, 2004, 2006). Older protocols on generating transgenic plantlets involved embryonic Type 3 callus with morphogenesis proceeding via indirect embryogenesis, which took as long as 36 weeks (Snyman et al., 2001). A more recent protocol uses leaf disks containing floral

initials as recipient material, with ensuing plant regeneration via direct embryogenesis within only 14 weeks (Snyman et al., 2006).

The identification and study of the structure and function of gene sequences has grown in leaps and bounds in the last 20 years. One of the most powerful tools is the development of massive public databases easily accessible via the Internet. The online publishing of sequenced genomes makes it easy to isolate genes of interest from specific organisms. Bio-informatic tools, whether free online or in the form of subscription software, make it possible to use these online sequence data and study them *in silico*. A vast amount of information can be derived using these constantly improving tools and algorithms.

Expressed Sequenced Tags (ESTs) are also available online. Derived from cDNA libraries, these ESTs provide an invaluable resource to study genes in non-sequenced complex organisms like sugarcane (Watt et al., 2010). The largest of these EST collections are in the SUCEST database, which contains over 40 000 putative transcripts (Grivet and Arruda, 2001; Waclawovsky et al., 2010). Databases like this help to identify genes encoding enzymes, transcription factors and protein kinases involved in determining agronomically important traits. These ESTs were used to develop custom cDNA microarrays, which are used to study gene expression associated with specific metabolic pathways. The Affymetrix Sugarcane Genome GeneChip, for example, was used to study sucrose metabolism and transport in sugarcane (Casu et al., 2003). This has proven to be an invaluable resource in studying the pathways and gene expression involved in sugar accumulation, as well as sugar sensing and signalling in mediating the source-sink relationship in sugarcane (Casu et al. 2004; McCormick et al., 2006; McCormick et al., 2009).

A range of molecular resources have been developed and identified for improving sugarcane. Most of the traits that breeding programmes seek to improve are quantifiable, such as sucrose concentration, number of stalks, fibre content and resistance to pests. Sugarcane has a highly polyploid and aneuploid genome with 10-12 copies of each chromosome and possibly the same number of copies for each gene (Butterfield et al., 2001; Souza et al. 2010). The monoploid genome is considered to be between 760-926 Mbp (D'Hont and Glaszman, 2001); twice that of rice (386 Mbp), similar to sorghum (760 Mbp), but smaller than maize (2500 Mbp). Sugarcane's genome sequence is still not publicly available, but is currently being annotated by a consortium of laboratories in Brazil and Australia, with additional contributions from SASRI. Obtaining the fully sequenced genome will provide a wealth of information to speed up research into sugarcane physiology and trait improvement. Due to sugarcane's high genetic similarity to other sequenced cereal species like sorghum and maize, synteny can be used to help with map-based isolation of genes, identification of regulatory networks, cis regulatory elements and promoters (Waclawovsky et al., 2010).

Increasing sucrose stored in sugarcane plays, as mentioned before, a crucial role in yield improvement of sugarcane. Areas that are of importance for increasing sucrose accumulation in sugarcane are the rate of photosynthesis, enzymes involved in sucrose synthesis pathways, rate of phloem loading and transport to the ripening stalk, carbon partitioning within the stem, vacuoles and also rate of sucrose remobilization to support vegetative growth (Grof and Campbell, 2001; Govender, 2008).

In addition, isolation of regulatory and gene elements within sugarcane will also enable sugarcane to be manipulated with elements native to the crop. This 'cisgenic' approach will

be different than transgenic manipulation due the use of endogenous genetic sequences, which may deregulate the commercialisation of genetically altered sugarcane (Jacobsen and Schouten, 2009). Successful use of this approach has been recently reported in apples where transformed plants contained only genetic elements derived from apple (Vanblaere et al., 2011). These plants are in essence the same as traditionally bred apple cultivars. However using *in vitro* propagation and molecular manipulation may reduce time, labour and costs usually involved in breeding.

Two projects will be addressed in this thesis. The first concerns utilising transgenic techniques in an attempt to increase sucrose storage in vacuoles in sugarcane callus cells by expressing an *Arabidopsis* vacuolar pyrophosphatase (*AVP-3*) gene in sugarcane. The second project is the development of novel sugarcane transformation technology. The goal here is to build a sugarcane transformation vector consisting entirely of sugarcane DNA sequences. This technology aims to hasten and deregulate the process of GMO sugarcane commercialisation.

Chapter 2

Literature Review

2.1 Source to sink sucrose metabolism in plants

2.1.1 Sucrose metabolism in the leaf mesophyll

Plants are sessile autotrophic organisms that utilise light and water in the process of photosynthesis to produce ATP (adenosine tri-phosphate) and NADP (nicotinamide adenine dinucleotide). These molecules provide energy to fix absorbed carbon dioxide (CO₂) into triose phosphates (TPs), such as glyceraldehyde-3-phosphate (G3P), via the reductive pentose phosphate cycle (otherwise known as the Calvin Cycle). Fixed carbon has many fates, one of which is the production of the disaccharide sucrose (β -D-fructofuranulosyl-(2 \rightarrow 1)- α -D-glucopyranoside). Carbon metabolism in plants has been studied for many years and, with the advance of molecular genetic techniques, much progress has been made in understanding how the enzymes involved influence particular pathways. Most of this work has been performed in leaves of the model plant *Arabidopsis thaliana*, or in seeds and tubers of starch storing crops such as potato, maize and rice. Despite their agronomic importance, less work has been performed on sucrose storing plants. The rest of this section will describe what is known about leaf sucrose metabolism drawing on data from all species, but highlighting results from sugarcane where possible.

A simplified pathway of leaf carbon metabolism is shown in Figure 1. Photosynthate is transported out of the chloroplasts in the form of a variety of sugars by a number of transporters depending on the environmental conditions. During the day triose phosphates

(TPs) are manufactured in the Calvin Cycle and can be exported by a triose phosphate/phosphate translocator (TPT) out of the chloroplast (Fliege et al., 1978; Flügge, 1999; Linka and Weber, 2011). Within the plastid TPs can also be used to synthesise starch, which acts as a transient carbon store that is degraded to soluble sugars during the dark period (Kaiser and Heber, 1984; Weber et al., 2000). At night the starch is mobilised to glucose and maltose which are then exported into the cytosol by the pGlcT (Weber et al., 2000) and MEX1 (Niittylä et al., 2004) transporters respectively (Fig.1).

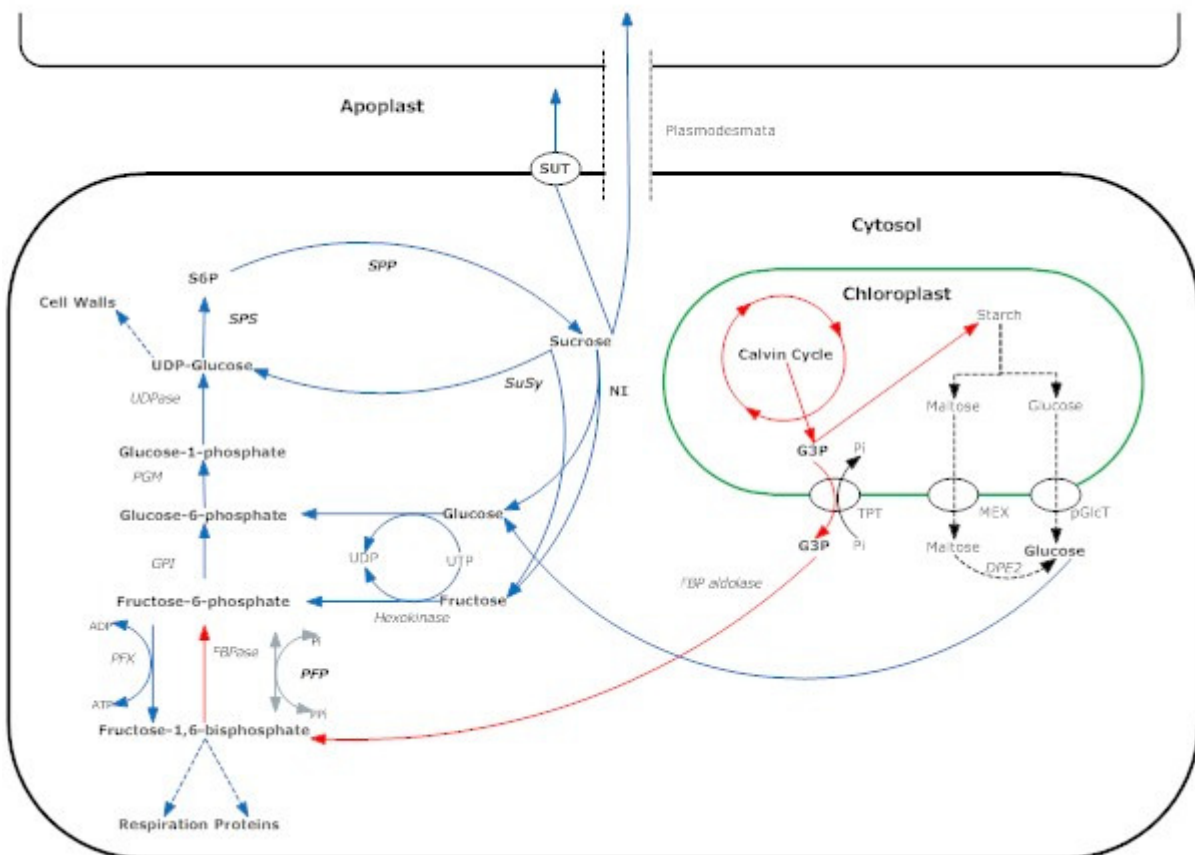


Figure 1: Simplified overview of leaf sucrose metabolism. Triose phosphates are generated by the Calvin Cycle within the chloroplasts, from where they are transported into the cytosol to be converted into sucrose. This sugar is then moved out of the cell into the apoplast via sucrose transporters (SUTs) or to other cells through plasmodesmata. Red arrows indicate reactions in the day, dashed black arrows show reactions at night and blue arrows show reactions that occur both during the day and at night. Acronyms of enzymes and metabolites included in the figure are G3P (glyceraldehyde-3-phosphate), TPT (triose phosphate

transporter), DPE2 (disproportionating enzyme 2), FBPase (fructose-1,6-bisphosphatase), FBP aldolase (fructose-1,6-bisphosphate aldolase), Pi (inorganic phosphate), PPi (pyrophosphate), PFP (pyrophosphate dependent phosphofructokinase), PFK (ATP dependent phosphofructokinase), GPI (glucose-6-phosphate isomerase), PGM (phosphoglucomutase), UDP (uridine 5-diphosphate-glucose), UDPase (UDP glucose pyrophosphorylase), SPS (sucrose phosphate synthase), S6P (sucrose-6-phosphate), SPP (sucrose-6-phosphate phosphatase), SuSy (sucrose synthase).

Within the cytosol these sugars are converted to sucrose by a series of enzymatic steps (Fig.1). Modelling of the pathway using known kinetic parameters of the different enzymes has indicated that two enzymes, namely fructose 1,6-bisphosphatase (FBPase) and sucrose phosphate synthase (SPS), exhibit a high degree of control over sucrose levels (Grof and Campbell, 2001). The extent to which FBPase and SPS contribute to control the synthesis pathway changes depending on environmental conditions. Limiting conditions such as low CO₂, radiation flux or photosynthetic rates result in FBPase having a greater level of control by shifting focus to providing sufficient metabolites for efficient Calvin Cycle turnover. Under non-limiting conditions, when photosynthetic rates are higher, SPS seems to exert more control and thus have a greater influence on maximum flux (Stitt 1989; Stitt and Quick, 1989; Huber and Huber, 1996).

Two isoforms of FBPase exist localised in the chloroplast (cpFBPase) and cytosol (cyFBPase), both of which convert fructose-1,6-bisphosphate (FBP) to fructose-6-phosphate (F6P) (Serrato et al., 2009). cpFBPase helps with Calvin Cycle turnover and provides substrate for starch synthesis (Fridlyand et al., 1999; Serrato et al., 2009). cyFBPase is more involved in sucrose synthesis by catalysing the first irreversible reaction of the conversion TPs into sucrose (Daie, 1993; Serrato et al., 2009). Antisense repression

of cyFBPase in potato (Zrenner et al., 1996) and *Arabidopsis* (Strand et al., 2000) leaves resulted in increased starch and decreased sucrose synthesis.

Both SPS and SuSy are able to synthesise sucrose, with SPS playing a more prominent role in leaf sucrose synthesis. In *Arabidopsis*, SuSy appears to have little control over leaf sucrose concentrations (Barratt et al., 2009). Leaf SPS activity on the other hand has been determined to have a positive correlation with leaf sucrose content of tomato, sugarcane and *Arabidopsis* (Worrel et al., 1991; Grof et al., 1998; Signora et al., 1998; Murchie et al., 1999). Anti-sense SPS *Arabidopsis* leaves had lower sugar levels and no significant reduction in starch (Strand et al., 2000), suggesting that SPS is under the control of other sensing and regulatory mechanisms. The extent of SPS control over changes in carbon metabolism is supported by over-expression studies that were performed using tomato plants. Expression of the maize *SPS* gene, linked to the rubisco small subunit (*rbcS*) promoter resulted in a six fold increase in leaf SPS activity in addition to increased photosynthesis and sucrose synthesis (Worrel et al., 1991). Furthermore, expression of maize *SPS* in tomato, using the *CaMV 35S* promoter, resulted in a twelve fold increase in leaf SPS activity (Micallef et al., 1995).

Four *SPS* genes have been identified in *Arabidopsis* (Langenkämper et al., 2002) and five in rice (Okamura et al., 2011). Some of the genes are light-dependent and only express in leaves (Chávez-Bárcenas et al., 2000; Harmer et al., 2000). Enzyme activities are greatly influenced by post-translational modification, especially phosphorylation by a number of protein kinases, including the sucrose non-fermentable related kinase (SnRK1) and a camodulin-like domain protein kinase (CDPK), as well as interactions with 14-3-3 regulatory proteins (Huber and Huber, 1996; Toroser et al., 1998; Huang and Huber, 2001;

Pagnussat et al., 2002; Hrabak et al., 2003; Lunn and MacRae, 2003). In its inactive form, a phosphorylated SPS is dephosphorylated by a 2A protein phosphatase to become active. The active form has a much higher affinity to its substrate and is less likely to be inhibited by Pi (inorganic phosphate) (Stitt et al., 1988; Huber and Huber, 1996; Trevanion et al., 2004).

The enzyme sucrose-6-phosphate phosphatase (SPP) catalyses the final step in sucrose synthesis by dephosphorylating sucrose-6-phosphate (S6P) (Fig.1) (Lunn et al., 2000). It has been hypothesised that SPS and SPP form a complex in which sucrose is produced directly from UDP Glucose (UDPGlc) and F6P (Lunn and MacRae, 2003). Repression of SPP activity in tobacco via RNA interference (RNAi) in leaves resulted in lower photosynthetic activity and growth, as well as the accumulation of starch and S6P (Chen et al., 2005). However, changes in sucrose levels only occurred after repression of 90% of SPP activity, indicating less control of this enzyme over leaf carbon metabolism than SPS (Chen et al., 2005). In many, but not all, plants sucrose is the most important form of soluble carbon that is transported in the phloem, however, prior to this long distance transport it has to be unloaded from the leaves and moved into the phloem.

2.1.2 Phloem loading

Export mechanisms to the phloem are required to alleviate increasing levels of sucrose in the source leaf tissue, otherwise saturation occurs, which leads to feedback inhibition of photosynthesis and sucrose production (Riesmeier et al., 1992; Riesmeier et al., 1994; Strand et al., 2000; Serrato et al., 2009). The source includes parts of the plant that are photosynthetically active, such as leaves, which supply carbohydrates to sinks, where

processes such as growth, respiration and storage occur (McCormick et al., 2009). Sucrose can be exported from photosynthetic mesophyll cells in two ways, namely symplastically through cell-to-cell connections termed plasmodesmata or apoplastically via sucrose transporters (SUTs) situated on the plasma membrane (Lalonde et al., 2003). Symplastically exported sucrose moves due to sucrose concentration gradients between the mesophyll and the phloem (van Bel, 2003) (Fig.2). Sucrose can also move into the apoplast through H⁺/sucrose symport or antiport transporters either by facilitated diffusion or active transport (Fig.2). Evidence for this sucrose transport comes from plants lacking specific sucrose transporters, which results in impaired sucrose export from the leaves (Lemoine et al., 1996; Gottwald et al., 2000; Chincinska et al., 2008). These SUTs are powered by differences in pH between the cell and apoplast or by active proton pumps situated on the cell membrane which create pH differences to facilitate sucrose transport out of the cell (Lalonde et al., 2003; Sauer, 2007; Wind et al., 2010). In leaves sucrose may enter the phloem by two different methods. It can enter via diffusion into companion cell/sieve element complexes from the mesophyll through plasmodesmata (Turgeon and Ayre, 2005; Schulz, 2005), or be imported directly from the apoplast via SUTs (van Bel and Gamalei, 1992; Sauer, 2007).

2.1.3 Phloem unloading

Sucrose is transported through the phloem towards sink organs and needs to be unloaded before it can enter the cells within the sink. This can again be done either symplastically through plasmodesmata or apoplastically via SUTs. Unloading by these two routes depends upon the species, developmental stage and structure of sink tissues.

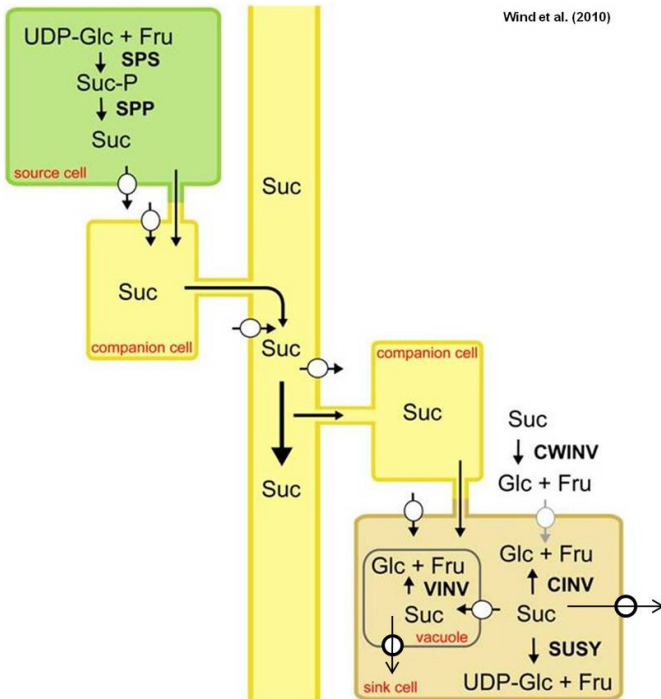


Figure 2: General overview of source-to-sink sucrose flow and within plants [adapted from Wind et al. (2010)]. Sucrose is produced in the source tissue and transported into the companion cells symplastically via plasmodesmata and/or apoplastically via sucrose transporters. It is then transported into the phloem and subsequently moves towards the sink tissues. ($\ominus \rightarrow$) indicates a sucrose transporter and direction of transport. Acronyms used for metabolites and enzymes in this figure are UDP-Glc (UDP Glucose), Fru (fructose), Suc (sucrose), Glc (glucose). Suc-P (sucrose-6-phosphate), SPS (sucrose phosphate synthase), SPP (sucrose phosphate phosphatase), CWINV (cell wall invertase), CINV (cytosolic invertase), SuSy (sucrose synthase), VINV (vacuolar invertase).

Sugarcane unloads sucrose from the phloem through a combination of plasmodesmata and SUTs and the mode of unloading is influenced by the structure of sink tissues. Vascular bundles become increasingly lignified as the stem matures, making it difficult for sucrose to be exported directly into the apoplast surrounding the storage parenchyma (Jacobsen et al., 1992). By using tracer dyes it was determined that in mature internodes sucrose moved from the phloem through the vascular bundles via symplastic connections, towards the first layer of the mature internode storage parenchyma cells (Rae et al.,

2005a). It is suggested that sucrose is then transported out of this first cell layer into the apoplast by SUTs (Welbaum and Meinzer, 1990; Rae et al., 2005b).

Apoplastic fluids in the mature internodes contain high concentrations of sucrose, implying that this is transported from the cells surrounding the vascular bundles, into the apoplast (Welbaum and Meinzer, 1990; Rae et al., 2005b). It has been noted that increased apoplastic unloading and sucrose accumulation coincided with increased expression of hexose (Filion et al., 1999) and sucrose transporters (Davies, 1999). In sugarcane a sucrose transporter ShSUT1 has been found to be localised around the vascular tissues, suggesting active sucrose transport into the apoplast (Rae et al., 2005a). The role of these transporters in phloem unloading in sugarcane has, however, not yet been determined.

2.2 Sucrose metabolism in sugarcane culm

Sucrose metabolism and accumulation in sugarcane stems is a very important research topic due to its industrial importance. A simplified model of the pathway of sucrose metabolism in sugarcane culm is shown in Figure 3. Using mathematical modelling Rohwer and Botha (2001) determined a number of possible sites for molecular manipulation to increase sucrose levels within the sugarcane culm. They suggested decreasing cytosolic invertase and PFP activity, as well as increased sucrose transport and subsequent storage in the cell vacuole. Research conducted on these possible targets in sugarcane and other plant species will be discussed in this section.

2.2.1 Invertases

As mentioned above sucrose is transported into sugarcane sink cells from the apoplast through sucrose or hexose transporters (Fig.3). Hexose transporters are able to utilise break-down products of sucrose, glucose and fructose, which result from its cleavage by the group of enzymes called invertases (β -Fructofuranosidase). Plants contain many genes encoding invertases, for example *Arabidopsis* contains 17 and rice 19 (Ji et al., 2005), however the number of genes in sugarcane still needs to be determined.

Generally the genes encode enzymes that can be divided into three isoform types. These are characterised by sub-cellular location, solubility and pH optima (Grof and Campbell, 2001). There are two types of acid invertases distinguished as soluble and insoluble forms with a pH optimum between 4.5 and 5 (Sturm, 1999). Insoluble invertase, known as cell wall invertase, is linked to the cell wall with the catalytic subunit facing towards the apoplast, which cleaves apoplastic sucrose to products which can be utilised by hexose transporters (Xu et al., 1996) (Fig.3). In many species it has been demonstrated that apoplastic invertases play a significant role in influencing the sink-source relationship.

Yeast acid invertases (YI) were expressed in separate plant species, which elucidated many details on the role of the enzyme and its contribution in photoassimilate transport (Stitt et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992; Sonnewald et al., 1997; Tymowska-Lalanne and Kries, 1998). The effects differed between the species, but generally resulted in adverse effects on the plants, demonstrating the importance of invertases in carbon partitioning in the sink tissues.

The soluble acid isoform is located in the vacuole and is termed vacuolar acid invertase (VAI) (Leigh et al., 1979; Avigad, 1982) (Fig.3). VAI is highly active in immature sugarcane internodes, but becomes less so as the internodes mature (Zhu et al., 2000; Rae et al., 2011). This enzyme is needed in young tissues due to its role in cleaving vacuolar sucrose in order to feed the high demand for hexoses in growing tissues. Furthermore this enzyme enables the remobilisation of sucrose from storage when the plant is under biotic or abiotic stresses.

The third invertase isoform is known as neutral invertase (NI). It is cytosolic and works optimally at pH 7 (Sacher et al., 1963). In sugarcane this enzyme has been isolated from leafsheaths (Sampietro et al., 1980), suspension cultures (Wendler et al., 1990) and immature sugarcane tissues (Glasziou, 1961; Sacher et al., 1963; Bindon and Botha, 2002). Suppression of NI in sugarcane via an antisense construct lead to decreased activity, increased sucrose concentrations and heightened SuSy activity in suspension cultures and the immature internodes of glasshouse plants (Joubert, 2006; Rossouw et al., 2007). The reduction of NI activity in plants was less pronounced than in suspension cultures; however increased sucrose levels and SuSy activity were accompanied with lower growth rates (Joubert, 2006). Furthermore NI activity decreased further as internodes matured. Glucose and fructose concentrations, as well as growth rates in both suspension cultures and glasshouse plants decreased (Rossouw et al., 2007). This resulted in reduced vitality in transgenic plants due to fewer hexoses being available for respiration and growth. The increased SuSy activity probably occurred to compensate for the lowered NI activity by taking over the bulk of sucrose hydrolysis and cycling.

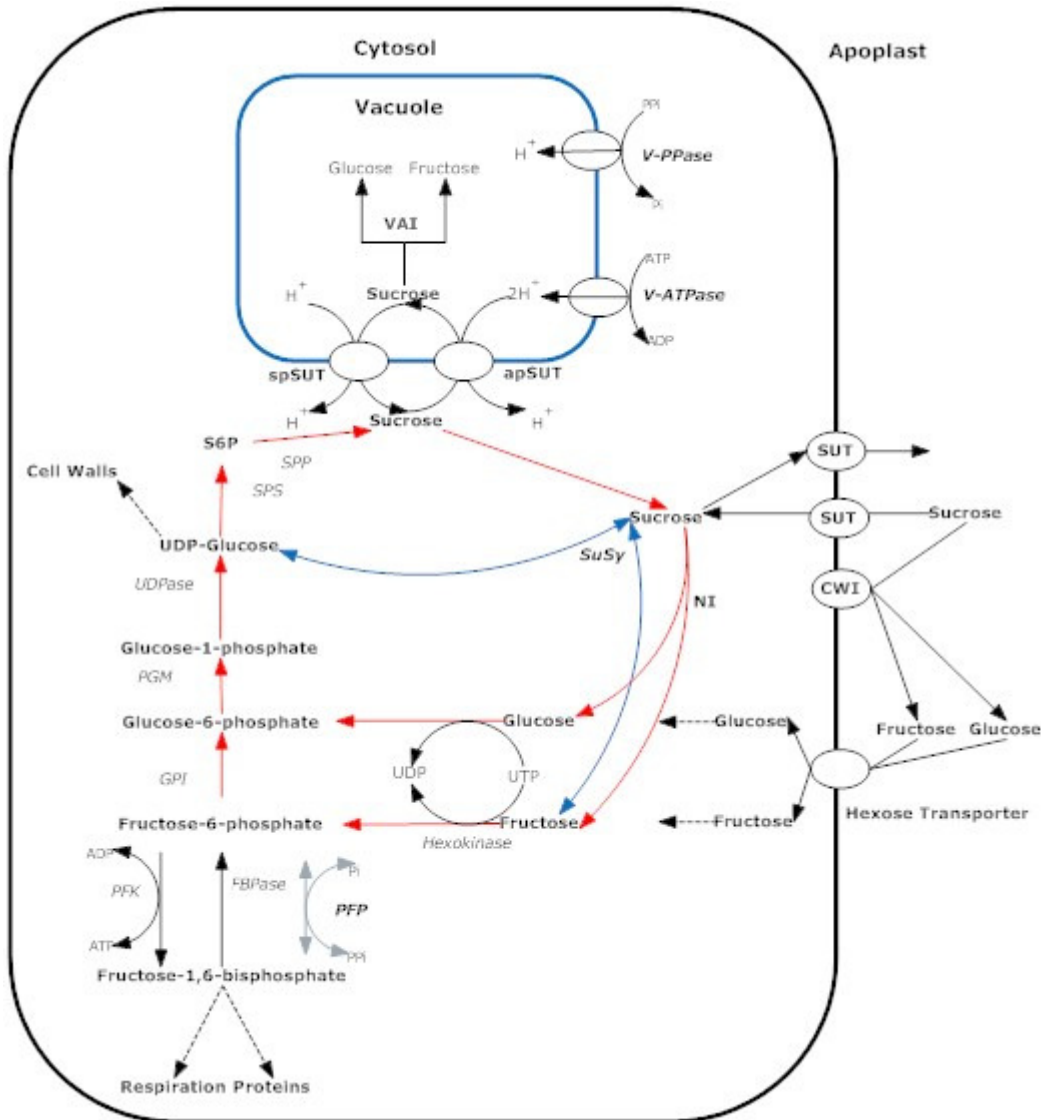


Figure 3: Overview of sucrose metabolism and sugar cycling in sugarcane sink tissue cells. Sucrose can either be imported or exported from sink parenchyma cells via sucrose transporters (SUT) or cleaved by cell wall invertases (CWI) into fructose and glucose, which are transported into the cell by hexose transporters. These hexoses are used for respiration and biosynthesis or resynthesised into sucrose by SPS. Sucrose is moved into the vacuole by H^+ /sucrose antiporters (apSUT) and is either stored, or cleaved by vacuolar acid invertases (VAI). It can also be remobilised out of the vacuole into the cytosol by H^+ /sucrose symporters (spSUT). Sucrose can be broken down in glucose and fructose by neutral invertase (NI); or into UDP-glucose and fructose by via the reversible action of sucrose synthase (SuSy). Sugar cycling reactions are indicated by red arrows. The reversible reaction catalysed by SuSy is shown by blue arrows. Abbreviations: G3P (glyceraldehyde-3-phosphate), FBPase (fructose-1,6-bisphosphatase), Pi (inorganic phosphate), PPi (pyrophosphate), PFK (ATP dependent phosphofruktokinase), PFP (pyrophosphate dependent phosphofruktokinase), PFK (ATP dependent

phosphofructokinase), V-PPase (vacuolar pyrophosphatase), V-ATPase (vacuolar ATPase), ATP (adenosine tri-phosphate), ADP (Adenosine di-phosphate), GPI (glucose-6-phosphate isomerase), PGM (phosphoglucomutase), UDP (uridine 5-diphosphate-glucose), UDPase (UDP glucose pyrophosphorylase), SPS (sucrose phosphate synthase), S6P (sucrose-6-phosphate), SPP (sucrose-6-phosphate phosphatase), SuSy (sucrose synthase).

2.2.2 SuSy

In sugarcane SuSy activity is higher around the vascular bundles and companion cells of the mature internodes than that of the young internodes and tends to be shifted towards sucrose breakdown (Buczynski et al., 1993; Nolte and Koch, 1993; Wang et al., 1994; Geigenberger et al., 1997; N'tchobo et al., 1999; Botha and Black, 2000; Grof and Campbell, 2001; Schäfer et al., 2004b). SuSy catalyses a reversible reaction where sucrose can either be cleaved to, or synthesised from fructose and UDPGlc. Increased SuSy activity in tobacco (Coleman et al., 2006), wheat (Xue et al., 2007), *Arabidopsis* (Park et al., 2008) and poplar trees (Coleman et al., 2009) resulted in increased sink strength and heightened cellulose and fibre production, the last of which may be due to increased levels of UDPGlc, which acts as a substrate for cellulose biosynthesis. This suggests that this enzyme plays an important role in sucrose accumulation and fibre biosynthesis.

Both SuSy and SPS take part in carbon cycling within the sugarcane culm. SuSy works mainly in the sucrose break-down direction, while SPS works in that of the synthesis (Botha and Black, 2000; Haigler et al., 2001). SPS activity in sugarcane suspension cultures was found to increase during sucrose accumulation (Wendler et al., 1990) and is three fold more active than the degradative reaction catalysed by SuSy in mature

internodal tissues (Botha and Black, 2000). This makes sense in light of the high amounts of biomass and fibres that are synthesised within the mature sugarcane internodes (Smith, 2008), as well as the increased SuSy activity in these tissues (Schäfer et al., 2004b). SPS ensures that fructose levels do not become too high, which may inhibit SuSy activity, and that the abundant UDPGlc is funnelled towards sucrose and fibre biosynthesis.

2.2.3 Sugar cycling

A continuous cycle of sucrose breakdown and synthesis called a futile cycle occurs within the cytosol of sugarcane sink cells (Fig.3). Sucrose is synthesised by SPS and SPP and broken down again by the activities of invertases and SuSy (Fig.3) (Nguyen-Quoc and Foyer, 2001). A study of carbon metabolism in maize roots using ^{13}C and ^{14}C radio-labelled glucose showed that almost 70% of ATP produced via respiration was consumed in this cycle (Dieuaide-Noubhani et al., 1995). This demonstrates the high amount of energy the plant invests in sucrose turnover. It enables plasticity in sucrose metabolism to quickly shift towards the production or breakdown of specific metabolite depending on the needs dictated by environmental and internal metabolic changes and demands (Moore, 1995; Fernie et al., 2002).

In sugarcane it was shown that sugar cycling between triose phosphates and hexose phosphates decreased as internodes matured and sucrose levels increased (Bindon and Botha, 2002). Other studies confirmed this by showing that lowered PFP expression resulted in an inhibition of triose phosphate to hexose phosphate cycling, thus increasing the amount of hexose phosphates and sucrose in the immature internodes (Groenewald and Botha, 2001; Groenewald and Botha, 2008; Van der Merwe et al., 2010). Flux

experiments on anti-sense PFP plants noted more rapid cycling in the transgenic plants. Furthermore an increase in UDPGlc and a major decrease in UDP was also noted (Van der Merwe et al., 2010). UDPGlc and G6P are known to allosterically activate SPS, thus stimulating sucrose synthesis (Amir and Preiss, 1982; Doehlert and Huber, 1983; Kalt-Torres et al., 1987; Reimholz et al., 1994). These changes in UDP and UDPGlc may also have reverted SuSy activity towards sucrose production (Van der Merwe, 2010). Lowered PFP activity also resulted in an increase in fibre in all tissues (Groenewald and Botha, 2008; Van der Merwe et al., 2010), which might have been due to the increase in UDPGlc, a precursor of cellulose synthesis (Groenewald and Botha, 2008).

2.2.4 Sink vacuole

Sucrose levels in the cytosol of these cells are usually low, which, in turn, drives sucrose uptake into the parenchyma from the apoplast or symplast. Most of the cell sucrose is stored within the vacuole where it can be easily mobilised. Approximately 90% of a plant cell's volume is occupied by the vacuole (Maeshima, 2001) and this organelle regulates processes such as recycling of cellular components, cytosolic homeostasis, space filling and storage of inorganic ions, organic acids and sugars (Hedrich and Schroeder, 1989; Taiz, 1992; Maeshima et al., 1996). In order to do the above, many transport proteins are localised to the tonoplast membrane allowing metabolite exchange. Those involved in sucrose transport are outlined below.

2.2.5 Vacuolar sucrose transport

Many tonoplast localised transporters, including those involved in sucrose transport, need a transmembrane electrical gradient and pH differences between the vacuole and cytosol to function. Two types of tonoplast membrane vacuolar transport proteins are involved in creating this, namely the H⁺-ATPase (V-ATPase, EC 3.6.1.3) and H⁺-PPase (V-PPase; EC 3.6.1.1) (Fig.3) (Sze 1985; Rea and Sanders, 1987; Rea and Poole, 1993; Maeshima, 2001; Swart, 2005; Krebs et al., 2010). Both of these proteins transport protons into the vacuole using either ATP or pyrophosphate (PPi) as an energy source, thus generating a pH difference across the tonoplast between the cytosol and vacuole lumen (Rae et al., 2005a) (Fig.3). This H⁺-gradient powers secondary transporters to drive uptake of numerous metabolites and sucrose into the vacuole against a concentration gradient (Hedrich and Schroeder, 1989; Taiz, 1992; Martinoia et al., 2007; Krebs et al., 2010). A comparison of different sugarcane cultivars found a positive correlation between V-PPase activity and sucrose content indicating that it may play an important role in sucrose storage (Swart, 2005).

Secondary transporters localised on the tonoplast include SUTs, which belong to the major facilitator superfamily (MFS), as well as being distantly related to hexose transporters (Kühn and Grof, 2010). A multitude of sucrose transporters have been isolated and characterised in plants. The first to be isolated was the *SoSUT1* gene from spinach (Riesmeyer et al., 1992). Sucrose transporters are subdivided into five major clades, SUT1-SUT5, which differ from each other based on substrate affinity, sub-cellular and plant organ location, plant sub-species, or whether they are isolated from monocot or dicot species (Kühn and Grof, 2010). For example *Arabidopsis* contains nine genes

(Arabidopsis Genome Initiative, 2000), whereas rice has five (Aoki et al., 2003). Some of these transporters are not limited to sucrose transport, but also transport other molecules like maltose and a multitude of glucosides (Chandran et al., 2003; Sivitz et al., 2007).

When sucrose is remobilised out of the vacuole, it is done so via H⁺/Sucrose symporters (Fig.3) (Boorer et al., 1996; Carpaneto et al., 2005; Carpaneto et al., 2010; Eom et al., 2011). Vacuoles in photosynthetic tissues also transiently store sucrose in addition to starch and need to be exported from the cells by SUTs. A H⁺/Sucrose symporter AtSUT4 isolated from *Arabidopsis* (Weise et al., 2000), belonging to clade 4 of the sucrose transporters, was found to be expressed in companion cells (Schulze et al., 2003) and the tonoplast of leaf mesophyll cells (Endler et al., 2006). A recent study also found a rice H⁺/Sucrose symporter (OsSUT2) localised on the tonoplast, which is involved in sucrose export from the vacuole (Eom et al., 2011). A mutant plant *ossut1* showed a major decrease in sugar export from leaves to the sink, as well as accumulation of sugars in the leaves (Eom et al., 2011).

In sucrose accumulating species like sugarcane and sugar beet, H⁺/Sucrose antiporters situated on the tonoplast membrane are thought to play a critical role in transporting sucrose into the vacuole (Fig.3) (Willenbrink, 1987; Getz and Klein, 1995; Schulz et al., 2011). A gene encoding for such an antiporter has recently been identified in *Arabidopsis* (Schulz et al., 2011), although in sugarcane these genes still need to be identified (Kühn and Grof, 2010). As mentioned above, these transporters need a proton gradient between the cytosol and lumen in order to function. Manipulation of this gradient or transport activity may result in increased sucrose transport across the tonoplast into the vacuole.

2.3 Development of a cisgenic transformation technology

Genetically modified (GM) crops cover a worldwide area of 148 million hectares and are worked by 15.4 million farmers in 29 countries (James, 2010). Their use has grown steadily with a global annual growth of 10%, in which developing countries are growing significantly faster than industrialised countries. The USA has the highest hectareage at 66.3 million, while that of South Africa is about 2.2 million, being mostly maize, soybean and cotton (James, 2010). Europe, in comparison, shares only a small percentage of this. Spain has the highest amount (100 000 hectares), while other countries (Portugal, Germany, Sweden, Poland, Czech Republic, Slovakia and Romania) grow less than 50 000 hectares each.

Commercialisation of transgenic crops is expensive and takes years to gain approval. The first GM crop to be sold to consumers was the Flavr Savr tomato in 1994, which was developed by the company Calgene. However it was not commercially successful due to business inexperience, financial and processing difficulties (Kramer and Redenbaugh, 1994; Bruening and Lyons, 2000).

Since then many GM plant varieties have been successfully developed and marketed. The traits that have been commercialised include resistance to broad spectrum herbicides, as well as to insect predation. These allow farmers to grow plants without using as many weed or insect control agents. However, until now no GM plants with altered output traits (such as increased sucrose concentrations) have been commercialised. One of the reasons for this is public resistance, especially in the EU (European Union), to GM technology and this has led to the slow adoption of some potentially lifesaving plants. One

example of this is Golden Rice, where development started in the 1990s. It suffered from over-regulatory stalling, as well as high commercialisation costs and will only be approved for release in 2013 (Burkhardt et al., 1997; James, 2010). This transgenic rice contains high amounts of β -carotene, the precursor for Vitamin A. In some developing countries where Vitamin A deficiency is a major health problem, this crop could already have been used to help alleviate this problem (Burkhardt et al., 1997; García-Casal et al., 1998; James, 2010).

In South Africa, sugarcane has been earmarked to play a major role in its renewable energy program, i.e. biofuel; however, currently no GM sugarcane is cultivated commercially in South Africa (African Centre for Biodiversity, 2010). There are many reasons for this, one of them being local consumer attitudes, due to misinformation and civil and public society groups that oppose GM. These attitudes are also echoed by international trading partners such as the EU and Japan, which import a large percentage of South African sugar. Segregation of GM and non-GM sugar during transport is one of the major concerns.

Sugarcane is also propagated vegetatively, creating problems with selling of “seed” by agrochemical companies. An additional concern in regulating GM sugarcane is due to the transformation method. Biolistic transformation inserts DNA randomly into the sugarcane genome, which means that control of gene insertion is limited. This may result in unexpected consequences in gene expression; however, this normally is dealt with during *in vitro* selection and field trials.

Another reason for the slow adoption of commercially cultivated GM sugarcane is that the financial and legal negotiations between the South African government, foreign governments (like Brazil) and international businesses are not yet complete. The above parties are currently working on setting up a strong platform for the future production of agro-biofuels in South Africa (African Centre for Biodiversity, 2010)

The commercialisation of genetically engineered organisms has been a point of intense public debate since the mid-1990s. Existing preconceptions hamper public confidence in purchasing products from genetically modified organisms (GMOs). A high profile incident in Britain included the public announcement that lab rats died after eating GM potatoes, resulting in negative media attention and public mistrust of GM foods (Enserink, 1998). Bio-safety concerns are some of the biggest issues in the GM debate. These include cross-pollination with wild type relatives, destruction of natural diversity, toxin build-up in pesticide producing crops, increases in weed and pest resistance and long-term effects on environmental and human health (Sandermann, 2006; Lammerts van Beuren et al., 2007).

Although there are genuine concerns regarding the testing and commercialisation of transgenic crops, there is still a prodigious amount of misinformation about them. Fighting these misconceptions has proven to be a difficult task, but this also presents unique challenges and opportunities for plant biotechnologists. A market survey in Mississippi indicated that 81% of the subjects questioned would eat a vegetable with an extra gene from the same vegetable, while only 14% would eat one with an extra gene of a virus (Lusk and Sullivan, 2002). If crop plants can be transformed with their own genes (a cisgenic approach), this may address many bio-safety concerns and help possible deregulation of the resultant cultivars.

Current genetic modification relies mostly on the transgenic approach where DNA from another species is introduced into a plant. Traditional plant breeding methods, in contrast, rearrange genomic material of plants within the same sexual compatibility group. If a plant could be genetically modified using DNA from within the same sexual compatibility group, using molecular techniques, it would be possible to create an 'intragenic' plant (Rommens, 2004). This is essentially the same as plant breeding, except more precise and efficient. This process may also help to avoid breeding problems such as inbreeding depression and linkage drag, which occur when unwanted traits are transferred to a new cultivar (Charlesworth and Charlesworth, 1987; Brown et al., 1989; Song et al., 2003).

Intragenic plants differ from the wild-type (WT) because they contain intragenes, which are composed of functional parts of genes found in either the same species or from a crossable species. In addition, cisgenic plants contain cisgenes that are full genes, containing promoters, terminators and introns. These genetic resources already belong to the traditional breeders' gene pool and, in the case of cisgenes, are products of natural evolution (Jacobsen and Schouten, 2009).

A cisgenic approach has started to be developed in a number of species, for example in strawberries (Schaart, 2004), potatoes (Rommens et al., 2006), melons (Benjamin et al., 2009) and apples (Joshi et al., 2011, Schaart et al., 2011; Vanblaere et al., 2011). These manipulations all included efforts to increase resistances to natural pests using endogenous resistance genes with their terminators and promoters. However, transgenic methods were first used as proof of concept for the functionality of these sequences. Melons were transformed with a native glyoxylate aminotransferase encoded by the genes *At1* and *At2*; however the *CaMV35S* promoter drove the expression. The resultant

transgenic plants did show increased resistance to *Pseudoperonospora cubensis* (Benjamin et al., 2009) and the next step would be to use a melon promoter to drive expression.

In strawberries such an approach was also followed by first isolating and assessing a gene-of-interest and regulatory sequences, as well as the development of a “backbone free” transformation method (Schaart, 2004). The *PFIP* gene, which conferred increased resistance to *Botrytis cinerea* and an expansin-2 (*FaExp2*) promoter, which showed GUS expression in fruit, were isolated (Schaart, 2004). An *Agrobacterium* transformation method was also developed which eliminated any vector backbone or marker gene integration, using a recombinase-mediated removal of DNA sequences (Schaart, 2004).

An example of a full cisgenic plant included potato tubers in which the native genes polyphenol oxidase (*Ppo*), *Glucan Water Dikinase* (also known as *R1*) and phosphorylase-L (*PhL*) were silenced using a multi-gene stacking anti-sense approach (Rommens et al., 2006). These genes included their native promoters and terminators. Cisgenic plants which contained no vector backbone were screened and selected via PCR. This approach resulted in improved black spot bruise resistance, reduced cold-sweetening, enhanced frying performance and less processing-induced acrylamide accumulation (Rommens et al., 2006).

In a recent study (Vanblaere et al., 2011) apple plants were transformed with a native *HcrVf2* gene (Acc: AJ297740), which has been shown to be involved in increased resistance against the fungus *Venturia inaequalis* (Lespinasse, 1989; MacHardy, 1996).

The plants were transformed via the marker free transformation method described by Schaart (2004) and the *HcrVf2* gene included the native promoter, coding region and terminator; however it did not confer increased fungus resistance in comparison to traditionally bred cultivars (Vanblaere et al., 2011).

Currently the commercial use of cisgenic or GM plants in Europe is restricted by Directive 2001/18/EC, which is used to determine whether a plant is GM or not (Anonymous, 2001). The International Cartagena Bio-safety Protocol, however, does not exclude cisgenic plants as non-GM (Jacobsen and Schouten, 2009; <http://bch.cbd.int/protocol/>, 2011). This protocol defines GMOs as “living modified organisms, which means, any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology”. It also defines modern biotechnology as “*in vitro* nucleic acid techniques including recombinant deoxyribo nucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or fusion of cells beyond the taxonomic family, that overcome natural physiological reproductivity or recombination barriers and are not techniques used in traditional breeding and selection” (<http://bch.cbd.int/protocol/>, 2011).

South Africa is also a signatory of this protocol with two added national acts, the Genetically Modified Organisms Act (Act 15, 1997) and the Genetically Modified Organisms Amendment Act (Act 23, 2006) (Mayet, 2007; www.gov.za, 2011). The current South African definition of GMO is “an organism the gene or genetic material of which has been modified in a way that does not occur naturally through mating or natural recombination or both” (www.gov.za, 2011). Determining whether if something is GMO, one has to look at the technology used and the source of genetic material. By using genetic material usually available to the traditional breeders’ genepool and within the taxonomic

family, one might see a cisgenic plant as non-GM (Jacobsen and Schouten, 2007). One may argue that cisgenic plants are legally different from transgenic due to the use of endogenous genes. This might in the future lead to a revision on the legal definition of cisgenic plants, which would deregulate and speed up the process of their commercialisation.

2.4 Objectives

This project is divided into two parts. The first utilises transgenic technology to express an *Arabidopsis V-PPase* gene (*AtV-PPase*) (Acc: M81892.1) in sugarcane to examine whether it leads to increased sucrose accumulation.

The second part is to start to develop cisgenic technology for sugarcane transformation by isolating strong constitutive promoters and a selectable marker gene.

Chapter 3

Expression of an *Arabidopsis V-PPase* in sugarcane callus

3.1 Introduction

Plant species such as sugarcane, sugar beet and sweet sorghum can store high concentrations of sucrose in their sink organs, whereas most other plants store excess carbon or reserve energy in the form of starch, proteins or lipids (Wu and Birch, 2007). In sugarcane sink tissues the vacuole has been suggested to play a critical role in sucrose accumulation (Rae et al., 2009). This organelle fills up to 90% of the cell volume and acts as storage for a range of metabolites including sucrose. The sucrose storing potential of sugarcane has been estimated to be more than double the current levels that commercial varieties accumulate (Wu and Birch, 2007) and it has been suggested that targeting more storage within the vacuole would be one way to achieve this (Rohwer and Botha, 2001).

Plants are able to determine their carbohydrate status using various sugar sensing mechanisms. Of those mechanisms that have been elucidated [for example hexokinase (Cho et al., 2010), SnRK (Rolland et al., 2006) and trehalose 6-phosphate (Paul et al., 2010)] none are known to be able to sense the carbohydrate status of the vacuole. It might be assumed, therefore, that if vacuolar sucrose concentrations are increased the plant will be unable to sense this. The plant will, however, maintain cytosolic sucrose concentrations by increasing supply from source leaves. This model has been tested by expressing sucrose isomerase in sugarcane and targeting it to the vacuole. This enzyme isomerises sucrose into isomaltulose (α -D-glucopyranosyl-1,6-D-fructofuranose), which is resistant to invertase activity. Isomaltulose concentrations up to 530 mM were detected in these plants

without a significant decrease in sucrose concentrations (Wu and Birch, 2007; Wu and Birch, 2010).

One way of increasing sucrose storage within the vacuole would be to promote the transportation of this sugar from the cytosol into the vacuole. As was discussed in Chapter 2 tonoplast sucrose transporters (SUTs), rely on a proton gradient to function. Manipulation of this should influence the activity of SUTs and change rates of sucrose exchange between the lumen and cytosol. Increasing the gradient might be achieved by engineering a greater amount of tonoplast H⁺-PPase or H⁺-ATPase activity.

Vacuolar H⁺ translocating inorganic pyrophosphatase (V-PPase) is a proton pump situated on the tonoplast membrane of the vacuole (Fig.4) (Sze, 1985; Rea and Sanders, 1987; Rea and Poole, 1993; Maeshima, 2000). V-PPase hydrolyses PPI to Pi, allowing transport of a proton into the vacuole, thus acidifying the lumen and creating a proton gradient (Fig.4) (Rae and Poole, 1993). This activates secondary transport mechanisms on the tonoplast membrane. In sugarcane one such secondary mechanism is an H⁺/sucrose antiport (Fig.3 and Fig.4) (Grof and Campbell, 2001; Rae et al., 2005; Swart, 2005), which is responsible for importing sucrose from the cytosol, into the vacuole (Maeshima and Yoshida, 1989; Nakanishi and Maeshima, 1998; Grof and Campbell, 2001; Rae et al., 2005a; Swart, 2005).

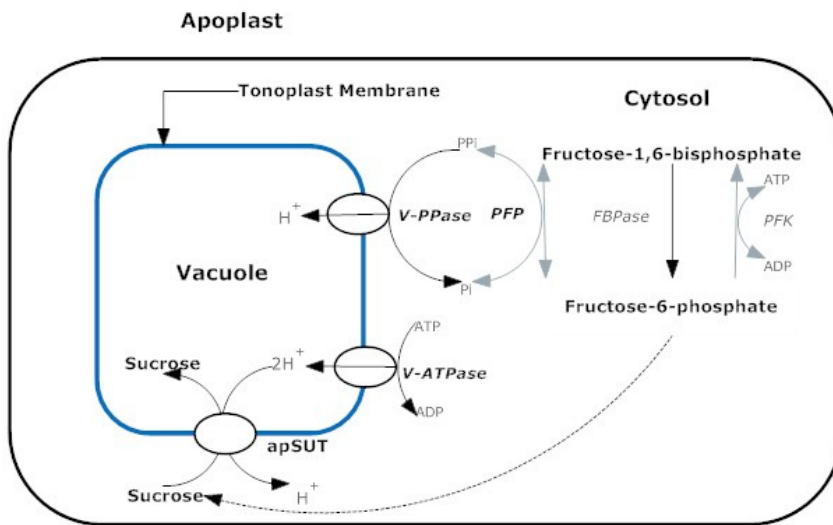


Figure 4: Interaction between selected tonoplast transporters and enzymes involved in cytosolic sucrose metabolism. Sucrose is moved into the vacuole by H^+ /sucrose antiporters (apSUT). Abbreviations: Pi (inorganic phosphate), PPI (pyrophosphate), PFP (pyrophosphate dependent phosphofructokinase), PFK (ATP dependent phosphofructokinase), V-PPase (vacuolar pyrophosphatase), V-ATPase (vacuolar ATPase), ATP (Adenosine tri-phosphate), ADP (Adenosine di-phosphate).

A large amount of PPI is produced in the cytosol during growth and development of young tissue. It is necessary for the cell to remove the excess PPI to avoid feedback inhibition of a number of biosynthetic enzymes, including those involved in DNA and RNA synthesis. Regulation of cytosolic PPI concentrations is important for normal plant growth and metabolism as demonstrated by expression of an *E. coli* inorganic pyrophosphatase in the cytosol of tobacco and potato which resulted in leaves containing increased soluble sugars and which negatively affected growth (Sonnewald et al., 1992). In addition to this it has recently been demonstrated that V-PPase activity is necessary for the normal development of *Arabidopsis* plants due to its ability to remove excess PPI from the cytosol (Ferjani et al., 2011).

In tissues of young sugarcane plants H⁺-PPase activity is normally greater than that of H⁺-ATPase. As plants mature, however, less PPi is manufactured and fewer metabolites are transported into the vacuole, so H⁺-PPase activity normally decreases to below that of V-ATPase (Maeshima et al., 1996; Shiratake et al., 1997).

In this study a *V-PPase* gene from *Arabidopsis* will be expressed in sugarcane. It is hoped that this will lead to improved sucrose storage by increasing the proton gradient across the tonoplast membrane, as well as by influencing sucrose synthesis within the cytoplasm.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

All chemicals, reagents and enzymes used were obtained from Sigma-Aldrich (St. Louis, MO, USA), Fermentas (Hanover, MD, USA), Qiagen (Dusseldorf, Germany), Merck Chemicals (Pty) Ltd. (295 Davidson Rd, Wadeville, Gauteng, RSA), Promega (Madison, WI, USA), Zymo Research (Orange, CA, USA) and Conda (Madrid, Spain). All primers were designed using Primer3Plus (Untergasser and Nijveen, 2007) and made by Inqaba Biotech (Pretoria, South Africa) and Integrated DNA Technologies (Coralville, IA, USA).

3.2.2 DNA and RNA isolation

Leaves were harvested from *Arabidopsis thaliana* Colombia 0 plants, frozen in liquid nitrogen and stored at -80°C until use. DNA was isolated from the plant tissue using a modified method by Porebski and Bailey (1997). The leaf material was ground to a fine powder with a mortar and pestle that had been pre-cooled with liquid nitrogen. 1.2 mL of extraction buffer (2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl, 2 M NaCl, 25 mM EDTA, 0.05% (v/v) spermidine, 3% (v/v) β -mercaptoethanol) at 65°C was added to each tube containing approximately 200 mg of the frozen powdered plant material. This was gently mixed and incubated at 65°C for 10 min. The product was centrifuged at 16 000 xg at room temperature and the supernatant transferred to a new micro-centrifuge tube. An equal volume of chloroform/isoamylalcohol (24:1) was added and the tube was then mixed and centrifuged for 10 min at 16 000 xg at 4°C. Following transfer of the supernatant to a new tube, this step was repeated. The supernatant was transferred to a new 2 mL tube

and ethanol was added to a final volume of 1.6 mL. This was then incubated at -20 °C overnight, after which the tubes were centrifuged for 20 min at 16 000 *xg* whereupon the supernatant was discarded. The pellet was washed with 500 µL of 70% (v/v) ethanol and the tubes were centrifuged for 10 min at 16 000 *xg* at room temperature. The resultant pellets were dried in a vacuum and re-suspended in 50 µL TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with 0.2 mg/mL RNase A.

RNA was extracted from the plant tissue using a modified method from Malnoy et al. (2001) and Hu et al. (2002). The leaf material was ground to a fine powder with a mortar and pestle that had been pre-cooled with liquid nitrogen. 1 mL pre-heated (65 °C) extraction buffer (2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl, 2 M NaCl, 25 mM EDTA, 0.05% (v/v) spermidine, 3% (v/v) β-mercaptoethanol) was added to each tube containing approximately 200 mg of the frozen powdered plant material. This was mixed vigorously for 30 s and incubated at 65 °C for 10 min. The product was centrifuged at 16 000 *xg* at room temperature and the supernatant transferred to a new 2 mL micro-centrifuge tube. An equal volume of chloroform/isoamylalcohol (24:1) was added to the tube which was then vortexed and centrifuged for 10 min at 16 000 *xg* at 4 °C. The supernatant was added to a new tube and the chloroform/isoamylalcohol extraction was repeated. LiCl (final concentration: 2 M) was added to the supernatant and incubated overnight to precipitate the RNA. This was centrifuged for 30 min at 16 000 *xg* at 4 °C and the supernatant was discarded before the RNA pellet was washed with 70% v/v ethanol. The solution was centrifuged again for 10 min and the pellet re-suspended in 30 µL nuclease free distilled water (dH₂O) and stored at -80 °C.

3.2.3 Amplification using polymerase chain reaction and isolation of *AtvPPase* gDNA and cDNA

mRNA was synthesised from total RNA with the RevertAid™ H First strand Synthesis cDNA kit (Fermentas) using OligodT₁₈ primers as specified by the manufacturer. The sequence of an *Arabidopsis V-PPase* isoform (*AVP-3*) (accession number: M81892.1) was used to design primers for polymerase chain reaction (PCR) amplification. PCR amplification was performed using a *Pfu* or *Taq* DNA Polymerase kit according to the specific manufacturer's instructions. The PCR machine used was the GeneAmp PCR System 9700 (Applied Biosystems).

3.2.4 Separation of DNA fragments by gel electrophoresis

5 µL of each of the PCR amplification products were loaded into pre-set wells of an agarose gel (0.8% agarose (w/v), TBE buffer [5.4 g/L Tris base, 2.75 g/L Boric acid, 0.465 g/L EDTA (pH 8.0)] containing 0.0002 mg/mL ethidium bromide. The gel is submerged in TBE buffer and DNA fragments separated within the gel at 120 mV for an hour. Separating fragments could be viewed under UV-light using the Alpha Imager 2000 (Alpha Innotech). Depending on the separation of DNA fragments, the corresponding correct PCR products were purified using the Fermentas PCR Purification kit according to the manufacturer's specifications.

3.2.5 Isolation of DNA from gel

Pieces of TBE gel containing DNA were removed using a sterile scalpel blade, and DNA purified from the gel via the Fermentas Gel Purification Kit, according to the manufacturer's specifications.

3.2.6 Ligation of PCR product and selection of plasmids containing inserts

PCR products amplified using *Taq* DNA polymerase were ligated into pGEM-T Easy using the pGEM-T Easy Vector System Kit (Promega) according to the manufacturer's instructions. The vectors were transformed into DH5 α *E. coli* and transformants were selected on solid LB (Luria Bertani) media (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl) containing 40 μ g/mL X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 0.1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) and 50 μ g/mL ampicillin. Putative positive clones were selected based on the inability to metabolise X-Gal and used to inoculate 2 mL LB ampicillin cultures.

3.2.7 Isolation of plasmid DNA

Bacterial cultures were centrifuged at 16 000 xg for 1 min. After discarding the supernatant the resulting pellet was re-suspended in 200 μ L STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0, dH₂O, 1 mg/mL lysozyme). The suspension was then incubated at 100 °C for 2 min and centrifuged at 16 000 xg for 10 min, after which the pellet was removed and 200 μ L isopropanol was added to the supernatant. The mixture was cooled on ice for 10 min to precipitate the plasmid DNA, which was isolated

by centrifugation at 16 000 xg for 10 min. The supernatant was discarded and the pellet washed with 700 μL 70% (v/v) ethanol. After discarding the supernatant, the pellet was dissolved in 50 μL TE buffer including 40 $\mu\text{g}/\text{mL}$ RNase and stored at -20°C .

3.2.8 Restriction digests

Plasmid DNA was digested with a chosen restriction enzyme according to the manufacturers' instructions (Fermentas).

3.2.9 DNA sequencing

Inserts in the vectors were sequenced at the Central Analytical Facility (CAF), Stellenbosch University or Inqaba Biotech, Pretoria.

3.2.10 Initiation and maintenance of embryogenic sugarcane callus

A number of mature wild-type NCo310 cultivar sugarcane plants were harvested from a patch growing outside the laboratory of the Institute for Plant Biotechnology, Stellenbosch University. These plants grew under natural conditions of the Stellenbosch area. The harvested stalks were washed continuously with 100% ethanol while leaves and old stem tissue were removed with a sterilized pruning scissor (Felco). Further leaf removal and sterilisation was done in a laminar flow while spraying with 70% ethanol. Upon the removal of the last leaf, the soft bendable inner-leaf was cut into small disks using sterile forceps and a knife. The disks were transferred, using sterile forceps and a knife, to a petri dish containing MSC_3 media (4.43 g/L MS [Murashige and Skoog, 1962] salts and vitamins

[Highveld Biological (PTY) LTD, Lyndhurst, South Africa], 20 g/L sucrose, 0.5 g/L casein, 3 mg/L 2,4-Dichlorophenoxyacetic acid [2,4D synthetic auxin], 2.22 g/L gelrite [pH.6]) and incubated for six weeks in the dark incubation room at 28°C. After every 2 weeks actively growing callus were transferred onto fresh MSC₃ media and left to grow in the dark room again.

3.2.11 Transformation of sugarcane callus

Sugarcane was co-bombarded with a transformation vector and the selection vector pEmuKN. Bombardment and selection procedures were done as described by Birch and Bower (1992) and Bower et al. (1996).

Small actively growing sugarcane callus were collected from the embryogenic callus initiation plates, using sterile forceps and a knife, and transferred to fresh MSC₃. The callus was incubated at 28°C in the dark for 4 days. Small pieces of callus were transferred to plates containing MSC₃Osm media (MSC₃ media, 0.2 M sorbitol, 0.2 M mannitol) and incubated for 4 hours at 28°C in the dark.

5 mg tungsten powder (Bio-Rad) (0.7 micron per particle) was sterilized with 400 µL ethanol and rinsed three times with sterile dH₂O. The tungsten was re-suspended in 50 µL Milli-Q water (MQ) (Millipore). After this 10 µL of 1 µg/µL plasmid DNA (1:1 ratio when using two plasmids) were precipitated on the tungsten by the addition of 50 µL 2.5 M CaCl₂ and 20 µL 0.1 M Spermidine. The microcentrifuge tube containing the mixture was put on ice to precipitate the DNA onto the tungsten.

The gene gun (custom built, Institute for Plant Biotechnology) was put in the laminar flow and disinfected with 70% (v/v) ethanol. Outlet pressure at the helium cylinder was adjusted to 1000 kPa and the solenoid timer to 0.05 seconds. A 100 μ L of the supernatant was taken off the precipitation mixture and the remaining mixture was re-suspended. 5 μ L of the precipitation mixture was placed into the centre of the support screen in a 13 mm stainless syringe filter holder. The MSC₃Osm, containing the callus, was put onto the central circle within the gene gun. After this, the door was closed to create a vacuum in the gene gun. When the pressure gauge indicated a pressure of 80 kPa, the tungsten was fired into the callus.

The bombarded callus was put back onto the MSC₃Osm and four hours later transferred onto fresh MSC₃ medium, which was left to incubate for two days in the dark. After incubation the callus was transferred to MSC₃ media containing 50 μ g/mL geneticin (G418) and further incubated in the dark. The callus was sub-cultured every 2 weeks onto fresh MSC₃ geneticin media until transgenic calli could be identified.

Positive clones were put onto fresh MS geneticin media (4.43 g/L MS salts and vitamins, 20 g/L sucrose, 2.22 g/L gelrite [pH.6], 50 μ g/mL geneticin) and incubated at 16h/8h day/night cycles at 24°C. These were left to grow for 4 weeks, subculturing every two weeks onto fresh media. Plantlets resulting from this were transferred into pots containing containing vermerculite (Rosarium, South Africa), potting soil and sand in a ratio of 1:1:1 and left to grow in the glasshouse. These plantlets were hardened off to glasshouse conditions for two weeks and left to grow. Plants were watered thrice a week and grew under natural day/night cycles at 25°C.

3.2.12 Determination of soluble sugar

50 mg fresh weight of callus from each transgenic line was stored in 1.5 mL microcentrifuge tubes and frozen in liquid nitrogen. 500 μ L buffer (20 % [v/v] HM buffer [100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH.7.8), 20 mM MgCl_2], 80% [v/v] absolute ethanol) was added to each sample and incubated overnight at 80°C. The vials were centrifuged at 12 000 xg for 10 min and the supernatant transferred to a new tube. Glucose, fructose and sucrose concentrations were determined spectrophotometrically using an enzymatic method described by Bergmeyer and Bernt (1974).

3.3 Results and Discussion

3.3.1 Isolation of the *AtV-PPase* cDNA

In order to increase V-PPase activity in sugarcane it was decided to express an *Arabidopsis* isoform in transgenic plants. A list of primers used in the isolation of the *Arabidopsis* cDNA for construction of the transformation plasmid is provided in Table 1. Attempts were made to amplify and isolate the entire *AtV-PPase* cDNA from *Arabidopsis*; however, none of these resulted in the amplification of PCR error free sequences. Primers were designed to bind in 5'-UTR upstream of the ATG site and 3'UTR downstream of the TAA site, respectively.

Table 1: Primers designed and used in PCR amplification and sequencing to isolate the *AtV-PPase* gene.

Chapter 3 <i>VPPase</i> Primers		
Gene	Name	Sequence
Full <i>VPPase</i> cDNA 1	Tobie_VPPase_FWD	GCATGGTGGCGCCTGCTTTGTT
	Tobie_VPPase_REV	GCTTAGAAGRACTTGAAAAGGAT
Full <i>VPPase</i> cDNA 2	AtVPPase2_FWD	CTCTTATACGGAGGAGAGAAGATGG
	AtVPPase2_REV	TCTTCTCCCTCGGATTGAG
<i>VPPase</i> sequencing primers 1	Seqprim_FW	CAATGTTGGTGACATTGCTGG
	Seqprim_REV	CCAGCAATGTCACCAACATTG
<i>VPPase</i> sequencing primers 2	Seqprim2_FW	TGGCAGAGAACCAGTAAGGGA
	Seqprim2_REV	TCTTCTCCCTCGGATTGAG
<i>VPPase</i> gDNA fragment	VPa_FW	CGGAGGAGAGAAGATGGTG
	Vpa_REV	CCATGGAAGACCCACCAA
<i>VPPase</i> cDNA fragment	VPb_FW	GTCTTCATGGCTCTCTTTGG
	VPb_REV	TCTTCTCCCTCGGATTGAG
<i>Ubiquitin-VP</i> fusion fragment in sugarcane	VP_RTPCR_F	GCTGAGATTCAGACTGCTATTTCC
	VP_RTPCR_R	TAGATCCCACCAACACG

In two experiments a nonsense mutation and nucleotide substitution occurred respectively. The nonsense mutation (*AtV-PPase A*) was due to a nucleotide deletion base pair 169, while the substitution mutation would have resulted in an amino acid change (*At-VPPase B*) at amino acid 491 from leucine to proline (Fig.5). It is likely that such a change would have affected protein activity and stability due the known ability of proline to introduce a kink in the polypeptide conformation (Williamson, 1994).

<i>AtV-PPase A</i>	TCTGACCTCGGCGCATCGTCTTCCGGTGGAGCTAACAAATGGGAAGAAT-GATACGGTGAT
M81892.1	TCTGACCTCGGCGCATCGTCTTCCGGTGGAGCTAACAAATGGGAAGAATGGATACGGTGAT
<i>AtV-PPase B</i>	TATGTATGGTGTGCTGTTGCTGCTCCTEGTATGCTCAGTACCATTGCCACTGGTTTGGC
M81892.1	TATGTATGGTGTGCTGTTGCTGCTCTTEGTATGCTCAGTACCATTGCCACTGGTTTGGC

Figure 5: Sequence determined following PCR amplification of the *AtV-PPase* cDNA (M81892.1). *AtV-PPase A* contained a base pair deletion leading to frame-shift mutations, while *AtV-PPase B* contained a nucleotide substitution, which would have resulted in an amino acid substitution of leucine to proline. Red boxes outline the mutation sites.

It was hypothesised, therefore, that expression of the cDNA in *E.coli* might be detrimental to the growth of the bacteria. To overcome this, a strategy was developed to include an intron into the construct (Fig.6). This would stop production of protein in *E. coli* encoded by the DNA due to the inability of bacteria to splice out introns; however, this should not affect translation of a fully functioning protein *in planta*. PCR was used to amplify the entire first exon along with the first intron and 460 bp of the second exon from *Arabidopsis* gDNA. This 916 base pair fragment, containing a unique *Nco* I site in the second exon, was ligated into pGEM-T Easy (Fig.7). For the remainder of the construct PCR was performed using cDNA as a template. Primers were designed to amplify a region just upstream of the *Nco* I site and the end of the cDNA. The resulting 1616 bp fragment was also ligated into pGEM-T Easy (Fig.7). The intron-containing 916 bp fragment was restricted out of the vector, using *Nco* I and ligated into the *Nco* I restriction site of the vector containing the 1616 fragment. Ligation was done using the Fermentas T4-Ligase Kit according to the manufacturer's instructions.

Following this the resultant gene construct was restricted out of pGEM-T Easy using *EcoR* I and ligated into the same site, in sense orientation, in the pUBI 510 vector (ECACC: 00042603) (Odell et al., 1985; Christensen et al., 1992; Groenewald et al., 2000; Sooknandan et al., 2003) to make the vector VP-pUBI (Fig.8A).

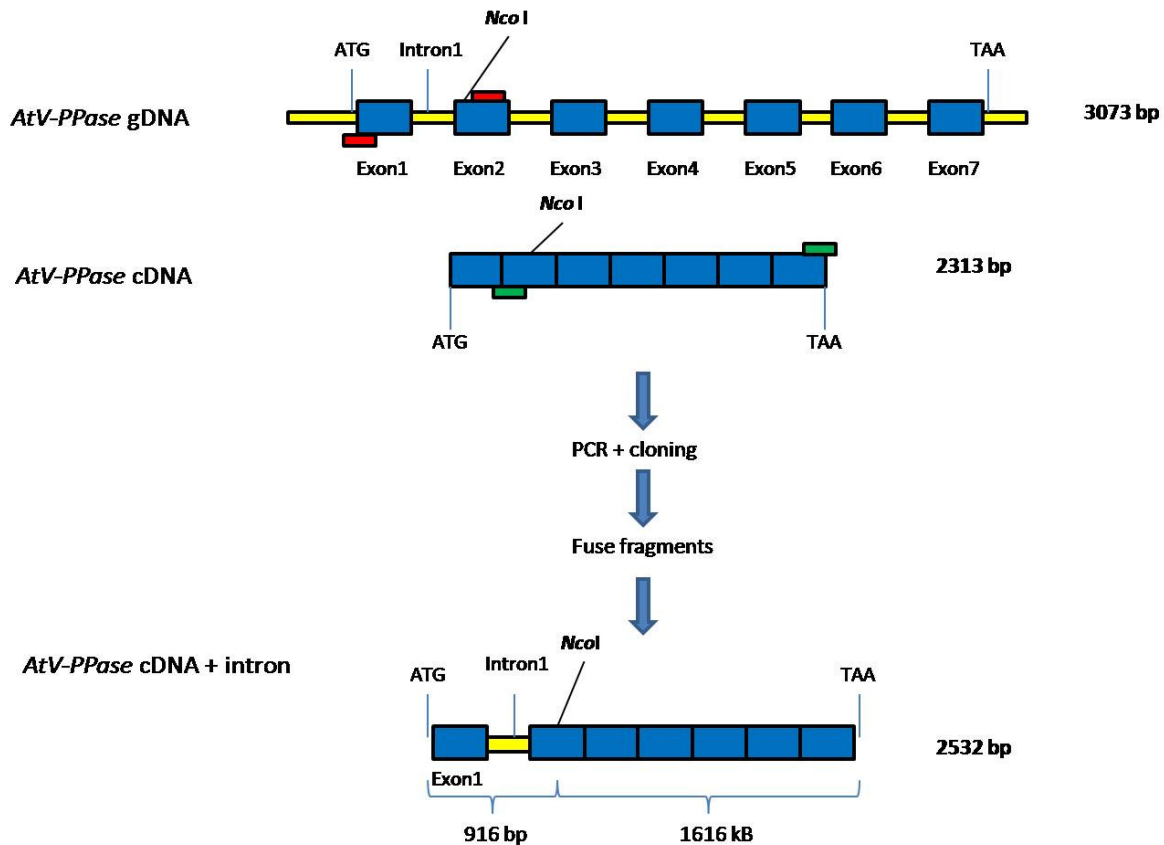


Figure 6: Strategy to include an intron in the construct. The red and green bars indicate where primers bound during PCR. A 916 bp fragment of *AtV-PPase* was amplified from *Arabidopsis* gDNA and fused to a 1616 bp fragment amplified from cDNA.

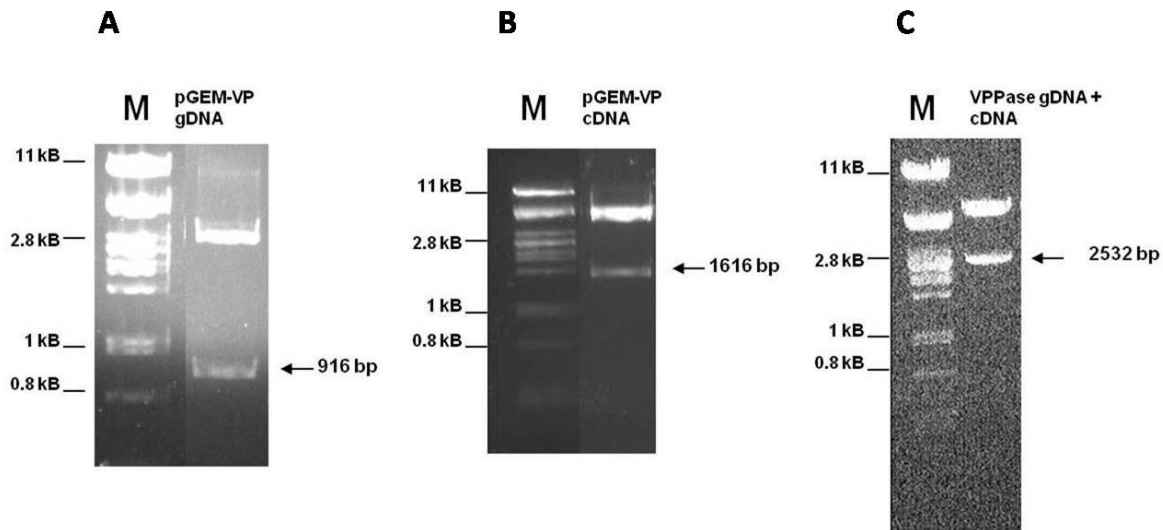


Figure 7: V-PPase gDNA and cDNA fragments. Lane M = λ DNA digested with *Pst*I. **(A)** pGEM-VP gDNA = pGEM-T Easy vector containing the *AtV-PPase* gDNA fragment (916 bp) digested with *Eco*R I. **(B)** pGEM-VP cDNA = pGEM-T Easy vector containing the *AtV-PPase* cDNA fragment (1616 bp) digested with *Eco*R I. **(C)** VPPase gDNA+cDNA = pGEM-T Easy vector containing the fused *AtV-PPase* gDNA and *AtV-PPase* cDNA fragments (2532 bp) digested with *Eco*R I.

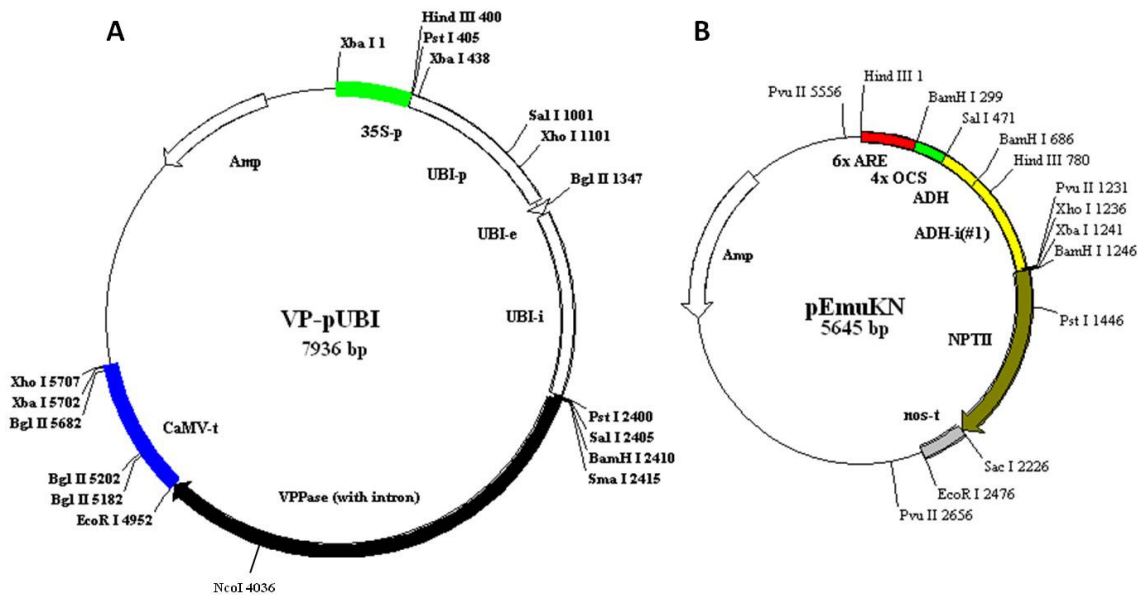


Figure 8: Sugarcane transformation vectors. **(A)** VP-pUBI containing the *Arabidopsis V-PPase* gene construct (*AtV-PPase*) (7936 bp). **(B)** pEMuKN vector (5645 bp) used for geneticin selection.

3.3.2 Analysis of transgenic sugarcane callus

Sugarcane callus was transformed biolistically using the VP-pUBI and pEmuKN vectors, after which it was placed on selection media containing geneticin. Following 14 weeks on selection medium, gDNA was extracted from callus. PCR was performed on this using primers that bound on either sides of the *AtV-PPase* intron, to check for the insertion of the gene. Several positive transgenic clones were found (Fig.9).

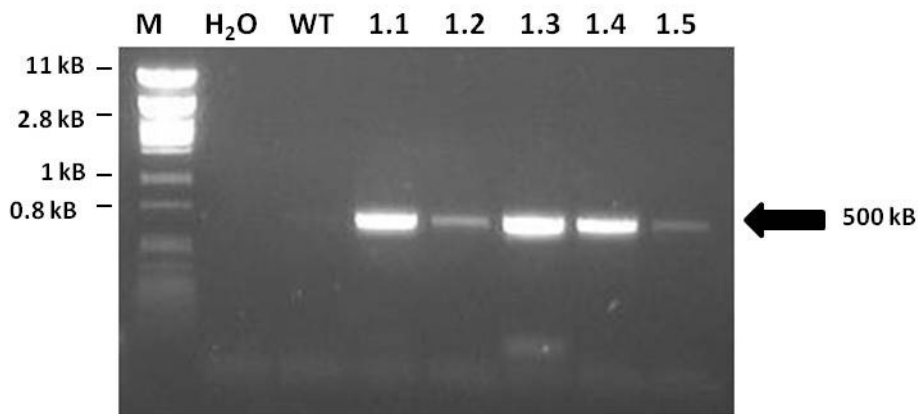


Figure 9: Transgenic *AtV-PPase* sugarcane callus. Lane M = λ DNA digested with *Pst* I. H₂O = water control. WT = wild type NCo310 sugarcane genomic DNA. Lines 1.1-1.5 = Transgenic sugarcane callus containing the *AtV-PPase* construct.

3.3.3 Sugar assays on transgenic *AtV-PPase* lines

The transgenic callus was grown on MSC₃ geneticin media for 14 weeks before soluble sugars were extracted from transgenic callus and concentrations of glucose, fructose and sucrose were determined enzymatically (Fig.10).

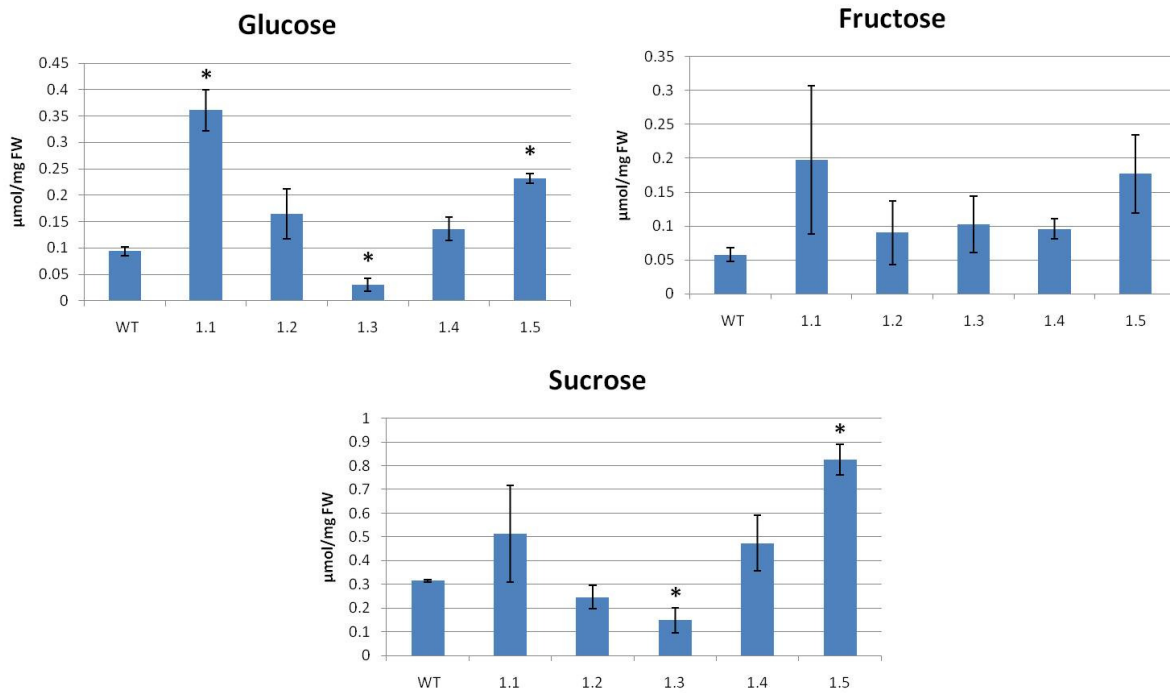


Figure 10: Soluble sugar concentrations [$\mu\text{mol/mg}$ fresh weight (FW)] of callus from wild-type NCo310 and five independent transgenic lines containing the *AtV-PPase* construct. An asterisk denotes significant (<0.05) changes in comparison to wild type. Error bars were calculated using standard error of the mean (SEM) between concentrations of three biological replicates per line. Significant changes (*) (<0.05) were determined using a t-test.

In only one line (1.5) a significant increase in sucrose was observed compared with wild type NCo310 (Fig.10). However, the resultant plantlets from line (1.5) did not produce any chlorophyll, possibly due to somaclonal variation.

There were no consistent changes in soluble sugars in the other transgenic lines; however the results need to be checked in a number of ways. Firstly, expression of the transgene needs to be shown. This could be performed using semi-quantitative reverse transcriptase PCR (sqRT-PCR) on cDNA manufactured from RNA isolated from the plants. Time constraints did not allow this, but the information could be used for two purposes. Firstly to

show that the transgene is being expressed, implying that protein would be translated and secondly to examine whether the intron has been spliced out. This could be shown by using PCR primers located either side of the intron that would give different sized PCR products depending on the presence or absence of the intron. The intron incorporated into the construct came from, the dicotyledenous plant *Arabidopsis thaliana*. As sugarcane is a monocotyledonous crop it is possible that it does not recognise the splice sites. However an intron from the dicot castor bean was successfully spliced in transgenic rice protoplasts and plantlets (Tanaka et al., 1990), suggesting that this may also be possible in sugarcane.

In addition the callus should be regenerated into transgenic plants which should be analysed. The transformation event was performed using embryonic callus and it isn't clear if its metabolism would be affected in the same way as that of culm tissue and so it is possible that, although sucrose concentrations weren't increased significantly, they might be in transgenic plants. Time constraints again made this impossible.

3.4 Conclusion

Sugarcane was successfully bombarded with *AtV-PPase* and transgenic material was obtained. No significant differences in sugar concentrations were observed in transgenic callus, although these plants still need to be studied in more detail.

Chapter 4:

Development of a Cisgenic sugarcane transformation technology

4.1 Introduction

Traditional ways of improving major food crops has been through extensive breeding programs. It can, however, take many years of crossing, selection and field trials before new cultivars are available for commercial use. With the advent of modern genetic transformation techniques and *in vitro* propagation methods it is possible to be more selective of the genetic resources that are manipulated and transferred. An important tool for genetic manipulation is the transformation vector, which is composed of different genetic elements. It has to have a functioning promoter (either constitutive or tissue specific) to drive gene expression. Another element needed is a terminator to ensure that only the gene sequence between promoter and terminator is expressed. Finally it is also important to have an effective selection system to discern between transformed and non-transformed plants. Traditionally these elements have been provided from species separate from the one being transformed, a transgenic approach. Alternatively cisgenic technology would use DNA only from the species that is being transformed to accomplish this, the advantage being that it may help in decreasing the burden of regulatory approval.

As was described in Chapter 2, cisgenic manipulation of plants has been developed for several species (Schaart, 2004; Rommens, 2006; Vanblaere et al., 2011); however this is not yet the case for sugarcane. For the development of a cisgenic or an intra-genetic plant one needs to isolate and assess the various components of a transformation vector. In this chapter the development of a promoter and selection system will be addressed.

An important facet of genetic engineering in sugarcane is the isolation of suitable promoters to drive transgene expression. Promoters are important cis-acting regulatory regions which control the transcription of flanking genes into mRNAs, which are subsequently translated into proteins (Datla et al., 1997). To identify promoters useful for a particular biotechnological purpose, transcripts showing the desired tissue expression pattern have to be identified. The promoter upstream of the coding sequence can then be isolated from genomic DNA (gDNA) and functionally assessed for its transcriptional activity *in planta* (Watt et al., 2010). Only a few promoters have been demonstrated to constitutively and strongly express in mature transgenic sugarcane and other important monocot crop species. These include the *CaMV 35S*, rice actin, maize ubiquitin *Ubi-1* and EMU promoters (McElroy et al., 1990; Gallo-Meagher and Irvine, 1993; Rathus et al., 1993; Christensen and Quail 1996; Grof et al., 1996; Elliot et al., 1998; Yang et al., 2003; Govender, 2008; Lu et al., 2008; He et al., 2009).

Several attempts have been made to identify sugarcane promoters that can drive gene expression in transformed sugarcane plants. Initially individual promoters were isolated based on their expression patterns. For example two stem-specific promoters - *DIRIGENT* (*SHDIR16*) and *O-METHYLTRANSFERASE* (*SHOMT*) - were identified via transcription profiling. These promoters were isolated and fused to a *GUS* gene and were found to be stably expressed in the stem tissues of mature sugarcane, maize, rice and sorghum (Damaj et al., 2010). At the Institute for Plant Biotechnology it was determined using expression studies that UDP-glucose dehydrogenase transcripts were present in all sugarcane tissues. This led to the isolation of the 5' untranslated region (5'-UTR) upstream of this gene and was found to actively drive GUS expression in transgenic sugarcane callus and glasshouse grown plants (Van der Merwe, 2006). In addition an

intron was located within this region, which proved to be essential for strong *GUS* (β -*glucuronidase*) reporter gene expression. Unfortunately upon testing for sustained expression in field trial experiments *GUS* activity dropped to below detectable levels, indicating that the promoter was silenced (Van der Merwe, 2006).

More recently high throughput techniques have been used. These include screening of Bacterial Artificial Chromosome (BAC) libraries, using nucleotide sequences of genes that show appropriate expression profiles (Watt et al., 2010). However, progress was hampered by numerous difficulties. These were mostly due to the complex polyploid nature of the sugarcane genome, in which there are many pseudogenes with non-functional promoters and multiple alleles of genes with differing levels of transcriptional activity (Watt et al., 2010). Sugarcane promoters have also been identified using sequence from closely related species, such as *Sorghum bicolor* and *Zea mays*. Due to the availability of genome sequence from these plants, primers could be designed in a sequence-specific manner to target candidate promoter regions of differentially expressed genes; however no functional promoters have been isolated thus far (Watt et al., 2010).

Numerous other groups have had similar problems due to post-transcriptional gene silencing. Two sugarcane poly-ubiquitin promoters *Ubi4* and *Ubi9* were tested in both rice and sugarcane for expression using the *GUS* reporter gene. In rice, *Ubi9* was found to be highly expressed in callus and mature plants, comparable to the maize *Ubi-1* promoter (Wei et al., 2003). In sugarcane both *Ubi9* and *Ubi4* expressed in transformed callus after bombardment, however as plants matured both became silenced (Wei et al., 2003). Another promoter encoding for a MYB transcription factor was found to have 8 alleles, however only 3 of these led to accumulation of mRNA. Promoters isolated from the active

alleles were tested for expression in sugarcane via GFP (Green Fluorescent Protein) studies, and although they did express soon after bombardment, expression was silenced in mature plants (Mudge et al., 2009).

Gene silencing is a problem when expressing transgenes in sugarcane and can be induced through the integration of multiple copies of a gene in plants during transformation (Van der Krol et al., 1990). Current sugarcane cultivars are complex polyploids and, as mentioned in Chapter 1, are products of hybridisation of two polyploid cultivars. In many experiments it has been found that transgenes are silenced through a process called homology-dependent gene silencing (HDGS) (Matzke and Matzke, 1998). This is a generic term for the two types of gene silencing, namely transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Vaucheret et al., 1998). TGS occurs when gene transcription is inhibited via promoter methylation due to interacting genes sharing sequence homology in their promoter regions. PTGS occurs when there is homology in the transcribed product, namely the RNA. Transcription rate is not affected, but the formation of double-stranded RNA induces the degradation of homologous RNA sequences (Mette et al., 1999; Bass, 2000; Fagard and Vaucheret, 2000). Although it isn't clear why promoters often become silenced in sugarcane either, or both of these mechanisms, may be involved (Meyer and Saedler, 1996; Govender, 2008).

During the production of transgenic plants, it is essential to discriminate between transformed and non-transformed plant material. This is usually achieved using selectable marker genes, which confer a trait to the transformed plant that the non-transformed plant does not have. In plants these selectable markers are usually herbicide or antibiotic resistance genes. In a cisgenic transformation system the selectable marker is normally a

gene encoding a protein which is an herbicide target. The gene is mutated to encode a protein that is altered so that it becomes resistant to the herbicide. Currently no cisgenic selectable marker is available for sugarcane, so one has to be developed.

A range of imazapyr resistant sugarcane lines have been evaluated to determine the mechanism through which resistance occurs (Punyadee et al., 2007). Imazapyr forms part of the imidazolinone group of herbicides, which inhibit the enzyme acetolactate synthase (ALS) (EC 4.6.3.8) (Duggleby and Pang, 2000). They found that the most resistant sugarcane line K96-282 had an ALS activity 6.5 times higher than wild type suspension cells (Punyadee et al., 2007). However, they only isolated a partial clone of the sugarcane *ALS* gene (Genbank: EU243999.1). It was suggested that increased activity of ALS resulted in higher levels of resistance towards the herbicide (Punyadee et al., 2007).

The *ALS* gene encodes a polypeptide which is the first common enzyme in the biosynthetic pathway of the branched amino-acids leucine, isoleucine and valine (Chaleff and Mauvais, 1984; Yadav et al., 1986; Singh and Shaner, 1995). It catalyses the condensation of two pyruvate molecules to acetolactate, as well as the condensation of pyruvate and ketobutyrate to acetohydroxybutyrate (La Rossa and Schloss, 1984; Schloss et al., 1985; Yadav et al., 1986; Singh and Shaner, 1995). ALS is encoded in the nucleus, but the protein is targeted to the chloroplasts (Corbett and Tardif, 2006).

Two classes of herbicides act on this enzyme, which include the sulfonylureas (La Rossa and Schloss, 1984) and imidazolinones (Shaner et al., 1984; Duggleby and Pang, 2000). These herbicides act on the catalytic region of the ALS enzyme, as a competitive inhibitor

for pyruvate (Chaleff and Mauvais, 1984; McCourt et al., 2006) resulting in the inactivation of the enzyme and the subsequent halting of branched amino acid synthesis.

A range of different mutations in the *ALS* genes in *Escherichia coli* (Yadav et al., 1986), *Saccharomyces cerevisiae* (Falco and Dumas, 1985; Yadav et al., 1986) and plants (Chaleff and Ray, 1984; Chaleff and Mauvais, 1984; Lee et al., 1988; Kochevenko and Willmitzer, 2003) have been identified to confer resistance to the sulfonylurea herbicides. Single nucleotide changes in some plant *ALS* genes leads to an amino acid substitution, which results in an enzyme less sensitive to inhibition.

A mutant *ALS* allele from *Arabidopsis* (*csr1-1*) was used as a selectable marker in a rice transformation experiment (Li et al., 1992). The transgenic rice was both fertile and had a high resistance to chlorosulfuron. The gene acted as an effective selectable marker by being able to select even at chlorosulfuron concentrations of 10 nM. This suggested that *ALS* inhibition and protein transport to the chloroplast is conserved between dicots and monocots (Li et al., 1992) and that such a gene could be used as an effective selectable marker in monocots.

Reports of *ALS*-inhibitor resistance abound in literature. It has been noted in canola (Swanson et al., 1989), soybean (Sabastian et al., 1989), maize (Newhouse et al., 1991; Bailey and Wilcut, 2003), wheat (Newhouse et al., 1992), cotton (Rajasekaran et al., 1996), sugar beet (Wright and Penner, 1998a,b), rice (Bae et al., 2002) and tobacco (Kochevenko and Willmitzer, 2003). Herbicide resistance was suggested to be attributed to an altered *ALS* enzyme, which resulted in reduced sensitivity to the herbicides.

4.1.1 Aims and objectives

This chapter will be divided into two parts. The first section will concern the isolation and assessment of the activity of sugarcane promoter sequences. The second will concern the development of a selection system using the herbicide chlorsulfuron and a mutated *als* allele to provide resistance against it. This is to provide a proof of concept for using chlorsulfuron as a selection system in sugarcane. If it is successful then the native *ALS* gene could be isolated and amino acids known to confer resistance against chlorsulfuron in other species mutated using site directed mutagenesis.

4.2 Materials and Methods

4.2.1 Isolation and assessment of sugarcane promoters

4.2.1.1 Isolation of putative promoter regions from sugarcane gDNA

Genomic DNA (gDNA) was extracted from leaves of the NCo310 sugarcane cultivar as described in Chapter 3. Primers (Table.2) were designed to amplify out 1.5 kB to 2.0 kB of the 5'-UTR adjacent to a range of candidate genes from sugarcane gDNA.

Table 2: List of primers used attempting to amplify 5'-UTR of candidate genes.

Gene 5'-UTR	Name	Sequence
5'UTR- <i>SH2</i> region	SH2_F	AAACGGTAATCATAAGAGGGAGA
	SH2_R	ATCTCCTCCAACACTACTAGACACA
5'UTR- <i>Adh1</i> region	Adh1_F	GCTGAATAAAATGACCTAGAAACACA
	Adh1_R	TCACGGACCTTTGCACTTG
5'UTR- <i>ubi9</i> region	ubi9(1)_F	AACTGCAGTCCTTGCCTC
	ubi9(1)_R	ACT TCAAAAACATTAGCTAACACACA
	ubi9(2)_F	AAACCTAGGCGTCGCTTCACTTAT
	ubi9(2)_R	AAACCTAGGCAAACGTCAACAAGCAA
5'UTR- <i>ubi</i> region	ubi(3)_F	CGGCGTCGCTTCACTTAT
	ubi(3)_R	CTGCAAACGTCAACAAGCAA
5'UTR- <i>Tub</i> region	Tub(1)_F	CAGTGCGGCAACCAGATC
	Tub(1)_R	TTTTGGGTGTCACATCGGAT
	Tub(2)_F	CAGTGCGGCAACCAGATC
	Tub(2)_R	TTTTGGGTGTCACATCGGAT
5'UTR- <i>Act</i> region	Act(1)_F	AAACCTAGGGAGCCTTTTCGGTTTC
	Act(1)_R	AAACCTAGGCGTCAGCCATTCTCA
	Act(2)_F	GTGGAGCCTTTTCGGTTTC
	Act(2)_R	CGTCAGCCATTCTCAACTATTC

5'UTR- <i>SHDIR16</i> region	SHDIR16_F	GCTTCCTCCAACCTTCTCGTC
	SHDIR16_R	ACTAGTTATGGCAGCTACCGTTT
5'UTR- <i>SHOMT</i> region	SHOMT_F	CTTCAGTGCAGAATTCATACCAA
	SHOMT_R	GCACGGCTTCTGTTTTTGC
5'UTR- <i>APX</i> region	APX_F	TGGGCATGAATGATTTGAAG
	APX_R	TGCTTATGCAGAATTGTCTCTCTT
5'UTR- <i>PGD1</i> region	PGD1_F	TAGGCCATCCTCTAGGAAGTTTT
	PGD1_R	CTCGGCGATGTTGAGAGC
5'UTR- <i>RIGIB</i> region	RIGIB_F	GAGCTTTGCTGTAGGTAGAAAATG
	RIGIB_R	AGTATTTGGACCCGGTGAAA
5'UTR- <i>EIF</i> region	EIF5_F	AGTGGTGGGGTCGGAGAT
	EIF5_R	GCGGAATAAATAATGAACTATAACATC

4.2.1.2 Construction of a vector for sugarcane bombardment

PCR products of the 5'-UTR regions were ligated into the pGEM-T Easy vector for sequencing. Inserts were then isolated following digestion with *EcoR* I and separation from the vector by TBE gel electrophoresis, after which it was ligated into the *EcoR* I site of a vector containing the GFP reporter gene. Vectors with the insert in the correct orientation were identified through restriction digests with *Sac* I.

4.2.1.3 Biolistic bombardment of sugarcane callus

Sugarcane callus was co-bombarded with the pGFP SK- vector containing a promoter and the pEmuKN vector. As a positive control, the pGEM-GFP-ubi vector, which contains the maize *Ubi-1* promoter, was co-bombarded into sugarcane callus. The procedure for bombardment followed as stipulated in Chapter 3.

4.2.1.4 Transient GFP analysis of transgenic sugarcane callus cells

After two days the bombarded sugarcane was viewed under a florescent light microscope using the Leica MZFLIII microscope to check for GFP activity. Calli were illuminated with UV-light and viewed through the GFP Plant Fluorescence filter set (excitation filter: 470/40 nm; barrier filter: 525/50 nm). Positive calli were selected based on visualisation of fluorescence and put on MSC₃ media containing geneticin for selection. The calli were sub-cultured every two weeks on fresh MSC₃ with geneticin for a further 14 weeks.

4.2.1.5 Analysis for continued GFP production in putative transgenic callus

Following the selection period, GFP analysis was done on these putative transgenic calli, using a Leica MZFLIII microscope and filters as indicated above. Putative calli were also transferred onto MS media to generate plantlets and left to grow for 4 weeks, subculturing every two weeks onto fresh media. These were grown at 16h/8h day/night cycles at 24 °C. Following this, plantlets were transferred to pots containing vermerculite, potting soil and sand in a ratio of 1:1:1. These plantlets were hardened off to glasshouse conditions for two weeks and left to grow. Plants were watered thrice a week and grew under natural day/night cycles at 25 °C.

4.2.1.6 Analysis for insertion of GFP gene into sugarcane genomic DNA

gDNA was extracted from putative transgenic callus following the protocol in Chapter 3. PCR was performed on the gDNA extracts to check for the insertion of the GFP gene into sugarcane gDNA.

4.2.1.7 Immunoblots

Total protein was extracted from transgenic callus. 50 mg fresh weight (FW) callus of each sample line was put into 1.5 mL microcentrifuge tubes and frozen in liquid nitrogen. Samples were ground in 400 μ L protein extraction buffer (50 mM Tris, pH.7; 5 mM EDTA, 0.1% Triton-X, 1 mM β -Mercaptoethanol, *SIGMAFAST* protease inhibitor cocktail [Sigma]). All samples were centrifuged at 16 000 xg for 5 min at 4 $^{\circ}C$ and the supernatant transferred to a new tube. All samples were stored at -20 $^{\circ}C$ until further use.

Protein concentrations were determined using an assay based on the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. 20 μ g total protein from each sample was denatured according to Laemmli (1970) and separated by 10% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis).

The gel was blotted onto nitrocellulose membrane in a semi-dry transfer cell (Bio-Rad Trans-blot SD). The membrane was incubated overnight in blocking buffer (phosphate buffered saline [PBS] [8 g NaCl, 0.2 g KCl, 1.44g Na_2HPO_4 , 0.24g KH_2PO_4 in 1 L, pH 7.4], 3% (w/v) milk powder, 0.1% (v/v) Tween 20).

Membranes were washed three times for 5 min each time in PBS + 0.1% (v/v) Tween 20 (PBS-Tween) before being incubated in PBS-Tween including Anti-GFP antibody raised in rabbit (1:1000 dilution) (Invitrogen) for 2 hours at room temperature. The membranes were washed a further three times with PBS-Tween, after which they were incubated in PBS-Tween plus Anti-Rabbit IgG Alkaline Phosphatase antibody (1:7500 dilution) (Sigma) for 2 hours at room temperature. After the final wash, membranes were incubated for 10 min in

the dark in a solution containing *SIGMAFAST* BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablet (Sigma) dissolved in 10 mL water. The reaction was stopped by submerging the membranes in water.

4.2.2 Development of a cisgenetic system based on herbicide resistance

4.2.2.1 Isolation of a tobacco mutant ALS gene

Seeds of the *rchl 6.6* chlorosulfuron resistant tobacco line (Kochevenko and Willmitzer, 2003) were sterilised using a 1.5% (v/v) sodium hypochlorite solution and rinsed three times with sterile dH₂O. They were plated out on MS media and left to germinate before being transferred to soil and allowed to grow for 4 weeks.

RNA was extracted from mature plant leaves using the method described in Chapter 3. mRNA was synthesised from total RNA using the Fermentas RevertAidTM H First strand Synthesis cDNA kit according to the manufacturer's specifications.

PCR was performed using the *pfu* DNA polymerase enzyme (Fermentas) according to the manufacturer's specifications. Primers designed were NbALSBamHI_FW (AAGGATCCTTCGTCTCTCACT) and NbALSBamHI_REV (AACCTAGGAGCTCTGTAGCAC) for PCR amplification from cDNA. These primers also incorporated a *Bam*H I restriction site (underlined) on both ends of the PCR product. Following this, the PCR product was purified using the Fermentas PCR Purification Kit.

After confirmation by sequencing, the insert was restricted using *Bam*H I from the pBluescript SK- vector and ligated into a sugarcane transformation vector pUBI510.

4.2.2.2 Sugarcane transformation using ALSB-pUBI and selection using chlorsulfuron

Sugarcane callus was bombarded using the method described in Chapter 3. The transformed callus was put onto MSC₃ medium containing 3.6 µg/L chlorosulfuron and incubated in the dark. The bombarded callus was sub-cultured onto fresh MSC₃ chlorosulfuron media every 2 weeks for 8 weeks.

4.2.2.3 Secondary selection phase

Putative transgenic calli were transferred to MS media containing 3.6 µg/L chlorosulfuron and left to grow for 4 weeks, subculturing every two weeks onto fresh media. These were grown at 16h/8h day/night cycles at 24°C. Following this, plantlets were transferred to pots containing vermerculite, potting soil and sand in a ratio of 1:1:1. These plantlets were hardened off to glasshouse conditions for two weeks, afterwhich they sprayed with 300 µM chlorosulfuron, once every 2 weeks for 4 weeks. Plants were watered thrice a week and grew under a 16h/8h day night cycle at 24°C.

4.2.2.4 Testing for gene insertion

Genomic DNA was extracted from 30 putative transgenic plants and PCR was performed to determine if the *alsb* gene was inserted into the genome.

4.2.2.5 Isolation of a partial sugarcane ALS gene

RNA was extracted from mature sugarcane plant leaves using the method described in Chapter 3. mRNA was synthesised from total RNA using the Fermentas RevertAid™ H First strand Synthesis cDNA kit according to the manufacturer's specifications.

A partial sugarcane *ALS* sequence (Genbank: EU243999.1) (*SoALS*) was amplified from sugarcane mRNA using the primers SoALS_2F: CCAAGGCAGTGGTTGTCTT and SoALS_2R: CCATCACCATCCAGGATCA. PCR was performed using the Biorline *Taq* Polymerase Kit (Biorline) according to the manufacturer's specifications.

The PCR product was ligated into pGEM-T Easy, sequenced and sent off to SASRI to obtain the full sugarcane *ALS* sequence(s) via hybridisation screening of BAC libraries.

4.3 Results and Discussion

4.3.1 Isolation and assessment of a sugarcane promoter

4.3.1.1 Construction of the GFP reporter plasmid

A vector for testing the activity of the isolated promoter regions in sugarcane was constructed. The reporter vector included a pBluescript SK- (Agilent Technologies) backbone with the *GFP* reporter gene and its *nos* terminator integrated into the vector's multiple cloning site between the *Cla* I and *Eco*R I restriction sites. This vector was termed pGFP SK- (Fig.11).

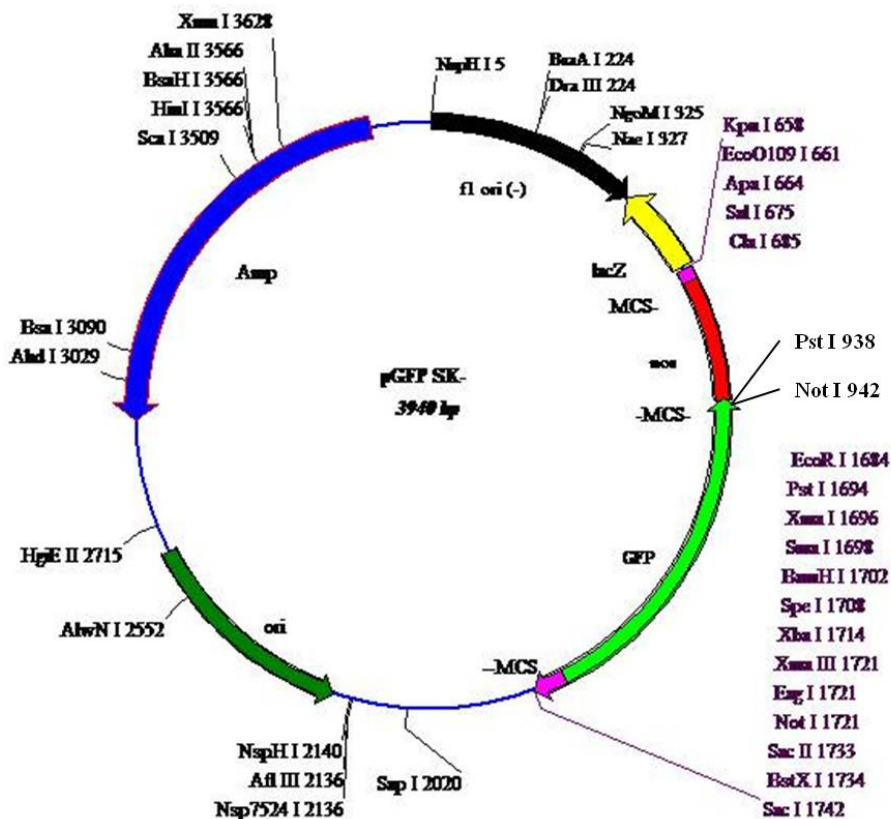


Figure 11: The GFP reporter vector used to assess promoter activity in sugarcane. A *GFP* reporter gene fused to a *nos* terminator ligated into a pBluescript SK- vector.

4.3.1.2 Isolation of promoter regions

Promoter regions were identified through searching for possible candidates from existing literature and online sources. The initial experimental approach was to use gDNA sequences from sorghum to design primers that allow PCR amplification of promoters from sugarcane gDNA. The sorghum genome has been sequenced allowing easy primer design and has been shown to be very similar to that of sugarcane meaning that this strategy is feasible (Dufour et al., 1997).

Several candidate genes were chosen for isolation of promoter regions in the hope that they would provide strong, constitutive expression. The 5'-UTRs of these are listed in Table.3. The reason for choosing some of these genes is due to their known, strong expression, in other species. The maize *Ubi-1* (Christensen and Quail, 1996) is commonly used in sugarcane transformation and 5'-UTRs of rice *ubiquitin* genes have proven to confer strong constitutive expression to both rice and sugarcane (Wang et al., 2000; Liu et al., 2003; Sivamani and Qu, 2006; Lu et al., 2008). Even though the sugarcane *ubi9* and *ubi4* promoters were silenced in sugarcane (Wei et al., 2003), it was reasoned that promoters from other *ubi* alleles may not become silenced.

The 5'-UTRs of rice *act1* and *actin2* genes have proven very successful in rice transformation and showed strong constitutive expression (McElroy et al., 1990; He et al., 2009). Isolating these regulatory sequences from sugarcane may also result in strong constitutive expression. Tubulin genes are known as constitutively expressed within plants (Fiume et al., 2004) and its 5'-UTR was considered as good candidates for constitutively expressed promoters. In a survey of rice regulatory sequences a candidate was isolated,

using real-time quantitative PCR (RT-qPCR) and *GFP* expression analysis, and identified an *Ascorbate peroxidase* (*APX*) 5'-UTR for strong constitutive expression in rice (Park et al., 2010).

Furthermore the *eukaryotic translation initiation factor 5A-2* (*EIF*) promoter is a weak constitutive promoter, which may be used if gene expression is needed at a low level, for example for the expression of potent insecticides (Park et al., 2010).

Some of the candidates are not constitutively expressed, however a wide variety of promoters are needed for genetic engineering. Tissue specific promoters are used when expression is only required in specific parts of the plant, such as leaves, roots or the stem. Amplification of promoters from sugarcane stem-specific genes, such as *SHDIR16* and *SHOMT* (Damaj et al., 2010), were thus also attempted. A phylogenetic survey in monocots conducted by Guo and Moose (2003) suggested that the 5'-UTR of *Sorghum bicolor* *ADP-glucose pyrophosphorylase subunit SH2* (*SH2*) and *S. bicolor* putative *alcohol dehydrogenase* (*Adh1*) genes were also a target for promoter isolation due to possible strong tissue specific expression. Rice promoters from *6-phosphogluconate dehydrogenase* (*PGD1*) showed strong expression in rice flowers and mature roots; and *actin1* (*RIBIG*) in rice seeds (Park et al., 2010).

Table 3: Candidate plant genes looked at for amplification of the 5'UTR region and subsequent GFP expression analysis.

Gene	Description	Size (kB)	References
<i>ubi9</i>	Partial <i>ubi9</i> poly-ubiquitin gene (Primers designed on sorghum template)	1.5	Wei et al., 2003
<i>SH2</i>	<i>S. bicolor</i> ADP-glucose pyrophosphorylase subunit SH2	1.5	Guo and Moose, 2003
<i>Adh1</i>	<i>S. bicolor</i> putative alcohol dehydrogenase	1.5	Guo and Moose, 2003
<i>ubi9(2)</i>	<i>ubi9</i> poly-ubiquitin gene (Primers designed on sorghum template) second round	1.5	-
<i>Act</i>	Actin (Primers designed on sorghum template)	1.5	-
<i>Tub</i>	Tubulin (Primers designed on sorghum template)	1.5	-
<i>SHDIR16</i>	<i>Saccharum</i> hybrid DIRIGENT	2.6	Damaj et al., 2010
<i>SHOMT</i>	<i>Saccharum</i> hybrid O-METHYLTRANSFERASE	2.9	Damaj et al., 2010
<i>APX</i>	Rice Ascorbate peroxidase – strong constitutive	2	Park et al., 2010
<i>PGD1</i>	Rice 6-phosphogluconate dehydrogenase - strong constitutive	2	Park et al., 2010
<i>RIGIB</i>	Rice actin1 - strong constitutive	1.9	Park et al., 2010
<i>EIF</i>	Rice eukaryotic translation initiation factor 5A-2 - weak constitutive	1.9	Park et al., 2010

Using PCR with the primers designed based on the sorghum genome (Table.2), only products thought to represent the 5'-UTRs of *Adh1*, *SH2* and *SHDIR16* (Fig.12) could be amplified. However sequence information showed that the *Adh1* PCR product had homology to a putative sorghum reverse transcriptase while that from *SHDIR16* could not, after many attempts, be isolated and ligated into a vector. Of the amplified products, only the *SH2* 5'-UTR could be confirmed by sequencing, which had a 92% similarity to that of the 5'UTR of sorghum and maize *ADP glucose pyrophosphorylase* genes, indicating successful amplification (Fig.13).

SH2 encodes the large subunit of ADP glucose pyrophosphorylase which is involved in starch biosynthesis (Hannah, 1997; Preiss, 1997; Lloyd et al., 2005; Zeeman et al., 2010). The promoter of this gene is a strong tissue specific promoter to the endosperm of monocot seeds like rice, maize and wheat (Bhave et al., 1990; Anderson et al., 1991; Chen et al., 2003; Smidansky et al., 2002; Smidansky et al., 2007), although promoters from ADP-glucose pyrophosphorylase genes from other species have been shown to be either constitutive, with especially high expression in sink tissues (Ursula et al., 1995). As it was the only promoter successfully isolated, it was decided to continue with the assessment of its activity in sugarcane callus.

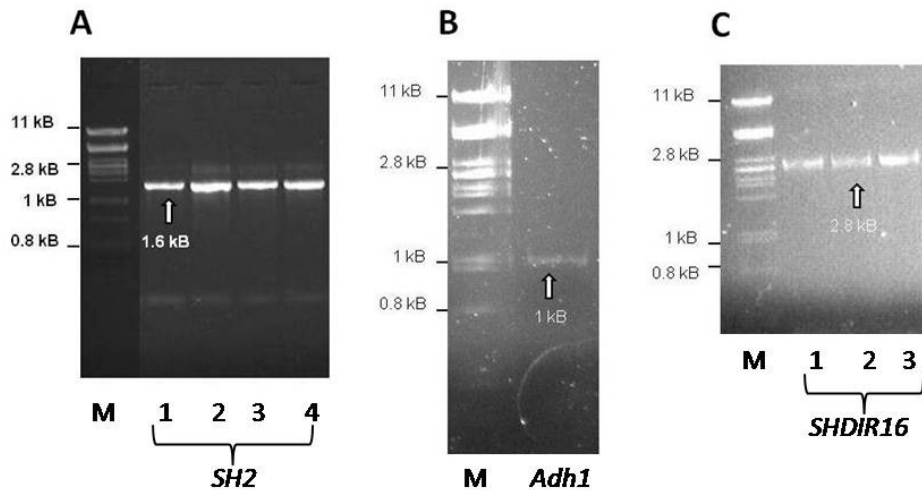


Figure 12: PCR amplification of 5'-UTRs of genes *SH2*, *Adh1* and *SHDIR16*. Lane M = λ DNA digested with *Pst*I and arrows indicate where the 5'-UTR has separated. **(A)** Lanes 1-4 = 5'-UTR of the *SH2* gene (1.6 kB). **(B)** Lane Adh1 = 5'-UTR of the *Adh1* gene (1 kB). **(C)** Lanes 1-3 = 5'-UTR of the *SHDIR16* gene (Damaj et al., 2010) (2.8 kB).

```

5' _TCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCG
AATTCAGTAGTGATTAACCGTAATCATAAGAGGGAGAGCCATGCGGCGTA
TGTCCTCAAAGGCACCTTTGGCGTTATCTGAAACCAATATCGCTCTCTGTTA
GTTTATCTTGCATGAACGTTTGTGGAAACTACTAGCTTACAAGCATTAGTG
ACAGCTCAAAAAAAGTTATTTCTGAAAGGTTTCATGTGTACCGTGGAAAA
TGTTGCCAACTCAAACACCTTCAATATGTTGTTTGCACGCAAATTTCTCTG
GAAGAAAGGTGTCTAAAACCTATGAACGGTTACAGAAAGGTACAAACCAG
GCTGTGCATTTTGGAAAGTATCATCTATAGTCTGTTGAGGGGAAAGCCGCAC
GCCAAAAGTTATTTACTGAGAAACAGCTTCAACACACAATTGTCTGCTTTAT
GATGGCATCTCCACCCAGGCACCCACCATCACCTCTCTCTCGTGTCTGTTT
ATTTTCTTGCCCTTTCTGCTCATAAAAATCATTAAAGAGTTTATAAACACCC
ATAGGCATACCAATATGCACATCTATTAATGGCCAGCGGATCATCTTCCT
ACTCTTTAGTTATTTATTTGTTTGAATAATATGTCCTGCACCTAGGGAGCTC
GTGCACAGTACCAATGCATCTTCATTAAATGTGAATTTCAAAAAGGAAGTA
GGAACCTATGAGAGTATTTTCAAATAATAAATGGCTTCTATATGTTT
ATACCAAAGGCCAAGGGCAAAAATGGAAATGCTAATGATGGTTGGCTGCGT
GAGTCTGTGCGATTACTTGCAGAGAAATGTGAACCTTTGTTTCTGTGCGTGA
CATAAAACCAAAAGCTTCTAGCCTCTTTCACGGTACTTGCACTTGAAGAA
ATGTGAACCTCCTTTTCATTTCTGTATGTGGACGTAACGCAAAGCATCCAGG
CTTTTTCATGGTTGTTGATGTCTTGCACAGTCCATCACCACCTGTATGCC
CTCCTCCCCTCTATATAAGCACATCAACAGCACCAGCAATTAGACACAGGC
AGCAATTGGAGCCACAAGAACACTTAGGGAGGCAAGTGGCATTTGATCTT
GCAGCCACCTTCGTGTTCTGTTGTAAGTATACATTCCCTTGCCATCTTTAT
TTGTTAGCTTAATTCGTAATTGGGAAGTATTAGTGGAAAGAGGGTGAGATG
CTATCATCTATGCACCTGCAAAATGCATCTGACGTTATACAGGTGGCTTTG
TATAGAATTGCTCCATTCTTGCCAAACATATACTGCAAGGTATATGCCTAG
TTGCATCAAAGTTCTGTTTTTTCATTCTAAAAGCATTTTATTGGCATGTGAT
TTTTGTCCATGGGGGAAGGAGATCTGCTTTGGTTATTTTGTCTTGAGGAGCA
TTCTTCATGAGTCCAGTTTATGGAAGTAGTAAATCTCGGTTTGGTCATAA
GATGTCATATTGTAGCGCAAACATATATACTTAAATGTTCAATTCATAGT
AAATGTTCCCTTGCTGTAAGAATGCTACTCATTTATTTGAGTTGCAGG
TGTGCTAGTAGTTGGAGGAGATAATCGAATTCCCGCGGCCCCATGGCGG
CCGGGAGCAT_3'
    
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Figure 13: Nucleotide sequence of the SH2 promoter region PCR amplified from sugarcane gDNA.

4.3.1.3 Transient GFP analysis

Two days after sugarcane biolistic transformation with the pGFP SK- vector containing the *SH2* 5'-UTR (SH2-pGFP) (Fig.14), calli were screened for fluorescence on the basis that this would be due to transient *GFP* expression (Fig.15). The positive control callus containing *Ubi-1* promoter fluoresced strongly. Roughly 720 calli were screened in total using a fluorescent microscope and 42 clones showed weak fluorescence. Putative positives were transferred to MSC₃-geneticin selection media.

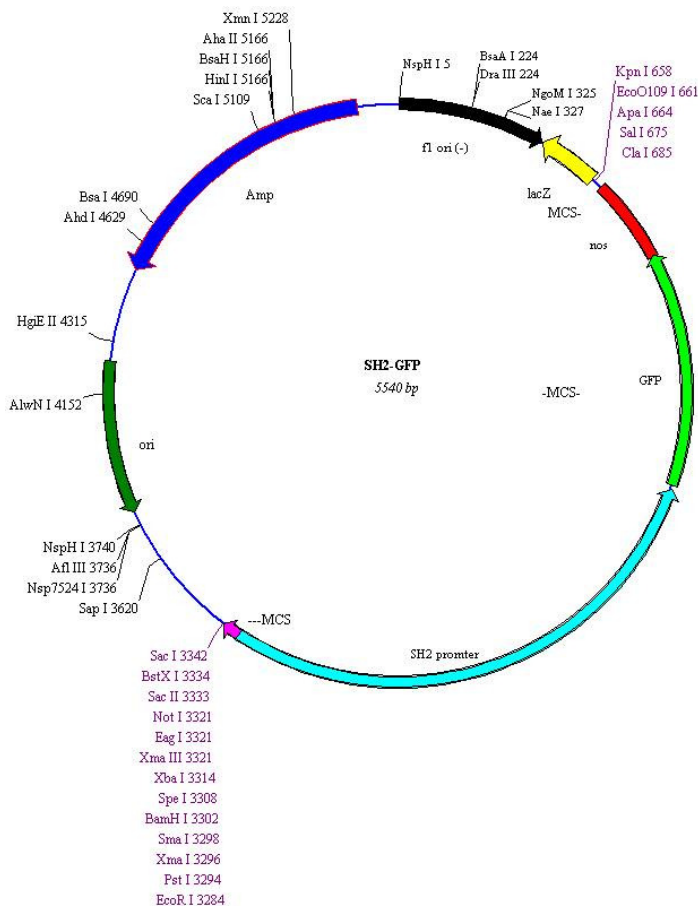


Figure 14: The sugarcane transformation and reporter gene vector pGFP SK- containing the 5'-UTR of the *SH2* gene.

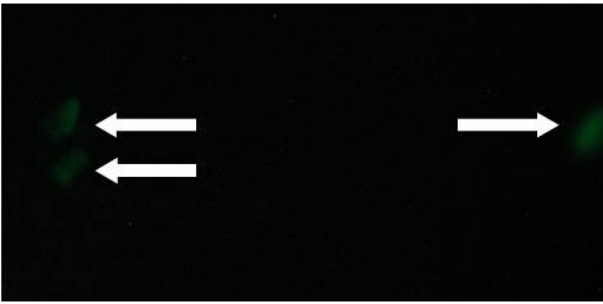


Figure 15: Weak transient GFP production in sugarcane callus. White arrows indicate SH2-GFP bombarded callus cells producing fluorescence. WT NCo310 callus did not fluoresce, while the positive control (*GFP* reporter gene linked to the strong constitutive *Ubi-1* promoter) callus fluoresced strongly.

4.3.1.4 Incorporation of the *GFP* cDNA into the sugarcane genome

After a selection period of 8 weeks, PCR was performed on putative transgenic calli, which indicated 5 transgenic lines containing the *GFP* gene (Fig.16).

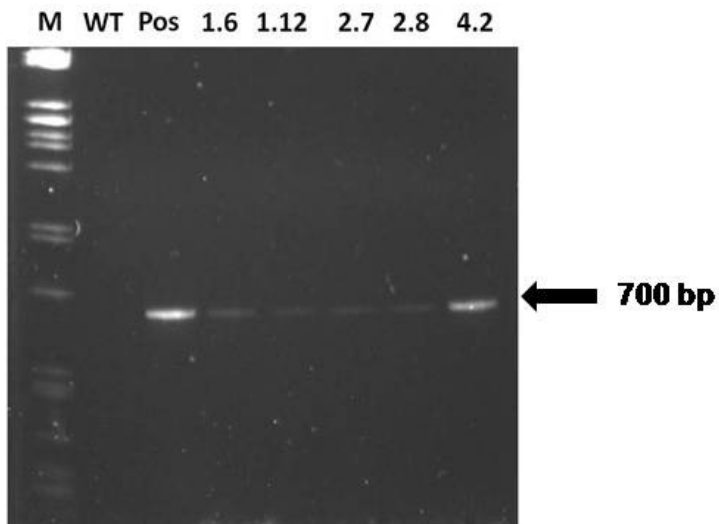


Figure 16: PCR confirmation of *SH2* transgenic sugarcane. 0.8% (w/v) agarose TBE electrophoresis gel containing PCR products from gDNA extracted from lines 1.6, 1.12, 2.7, 2.8 and 4.2 as PCR template showing the presence of the *GFP* reporter gene (700 bp); Lane M = λ DNA digested with *Pst* I. WT = wild-type NCo310 sugarcane gDNA. Pos = SH2-pGFP plasmid DNA.

4.3.1.5 Detection of GFP using immunoblots

A protein immunoblot was performed to demonstrate that the fluorescence noted in the calli corresponded to the presence of GFP protein. However, although GFP protein could be found in the positive control (sugarcane expressing GFP under control of the maize ubiquitin promoter), it could not be detected in the SH2-GFP transgenic lines (Fig.16) in five independent experiments (Fig.17), this led us to hypothesise that the *SH2* promoter region was either silenced or does not express in callus tissue. These results are similar to previous studies where, following bombardment of sugarcane callus with sugarcane promoter regions, transient *GFP* expression was found. However, in plants expression did not continue probably due to TGS or PTGS (Wei et al., 2003; Mudge et al., 2009).

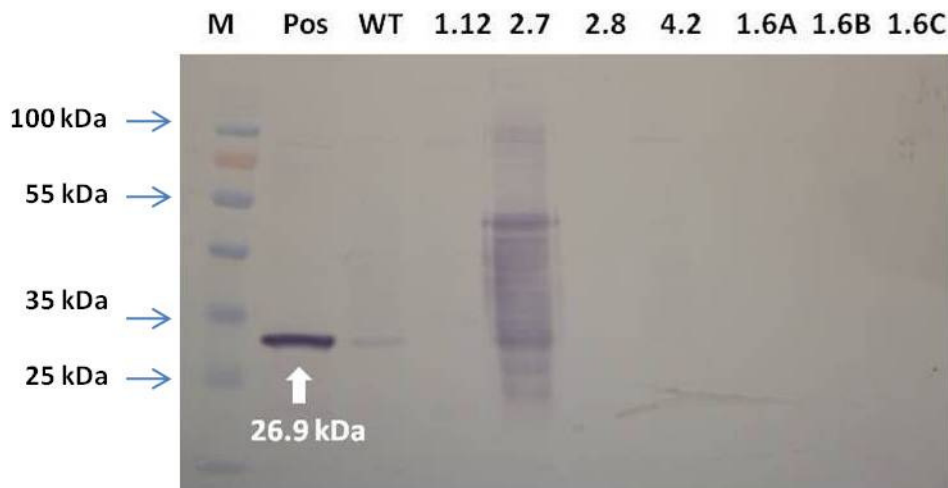


Figure 17: Immunoblot to detect GFP of sugarcane callus transformed with the SH2-GFP construct. Lines are 1.12, 2.7, 2.8, 4.2, 1.6 A, B & C; Lane M = PageRuler™ prestained protein ladder (Fermentas); Pos = Positive control for the presence of the GFP protein (26.9 kDa) from the transgenic calli containing the *GFP* reporter gene linked to the strong constitutive *Ubi-1* promoter; WT = Wild type NCo310. Some cross contamination in the WT and non-specific staining occurred with line 2.7.

Although it seems that no GFP protein is present in these lines, it should be pointed out that these samples were from callus. It is possible that if the callus was regenerated into plants, then expression might be found. The presence of GFP transcripts will still be tested to examine whether the *SH2* promoter drives GFP expression in transgenic plants.

4.3.1.6 Isolation of strong constitutive gene for hybridisation studies

In a further attempt to identify promoters for use in sugarcane; partial cDNA sequences of strong constitutively expressed genes from were amplified using PCR. These sequences are currently being used as probes to screen sugarcane BAC libraries to help identify and isolate their corresponding promoters. Due to sugarcane's aneuploid genome there may be ten to twelve copies, these genes may have up to twelve different promoters driving expression of separate alleles. It is anticipated that through assessing promoter regions from these different alleles that it will be possible to isolate a promoter that will result in continued strong constitutive expression in sugarcane.

cDNAs that were chosen for this are products of the *ubiquitin9*, *β -actin*, *β -tubulin* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* genes. The last three were chosen due to their use as reference genes in RT-qPCR and having strong constitutive expression in sugarcane (Bustin, 2000; Iskandar et al., 2004). One partial cDNA that was isolated was the sugarcane ubiquitin gene (Fig.18); however the other three genes (*GAPDH*, *tubulin* and *actin*) failed to be amplified. These are currently being isolated at SASRI (Bernard Poitier, personal communication).

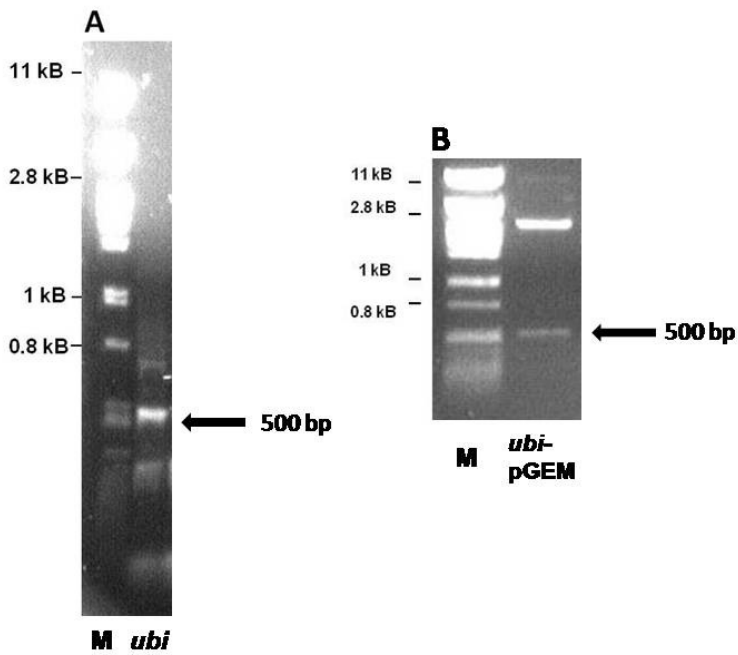


Figure 18: Partial sugarcane *ubiquitin* cDNA. (A) PCR amplification product of a part of the sugarcane ubiquitin (*ubi*) gene (500 bp); Lane M = λ DNA digested with *Pst*I. (B) *ubi*-pGEM = *Eco*R I digested plasmid DNA of the pGEM-T Easy vector containing *ubi* fragment, which separates at 500 bp; Lane M = λ DNA digested with *Pst*I.

4.3.2 Development of novel sugarcane selection system based on herbicide resistance

This experiment was done as an initial step towards the development of a selectable marker gene consisting entirely of sugarcane DNA. The mutant tobacco *ALS SurB* isoform (*alsb*) from the tobacco *rchl 6.6* line was introduced into sugarcane callus to check for the development for chlorosulfuron resistance. If successful, a sugarcane *ALS* gene will be isolated from sugarcane, mutated and transformed into sugarcane to check for herbicide resistance.

4.3.2.1 A partial sugarcane ALS gene for hybridisation studies

Only a partial sugarcane *ALS* gene (Genbank: EU243999.1) (*SoALS*) is available online. *SoALS* was amplified from sugarcane mRNA and was ligated into pGEM-T (Fig.19). This is currently being used as a probe to screen SASRI sugarcane BAC libraries to obtain the full sugarcane *ALS* gene. SASRI has offered to assist by doing the hybridisation studies (Bernard Poitier, personal communication).

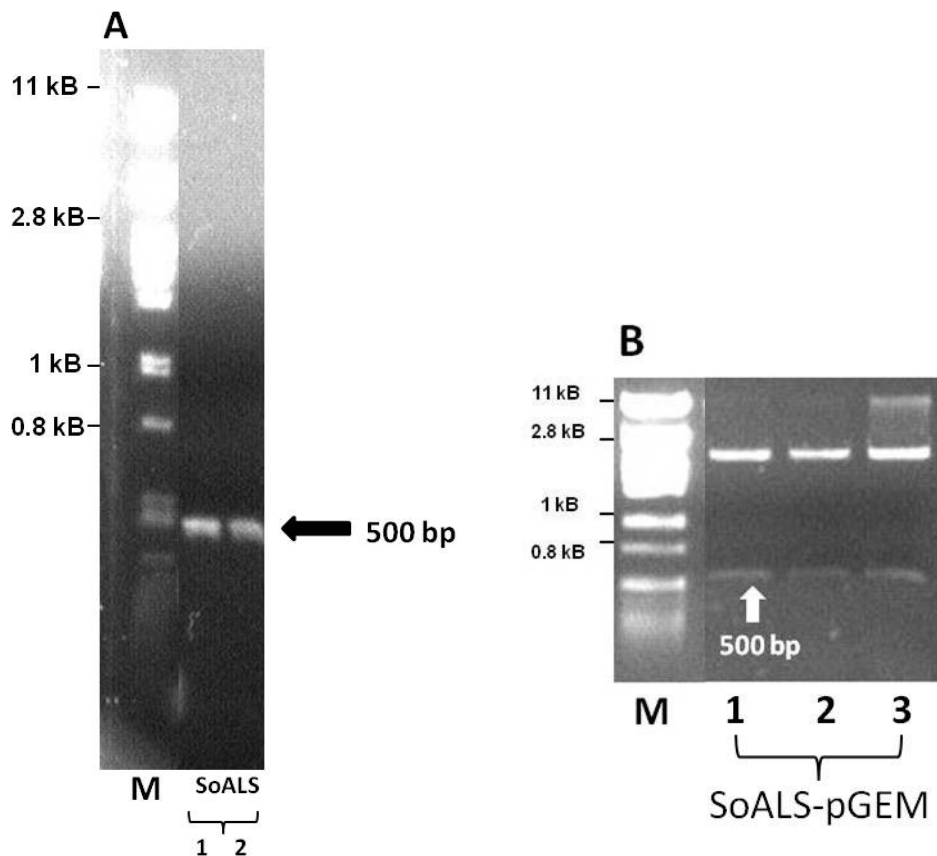


Figure 19: Partial sugarcane *ALS* cDNA **(A)** PCR amplification of *SoALS*. Lanes 1 and 2 = 500 bp *SoALS*; Lane M = λ DNA digested with *Pst*I. **(B)** Enzymatic digest of pGEM-Easy containing *SoALS* using *EcoR* I. Lanes 1-3 = 500 bp *SoALS*; Lane M = λ DNA digested with *Pst* I.

4.3.2.2 Amplification of the *alsb* mutant cDNA from a chlorosulfuron mutant tobacco line

Tobacco plants in which the *alsb* gene has been mutated (Line *rchl 6.6*; Kochevenko and Willmitzer, 2003) to make it resistant to chlorosulfuron were grown in the glasshouse at the IPB. cDNA was manufactured from RNA and PCR was used to amplify the *alsb* cDNA (Fig.20).

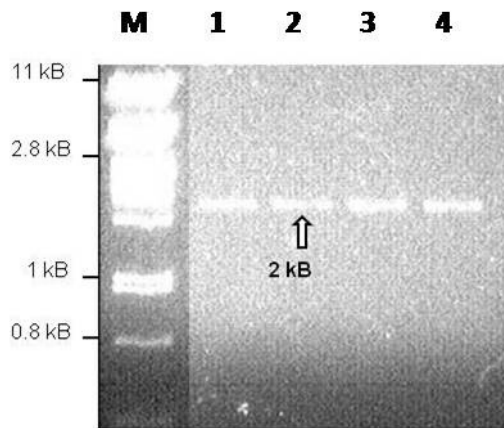


Figure 20: PCR amplification of the *alsb* cDNA from the chlorosulfuron resistant tobacco line *rchl6.6*. Lanes 1-4 = *alsb* gene (2 kB); Lane M = λ DNA digested with *Pst* I.

The gene was ligated into the *Sma* I site of the pBluescript SK- vector. These were digested with *Bam*H I to show the presence *alsb* gene within the vector.

The PCR product was sequenced and results indicated that the single nucleotide base pair change at base pair 1719 (TGG to TIG), which result in a Trp-573-Leu amino acid substitution, was present (Fig.21). Following this, the insert was restricted using *Bam*H I from the pBluescript SK- vector and into the *Bam* HI restriction site of the pUBI 510 vector to give ALSB-pUBI (Fig.22). Correct orientation was checked using *Eco*R I.

```

5`_TTTCGTCTCTCACTGCTCTCATTCAACAATAATGGCGGCGGCTGCGGCGGCTCCATCTCCCTCT
TTCTCCAAAACCCCTATCGTCCCTCCTCCAAATCCTCCACCCTCCTCCCTAGATCCACCTTCCCT
TTCCCCACCACCCCCACAAAACCACCCACCACCCCTCCACCTCACCCCCACCACATTCACAGC
CAACGCCGTCGTTTTACCATCTCCAATGTCATTTCCACTACCCAAAAAGTTTTCCGAGACCCAAAAA
GCCGAAACTTTTCGTTTCCCGTTTTGGCCCTGACGAACCCAGAAAGGTTCCGACGTTCTCGTGGAG
GCCCTCGAAAGAGAAGGGGTTACGGACGTTTTTTCGCTACCCAGGCGGCGCTCCATGGAGATTAC
CAAGCTTTGACGCGCTCAAGCATCATCCGCAACGTGCTACCACGTCACGAGCAGGGTGGTGTCTTC
GCCGCTGAGGGTTACGCACGCGCCACCAGGCTTCCCGGCGTTTTGCATGACCCTCCGGCCCTGGC
GCCACCAATCTCGTCAGTGGCTTCGCGGACGCCCTACTGGATAGCGTCCCATTTGTTGCTATAACC
GGTCAAGTGCCACGTAGGATGATCGGTACTGATGCTTTTTCAGGAAACTCCGATTGTTGAGGTAAC
AGATCGATTACCAAGCATAAATTATCTCGTTATGGACGTAGAGGATATTCCTAGGGTTGTACGTGAG
GCTTTTTTCTTGGCAGATCGGGCCGGCTGGCCCTGTTTTGATTGATGTACCTAAGGATATTTCAG
CAACAATGGTGATACCTGACTGGGATCAGCCAATGAGGTTGCCTGGTTACATGTCTAGGTTACCT
AAATTGCCCAATGAGATGCTTTTAGAACAAATTGTTAGGCTTATTTCTGAGTCAAAGAAGCCTGTT
TTGTATGTGGGGGTTGGGTGTTTCGCAATCGAGTGAGGAGTTGAGACGATTCTGGAGCTCACCGGT
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ATGTTGGGTATGCATGGTACTGTTTATGCTAATATGCTGTGGACAGTAGTGATTTATTGCTCGCA
TTTGGGGTGAGGTTGATGATAGAGTTACTGGAAAGTTAGAAGCTTTTGCTAGCCGAGCGAAAATT
GTTACATTGATATTGATTACGCTGAGATTTGAAAGAACAAAGCAGCCTCATGTTCCATTTGTGCG
GATATCAAGTTGGCGTTACAGGGTTGAATTCGATATTGGAGAGTAAGGAAGTAAACTGAAGTTG
GATTTTTCTGCTTGGAGGCAGGAGTTGACGGTGCAGAAAGTGAAGTACCCGTTGAATTTAAAACT
TTTGGTGATGCTATTCCTCCGCAATATGCTATCCAGGTTCTAGATGAGTTAACTAATGGGAGTGCT
ATTATAAGTACCGGTGTTGGGCAGCACCAGATGTGGGCTGCTCAATATTATAAGTACAGAAAGCCA
CGCAATGTTGACATCTGGTGGATTAGGAGCGATGGGATTTGGTTTTGCCCGCTGCTATTTGGTGCG
GCTGTTGGAAGACCTGATGAAGTTGTGGTTGACATGATGGTGATGGCAGTTTCATCATGAATGTG
CAGGAGCTAGCAACTATTAAGGTGGAGAATCTCCAGTTAAGATTATGTTACTGAATAATCAACAC
TTGGGAATGGTGGTTCAATTGGAGGATCGGTTCTATAAGGCTAACAGAGCACACACATACCTGGGG
AATCCTTCTAATGAGGCGGATCTTTCTAATATGTTGAAATTTGCAGAGGCTTGTGGCGTACCT
GCTGCGAGAGTGACACACAGGATGATCTTAGAGCGGCTATTCAAAGATGTTAGACACTCCTGGG
CCATACTTGTGGATGTGATTGTACCTCATCAGGAACATGTTCTACCTATGATTCCCAGTGGCGGG
GCTTTCAAAGATGTGATCACAGAGGGTGACGGGAGAAAGTTCTATTGACTTTGAGGTGCTACAGAG
CT_3`
    
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Figure 21: Nucleotide sequence of the *alsb* gene, which includes the substitution mutation at basepair 1719 (TGG to TTG) (underlined and bolded) corresponding to the *Hra* mutation.

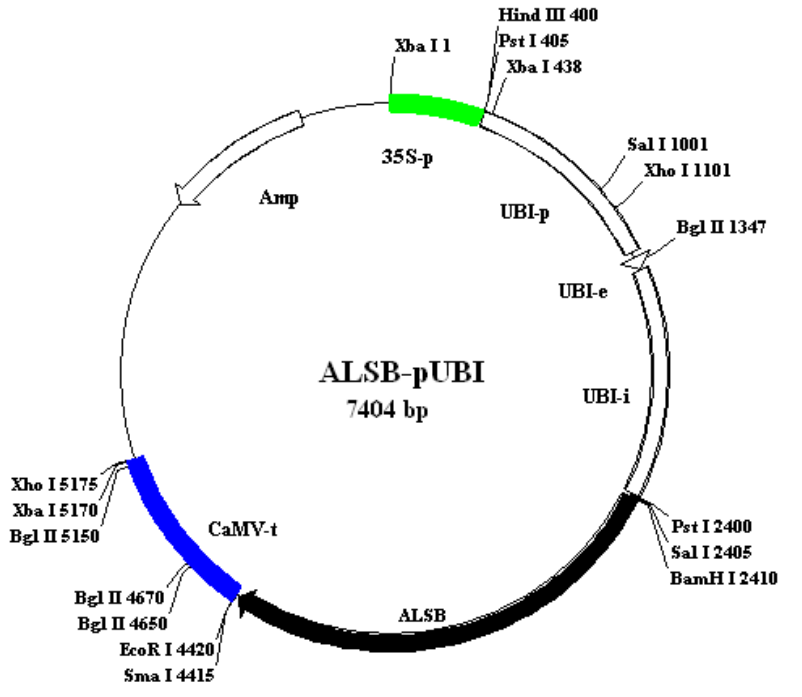


Figure 22: The pUBI 510 vector containing the *alsb* gene.

4.3.2.3 Selection of putative transgenic plantlets

The mutated cDNA was ligated into a transformation construct between the *Ubi-1* promoter and *CaMV-t* terminator and used to bombard sugarcane callus. A kill curve to identify the optimal chlorosulfuron concentration had been performed previously by Dr. Christell van der Vyver (Institute of Plant Biotechnology, Stellenbosch University, South Africa). However the initial herbicide selection process for putative transgenics proved ineffective. When using geneticin for selecting transgenic calli, normally 1% of the total bombarded calli survives. After 8 weeks on chlorosulfuron selection 20% of the bombarded calli survived and they did not show sufficient new growth to easily differentiate between transgenic and non-transgenic. Putative transgenic calli were, based on faster growth, nevertheless, regenerated into plantlets on MS media containing chlorosulfuron to eliminate additional non-rooting non-transgenic clones. The herbicide had little effect on the rooting ability of the putative transgenic clones and 105 regenerated plantlets were transferred to pots containing soil in the glasshouse and sprayed with 300 μM chlorosulfuron once every two weeks. This regime substantially decreased the number to putative transgenics from 105 to 30.

4.3.2.4 Testing for transgenic sugarcane

After the selection process, the 30 surviving putative transgenic plants were tested for the presence of the *alsb* gene via PCR. Six lines were found to be positive transgenics for the maize ubiquitin promoter (*Ubi-1*)-*alsb* fusion (Fig.23), of which one (line 55A) died shortly after it was tested for the transgene.

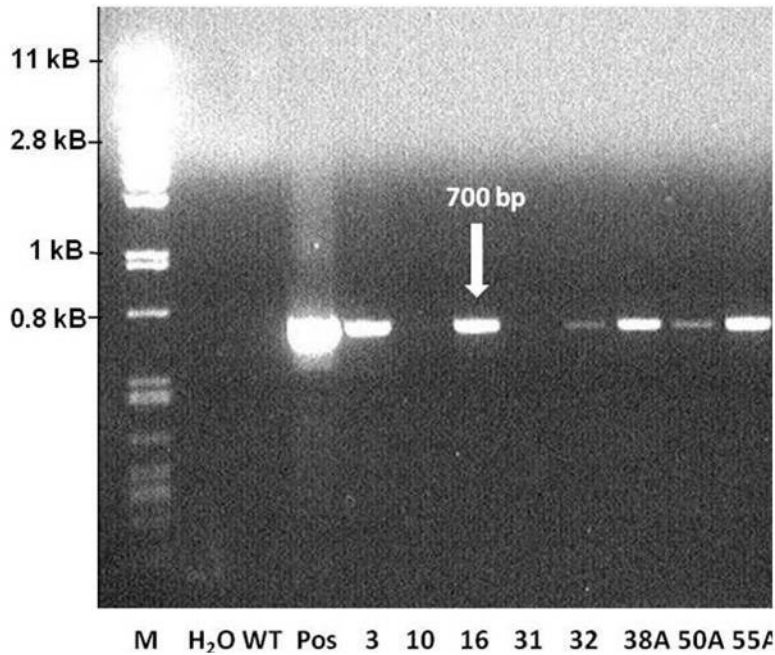


Figure 23: PCR amplification of a fragment of the fused ubiquitin promoter (*ubi*) and mutant acetolactate synthase B (*alsb*) gene from gDNA extracted from transformed ALS-pUBI sugarcane plants. Lane M = λ DNA digested with *Pst* I. H₂O = water control. WT = wild-type NCo310 sugarcane cultivar. Pos = positive control, ALS-pUBI vector. Lines 3, 16, 32, 38A, 50A and 55A were positive transgenic plants. Lines 10 and 31 were negative.

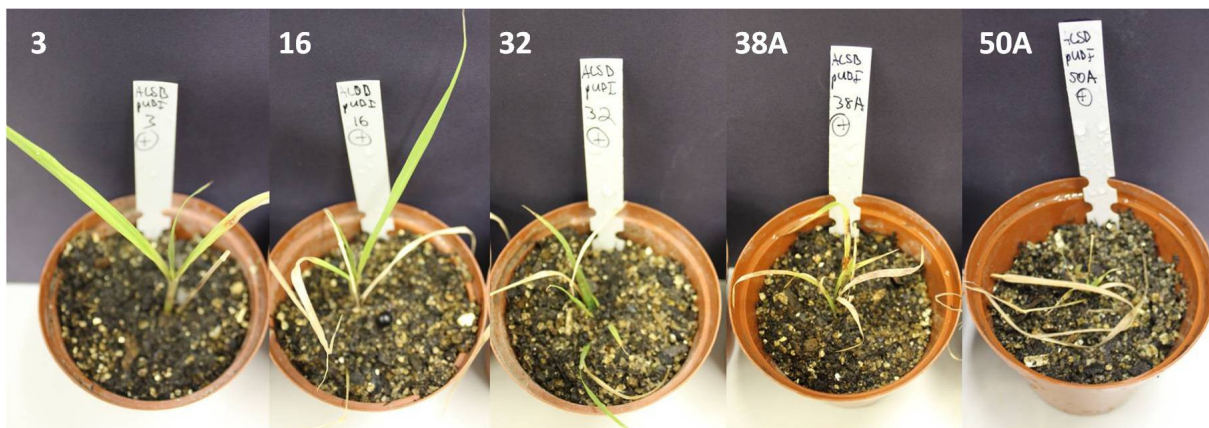


Figure 24: Positive transgenic *alsb* sugarcane lines (6 weeks old). Line 3 indicated a strong growth phenotype in comparison to the other transgenic lines. Line 16 was smaller than 3, but had similar leaf growth and a shorter stem. Lines 32 and 38A showed some growth however, no stem formation. Line 50A had a severe stunted growth phenotype with small thin leaves and no stem growth. No control plants (NCo310) survived the spray selection process.

Visual inspection of the plants indicated that there were differences in form with respect to leaf formation and growth virility. Phenotypes differed greatly within the lines meaning that no significant difference growth between transgenic and non-transgenic plants could be found (Fig.24).

The effectiveness of the *alsb* in sugarcane transformation is not clear. The data from this study suggests that *alsb* does not allow effective selection in tissue culture when chlorosulfuron is used. Some selectivity appears to be present when regenerated plants are sprayed with chlorosulfuron, however, due to the lack of effectiveness in tissue culture it would be feared that the surviving plants are chimeric, consisting of transformed and untransformed cells. In addition the selectivity by spraying is not complete, with only six of the surviving thirty plants testing positive for insertion of the *alsb* cDNA (Figure 24).

Currently a second round of sugarcane callus transformed with ALSB-pUBI is growing on an optimised selection media, which may reduce the number of putative *in vitro* transgenics, reducing both somaclonal variation between putative clones and labour. Somaclonal variation refers to genetic and phenotypic variation among clonally propagated plants of a single donor clone (Kaepler et al., 2000). This can be induced in tissue culture where the wild-type plant can develop resistance to a herbicide and/or the loss of growth and reproductive vigor at later stages in development (Kaepler et al., 2000). Reducing the chances of this by an effective selection system may provide more conclusive data in regards to the effectiveness of *alsb* as a selection marker; however, it is suggested that other selectable marker genes should be assessed. Currently, other candidate selectable markers are being assessed (Christell van der Vyver, IPB, Personal Communication). One of these is the *Protoporphyrinogen IX oxidase (PPO)* gene. PPO catalyzes the oxidation of

protoporphyrinogen to protoporphyrin IX. This enzyme is the target site for a number of herbicides across the diphenylethers and oxidazoles herbicide chemistries. Preliminary data (results not shown) suggest that a combination of *PPO* expression and the use of a diphenylether herbicide (fomesafen) may be used a system for the development of cisgenic sugarcane.

4.4 Conclusion

The focus of this chapter was the development of a cisgenic transformation system for sugarcane. The initial step in this project was to isolate and assess a functioning promoter from sugarcane. A single promoter *SH2* was assessed and appears not to drive expression in sugarcane callus, possibly due to silencing. Other promoters, namely *ubiquitin*, *actin*, *tubulin* and *GAPDH* are currently being identified and isolated at SASRI and will be assessed in future. The isolation of functioning promoters will be simplified following the publication of the sugarcane genome and should help in the development of this part of the project.

The second part of developing a cisgenic transformation technology was to develop a high throughput selection system. A tobacco mutant *alsb* gene was expressed in sugarcane callus to assess whether it confers chlorosulfuron resistance. Following bombardment, regenerated plants were isolated, but sugarcane appears to have high resistance to chlorosulfuron and many of them were not transgenic. This indicates that this strategy will not be successful, and other selection systems need to be assessed.

Chapter 5

General Discussion

In this thesis two things were attempted; firstly to increase the sucrose yield of sugarcane and secondly to develop a novel sugarcane transformation technology based on endogenous genetic sequences.

This agronomically important crop accumulates high amounts of sucrose within the mature internodes of the stem. The vacuole of the parenchyma cells within these tissues was targeted as a possible site for genetic manipulation to increase sucrose accumulation (Rowher and Botha, 2001). The potential ability to store sucrose within this organelle has been determined to be more than twice that of current commercial cultivars (Wu and Birch, 2010). H⁺/Sucrose transporters situated on the tonoplast membrane of the vacuole rely on a proton gradient between the cytosol and lumen to pump sucrose into the lumen (Eom et al., 2011). A tonoplast bound H⁺/PPase (V-PPase) has been identified to be important in driving this gradient (Maeshima, 2000; Ferjani et al., 2011) and increased expression has been correlated with increased sucrose accumulation in the sugarcane culm (Swart, 2005). It was hoped that the proton gradient generated from increased V-PPase activity would increase the amount of sucrose transported into and stored within the vacuole. Increased activity of this protein may increase sucrose for a different reason. The protein will decrease P_i concentrations within the cytosol. Lowered P_i should decrease PFP activity and, as antisense PFP sugarcane plants accumulate more sucrose, should increase the amount that accumulates.

An *Arabidopsis V-PPase* (*AtV-PPase*) was chosen to be expressed in sugarcane callus. Initially it was attempted to isolate the full cDNA clone of this gene from *Arabidopsis* RNA, however, this proved impossible. Because of this a construct was produced containing an intron between two parts of the coding sequence. This was successfully transferred into a transformation vector and transgenic sugarcane was produced. Although transgenic callus did not show consistent changes in soluble sugar, this will be tested in regenerated plants.

The second project in this thesis concerned the development of a novel sugarcane transformation technology. This cisgenic approach differs from the conventional transgenic technology, as genetic constructs will be sugarcane derived. With the use of only endogenous sequences, it should be considered similar to traditionally bred cultivars, with the added benefits of *in vitro* propagation and the specificity of molecular manipulation (Rommens, 2004). It is hoped that such cisgenic plants will be more accepted by regulatory bodies in future and will more easily lead to the commercial cultivation of genetically modified sugarcane.

One promoter was isolated from sugarcane gDNA by designing primers based on a sorghum template. Transgenic plants transformed with a construct where this promoter drives expression of the *GFP* cDNA did not lead to stably transformed callus producing GFP protein. Currently other candidate promoters are being isolated using an alternative approach. This entails the isolation of fragments of strongly constitutive genes, which will be used to screen sugarcane BAC libraries. The screening is currently underway at SASRI and promoters of four candidate genes, namely *ubiquin9*, *β -actin*, *β -tubulin* and *GAPDH*, have been chosen. It is hoped that promoters from multiple alleles of each gene will be

isolated using this method, giving up to 48 putative promoters that could be assessed in sugarcane via GFP expression.

The second component of the cisgenic project was concerned with the development of a novel high throughput selection system for sugarcane transformation. A selectable marker (*alsb*), isolated from a chlorosulfuron resistant tobacco mutant, was assessed in sugarcane for the development of herbicide resistance. Although inconclusive, data suggests that *alsb* was not effective as a high throughput selection marker. This may change due to a second round of selection, on optimised media, for herbicide resistance. If this gene proves to be effective, a sugarcane *ALS* gene will be mutated to have a similar mutation corresponding to the tobacco. This will be assessed in sugarcane for the development of herbicide resistance and its effectiveness as a native sugarcane selection marker.

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